The N-terminal cysteine cluster is essential for membrane targeting of B/K protein

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B/K protein belongs to a family of C-terminal-type (C-type) tandem C2 proteins that contain two C2 Ca^{2+}-binding motifs at the C-terminus. Although other C-type tandem C2 proteins have been found to have a unique N-terminal domain that is involved in membrane anchoring (e.g. synaptotagmin) or specific ligand binding (e.g. rabphilin-3A and Doc2), no research has been conducted on the function of the N-terminal domain of B/K protein. In this study we showed that despite lacking a transmembrane domain, both native and recombinant B/K proteins are tightly bound to the membrane fraction, which was completely resistant to 0.1 M Na_2CO_3, pH 11, or 1 M NaCl treatment. Deletion and mutation analyses indicated that the cysteine cluster at the N-terminal domain (consisting of seven cysteine residues, Cys-19, Cys-23, Cys-26, Cys-27, Cys-30, Cys-35 and Cys-36) is essential for the membrane localization of B/K protein. When wild-type B/K was expressed in PC12 cells, B/K proteins were localized mainly in the perinuclear region (*trans*-Golgi network), whereas mutant B/K proteins carrying Cys-to-Ala substitutions were present in the cytosol. Based on our findings, we propose that the N-terminal domain of B/K protein contains a novel cysteine-based protein motif that may allow B/K protein to localize in the *trans*-Golgi network.

Key words: C2 domain, C-type tandem C2 protein, Cys cluster, synaptotagmin.

INTRODUCTION

The C-terminal-type (C-type) tandem C2 protein family is defined as consisting of proteins that contain two C2 Ca^{2+}-binding motifs (named the C2A and C2B domains) [1,2] separated by a short linker (less than 50 amino acids) at the C-terminus [3,4], which are often found in proteins involved in Ca^{2+}-dependent transmitter secretion [5–7]. To date, four different subfamilies of C-type tandem C2 proteins have been reported (see Figure 1A), and they are distinguished from each other by their unique N-terminal sequences, which are essential for membrane anchoring or specific protein interactions. For instance, synaptotagmins (Syts) contain a single transmembrane domain at the N-terminus (reviewed in [5,8–10]): e.g. rabphilin-3A contains a rab-3A-binding domain [11], Doc2 proteins contain a Munc13-1-interacting domain [12–14] and Syt-like proteins (Slps) contain an Slp homology domain [15]. The rab-3A-binding domain of rabphilin-3A and the Munc13-1-interacting domain of Doc2α have been shown to regulate Ca^{2+}-dependent transmitter secretion [16,17]; however, an alternatively spliced isoform that lacks the unique N-terminal domain has recently been reported in several Syt isoforms (Syt VIΔTM) [18,19] and in the Slp family [15].

B/K protein (formally called Syt B/K) was originally identified as a Syt homologue selectively expressed in brain and kidney [20]. Since it lacks the above-described unique N-terminal domains as well as the known protein motifs, it should be classified as a fifth subfamily of C-type tandem C2 proteins. However, no information is available on the function of the N-terminal domain of B/K protein. In the present study, we first examined whether B/K protein is derived from an alternative splicing product of Syts or Slps by reverse transcriptase PCR or genomic sequence analyses and found that the *B/K* gene does not belong to either the *syt* or the *slp* gene family. We then showed that both native and recombinant B/K proteins are tightly bound to the membrane fraction and identified the N-terminal cysteine cluster as essential for specific membrane association [i.e. with the *trans*-Golgi network (TGN)]. Based on our findings, we discuss the mechanism of the membrane association of B/K protein.

MATERIALS AND METHODS

Materials

Ex *Taq* and recombinant *Taq* DNA polymerases were obtained from Takara Shuzo Co. (Shiga, Japan) and Toyobo Biochemicals (Tokyo, Japan), respectively. Polyclonal antibody against FLAG peptide was obtained from Zymed Laboratories (San Francisco, CA, U.S.A.). Horseradish peroxidase (HRP)-conjugated anti-(T7 tag) antibody was from Novagen (Madison, WI, U.S.A.). Anti-TGN38 antibody was from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-(Syt I) (SYA148) antibody was from StreeGen Biotechnologies Corp. (Victoria, BC, Canada). L-α-Phosphatidylcholine (PC), dipalmitoyl and L-α-phosphatidylserine (PS), dioleoyl, nocodazol and Brefeldin A were from Sigma (St. Louis, MO, U.S.A.). Wortmannin was from Calbiochem–Novachem Corp. (La Jolla, CA, U.S.A.). All other chemicals were commercial products of reagent grade. Solutions were made up in deionized water prepared with an

Abbreviations used: C-type, C-terminal-type; GST, glutathione S-transferase; HRP, horseradish peroxidase; PC, L-α-phosphatidylcholine; PS, L-α-phosphatidylserine; RACE, rapid amplification of cDNA ends; Slp, synaptotagmin-like protein; Syt, synaptotagmin; TGN, *trans*-Golgi network.

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The nucleotide sequence reported here is deposited in the DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under accession numbers AB069667 and AB069668.

