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Physical Association of KAB1 with Plant K^+ Channel α Subunits

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K^+ channel proteins contain four α subunits that align along a central axis perpendicular to membranes and form an ion-conducting pore. Recent work with K^+ channels native to animal membranes has shown that at least some members of this protein family also have four β subunits. These structural components of the holoenzyme each form tight associations with the cytoplasmic portion of an α subunit. We have cloned an Arabidopsis cDNA (KAB1) that encodes a polypeptide sharing 49% amino acid identity with animal K^+ channel β subunits. In this study, we provide experimental evidence that the KAB1 polypeptide forms a tight physical association with the Arabidopsis K^+ channel α subunit, KAT1. An affinity-purified KAB1 fusion protein was immobilized to a support resin and shown to sequester selectively the KAT1 polypeptide. In addition, polyclonal antibodies raised against KAB1 were shown to immunoprecipitate the KAT1 polypeptide as a KAT1–KAB1 protein complex. Immunoblot analysis demonstrated that KAB1 is expressed in Arabidopsis seedlings and is present in both membrane and soluble protein fractions. The presence of KAB1 (a soluble polypeptide) in both soluble and membrane protein fractions suggests that a portion of the total amount of native KAB1 is associated with an integral membrane protein, such as KAT1. The presence of KAB1 in crude protein fractions prepared from different Arabidopsis plant organs was evaluated. High levels of KAB1 protein were present in flowers, roots, and leaves. Immunoblot analysis of protein extracts prepared from broad bean leaves indicated that the KAB1 expression level was 80-fold greater in guard cells than in mesophyll cells. Previous studies of the in situ transcription pattern of KAT1 in Arabidopsis indicated that this α subunit is abundantly present in leaves and, within the leaf, exclusively present in guard cells. Thus, KAB1 was determined to be expressed in plant organs (leaves) and cell types (guard cells) that are sites of KAT1 expression in the plant. The in situ expression pattern of KAB1 suggests that it may associate with more than one type of K^+ channel α subunit. Sequence analysis indicates that KAB1 may function in plant K^+ channels as an oxidoreductase. It is postulated that β subunits native to animal K^+ channels act as regulatory subunits through pyridine nucleotide–linked reduction of α polypeptides. Although the KAB1 primary structure is substantially different from that of animal β subunits, amino acid motifs critical for this catalytic activity are retained in the plant β subunit.

INTRODUCTION

Recent work (Rehm and Lazdunski, 1988; Scott et al., 1994) has led to the first purification of a mammalian K^+ channel protein. Before this accomplishment, members of the superfamily of voltage-gated K^+ channel proteins were thought to be homotetramers composed of four pore-forming α subunits (Brown, 1993). Purification of the bovine brain cortex dendrotoxin receptor, with subsequent cloning of the cDNA encoding this polypeptide, identified it as the α subunit of the RCK5 K^+ channel (Reid et al., 1992). A low molecular mass (~40 kD) polypeptide copurified with the RCK5 pore-forming polypeptide and dissociated from the larger α subunit only when subjected to SDS-PAGE. Partial sequencing of this low molecular mass polypeptide led to the cloning of a corresponding cDNA (Scott et al., 1994). Subsequent work has identified this ~40-kD polypeptide (bovKvβ2) as one member of a protein family of K^+ channel β subunits in mammalian brain tissue sharing substantial sequence homology (Rettig et al., 1994).

Current models of voltage-gated K^+ channel structure (Parcej et al., 1992; Dolly et al., 1994; Jan and Jan, 1994) postulate that all or many members of this superfamily exist as heterooligomers composed of four pore-forming α subunits and four β subunits. Recent studies with some β subunits native to animal membranes suggest that these polypeptides may act as regulatory subunits of the holoenzyme. Coexpression of β subunit polypeptides with α subunits in Xenopus oocytes

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results in K^+ currents that have increased amplitude as well as altered voltage dependence, activation kinetics, or inactivation rates compared with expression of α subunits alone (Rettig et al., 1994; Chouinard et al., 1995; Majumder et al., 1995; McCormack et al., 1995; Morales et al., 1995). However, not all β subunits have been demonstrated to alter K^+ channel conductance parameters in the coexpression experiments (Rettig et al., 1994; Majumder et al., 1995; Morales et al., 1995). In some cases (Rettig et al., 1994; Scott et al., 1994), classification of a cDNA as encoding a K^+ channel β subunit has been based on sequence homology and/or demonstration of a physical association between a β polypeptide and a K^+ channel α subunit.

We have recently reported the sequence of an Arabidopsis cDNA (KAB1) that encodes a polypeptide sharing 49% amino acid identity and 70% similarity with the mammalian brain K^+ channel β subunit bovKvβ2 (Tang et al., 1995). In this article, we present evidence demonstrating a physical association of this putative plant K^+ channel β subunit with plant K^+ channel α subunits.

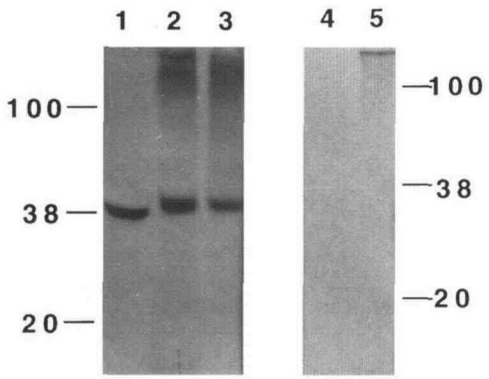

Figure 2. Immunological Identification of Native KAB1 Protein.

