Volume 249, number 2, 224–228 FEB 07190 June 1989

# Immunological evidence for a relationship between the dendrotoxin-binding protein and the mammalian homologue of the *Drosophila Shaker* $K^+$ channel

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Received 31 March 1989

Polyclonal antibodies were raised against two synthetic peptides from different parts of the predicted amino acid sequence of the mouse homologue (MBK1) of the *Drosophila Shaker* $K^+$ channel. The antibodies recognized the toxin-binding subunit of the dendrotoxin-binding proteins from rat and bovine brain. The results suggest that the dendrotoxin-binding protein is related to the expression products of the mammalian homologue of the *Shaker* gene.

$K^+$ channel; Mutation, shaker; Dendrotoxin; Immunological relationship; Glycosylation

## 1. INTRODUCTION

$K^+$ channels have been difficult to study due to their great variety and low abundance. The suggestion that the *Drosophila Shaker* locus might encode a voltage-dependent $K^+$ channel [1] initiated the isolation of the *Shaker* gene [2,3]. With this genetic approach a family of *Shaker* $K^+$ channels were identified and characterized [4–8]. Sequences from the *Drosophila Shaker* locus were used to isolate related cDNAs from mammalian brain libraries [9,10]. These mammalian cDNAs predicted channel proteins of approx. 56 kDa [9,10].

A parallel biochemical approach to voltage-dependent $K^+$ channels made use of dendrotoxin (DTX), $\beta$-bungarotoxin and mast cell degranulating peptide (MCD). These neurotoxins have been shown to block specifically a class of neuronal voltage-dependent $K^+$ channels [11,12]. The physiological importance of these channels is highlighted by the potent epileptogenicity of the toxins [13] and by the ability of MCD to induce hippocampal long-term potentiation [14]. DTX, MCD and $\beta$-bungarotoxin bind to a brain membrane protein [15–20] which is most likely identical with the $K^+$ channels that are physiologically blocked by the toxins [16,21]. This protein was recently purified from rat brain and named DMB protein [from D (DTX), M (MCD), B ($\beta$-bungarotoxin or binding)] [22,23]. DMB protein turned out to be a family of pharmacologically and structurally closely related proteins [23] which consist of at least two types of subunits of apparent molecular masses 80 and 38 kDa [22,23]. The 80 kDa subunit binds the toxin, and is glycosylated [19,24,25]; deglycosylation reduces its molecular mass to 65 kDa [25]. Because the voltage-dependent $K^+$ channels of the genetic and the biochemical approaches had similar molecular masses, we investigated the immunological rela-

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*Abbreviations:* SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DMB protein, dendrotoxin/mast cell degranulating peptide/$\beta$-bungarotoxin-binding protein; DTX, dendrotoxin; MCD, mast cell degranulating peptide



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00145793/89/$3.50 

tionship between DMB protein and the mammalian *Shaker* $K^+$ channel.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

DMB protein was purified from Triton X-100 extracts of rat and bovine brain membranes in three successive steps as described in [22]: ion-exchange chromatography was followed by affinity chromatography on a $DTX_I$ column and then affinity chromatography on a wheat germ agglutinin column. Elution of DMB protein from the $DTX_I$ affinity column was accomplished with 0.7 M KCl. This method has the advantage over the recently reported elution of DMB protein with dithiothreitol [26] in that the $DTX_I$ affinity column can be used repeatedly and is stable for several months. $DTX_I$, a member of the dendrotoxin family of neurotoxins, was purified from *D. polylebis* venom (Sigma) according to [27]. Goat anti-rabbit IgG antibody coupled to alkaline phosphatase was obtained from Sigma. Neuraminidase from *Arthrobacter ureafasciens* and glycopeptidase F from *Flavobacterium meningosepticum* were from Boehringer, Mannheim.

### 2.2. *Preparation of antibodies*

Two peptides predicted by a mouse $K^+$ channel cDNA clone (MBK1) [9] were chemically synthesized and purified by HPLC (peptide I, amino acids 1–27, N-terminal end; peptide II; amino acids 443–470 near the C-terminal end; see fig.2). The peptides were conjugated to keyhole limpet hemocyanin [28]. Polyclonal antibodies against this conjugate were raised in rabbits by Berkeley Antibody Co. (Richmond, CA). The titer of the antisera was evaluated by ELISA and dot-blot techniques for the conjugated peptides and by immunoblotting for the toxin-binding subunit of DMB protein. Using antibody dilutions from 1:50 to 1:1000, the antibodies against peptide I did not cross-react with peptide II and vice versa.