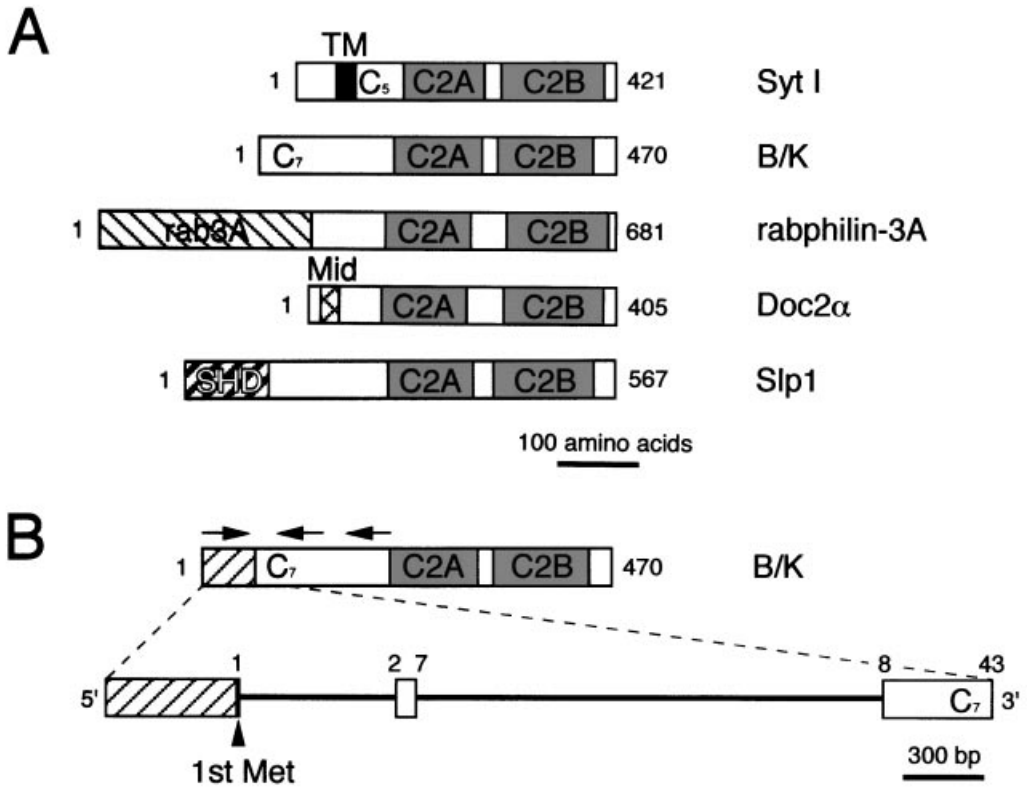

Figure 1 Schematic representation of the various C-type tandem C2 protein families

(**A**) Schematic representation of mouse Syt I (GenBank® accession no. BAA07040), B/K (AB069667), rabphilin-3A (JX0338), Doc2α (BAA23430) and Slp1 (BAB32651). Amino acid numbers are given on both sides. TM, transmembrane domain (black box); rab-3A, rab-3A-binding domain (hatched box); Mid, Munc13-1-interacting domain (cross-hatched box); SHD, Slp homology domain [15]; C_n, cysteine cluster, where *n* corresponds to the number of cysteine residues. (**B**) Organization of the partial mouse *B/K* gene. Exons and introns are indicated by boxes and solid lines, respectively. 5′-Non-coding regions are represented by hatched boxes. The numbers on the boxes (exons) indicate amino acid residues. The arrowhead and arrows indicate the positions of the first Met and primers used for DNA amplification, respectively.

Elix10 Water Purification System and Milli-Q Biocel A10 System (Millipore, Bedford, MA, U.S.A.).

Molecular cloning of mouse B/K cDNA

cDNAs encoding the N-terminal region of mouse B/K were amplified from Marathon-Ready mouse adult brain cDNA by 5′-rapid amplification of cDNA ends (RACE) (ClonTech, Palo Alto, CA, U.S.A.) as described previously [21]. The first 5′-RACE reactions were carried out using an adapter primer 1 (5′-CCATCCTAATACGACTCACTATAGGGC-3′) and the C2B lower primer designed on the basis of the mouse partial B/K sequence reported previously (5′-GGAATTCCATCCGCCGC-CAGTGGTTGG-3′, antisense, amino acid residues 433–439) [22]. The second RACE reactions were carried out using an internal adapter primer 2 (5′-ACTCACTATAGGGCTCGA-GCGGC-3′) and the C2A lower primer (5′-GGAATTCACTGG-GGATCAGGGCCTTCC-3′, antisense, residues 305–311). Both PCRs were carried out in the presence of Perfect Match PCR Enhancer (Stratagene, La Jolla, CA, U.S.A.) for 35 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 3 min. The first and second PCR products were purified from an agarose gel on a Micro-Spin column (Amersham Pharmacia Biotech, Little Chalfont, Bucks, U.K.) as described previously [21] and then inserted directly into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.). Both strands of the cDNA inserts were sequenced randomly with a Hitachi SQ-5500 DNA sequencer until a cDNA clone including the putative initiation methionine was obtained.