Shown is an immunoblot of the native Arabidopsis protein size fractionated on an SDS–polyacrylamide gel and probed with anti-KAB1 antiserum (lanes 1 to 3) or preimmune serum (lanes 4 and 5). Soluble protein (34 μg) was loaded in lanes 1 and 4. Membrane protein (146 μg) was loaded in lanes 2 and 5. Total protein (90 μg) was loaded in lane 3. Numbers at left and right indicate the migration of the molecular mass markers and are expressed in kilodaltons.

RESULTS

The Arabidopsis KAB1 cDNA encodes a polypeptide with a deduced molecular mass of 38.4 kD (Tang et al., 1995). The results shown in Figure 1 confirm that the KAB1 translation product is a 38.4-kD polypeptide. Protein in total cell lysate from *Escherichia coli* transfected with KAB1 in a PQE-30 expression vector (Hochuli et al., 1988) was size fractionated on SDS–polyacrylamide gels. Bacterial lysate was prepared from cells incubated in the absence or presence of isopropyl β-D-thiogalactopyranoside (IPTG) (Figure 1). The addition of IPTG to bacterial cultures resulted in the production of a 38.4-kD polypeptide corresponding to the overexpression of KAB1. The 200–amino acid C-terminal portion of KAB1 was overexpressed in *E. coli* in a fashion similar to that shown in Figure 1, affinity purified, and used to generate polyclonal anti-KAB1 antibodies.

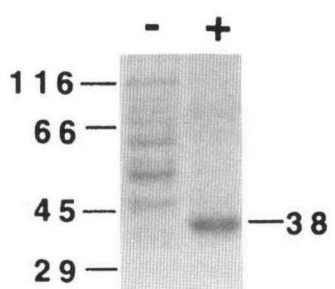

Figure 1. *Expression of the KAB1 Protein.*

Shown is a Coomassie Brilliant Blue R 250–stained SDS–polyacrylamide gel of total cell lysate from *E. coli* transfected with the recombinant PQE-30 plasmid containing the full-length KAB1 coding sequence. Cells were incubated in the absence (−) or presence (+) of IPTG. Equal amounts of bacterial culture were loaded in both lanes. Numbers at left indicate the migration of the molecular mass markers and are expressed in kilodaltons. The number at right highlights the KAB1 in vivo translation product.

Immunoblot analysis of Arabidopsis protein employing anti-KAB1 antiserum as a probe demonstrated the presence of the native KAB1 polypeptide in the plant (Figure 2). An ~38.4-kD polypeptide immunoreacted with the anti-KAB1 antiserum in preparations of total (lane 3), soluble (lane 1), and membrane (lane 2) protein, matching the molecular mass of the KAB1 cDNA translation product (Figure 1). This polypeptide was not immunoreactive with the preimmune serum (Figure 2, lanes 4 and 5). It is intriguing that the KAB1 protein appears in both membrane and soluble protein fractions. Hydropathy analysis of the deduced KAB1 sequence (Tang et al., 1995) indicated that it is hydrophilic, as is the case with β subunits native to mammals (Retting et al., 1994; Morales et al., 1995), whereas the pore-forming α subunit of K^+ channels is an integral membrane protein. The results shown in Figure 2 suggest that a substantial portion of native KAB1 protein is membrane bound. This finding offers indirect evidence consistent with the model that in situ, KAB1 is tightly associated with an intrinsic membrane protein, such as a K^+ channel α subunit. A portion of the β subunit protein native to mammalian brain remains tightly associated with K^+ channel α subunits until the protein complex is subjected to SDS-PAGE. When subjected to electrophoresis, the subunits resolve as separate polypeptide bands (Scott et al., 1994). Results presented in Figure 2 suggest that this may also be the case with plant β subunits. A substantial amount of KAB1 protein is also present in a soluble protein fraction prepared from Arabidopsis plants (Figure

2, lane 2). This finding suggests that some KAB1 may be present in situ either as a soluble protein or as a peripheral membrane protein (i.e., noncovalently associated with an integral membrane protein) that during purification is retained in the soluble protein fraction.

In a fashion similar to the evolving model of multisubunit K^+ channels, currents through Ca^{2+} channel pore-forming subunits have recently been shown to be modulated by a regulatory β subunit that is an integral component of the Ca^{2+} channel holoenzyme (Ruth et al., 1989; Pragnell et al., 1994). Expression of Ca^{2+} channel subunits as glutathione *S*-transferase (GST) fusion proteins led to the characterization of a physical interaction between the Ca^{2+} channel β and α subunits (Pragnell et al., 1994). GST fusion proteins can be affinity purified under mild conditions in a one-step procedure using glutathione Sepharose 4B (Frangioni and Neel, 1993). We followed a similar experimental strategy with KAB1, which we overexpressed as a GST fusion protein in *E. coli* and affinity purified from the total cell lysate.

The purified GST–KAB1 fusion protein was used in protein–protein interaction studies to demonstrate a physical association of KAB1 with a plant K^+ channel α subunit. For these studies, the GST protein alone was prepared by a method similar to that used for the fusion protein and served as a control treatment. The GST–KAB1 fusion protein or GST protein alone was immobilized on glutathione Sepharose 4B support resin. The plant K^+ channel α subunit KAT1 was translated and ^{35}S-labeled in vitro using a rabbit reticulocyte system. The ^{35}S-labeled KAT1 protein was incubated with purified, immobilized GST or GST–KAB1. Results of these binding experiments are shown in Figure 3. SDS-PAGE of the in vitro–translated KAT1 protein shows multiple bands in Figure 3 (lane 1). The highest molecular mass band appearing in lane 1 of Figure 3 is at ~78 kD, which corresponds to the molecular mass of the full-length sequence of the KAT1 polypeptide (Anderson et al., 1992). Bands appearing at lower molecular masses in lane 1 of Figure 3 likely are truncated translation products resulting from premature ribosome termination or alternative in vitro initiation sites (Tymms and McInnes, 1988). Autoradiography indicated that KAT1 bound to the GST–KAB1 fusion protein (Figure 3, lane 3). The control (GST protein) showed no sequestration of KAT1 (lane 2). These results demonstrate a physical association between KAT1 and KAB1.