### 2.3. *SDS-PAGE and immunoblots*

Aliquots from various steps in the DMB protein purification were precipitated according to [29] and analyzed on 8 or 10% SDS-PAGE [30]. The proteins of the gel were either silver-stained [31] or electrotransferred onto nitrocellulose [32]. After staining for protein with Ponceau S, the blots were blocked and incubated with one of the anti-MBK1 antibodies (dilutions 1:50–1:1000). Blots were developed with a goat-anti-rabbit IgG coupled to alkaline phosphatase (dilution 1:3000).

### 2.4. *Deglycosylation*

For treatment with neuraminidase and glycopeptidase F, DMB protein was precipitated according to [29] and resolubilized in 0.1% (w/v) SDS at 95°C. After addition of Triton X-100 (final concentration 0.2%, w/v) and dithiothreitol (final concentration 7 mg/ml) the samples were incubated with neuraminidase alone or neuraminidase followed by glycopeptidase F as described in [25]. Enzyme reactions were stopped with Laemmli sample buffer.

## 3. RESULTS AND DISCUSSION

DMB protein was purified from bovine brain using a protocol identical to that used for DMB protein from the rat [22]. In both species DMB protein was composed of at least two types of subunits. The toxin-binding subunit of the bovine DMB protein had an apparent molecular mass of 74 kDa in 8% SDS-PAGE (fig.1). The apparent molecular mass of the toxin-binding subunit of DMB protein in SDS-PAGE depended on the percentage of acrylamide used [25]. For the rat, it was 80 kDa in 8% SDS-PAGE and 90 kDa in 10% SDS-PAGE; for the bovine form it was 74 and 83 kDa, respectively ([25]; not shown). No difference between the two species was observed for the molecular mass of the 38 kDa subunits. In fig.1 some proteolytic breakdown products of the 38 kDa subunits can be observed which also seem to be identical between the two species. Purified DMB protein of both species contained in addition to the 38 kDa subunit small and variable amounts of a peptide of 40 kDa. Using a slightly different protocol DMB protein from bovine brain was also purified by Parcej and Dolly ([26] and see section 2). In agreement with our results, a toxin-binding subunit of 75 kDa and small subunits of 37 and 42 kDa were found in 8% SDS-PAGE.

Polyclonal antibodies were raised against two synthetic peptides (designated I and II) from the


Fig.1. Silver-stained 8% SDS-PAGE with purified bovine DMB protein (lane 1, 180 ng) and rat DMB protein (lane 2, 60 ng). (M) Molecular mass markers (in kDa): phosphorylase *b* (97.4), bovine serum albumin (66.2) and ovalbumin (42.7). The positions of the toxin-binding subunits are indicated by arrowheads. The 38 kDa subunits are marked by black circles (●).

predicted amino acid sequence of the mouse MBK1 $K^+$ channel [9] (fig.2). In immunoblots of pure bovine and rat DMB protein, antibodies against both peptides recognized the toxin-binding subunits of 74 and 80 kDa, respectively (fig.3). The low molecular mass band which is faintly recognized by antibodies against peptide I (fig.3, lane 2) is probably a degradation product of the toxin-binding subunit and not the 38 kDa subunit as the latter has a slightly higher molecular mass and it is better focussed. The antibodies were used to follow the purification of DMB protein. Very little immunostaining was seen with the initial Triton X-100 extract of the brain membranes or after ion-exchange chromatography, the latter estimated to yield a 10-fold purified preparation (fig.4 and not shown). This highlights the low abundance of DMB protein in brain (200–400 fmol/mg membrane protein) [15,16,19] and the specificity of the antibodies. Specific immunostaining of the toxin-binding subunit was first prominent in blots of the eluate from the $DTX_I$ affinity column (fig.3, lane 1). No immunostaining was seen with the breakthrough of the $DTX_I$ column, even when large amounts of protein were loaded (fig.5, lane 7). Thus, all detectable immunoreactivity seems to be retained on the $DTX_I$ affinity column, suggesting that the mammalian *Shaker* $K^+$ channels are included in the DMB protein family.