Expression constructs

Full-length mouse B/K cDNA, including the open reading frame, was amplified from the Marathon-Ready mouse adult brain cDNA by using the following primers with appropriate restriction enzyme sites (underlined): 5′-CGGATCCATGTTGGAACCA-CTGAACGAG-3′ (Met primer, sense, amino acid residues 1–7) and 5′-GGAATTCTCAGGTCACCTCCAGCGAGG-3′ (C1 primer, antisense, residues 465–470). The purified PCR products were inserted into the pGEM-T Easy vector (named pGEM-T-B/K). The *Bam*HI/*Spe*I fragment of pGEM-T-B/K was inserted into the *Bam*HI/*Spe*I site of pEF-T7-Syt IX (or pEF-FLAG-Syt IX) as described previously [23–25]. pEF-T7-B/KΔN was similarly constructed by PCR using ΔN primer (5′-GCGGATCCA-TGGCCAGCCGGAGCAA-3′, residues 58–63) and SP6 primer as described previously [21]. Other expression constructs [pEF-T7-Syt I, pEF-T7-Syt I(CA), pEF-T7-Syt VIΔTM and pEF-FLAG-Syt XI] were prepared as described previously [18,21,25,26]. Plasmid DNA was prepared using Wizard-mini preps (Promega) or Maxi prep kits (Qiagen, Chatsworth, CA, U.S.A.).

Site-directed mutagenesis

pEF-T7-B/K(CA) was essentially produced by means of a two-step PCR technique as described previously [27], using the following pairs of oligonucleotides [T7 primer and CA primer 1, 5′-GGACTCATATGCCCTCTGAGCGGCGTGTTGGGCA-GTCCAGCCGGCCAG-3′ (left half); CA primer 2, 5′-AGGG-CATATGAGTCCAGCGCCGCCCAGT-3′, and SP6 primer (right half)]. The right and left halves were amplified separately by using pGEM-T-B/K as a template, and the two PCR fragments obtained were digested with *Nde*I (underlined above), ligated to each other and reamplified with T7 and SP6 primers. The PCR fragment obtained that encoded the mutant B/K was digested with *Bam*HI/*Spe*I and inserted into the *Bam*HI/*Spe*I site of pEF-T7-B/K as described above.

Isolation of the partial mouse *B/K* genomic DNA

The partial *B/K* gene around the initiation Met residue was amplified from adult mouse (ICR strain) liver using the following primers as described previously [28]: 5′-TGCTGAGTAGCA-TGACGA-3′ (sense) and 5′-CACTTCATCTTCACTGGACT-3′ (antisense, residues 37–43). Reactions were carried out in the presence of Perfect Match PCR Enhancer for 35 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 3 min. The purified PCR products were inserted directly into the pGEM-T Easy vector and sequenced as described above.

Miscellaneous procedures

Transfection of pEF-T7-Syt into COS-7 cells (5×10^5 cells/10 cm dish, the day before transfection) was carried out by using LipofectAmine Plus reagent according to the manufacturer's instructions (Life Technologies, Rockville, MD, U.S.A.) [26]. Subcellular fractionation of COS-7 cells (or brain), SDS/PAGE and immunoblotting with HRP-conjugated anti-(T7 tag) antibody were performed as described previously [21,25,29]. Transfection of pEF-FLAG-Syt or pEF-T7-Syt into PC12 cells and immunocytochemical analysis were performed as described previously [18,30,31]. In some cases, cells were treated with 10 μM wortmannin, 3.3 μg/ml nocodazol or 10 μg/ml Brefeldin A for 1.5 h before fixation. Glutathione S-transferase (GST) fusion proteins (GST–Syt I-C2A, GST–B/K-C2A and GST–B/K-C2B) were purified as described previously [22,32]. Preparation of liposomes consisting of PC and PS (1:1, w/w) and a phospholipid-binding assay were also performed as described previously [33,34]. The protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) using BSA as a reference. An antibody against the C2A domain of B/K proteins (anti-B/K) was produced by immunizing rabbits with purified GST–B/K-C2A, and was immunoaffinity purified subsequently by using antigen-immobilized beads as described previously [18].

RESULTS

Membrane localization of brain B/K protein in mouse brain

B/K cDNA was cloned originally as a homologue of Syt lacking a transmembrane domain [20]. Since B/K protein has several structural similarities in common with the Syt family (i.e. it contains an N-terminal cysteine cluster and C-terminal tandem C2 domains; see Figure 1A), the original B/K cDNA may have been derived from a novel *syt* gene by transmembrane-exon skipping, the same as Syt VIΔTM [18,19]. To test this possibility,