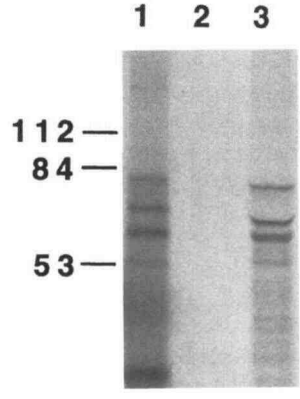

Figure 3. In Vitro Binding Assay of KAB1 and Radiolabeled KAT1.

Autoradiography of an in vitro–translated ^{35}S-labeled KAT1 protein subjected to SDS-PAGE is shown. Lane 1 contains an aliquot (5 μL) of the total translation product from the rabbit reticulocyte system used to generate KAT1. Aliquots (5 μL) of the in vitro–translated KAT1 protein were incubated with either the GST protein (lane 2) or GST–KAB1 fusion protein (lane 3) immobilized on glutathione Sepharose 4B before purification of the resin. The resin-associated protein was subjected to SDS-PAGE. Numbers at left indicate the migration of the molecular mass markers and are expressed in kilodaltons.

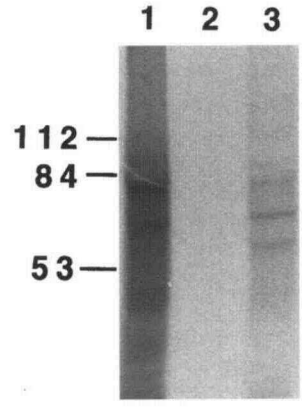

Figure 4. Immunoprecipitation of KAT1–KAB1 Protein Complex with the Anti-KAB1 Antibody.

Autoradiography of the in vitro–translated ^{35}S-labeled KAT1 protein subjected to SDS-PAGE is shown. An aliquot (5 μL) of the total translation product from the rabbit reticulocyte system used to generate KAT1 is shown in lane 1. Aliquots (5 μL) of the in vitro–translated KAT1 protein were incubated with total lysate of bacteria expressing either the GST protein (lane 2) or GST–KAB1 fusion protein (lane 3) before immunoprecipitation with the anti-KAB1 antiserum. KAT1–KAB1 protein complexes were reduced when diluted into sample buffer before size fractionation on an SDS–polyacrylamide gel. Therefore, in vitro–translated KAT1 should resolve on the gel as a separate band (in lane 3), even though it was immunoprecipitated as a KAT1–KAB1 protein complex. Numbers at left indicate the migration of the molecular mass markers and are expressed in kilodaltons.

Physical association of KAB1 with KAT1 was also evaluated using a second approach. ^{35}S-labeled KAT1 was incubated with total lysate from bacteria expressing either the GST–KAB1 fusion protein or GST alone (as a control). The protein complexes were then immunoprecipitated with the anti-KAB1 antibody. Autoradiography of the immunoprecipitated protein (Figure 4) again demonstrated a physical association between KAT1 and KAB1 (lane 3) that was not evident in the control (lane 2). Presumably, the anti-KAB1 antibody immunoprecipitated KAT1 because the K^+ channel α subunit formed a protein complex with KAB1.

It is evident from a comparison of results presented in Figures 3 and 4 that much more KAT1 translation product is sequestered by KAB1–resin complexes than as an immunoprecipitate. There

is less contrast between the intensity of the bands in lanes 1 and 3 of Figure 3 than in the corresponding lanes of Figure 4. This difference may be due to an excess of glutathione Sepharose 4B in the reaction mixture (relative to KAT1–KAB1 protein complexes), which is not matched by the titer of the anti-KAB1 antibody in the polyclonal antisera used for immunoprecipitation. When KAT1–KAB1 complexes are sequestered by the Sepharose beads, virtually all of the protein complexes are likely to be bound to the resin beads, which are provided in excess. In the immunoprecipitation experiment (Figure 4), the percentage of KAT1–KAB1 complexes sequestered would be a function of the antibody titer; not all protein complexes would necessarily be bound by the specific antibody in the antiserum.

The apparent association between KAB1 and KAT1 demonstrated in the experiments shown in Figures 3 and 4 might be explained by the ability of KAB1 to bind (nonspecifically) to any (hydrophobic) membrane protein. Control experiments were undertaken to address this possibility. The association of KAB1 with an H^+-ATPase channel-forming polypeptide was studied. Native membrane preparations expressing high levels of the (bacterial) proton pump bacteriorhodopsin (Krebs and Khorana, 1993) were incubated with a resin-immobilized GST protein or KAB1–GST fusion protein. Protein associated with the immobilized GST or KAB1–GST fusion protein was run on SDS–polyacrylamide gels. The presence of bacteriorhodopsin in the pool of sequestered protein was evaluated by conducting immunoblot analysis of the gels with an antibacteriorhodopsin antibody as a probe. Results (not shown) indicated that bacteriorhodopsin was not sequestered by either GST or GST-KAB1 protein. The protein–protein interactions between KAB1 and KAT1 (Figures 3 and 4) were not reproduced by nonspecific binding of KAB1 to a hydrophobic membrane protein that is not a K^+ channel subunit. The results presented in this report were obtained with use of two different methodologies and demonstrate that the KAB1 protein binds specifically to a plant K^+ channel pore-forming subunit in vitro.

