Vol. 191, No. 1, 1993
February 26, 1993
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 201-206

IMMUNOLOGICAL IDENTIFICATION OF THE *Shaker*-RELATED Kv1.3 POTASSIUM CHANNEL PROTEIN IN T and B LYMPHOCYTES, AND DETECTION OF RELATED PROTEINS IN FLIES AND YEAST

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Received December 30, 1992

SUMMARY: *Shaker*-related potassium (K^+) channel proteins contain sequences which exhibit remarkable conservation across species. We have generated polyclonal anti-peptide antibodies (Abs) which cross-react with peptide epitopes of several *Shaker*-related channels (Kv1.1, 1.2 and 1.3), in addition to a Kv1.3-specific Ab. The Kv1.3-*specific* Abs react with a protein expressed in human T-cells (Jurkat and PBLs), as well as in mouse T-cells (EL-4) and pre-B cells (230.37). The *cross-reactive* Abs detect the *Shaker* protein in *Drosophila melanogaster*, in addition to an immunologically related protein in the yeast *Saccharomyces cerevisiae*. Abs which recognize these shared epitopes could serve for the identification and biochemical characterization of *Shaker*-related proteins in diverse organisms.

Molecular cloning of mammalian voltage-dependent K^+ channels has revealed the existence of a large gene family comprising at least 16 members (see 1). Lymphocytes can express some of these channels, the predominant one being the type *n* channel which is encoded by the intronless Kv1.3 *Shaker*-subfamily gene (2, 3; for review see 4). B cells and pre-B cells express a channel biophysically similar to the type *n* K^+ channel; however, the expression of the Kv1.3 gene in these cells has not yet been established (5, 6, 7, 8). We report here the production of antipeptide antibodies cross-reactive with several *Shaker*-family proteins, and an absorbed fraction specific for the Kv1.3 protein. We have used affinity-purified Kv1.3-specific antibodies to demonstrate the expression of Kv1.3 protein in a mouse pre-B cell line, as well as mouse and human T-cell lines and human peripheral blood lymphocytes, and have shown that *Shaker* cross-reactive antibodies detect immunologically related proteins in *Drosophila melanogaster* and *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Peptide synthesis: Peptides were generously supplied by Drs. Douglas Hanson and Leonard G. Contillo (Pfizer Central Research, Groton, CT). Four peptides corresponded

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0006-291X/93 $4.00

to the putative extracellular region linking the S5 transmembrane segment to the pore region ("S5/P") of the mouse Shaker-family proteins Kv1.1 (VYFAEAEEAESHFSSIPDAF), Kv1.2 (VYFAEADERDSQFPSIPDAF) and Kv1.3 (AYFAEADDPSSGFNSIPDAF), and the *Shaw*-family protein Kv3.1 (IYYAERIGAQPNDPSASEHT). Two additional peptides corresponded to two other putative external loops of Kv1.3, those between S1/S2 (SPSQDVFEAANNSTSGAPSGASSFS) and S3/S4 (TLGTELAERQGNGQQAMSLAI).

Polyclonal antibodies: HPLC-purified peptides were coupled to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) at a ratio of 1:10 (w/w) using gluteraldehyde (9). Rabbit antisera were produced by the Berkeley Antibody Corporation (BAbCO, Richmond, CA), and antisera were assayed by ELISA using BSA-conjugated peptides as antigen, and HRP-conjugated anti-rabbit Ig (American Qualex, La Mirada, CA) as the second Ab.

Affinity purification of Abs: BSA-peptide conjugates were coupled to CNBr-activated Sepharose (Pharmacia, Piscataway, NJ) at about 1mg/ml. To produce affinity-purified *Shaker*-cross-reactive Ab, antiserum was passed over an immunoadsorbent bearing the Kv1.3 S5-P peptide, and bound Abs were eluted first with 0.1M glycine, pH 2.5, 0.5M KCl, and then with 0.1M triethylamine, pH 11.5, 0.5M KCl. To produce Kv1.3-specific antibody, antiserum was first exhaustively passed over an immunoadsorbent bearing the Kv1.1 S5/P peptide, and the final pass-through was then affinity-purified on a Kv1.3-bearing immunoadsorbent as above.

Cells: The human T cell line Jurkat (clone E6-1, ATCC, Rockville, MD), the mouse T cell line EL4, and the mouse pre-B cell line 230.37 (generously provided by Shiv Pillai, Harvard University, MA) were grown under standard conditions and harvested at densities of $0.5\text{-}0.8\times10^6$/ml. *Saccharomyces cerevisiae* strain YH82 (kindly provided by Dr. Suzanne Sandmeyer, U.C. Irvine) was grown in YT media and harvested at $OD_{600}\sim1$.