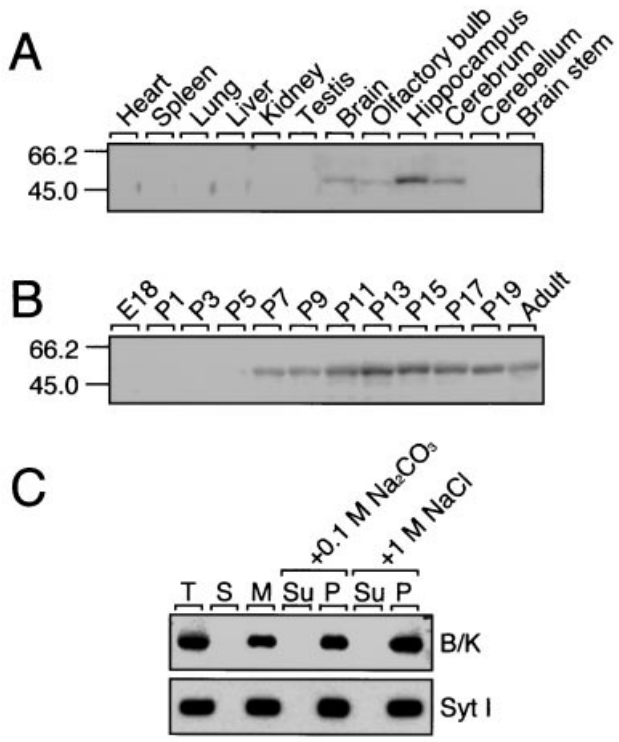

Figure 2 Tissue distribution of mouse B/K protein

(**A**) Distribution of B/K proteins in various adult mouse tissues. (**B**) Developmental expression of B/K proteins in mouse hippocampus. Tissue homogenates (15 μg of protein/lane) of mouse brain from different developmental stages [from embryonic day 18 (E18) and postnatal day (P) 1 to adult] were separated by SDS/PAGE (10% gels) and immunoblotted with anti-B/K-specific antibody. B/K proteins were expressed in rostral regions of the brain, especially in the hippocampus, and their expression increased towards adulthood. (**C**) Subcellular fractionation of mouse forebrain Syt I and B/K proteins. Membrane (M) and soluble (S) fractions were separated as described previously [18]. The membrane fraction was resuspended further in a buffer containing either 0.1 M Na_2CO_3, pH 11, or 1 M NaCl and incubated for 1 h at 4 °C. After centrifugation at 100000 ***g*** for 1 h, the supernatants (Su) and pellets (P) were recovered. Equal proportions of total tissue homogenates (T), the membrane fraction, the soluble fraction and the supernatants treated with 0.1 M Na_2CO_3, pH 11, or 1 M NaCl were subjected to SDS/PAGE (10% gels) and transferred to a PVDF membrane (Millipore). The blots were then immunoblotted with anti-Syt I- or anti-B/K-specific antibodies. Note that the membrane association of both brain Syt I and B/K is insensitive to these procedures.

we first examined the N-terminal region of mouse B/K cDNA by 5′-RACE and the mouse *B/K* genome (Figure 1B). However, we could only detect a single form of mouse B/K cDNA in brain corresponding to the previously reported rat *B/K* sequence [20], and we were unable to find a putative exon encoding a transmembrane domain in either the mouse or the human genomes (GenBank® accession no. AC010490). We therefore concluded that B/K protein belongs to a subfamily distinct from the known C-type tandem C2 protein families (Syt, rabphilin, Doc2 and Slp).

A B/K-specific antibody was produced and used to determine whether brain B/K proteins are soluble or membrane-associated, and the specificity of the anti-B/K antibody was checked by immunoblotting with recombinant proteins (mouse Syts I–XIII and B/K) expressed in COS-7 cells (results not shown). The anti-B/K antibody recognized a single band with an apparent M_r of approx. 50000 in selected regions of the brain, but not other tissues tested (Figure 2A). The B/K protein was preferentially expressed in rostral, phylogenetically younger brain regions (olfactory bulb, hippocampus and cerebrum), but very weakly in