Indirect evidence (Figure 2) has also been presented suggesting that KAB1 tightly binds to a native membrane protein in situ. This protein is possibly a K^+ channel α subunit. Further work was undertaken to examine the expression pattern of KAB1 in situ and to evaluate the putative interaction between KAB1 and KAT1. The presence of KAB1 in (total) crude protein fractions prepared from different Arabidopsis plant organs was checked by immunoblot analysis with anti-KAB1 antisera (Figure 5). High levels of a 38.4-kD immunoreactive polypeptide were detected in flowers (lane 1), leaves (lane 2), and roots (lane 3). Previous studies (Kochian et al., 1993) have indicated that KAT1 is primarily expressed in leaves rather than roots of Arabidopsis plants. Perhaps the KAB1 expressed in Arabidopsis roots is associated with a different K^+ channel α subunit. The α subunit AKT1 is selectively expressed in Arabidopsis roots (Kochian et al., 1993). Nakamura et al. (1995) have further characterized the transcription pattern of KAT1 as occurring exclusively in guard cells of leaves.

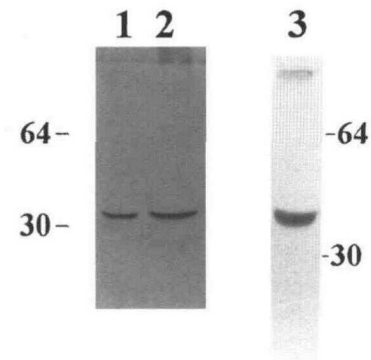

Figure 5. Expression of Native KAB1 in Different Arabidopsis Organs.

Protein isolated from organs of 6-week-old Arabidopsis plants was size fractionated on an SDS–polyacrylamide gel and subjected to immunoblot analysis by using anti-KAB1 antisera as a probe. Protein isolated from leaves (55 μg) and flowers (60 μg) was loaded in lanes 1 and 2, respectively. Results shown in lane 3 are from a different experiment: protein (50 μg) isolated from roots was loaded. Numbers at left and right indicate the migration of the molecular mass markers and are expressed in kilodaltons.

We attempted to examine the relative expression level of KAB1 in leaf mesophyll and guard cells, as shown in Figure 6. Total protein extracts were prepared from purified mesophyll and guard cell protoplasts of broad bean (Kruse et al., 1989; Li and Assmann, 1993). Immunodetection of the presence of KAB1 in broad bean mesophyll and guard cells was undertaken after SDS-PAGE by using anti-KAB1 antisera. A 38.4-kD polypeptide from broad bean demonstrated strong immunoreactivity with the antibody generated against the Arabidopsis-derived KAB1 protein (Figure 6A). We believe that this 38.4-kD polypeptide is a broad bean homolog of the 38.4-kD KAB1 protein from Arabidopsis. A comparison of the 38.4-kD bands immunoreactive with anti-KAB1 antibody in lanes 1 and 2 of Figure 6A indicates that the broad bean KAB1 homolog is expressed to a much greater extent in guard cells than in mesophyll cells. An attempt was made to quantify the relative abundance of the KAB1 homolog in mesophyll and guard cells. Densitometer scans of the 38.4-kD bands in lanes 1 and 2 of Figure 6A revealed relative optical densities of 0.5 and 4.5, respectively, for mesophyll and guard cell protein. Accounting for the amounts of protein loaded in lanes 1 and 2 of Figure 6A, the densitometer scans indicate that the relative abundance of the KAB1 homolog in guards cells is 80-fold greater than the level found in mesophyll cells.

Further studies suggest that this finding of an 80-fold higher expression level of KAB1 homolog in guard cells compared with mesophyll cells is an underestimate. In a subsequent experiment, immunoblot analysis of a gel that had varying levels of guard cell protein loaded in each lane (Figure 6A, lanes 3 to 5) was performed. Densitometer scans of the immunoreactive 38.4-kD bands in lanes 3 to 5 of Figure 6A revealed the

protein–optical density relationship depicted in Figure 6B. The analysis presented in Figure 6B indicates that at the level of protein loaded in lane 2 of Figure 6A, the protein–optical density relationship was clearly past the point of linearity. This analysis therefore indicates that the optical density of the 38.4-kD band in lane 2 of Figure 6A would yield an underestimate of the amount of KAB1 homolog present in the guard cell protein extract.

We conclude from the data presented in Figure 5 that KAB1 is expressed in leaves and from the data in Figure 6 that KAB1 (or its homolog) is present at much higher levels in guard cells than in mesophyll cells of leaves. Thus, we determined that KAB1 is expressed in plant organs (leaves) and cell types (guard cells) that are sites of KAT1 expression. This intriguing finding suggests an in vivo relationship between these two K^+ channel polypeptides; that is, they may comprise pore-forming and regulatory subunits of the K^+ channel holoenzyme in vivo.