Cell membrane extracts: Crude membranes were prepared from mammalian cells in the presence of protease inhibitors (10), and stored at -70°C until used. Membranes from *Saccharomyces* were prepared in the same manner after first disrupting the cell wall by vortexing with fine glass beads in sucrose buffer for 2 min.

Western and Northern blots: Western blotting was performed as described for the Enhanced Chemiluminescence System (Amersham, UK), with primary Abs used at a final concentration of 1-7 μg/ml. For Northern blots, 15μg per lane of total cellular RNA (11) was run on a formaldehyde-agarose gel prior to electroblot transfer to nylon membranes (Schleicher and Schuell, Keene, NH). Following hybridization with [^{32}P]-CMP labelled antisense cRNA from an 0.9 kb Kv1.3-specific *Sac*I-*Hind*III fragment containing the 3' end of the coding region (3), blots were washed at a final stringency of 0.1 X SSC and 0.1% SDS for 30 min at 72°C.

RESULTS

Identification of a 62 kDa Kv1.3 protein in human Jurkat T cells. *Figure 1A* shows an affinity-purified anti-Kv1.3 S5/P Ab reacting with a 62 kDa membrane protein in Jurkat T cells (*lane 1*). Ab binding was competitively inhibited by a 20-fold molar excess of the immunizing (Kv1.3 S5/P) peptide (*lane 2*), but not by irrelevant Kv1.3 peptides derived from the S1/S2 (*lane 3*) or S3/S4 (*lane 4*) extracellular loops.

Comparison of the S5/P peptide sequences of different channels (*Figure 1C*) suggests that the anti-Kv1.3 S5/P polyclonal Ab might cross-react with shared determinants in other *Shaker*-related channels; the specificity of such cross-reactivity is shown in the right-hand lanes of *Figure 1A*. A peptide derived from the S5/P region of the *Shaker*-related mouse Kv1.1 channel inhibits Ab binding to the 62 kDa protein (*lane 5*); similar inhibition was also seen with the Kv1.2 peptide (not shown), but the corresponding peptide from the *Shaw*-related mouse Kv3.1 channel did not block reactivity (*lane 6*). The anti-Kv1.3 Abs thus appear to be broadly *Shaker*-reactive, and detect a 62 kDa *Shaker*-related channel protein in Jurkat T cells.