The difference in molecular mass of the toxin-binding subunits between the rat and bovine pro-

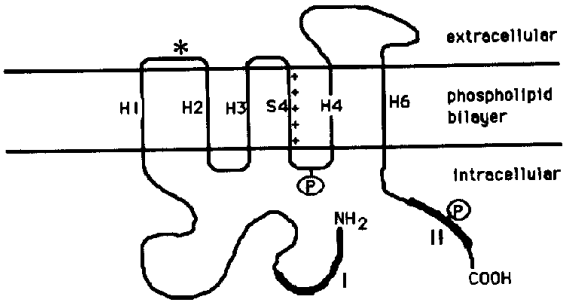


Fig.2. Model of the transmembrane structure of the MBK1 *Shaker* K+ channel protein. The positions of two potential intracellular phosphorylation sites (P), the S4 voltage sensor (+ + + + +) and a potential extracellular glycosylation site (*) are indicated. Bold lines (I and II) indicate the peptides used for antibody preparation.

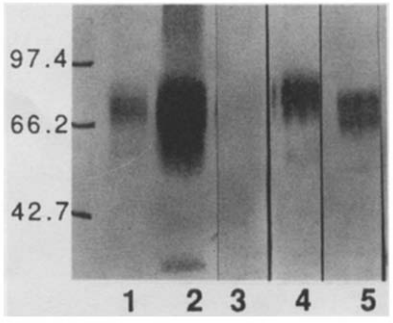


Fig.3. Immunoblots from 8% SDS-PAGE of partialy purified bovine DMB protein: eluate from the $DTX_I$ affinity column (lane 1: 2 μg protein), purified bovine DMB protein (lanes 2,3,5; 0.5, 0.5 and 0.4 μg protein, respectively) and purified rat DMB protein (lane 4, 0.1 μg protein). Incubation performed with (lanes): antibodies against peptide I; (3) preimmune serum, (5) antibodies against peptide II as described in section 2. Molecular masses (in kDa) are indicated.

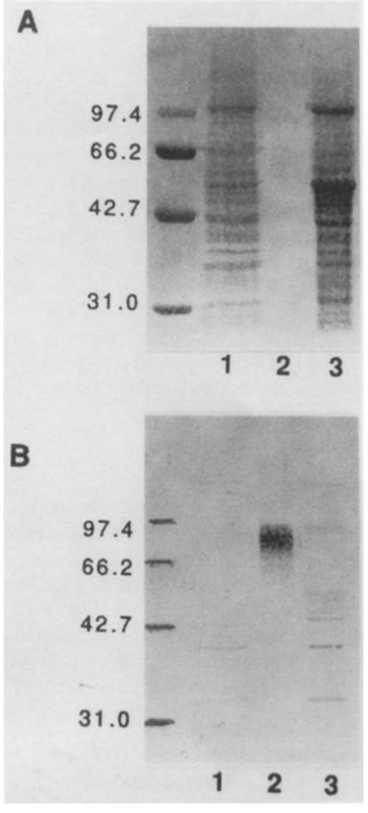


Fig.4. Immunoblot from 10% SDS-PAGE of Triton X-100 extract of bovine brain membranes (lane 1), purified bovine DMB protein (lane 2) and rat brain membranes (lane 3). (A) Protein pattern (Ponceau S staining), (B) immunostaining with antibodies against peptide I. Both panels show the same blot. Molecular masses (in kDa) are indicated.