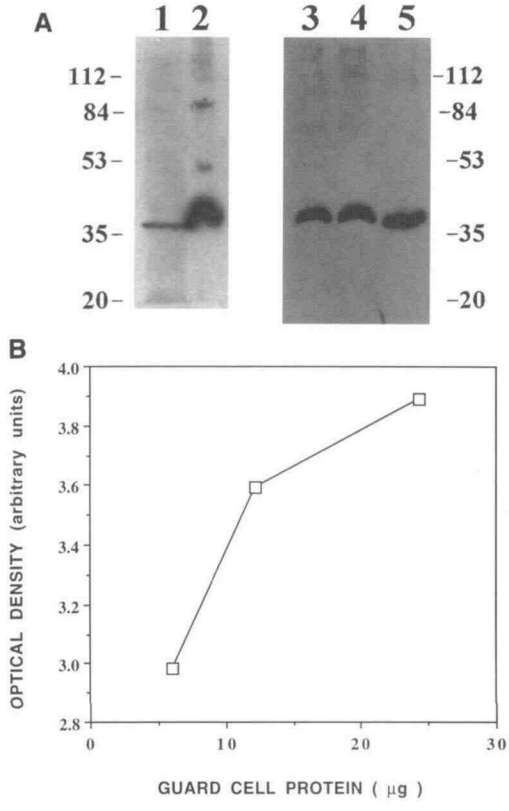

Figure 6. Analysis of the Relative Expression Level of a KAB1 Homolog in Mesophyll Cells and Guard Cells of Broad Bean Leaves.

(A) Immunoblot analysis of total protein in mesophyll cell (lane 1) and guard cell (lane 2) protoplasts. For guard cell protoplasts (lane 2), 60 μg of protein was loaded. Due to the faintness of the 38.4-kD immunoreactive band in SDS–polyacrylamide gels of mesophyll cell protein, 535 μg of protein from mesophyll cell protoplasts was loaded in this experiment (lane 1). Results from an additional experiment are shown in lanes 3 to 5. In this case, 6 (lane 3), 12 (lane 4), or 24 (lane 5) μg of protein from guard cell protoplasts was subjected to SDS-PAGE before immunoblot analysis with anti-KAB1 antisera.

(B) Densitometer scanning analysis of the 38.4-kD immunoreactive band in lanes 3 to 5 was undertaken whereby a line relating the intensity of the 38.4-kD band with the amount of guard cell protein was generated.

DISCUSSION

Purification of the first native K^+ channel protein led to the demonstration of a tight physical association between the pore-forming α subunit and an ~40-kD β subunit (Rehm and Lazdunski, 1988; Scott et al., 1994). Since the cloning of the cDNA encoding that K^+ channel β subunit, several other (animal) β subunits have been cloned (Rettig et al., 1994; Chouinard et al., 1995; Majumder, 1995; McCormack et al., 1995; Morales et al., 1995). However, the studies reported here represent a direct demonstration of a physical association between a newly identified, putative K^+ channel β subunit and a pore-forming α subunit. Based both on sequence homology of KAB1 to β subunits native to animal membranes (Tang et al., 1995) and on the demonstration in this report of a physical association between KAB1 and KAT1, we can conclude that at least some plant K^+ channel proteins have two distinct types of subunits. Of course, the assertion that plant K^+ channels contain β subunits leads to the obvious question of what physiological role KAB1 plays in K^+ channel function in vivo.

Evaluation of the presumably regulatory role KAB1 plays in plant K^+ channel function can be addressed most appropriately by coexpression of KAB1 with plant K^+ channel α subunits (KAT1) in oocytes followed by patch clamp analysis of the expressed K^+ currents. However, not all animal β subunits cloned to date have been successfully shown to alter α subunit current parameters when coexpressed in oocytes (Rettig et al., 1994; Morales et al., 1995). Future work with KAB1 should include attempts at coexpression with KAT1. However, comparison of the N-terminal portion of the KAB1 sequence with other β subunits highlights the likelihood that much more work is required to develop a clear understanding of the structure/function aspects of K^+ channel α and β subunit interaction.

The animal β subunit bovKvβ2 was identified as a component of K^+ channels because it copurified with the α subunit RCK5 (Scott et al., 1994). Cloning of the cDNA encoding bovKvβ2 from a bovine cerebral cortex expression library led to the identification of two bovKvβ2 homologs from rat brain, ratKvβ1 and ratKvβ2 (Rettig et al., 1994). Both ratKvβ2 and bovKvβ2 are 367 amino acids in length and share 99% amino acid identity. RatKvβ1 is a longer polypeptide; it has an N-terminal extension. The first N-terminal 72 amino acids of ratKvβ1 do not align with either ratKvβ2 or bovKvβ2, but the rest of ratKvβ1 is 85% identical to ratKvβ2 and bovKβd2 (Rettig et al., 1994).

When coexpressed, the β2 proteins did not alter α subunit function, whereas ratKvβ1 modulated α subunit currents (Rettig et al., 1994). Only ratKvβ1, with the N-terminal extension, modulated the activity of the K^+ channel α subunits RCK1 and RCK4. Rettig et al. (1994) speculated that the N-terminal extension of ratKvβ1 formed the "ball" of a ball-and-chain–type inactivation gate of the K^+ channel protein. They also provided some evidence suggesting that a cysteine residue in the N-terminal extension of ratKvβ1 was involved in both the physical interaction of ratKvβ1 with α subunits and the regulatory role of the β subunit in K^+ channel currents. Recent work has identified the sequences of three other β subunits: those from humans (hKvβ3; Majumder et al., 1995), ferret (ferKvβ3; Morales et al., 1995), and Drosophila (Hk; Chouinard et al., 1995). These three recently cloned β subunits (i.e., hKvβ3, ferKvβ3, and Hk) all have N-terminal extensions even longer than that of ratKvβ1, and in all cases the N termini contain cysteine residues. KAB1, on the other hand, has an N-terminal region even shorter than that of ratKvβ2 and bovKvβ2, and it contains no N-terminal cysteine residue (Tang et al., 1995). Thus, the demonstration in this report of a physical interaction between KAB1 and the plant K^+ channel α subunit KAT1 suggests that neither the N-terminal extension found in most β subunits cloned thus far nor the presence of an N-terminal cysteine residue is necessarily required for the binding of β subunits to α subunits.