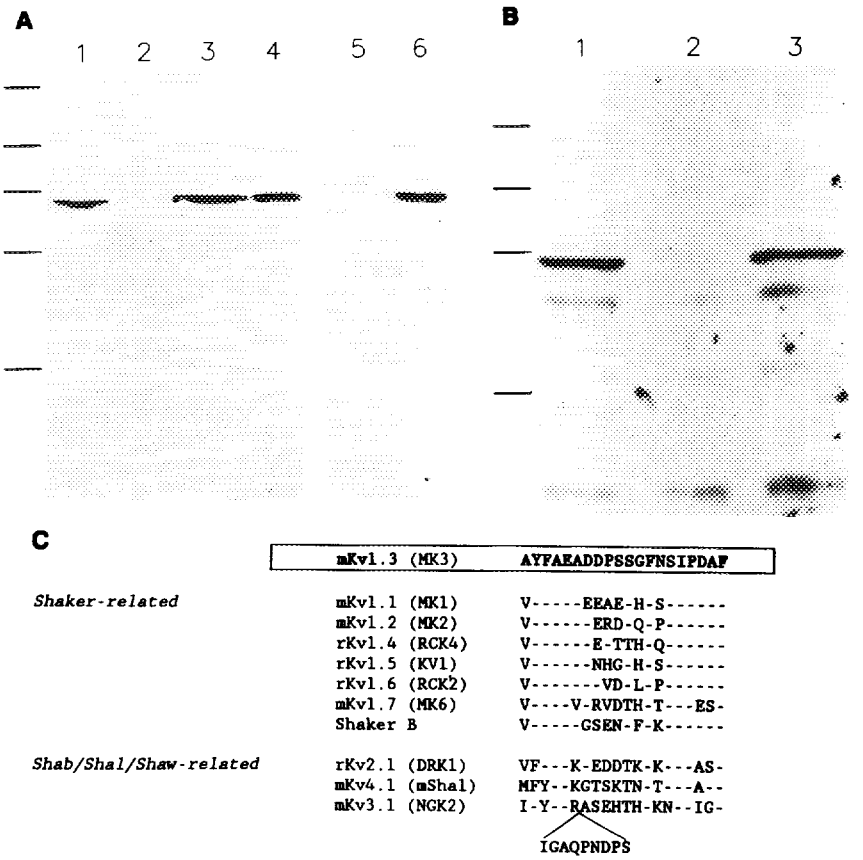

Figure 1. Western blots of human Jurkat cell membrane proteins. Blots of Jurkat cell membranes run on a polyacrylamide gel ($1x10^7$ cells/lane) were incubated with *(A) Shaker*-cross-reactive anti-Kv1.3 Ab alone (*lane 1*), or with a 20-fold molar excess of peptide Kv1.3-S5/P (*lane 2*), Kv1.3-S1/S2 (*lane 3*), Kv1.3-S3/S4 (*lane 4*), Kv1.1-S5/P (*lane 5*) or Kv3.1-S5/P (*lane 6*); (*B*) *Shaker-specific* Ab alone (*lane 1*), with Kv1.3-S5/P (*lane 2*) or Kv1.1-S5/P (*lane 3*). Bars indicate size standards, from top to bottom, of 200, 97, 69, 46 and 30 kDa.

The Kv1.3-*specific* Ab also recognized a 62 kDa protein in Jurkat cells (*Figure 1B*), and was specifically inhibited by the immunizing peptide (Kv1.3 S5/P) but *not* by the *Shaker*-related Kv1.1 S5/P peptide. We have also identified a similar-sized Kv1.3 protein in the mouse EL-4 T-cell line and in human peripheral blood T cells (not shown). The *Shaker*-related membrane protein detected by these antibodies in mouse and human T cells therefore appears to be Kv1.3 itself.

Detection of Kv1.3 transcripts and protein in the mouse pre-B cell line 230.37. We combined molecular and immunological methods to determine whether Kv1.3 was expressed in the mouse pre-B cell line, 230.37, which expresses type *n* K^+ channels (Cahalan, M. D., personal communication). First, Kv1.3-specific primers flanking the S1-S2 loop of Kv1.3 (3) were used in PCR to amplify the predicted 171 bp and 65 bp fragments from reverse-transcribed pre-B cell cDNA (not shown). Second, Northern blots using a

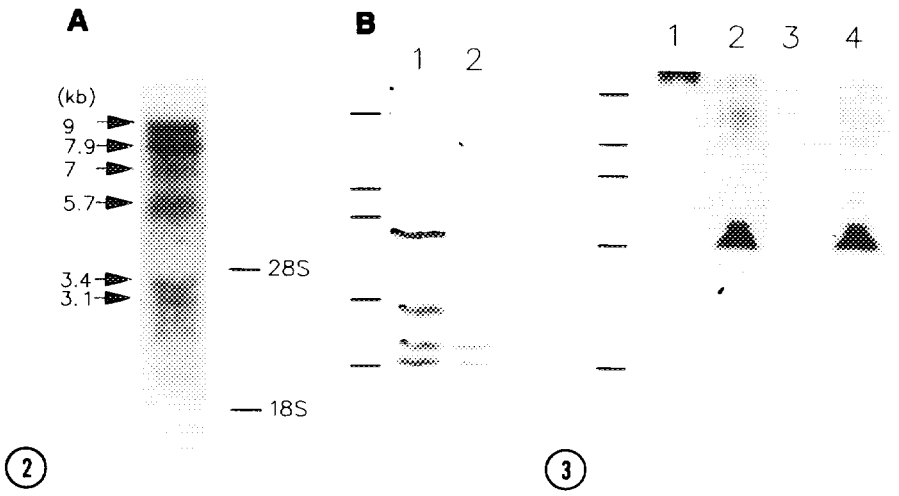

Figure 2. Expression of Kv1.3 mRNA and protein in the mouse pre-B cell line, 230.37. *(A)* Northern blot of total cellular RNA hybridized with a Kv1.3-specific cRNA probe. Positions of 28S and 18S rRNA are indicated. *(B)* Western blot of membrane proteins using *Shaker-specific* Ab alone (*lane 1*) or in the presence of peptide Kv1.3-S5/P (*lane 2*). Size standards are as in Figure 1.