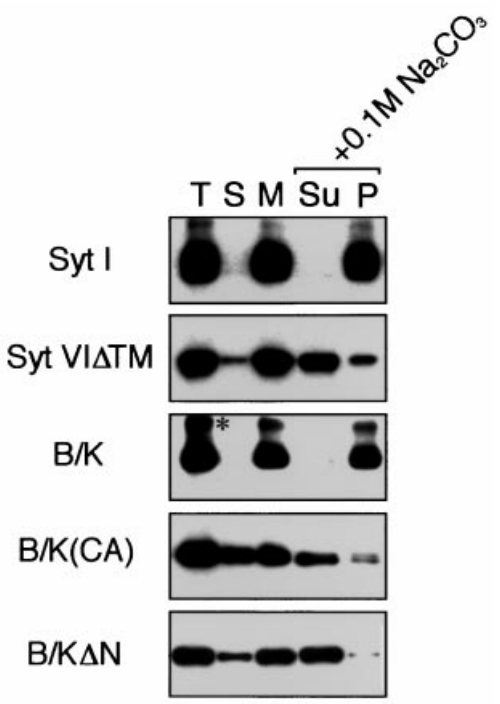

Figure 3 Subcellular fractionation of B/K protein expressed in COS-7 cells

Subcellular fractionation of COS-7 cells expressing pEF-T7-Syt I, pEF-T7-Syt VIΔTM, pEF-T7-B/K, pEF-T7-B/K(CA) or pEF-T7-B/KΔN (top to bottom panels, respectively). Membrane (M) and soluble (S) fractions were separated as described previously [18,25]. The membrane fraction was then resuspended in a buffer containing 0.1 M Na_2CO_3, pH 11, and incubated for 1 h at 4 °C. After centrifugation at 100000 ***g*** for 1 h, the supernatants (Su) and pellets (P) were recovered. Equal proportions of total tissue homogenates (T), the membrane fraction, the soluble fraction and the supernatants treated with 0.1 M Na_2CO_3, pH 11, were subjected to SDS/PAGE (10% gels) and transferred to a PVDF membrane. The blots were then immunoblotted with HRP-conjugated anti-(T7 tag) antibody. Note that the membrane association of B/K was insensitive to 0.1 M Na_2CO_3 treatment, as was the association of Syt I. By contrast, deletion of the N-terminal domain of B/K (B/KΔN) or Cys-to-Ala substitutions [B/K(CA)] resulted in dissociation from the membrane by 0.1 M Na_2CO_3, as observed for Syt VIΔTM. The asterisk indicates the post-translationally modified B/K protein at the cysteine cluster (see also Figure 5B).

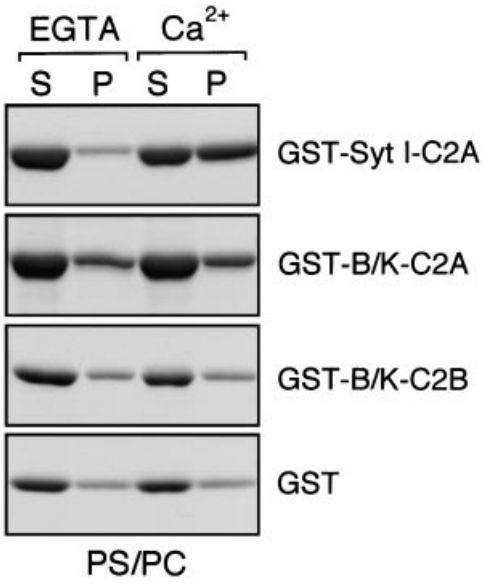

Figure 4 Phospholipid-binding properties of B/K C2 domains

Liposomes and GST fusion proteins were incubated in 50 mM Hepes/KOH, pH 7.2, in the presence of 2 mM EGTA or 1 mM Ca^{2+} for 15 min at room temperature. After centrifugation at 12000 ***g*** for 10 min, the supernatants (S, non-binding fraction) and pellets (P, phospholipid-binding fraction) were separated as described previously [33,34]. Equal proportions of the supernatants and pellets were subjected to SDS/PAGE (10% gels), and the gel was then stained with Coomassie Brilliant Blue R-250. Note that neither of the B/K C2 domains bound PS/PC liposomes, whereas the C2A domain of Syt I bound liposomes only in a Ca^{2+}-dependent manner. The bottom panel shows the negative control, GST.

caudal, phylogenetically older brain regions (cerebellum and brain stem) [35]. This expression pattern resembles that of the Syt I isoform, which is abundant in synaptic vesicles [36]. Although B/K mRNA was also detected in the kidney, it was impossible to detect B/K proteins under our experimental conditions. A discrepancy between the mRNA- and protein-expression profiles has often been found with regard to non-neuronal Syt isoforms ([37] and M. Fukuda, unpublished work), suggesting that B/K mRNAs are more preferentially translated in brain than in kidney. Expression of B/K protein was gradually increased until adulthood in parallel with synaptogenesis (Figure 2B).

The subcellular fractionation study indicated that brain B/K protein is tightly bound to the membrane fraction, the same as Syt I, an integral membrane protein of synaptic vesicles, although B/K protein itself has no transmembrane domain (Figure 2C) [20]. The membrane association of B/K protein is completely resistant to both 0.1 M Na_2CO_3, pH 11, and 1 M NaCl treatment (Figure 2C), which causes most peripheral membrane proteins to be released from the membrane, but it is sensitive to 1% Triton X-100 treatment (results not shown). In contrast, Syt VIΔTM proteins, which lack a transmembrane domain because of alternative splicing, were recovered in the soluble fraction and were highly sensitive to 0.1 M Na_2CO_3, pH 11, or 1 M NaCl treatment ([18], see also Figure 3).

The N-terminal cysteine cluster is essential for membrane localization of B/K protein

To elucidate the mechanism of the membrane association of B/K protein, T7-tagged B/K protein was expressed in COS-7 cells and their membrane association was examined by subcellular fractionation (Figure 3). Recombinant B/K protein was only recovered in the membrane fraction, the same as the native B/K protein and recombinant Syt I (Figure 3, top and middle panels). When the membrane fraction was treated with 0.1 M Na_2CO_3, the B/K protein was still attached to the membrane, the same as Syt I protein. In contrast, Syt VIΔTM proteins were easily released from the membrane fraction (Figure 3). To rule out the possibility that B/K protein is an integral membrane protein despite the absence of sufficiently long hydrophobic segments, we performed N-terminal antibody uptake experiments as described previously [25]. Cells expressing FLAG-tagged B/K protein, however, were unable to take up anti-FLAG antibody from the culture medium, whereas cells expressing FLAG-Syt XI or FLAG-Syt XIII took up the antibody efficiently (results not shown) [25]. Thus membrane localization of B/K protein is unlikely to be mediated by penetration of a hydrophobic segment into the membrane.