Some insight into KAB1 function in plants may be discerned from the apparent relationship between β subunit sequences and the aldo-keto reductase superfamily of proteins. Chouinard et al. (1995) and McCormack and McCormack (1994) have noted that K^+ channel β subunits share some sequence homology with members of a superfamily of enzymes that utilize reduced pyridine nucleotide as a cofactor and catalyze a broad range (i.e., with regard to substrate) of oxidoreductase reactions. Members of this enzyme superfamily are functionally diverse; nonetheless, they demonstrate substantial conservation of primary sequence related to the presence of secondary structural elements in these proteins. Specifically, amino acid motifs corresponding to the β sheets present in the α/β barrels formed as part of the tertiary structure of aldo-keto reductases are conserved (Chouinard et al., 1995).

Portions of some aldo-keto reductase sequences corresponding to sections of the polypeptides either at or near regions forming β sheet structural elements are shown in Figure 7. Corresponding regions of the animal K^+ channel β subunits ratKvβ1 and bovKvβ2 are aligned with these regions of the aldo-keto reductases. As pointed out previously (McCormack and McCormack, 1994), the alignment of aldo-keto reductases and K^+ channel β subunits shows a relatively high degree of conservation in these portions of the proteins (Figure 7). We note with interest that KAB1 shares substantial amino acid identity with ratKvβ1 and bovKvβ2 in regions in which the β subunit sequences have homology to the reductase sequences that form the β sheets. Alignment of the β subunit sequences with the regions of the reductases within or near regions forming β sheets indicates that 58 amino acids are found in most of the reductases that are also present in ratKvβ1 and bovKvβ2. (Only 39 of these 58 amino acids are in sections of the proteins shown in Figure 7.) Of these 58 amino acids, 44 are also conserved in KAB1 (i.e., 76% identity). Thus, KAB1 shows relatively high sequence homology with animal β subunits in regions of these proteins that show conservation with structural elements of aldo-keto reductases. Overall, the homology of KAB1 to bovKvβ2 is only 49% (Tang et al., 1995). Another structural feature of the aldo-keto reductases that is conserved in K^+ channel β subunits is the tyrosine residue, which confers catalytic activity on the reductases (McCormack and McCormack, 1994). This tyrosine residue is present in ratKvβ1, bovKvβ2, and KAB1 (at y-54), as shown in Figure 7. Sequence conservation is also maintained in aldo-keto reductases with regard to amino acid residues involved in pyridine nucleotide binding (Chouinard et al., 1995). Of the 15 residues fairly conserved among the reductases that are involved in binding this cofactor, 12 (i.e., 80%) appear in the KAB1 sequence (Figure 7). Thus, the analysis presented in Figure 7 suggests that KAB1 effects on K^+ channel α subunit function may be mediated by an oxidoreductase activity, with NAD(P)H binding to KAB1 and serving as the reductant. Of course, this analysis of structural motifs in the KAB1 sequence underlying the hypothesis that KAB1 function may be related to a reductase activity is highly speculative and should be viewed with caution. However, no experimental evidence has yet been generated that can provide a basis for the elucidation of the function of KAB1 in native plant K^+ channel proteins.

We conclude from the results presented in this study that KAB1 is a structural component of at least some plant K^+ channels. KAB1 is the first member of the K^+ channel β subunit protein family to be identified in plants. Members of this protein family have been shown to modulate K^+ channel function in mammals. We speculate, based on amino acid sequence comparisons, that KAB1 may act to alter the redox state of residues on α subunits (using the pyridine nucleotide as a cofactor) in carrying out regulatory functions.

METHODS

Protein Expression

The KAB1 protein was generated using several in vivo bacterial expression systems. The KAB1 coding sequence was generated using the polymerase chain reaction with oligonucleotides corresponding to the 3′ and 5′ ends of KAB1 as primers and an *Arabidopsis thaliana* Landsberg *erecta* cDNA library cloned into λZAPII (Tang et al., 1995) as template DNA. The KAB1 DNA was subcloned into either a PQE-30 (Qiagen, Chatsworth, CA) or pGEX (Pharmacia) plasmid. For the Qiagen expression system, the KAB1 coding sequence was fused in frame downstream of the six-histidine tag in the PQE-30 plasmid. This recombinant construct was transformed into the M15 host strain of *Escherichia coli* carrying the pREP4 repressor plasmid. After overnight culture in the presence of 200 μg/mL ampicillin and 25 μg/mL kanamycin, protein expression was induced by incubation in 2 mM isopropyl