Figure 3. Shaker-reactive membrane proteins in *Saccharomyces cerevisiae.* Western blot of membranes using IgG from preimmune serum (*lane 1*), and *Shaker*-cross-reactive Ab alone (*lane 2*) or in the presence of a 20-fold molar excess of peptide Kv1.3-S5/P (*lane 3*) or Kv3.1-S5/P (*lane 4*). Size standards are as in Figure 1.

Kv1.3-specific cRNA-antisense probe was shown to hybridize with multiple transcripts in 230.37 pre-B cells (*Figure 2A*). Similar-sized Kv1.3 transcripts have been detected in mouse thymocytes (12), human Jurkat T cells (13), and in human peripheral blood T cells (14). Since the coding region of the Kv1.3 gene is contained within a single exon (3,12,14), the multiple Kv1.3 transcripts probably arise from alternate splicing in the non-coding regions of the mRNA. Third, our Kv1.3-*specific* Ab reacted with a 62 kDa protein in membrane extracts of 230.37 cells (*Figure 2B*), and was also blocked by a 20-fold molar excess of the immunizing peptide. Together, these data demonstrate that the Kv1.3 protein is indeed expressed in pre-B cells and most likely functions as the type *n* channel in these cells.

Detection of related proteins in *Drosophila* **and** *Saccharomyces. Shaker* cross-reactive Abs were used to probe Western blots of extracts of *Drosophila melanogaster* heads (kindly provided by Dr. Diane O'Dowd, U.C. Irvine), and showed specific staining of three prominent bands at ~65, ~56 and ~54 kDa (not shown), a pattern similar to that detected by other *Shaker*-reactive Abs (15, 16, 17). We also used this Ab to probe membrane proteins of the yeast *Saccharomyces cerevesiae*, and detected two bands (a more intense one at ~48 kDa and a weaker one at ~50 kDa) as seen in *Figure 3, lane 2.* The *Shaker*-related Kv1.3 S5/P, but not the *Shaw*-related Kv3.1 peptide, inhibited antibody binding to both bands (*lanes 3 and 4*). *Saccharomyces cerevisiae* thus appears to express a protein which is immunologically related to the *Shaker*-subfamily channel proteins in mammals and flies.

DISCUSSION

We report here that polyclonal Abs specific for *Shaker*-related channels proteins can be used to characterize membrane proteins in widely divergent organisms. Affinity-purified Ab to a Kv1.3 peptide, which is cross-reactive with peptides of other channels in the *Shaker*-subfamily, bound specifically to a 62 kDa membrane protein in mouse and human lymphocytes, with several 55-65 kDa proteins in flies, and also with two 48-50 kDa membrane proteins in yeast. A Kv1.3-*specific* Ab produced by immunoabsorption specifically recognized the 62 kDa protein in mouse and human T lymphocytes, as well as mouse pre-B-cells. The size of this protein is slightly larger that the predicted mass of the gene product of human Kv1.3 (58,800 Da) based upon its deduced amino acid sequence. The additional mass may represent glycosylation or other post-translation modifications, although the molecular mass determined in this manner may be not be accurate, particularly in the case of integral membrane proteins (18).

Electrophysiological studies of yeast have revealed the existence of voltage-gated K^+ channels which resemble those of mammals (19, 20), as well as non-selective cation channels (4). Although the genes for these channels have not been isolated, anti-peptide Abs directed against the NH_2-terminus of the *Shaker* protein in *Drosophila* have been reported to bind to an 87 kDa membrane protein in yeast (22). We have identified two yeast membrane proteins of M_r~48-50 kDa which are specifically recognized by our broadly *Shaker*-reactive antibody. *S. cerevisae* therefore appears to express epitopes recognized by *Shaker*-subfamily reactive Abs, suggesting that yeast may express K^+ channels which are structurally related to *Shaker*.

Immunological reagents directed against K^+ channel-derived peptides should provide powerful tools for the isolation and biochemical characterization of these proteins, which are known to be expressed in a wide variety of mammalian cells. The reagents we describe may, in addition, aid in the identification of related proteins in evolutionarily diverse species.

ACKNOWLEDGMENTS

We thank Pfizer Inc., Groton CT, for providing us with K^+ channel peptides, and Dr. Luette Forrest for assistance with cell culture. This work was supported by a grant from Pfizer Inc., Groton, CT (KGC); USPHS grants AI24783 (KGC) and AI213166 (GAG); NIH Carcinogenesis Training Grant (5T32 CA09054, RHS); and the Cancer Research Institute, UC Irvine.

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