The phospholipid-binding capacity of the two C2 domains of B/K protein is also unlikely to be involved in this process, because neither C2 domain could bind PS/PC liposomes, irrespective of the presence of 1 mM Ca^{2+} (Figure 4). Consistent

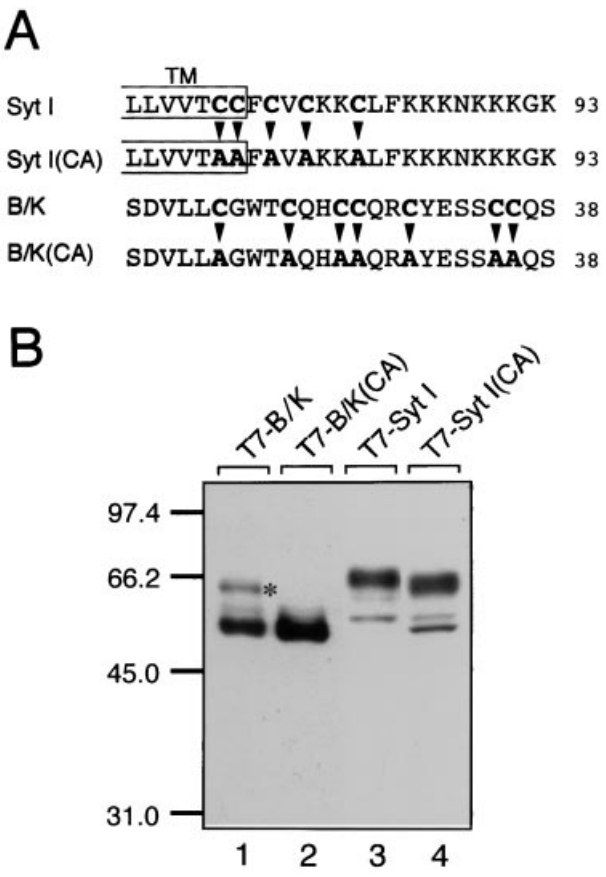

Figure 5 Comparison of the cysteine cluster in B/K and Syt I

(**A**) The alignment of the cysteine cluster (bold) in the N-terminal domain of B/K and Syt I. Arrowheads point to the Cys-to-Ala substitutions in B/K(CA) and Syt I(CA) mutants. Amino acid residue numbers are shown on the right. TM, transmembrane domain. (**B**) T7-B/K and T7-Syt I proteins were expressed in COS-7 and PC12 cells, respectively, and homogenized in 1% SDS with a 27-guage syringe. After addition of SDS sample buffer (+2% β-mercaptoethanol), the solubilized proteins were boiled for 3 min, subjected to SDS/PAGE (10% gels), and immunoblotted with HRP-conjugated anti-T7 tag antibody (1/10000 dilution). Note that the apparent M_r of the Syt I(CA) mutant (lane 4) was significantly lower than that of the wild-type protein (lane 3) due to the loss of fatty acylation [26]. By contrast, the apparent M_r of the B/K(CA) mutant (lane 2) was exactly the same as that of the major band of the wild-type protein, and the minor band of approx. 60 kDa in the wild-type protein was not visible in the mutant, suggesting that the cysteine cluster of at least some population of B/K proteins is post-translationally modified in COS-7 cells. The minor bands of around 50 kDa of Syt I (lanes 3 and 4) may represent degradation products [49].

with this, glutamate and aspartate residues involved in Ca^{2+}-binding have been mutated in the B/K C2 domains [2,20]. In contrast, the C2A domain of Syt I bound PS/PC liposomes only in the presence of Ca^{2+} (Figure 4, top panel), whereas the C2B domain of Syt I bound PS/PC liposomes irrespective of the presence of Ca^{2+} [23,38].

Next, we focused on the cysteine cluster (seven cysteine residues: Cys-19, Cys-23, Cys-26, Cys-27, Cys-30, Cys-35 and Cys-36) in the N-terminal domain (see Figures 1A and 5A). A similar cysteine cluster is located at the interface between the transmembrane and spacer domains in proteins of the Syt family, except Syt XII, and these cysteine residues are known to be fatty-acylated [39,40]. The fatty acylation is essential for stable SDS-resistant self-oligomerization [26,41]. Since fatty acylation of proteins is often involved in membrane association, we mutated these cysteine residues to alanine residues [B/K(CA) mutant] and expressed the mutant in COS-7 cells. The apparent M_r of mutant B/K(CA) protein was exactly the same as that of the major band of wild-type protein (Figure 5B, lanes 1 and 2), but a minor band of approx. 60 kDa was not found in the mutant protein (Figure 5B, lane 1, *), indicating that small proportions of B/K protein were post-translationally modified at the cysteine cluster. However, since we were unable to detect a 60 kDa immunoreactive band in brain, the post-translationally modified 60 kDa band in COS-7 cells may be an artifact resulting from forced over-expression or a unique event in non-neuronal cells. In contrast, the apparent M_r of Syt I(CA) mutant protein was lower than that of the wild-type protein because of the loss of fatty acylation (Figure 5B, lanes 3 and 4) [26]. Thus post-translational modification of the cysteine cluster of B/K protein is unlikely to be attributable to membrane localization. Nevertheless, the B/K(CA) or B/KΔN mutant proteins, lacking the N-terminal 57 amino acids, were also present in the soluble fraction and were easily released from the membrane fraction by 0.1 M Na_2CO_3 treatment (Figure 3, bottom two panels).