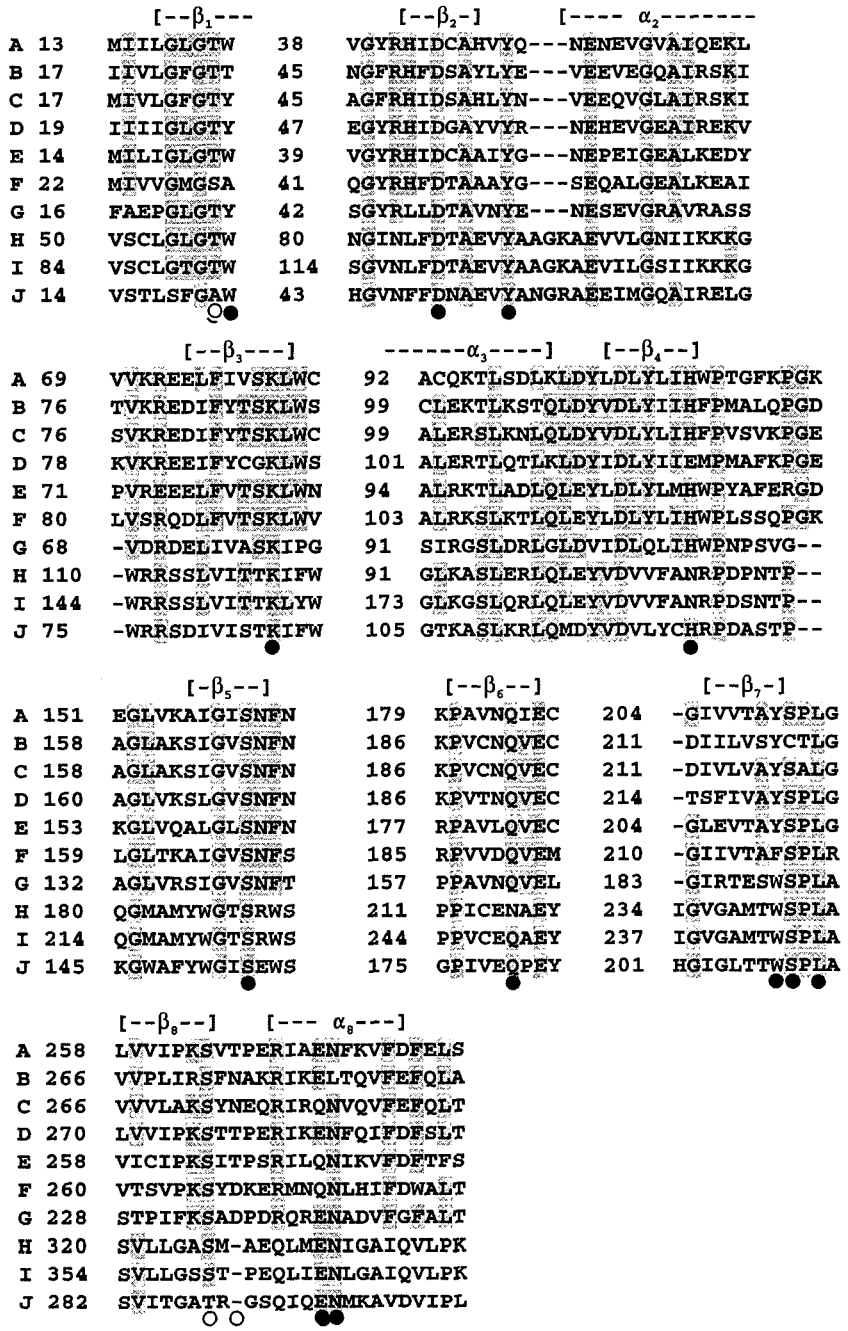

Figure 7. Regions of Sequence Homology Shared by Members of the Aldo-Keto Reductase Protein Superfamily and Selected K^+ Channel β Subunit Polypeptides.

Brackets above the sequences correspond to structural elements (α and β strands) of the β barrel secondary structure found in aldo-keto reductases, as delineated by McCormack and McCormack (1994). Only portions of the sequences within or near these structural elements are shown. Conserved regions of the aldo-keto reductases and K^+ channel β subunits (as identified by McCormack and McCormack, 1994) are shaded. Sequence information for the following aldo-keto reductases are shown. A is the human aldose reductase (SwissProt accession number P15121). B is the rat 3-α-hydroxysteroid reductase (SwissProt accession number P23457). C is human *trans*-1,2-dihydrobenzene-1,2-diol dehydrogenase (SwissProt accession number Q04828). D is rat 3-oxo-5-β-steroid 4-dehydrogenase (SwissProt accession number P31210). E is human aldehyde dehydrogenase (SwissProt accession number P14550). F is soybean NAD(P)H-dependent 6′-deoxychalcone synthase (SwissProt accession number P26690). G is *Corynebacterium* 2,5-diketo-D-gluconic acid reductase (SwissProt accession number P15339). The sequences identified as H, I, and J are bovKvβ2, ratKvβ1, and KAB1, respectively. For each polypeptide (A to J), numbers correspond to the position of the amino acid directly following. Circles denote amino acid residues of aldo-keto reductases that Chouinard et al. (1995) identified as being involved in pyridine nucleotide binding. Closed circles indicate that the pyridine nucleotide binding residue is conserved in the KAB1 sequence. Open circles indicate that the residue is not conserved in the KAB1 sequence.

β-D-thiogalactopyranoside (IPTG) for 3 hr. The pGEX2T plasmid was used to generate KAB1 as a glutathione *S*-transferase (GST) fusion protein. The recombinant pGEX-KAB1 construct was transformed into strain DH5α of *E. coli*. After overnight incubation, the bacterial culture was diluted 10-fold. After additional growth for 1 hr, the culture was brought to 1 mM IPTG, and the cells were cultured for an additional 3 hr. Total protein was solubilized in a solution containing 2% (v/v) sarkosyl and Triton X-100, 10 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM Na_2-EDTA.

The KAT1 protein was generated by using an in vitro translation system. KAT1 subcloned into the pSP64 poly(A) plasmid was provided by L. Kochian (Cornell University, Ithaca, NY). The TNT rabbit reticulocyte system (Promega) was used with 1 μg of circular plasmid DNA as template for in vitro transcription/translation. The SP6 promoter of the plasmid was used to initiate transcription, and the reaction mixture was incubated for 90 min at 30°C in the presence of 10 mCi/mL ^{35}S-methionine (1 Ci/μmol).

Antibody Production

A polyclonal antibody against the C-terminal 200 amino acids of the KAB1 polypeptide was raised in New Zealand white rabbits. Constructs for KAB1 protein production were prepared by subjecting KAB1 DNA to HindIII restriction digestion, yielding an ~1-kb 3′ terminus fragment of the sequence. This 1-kb KAB1 fragment was subcloned into the PQE-32 plasmid and used to generate a polypeptide in a fashion similar to that already described for the full-length KAB1 coding sequence. The KAB1 polypeptide fragment was affinity purified from the total cell lysate as follows. Harvested cells were incubated for 1 hr in lysate buffer (6 M guanidium-HCl, 100 mM NaH_2PO_4, and 10 mM Tris-HCl, pH 8.0) and then pelleted (10,000*g* for 15 min). Ni-nitrilo-tri-acetic acid resin (Qiagen) was equilibrated in lysate buffer, and 8 mL of a 50% (v/v) resin slurry was added to the supernatant. After incubation for 45 min, the resin was loaded onto a 1.6-cm-diameter column and washed with 10 column volumes of lysate buffer, followed by five column volumes of column buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0). The resin was then washed with column buffer at pH 6.3 until the A_{280} of the eluent was <0.01. The purified protein was eluted with 20 mL of 6 M guanidium-HCl and 0.2 M acetic acid, lyophilized, mixed with Freund's complete adjuvant, and used for immunizations.

Protein–Protein Interactions

For evaluation of the KAB1 and KAT1 interaction using the GST fusion protein system (Pharmacia) as an in vitro binding assay, solubilized (with sarkosyl and Triton X-100; see above) GST–KAB1 fusion protein or GST protein alone was incubated with glutathione Sepharose 4B resin (Pharmacia) and then washed six times with PBS buffer (138 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The affinity-purified and immobilized protein (GST or GST-KAB1) was resuspended and stored in storage buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM DTT, and 10% [v/v] glycerol). GST protein (control) or GST-KAB1 fusion protein (25 μg of purified protein) was diluted into 200 μL of binding buffer (20 mM Hepes-KOH, pH 7.9, 50 mM KC1, 2.5 mM $MgC1_2 \cdot 6H_2O$, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 10% [v/v] glycerol). Next, 3 μL of rabbit serum was added as a background protein source (Bengal et al., 1992), followed by the addition of 5 μL of in vitro–translated, ^{35}S-labeled KAT1 solubilized in 1% (v/v) Triton X-100. This mixture was then incubated overnight at 4°C with gentle shaking.

In the case of immunoprecipitation experiments, total bacterial lysate equivalent to 35 μg of protein from cells expressing either GST (control) or GST-KAB1 was incubated with ^{35}S-KAT1 under conditions described above for the GST fusion protein system. The protein mixtures were precleared by the addition of 30 μL of protein A–Sepharose 4B resin (Sigma) at 4°C for 1 hr. The resin beads were then pelleted, and the supernatant was brought to 500 μL with binding buffer. The anti-KAB1 antiserum (5 μL) was added, and the mixture was incubated overnight at 4°C with gentle shaking. Protein A–Sepharose 4B (30 μL) was then added, and the protein mixture was incubated for an additional 2 hr at 4°C.

The resin beads from both the in vitro binding and immunoprecipitation experiments were pelleted and washed five times with RIPA buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM Na_2EDTA, 0.2% [v/v] Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride). Both in vitro binding and immune complexes were reduced by boiling in SDS-PAGE sample buffer, and proteins were size fractionated on 10% SDS–polyacrylamide gels followed by autoradiography for 24 to 48 hr (Kodak X-OMAT-AR x-ray film).

Immunoblot Analysis

Protein was isolated from flowering 6-week-old Arabidopsis (ecotype Columbia) plants (i.e., either roots, flowers, stems, and leaves harvested separately or pooled together) by homogenization of 10 g of plant material in 20 mL of extraction buffer (PBS buffer, pH 7.4, with 2 mM Na_2 EDTA, 10 mM DTT, and 15 mM β-mercaptoethanol). After centrifugation (30,000*g* for 10 min [Sorvall SS34 rotor and RC5C centrifuge; Du Pont, Wilmington, DE]), the supernatant was used as soluble protein. Membrane protein was isolated from the same plants by using the protocol of Briskin (1986). Plant material (20 g) was homogenized in 40 mL of 250 mM sucrose, 70 mM Tris-HCl, pH 8.0, 3 mM Na_2EDTA, 15 mM β-mercaptoethanol, 5 mM DTT, and 0.5% (w/v) PVP 40,000. The homogenate was centrifuged at 13,000*g* for 15 min, and the pellet was discarded. The supernatant was centrifuged again at 80,000*g* for 30 min (SW40 rotor and L8-70M ultracentrifuge; Beckman, Fullerton, CA). The pellet was resuspended in a minimal volume of 250 mM sucrose, 1 mM Tris-Mes, pH 7.2, 5 mM DTT and used as a membrane protein fraction.

Native Arabidopsis protein was size fractionated on 10% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane in 20% methanol (v/v), 0.1% SDS (w/v), 192 mM glycine, 25 mM Tris-HCl, pH 8.3, on a transfer unit (Hoefer, San Francisco, CA). Nonspecific antibody binding was blocked by incubation of membrane in 5% (w/v) nonfat dry milk in TBS buffer (10 mM Tris-HCl, pH 7.4, and 155 mM NaCl). Anti-KAB1 rabbit antiserum (1:3000 dilution) was added, and the membrane was incubated overnight at 4°C with gentle shaking; this step was followed by washing three times in TTBS buffer (TBS with 0.05% [v/v] Tween 20). Immunoreactive bands were visualized with horseradish peroxidase secondary antibody (goat anti–rabbit IgG) conjugate (Pierce, Rockford, IL) and enhanced chemiluminescence detection (Amersham).

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