The N-terminal cysteine cluster is essential for specific membrane localization of B/K protein

Finally, we used immunocytochemistry to investigate whether the N-terminal cysteine cluster is involved in targeting B/K protein to specific membrane compartments. When the wild-type B/K protein was expressed in PC12 cells, it mainly localized in the perinuclear regions as well as in the form of small dots in the cytosol (Figure 6A). The perinuclear localization closely resembled that of Syt XI, which is mainly present in the Golgi [25], and closely overlapped that of TGN38, a marker for the TGN (Figure 6C) [31]. Since the exact Golgi or TGN localization was difficult to identify by immunocytochemistry alone, we combined it with exposure to drugs that are known to affect specific membrane compartments [30,31,42]. When cells were treated with wortmannin, which is known to alter the morphology of endosomes but not of the Golgi or TGN, B/K signals were unaffected and remained co-localized with TGN38 (results not shown). Nocodazol, which disrupts the microtubule-organizing centre, also had no effect on B/K localization (results not shown). When cells were treated with Brefeldin A, which causes redistribution of the Golgi markers to the endoplasmic reticulum and collapse of the TGN markers and early endosomal markers into the microtubule-organizing centre, B/K signals were still co-localized with TGN38 (Figure 6B), whereas Syt XI signals were distinct from the TGN38 signals (Figure 6D). These results strongly indicate that B/K protein is associated with TGN in PC12 cells. To our surprise, when expressed in PC12 cells B/K(CA) mutant protein was present throughout the cytosol (Figure 6E), indicating that the N-terminal cysteine cluster is essential for specific membrane (TGN) localization of B/K protein.

DISCUSSION

In the present study 5′-RACE and genomic analyses showed that B/K protein is a new subfamily of C-type tandem C2 proteins that is distinct from Syts, rabphilin, Doc2s and Slps [3–7] (Figure 1). B/K proteins contain the N-terminal cysteine cluster (consisting of seven cysteine residues) but lack a transmembrane domain and the known protein motifs. Like other C-type tandem C2 proteins [16,17], the N-terminal domain of B/K proteins seems to be important for expression of its function: the N-terminal cysteine cluster is essential for specific membrane (TGN)

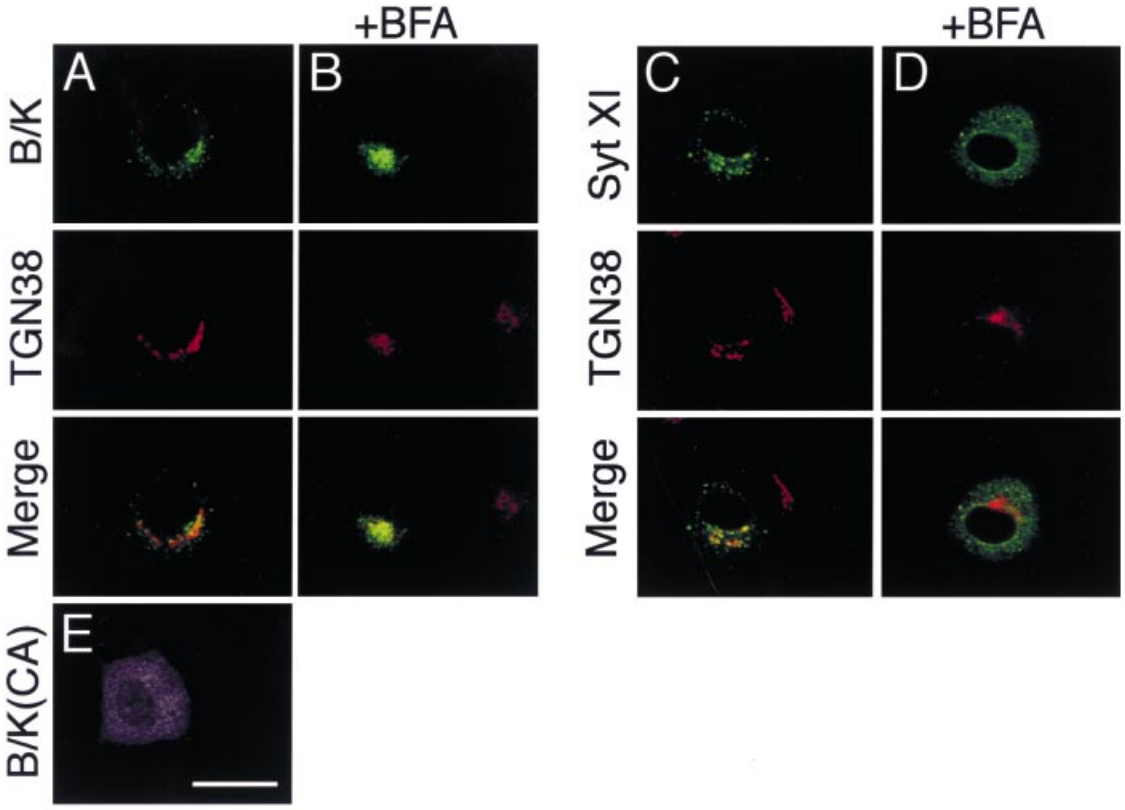

Figure 6 Subcellular localization of B/K and Syt XI in PC12 cells

PC12 cells expressing FLAG-B/K (**A**, **B**), FLAG-Syt XI (**C**, **D**) or FLAG-B/K(CA) (**E**) were fixed and permeabilized, and then stained with anti-FLAG (1/1000 dilution; green in the upper panels of **A**–**D** and in **E**) and anti-TGN38 antibodies (1/500 dilution; red in the middle panels of **A**–**D**). The bottom panels of (**A**)–(**D**) are superpositions of the top and middle panels. Note that both B/K and Syt XI were localized in the perinuclear region, which overlapped well with TGN38, but that their localizations were completely different after Brefeldin A treatment (+BFA; in **B** and **D**). Immunoreactivity was assessed with a fluorescence microscope (TE300; Nikon, Tokyo, Japan) attached to a laser confocal scanner unit CSU 10 (Yokogawa Electric Corp., Tokyo, Japan) and HiSCA CCD camera (C6790; Hamamatsu Photonics, Hamamatsu, Japan). Images were pseudo-coloured and superimposed with Adobe Photoshop software (version 4.0). The scale bar represents 20 μm.

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B/K          EPLNEGLLSRISDVLLCGWTCQHCC---QRCYESSCCQSSEDEVEILGPFFPAQTPPWLMASRSNDKDGDS  69
claudin-15   DPLYPGTKYELGPALYLGWSASLISILGGLCLCSACCCGSDEDPAASARRPYQAPVSVMPVATSDQEGDS 217

B/K          CCQRCYESSCCQSSEDEVEILGPFFPAQTPPWLMASRSNDKDGD  68
AL161576     CCSRCLQSYENDVAKVEKDLTGDNRSVLPQWLQNAKAND-DGD 207
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(*Arabidopsis thaliana* putative protein)

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B/K          SDVLLCGWTCQHCCQRCYESSCCQSSED-EVEILGPFFPAQTPPWLMASRSNDKDGDSVHTASDVP  72
DG kinase    TDPGLYGWRCA-WCQRCYHNDCYTRADSMEACDLGEFKDMIFPPYSFVAARTRDSMRLHLASITP 217
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(*Drosophila melanogaster* diacylglycerol kinase ε)

Figure 7 Three proteins that slightly resemble the cysteine cluster of B/K protein

The conserved cysteine residues are highlighted in bold. Amino acid residue numbers are given on the right.

localization in PC12 cells. Without this, B/K(CA) mutant protein cannot associate with TGN, and disperses in the cytosol (Figure 6E). A similar cysteine cluster was also found at the interface between the transmembrane and spacer domains of the Syt family proteins, except Syt XII [26], but the role of the cysteine cluster of Syts and B/K is completely different. The cysteine cluster of Syt proteins is fatty-acylated, which causes stable SDS-resistant homo- and hetero-oligomerization [26,41]. In contrast, most of the cysteine cluster of B/K protein is not post-translationally modified, and it is not involved in self-oligomerization (results not shown), but regulates B/K protein transport to TGN in PC12 cells. To our knowledge, B/K protein is the first C-type tandem C2 protein that is predominantly localized in the TGN of PC12 cells [4,18,25,30,43].

How does the cysteine cluster of B/K protein function in specific membrane targeting? We think that there are two possible answers. The first one is that post-translational modification (e.g. palmitoylation) of the N-terminal cysteine cluster allows B/K proteins to localize in membranes. However, this is unlikely because M_r shift was not observed even after Cys-to-Ala substitutions were performed (Figure 5B). Another, more likely, possibility is that the cysteine cluster functions as a protein-interaction site or a specific lipid-binding site, because cysteine residues play a crucial role in ligand binding in some protein motifs (e.g. the protein kinase C C1 and FYVE domains) [44–48]. Although the standard motif search has shown that the N-terminal domain of B/K protein does not contain any known protein motifs, we found three proteins in the PIR and SWISS-PROT protein databases that slightly resemble the cysteine cluster of B/K protein (see Figure 7).

One of them, *Drosophila* diacylglycerol kinase ϵ, is very interesting, because the similarity was observed in the putative C1 domain of diacylglycerol kinase, and four out of the seven cysteine residues are conserved between this protein and B/K protein. Thus it is tempting to speculate that the N-terminal domain of B/K protein contains an atypical type-C1 domain that may bind specific ligand. Alternatively, the N-terminal B/K protein may contain a novel cysteine-based protein motif. Further work is necessary to elucidate whether the N-terminal domain of B/K protein binds specific ligands. Potential ligands of this domain are being tested in our laboratory. Whether B/K protein is involved in vesicular transport from the TGN to specific membrane compartments is the next issue to be clarified.

In summary, we have shown that B/K protein is a subfamily of C-type tandem C2 proteins distinct from the Syt, Doc2 and Slp subfamilies. It has a unique N-terminal cysteine cluster that is responsible for specific membrane localization.

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