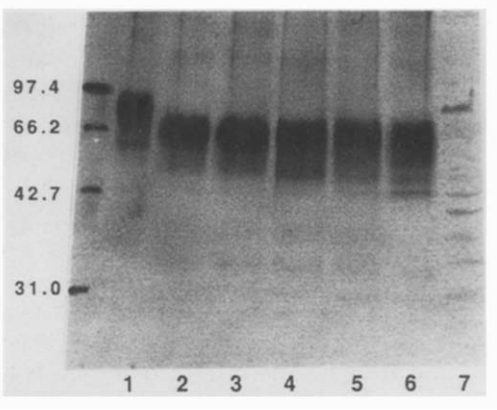


Fig.5. Immunoblot from 10% SDS PAGE of eluate (lanes 1–6, 2 μg protein per lane) and breakthrough (lane 7, 60 μg protein) of the $DTX_I$ affinity column. The eluate was treated with neuraminidase for 5 min (lane 2), 20 min (lane 3), or 90 min (lanes 4–6) and subsequently with glycopeptidase F for 20 min (lane 5) or 60 min (lane 6) as described in section 2. No enzyme treatment (control) in lane 1. The blot was developed with antibodies against peptide I. Molecular masses (in kDa) are indicated.

tein seems, at least partially, to be due to differential glycosylation. Treatment with neuraminidase reduces the molecular mass of the toxin-binding subunit of bovine DMB protein from 83 to 66 kDa in 10% SDS-PAGE (fig.5). Similar results were obtained with the toxin-binding subunit of rat DMB protein [25]. In contrast to the rat, however, the molecular mass of bovine toxin-binding subunit was not further reduced by an additional treatment with glycopeptidase F (fig.5), indicating a different structure of its sugar chain.

We have shown here that the purification method devised for rat DMB protein is applicable to DMB protein from other mammals. Apart from differential glycosylation, there may be little difference between mammalian DMB proteins. The major conclusion of this paper is that a strong immunological relationship exists between DMB protein and the mammalian homologue of the *Drosophila Shaker* $K^+$ channels. This conclusion is supported by the recent finding that DTX and MCD block $K^+$ currents induced in *Xenopus* oocytes by rat *Shaker* mRNA [33]. We suggest that some members of the DMB protein family are products of the mammalian homologue of the *Drosophila Shaker* gene.

*Acknowledgements:* H.R. is a stipend recipient of the Deutsche Forschungsgemeinschaft. The peptides used here were synthesized by Chris Turk at the UCSF Howard Hughes Medical Institute. L.Y. and Y.N. Jan supported both peptide synthesis and antibody production. We thank the Jans for their ongoing support and Mary Bascom for valuable help and encouragement. This work was supported by grants to B.L.T. from the Veterans Administration and the NIH (NS 27206-01).

## REFERENCES

[1] Jan, Y.N., Jan, L.Y. and Dennis, M.J. (1977) Proc. R. Soc. Lond. B. 198, 87–108.
[2] Papazian, D.M., Schwarz, T.L., Tempel, B.L., Jan, Y.N. and Jan, L.Y. (1987) Science 237, 749–753.
[3] Baumann, A., Krah, J., Mueller, R., Mueller, F., Seidel, R., Kecsemethy, N., Casal, J., Ferrus, A. and Pongs, O. (1987) EMBO J. 6, 3419–3429.
[4] Tempel, B.L., Papazian, D.M., Schwartz, T.L., Jan, Y.N., and Jan, L.Y. (1987) Science 237, 770–775.
[5] Timpe, L.C., Schwarz, T.L., Tempel, B.L., Papazian, D.M., Jan, Y.N. and Jan, L.Y. (1988) Nature 331, 143–145.
[6] Kamb, A., Tseng-Crank, J. and Tanouye, M.A. (1988) Neuron 1, 421–430.
[7] Iverson, L.E., Tanouye, M.A., Lester, H.A., Davidson, N. and Rudy, B. (1988) Proc. Natl. Acad. Sci. USA 85, 5723–5727.
[8] Schwarz, T.L., Tempel, B.L., Papazian, D.M., Jan, Y.N. and Jan, L.Y. (1988) Nature 331, 137–142.
[9] Tempel, B.L., Jan, Y.N., and Jan, L.Y. (1988) Nature 332, 837–839.
[10] Baumann, A., Grupe, A., Ackermann, A. and Pongs, O. (1988) EMBO J. 7, 2457–2463.
[11] Benoit, E. and Dubois, J.M. (1986) Brain Res. 377, 374–377.
[12] Stansfeld, C., Marsh, S., Halliwell, J. and Brown, D. (1986) Neurosci. Lett. 64, 299–304.
[13] Bidard, J., Gandolfo, G., Mourre, C., Gottesmann, C. and Lazdunski, M. (1987) Brain Res. 418, 235–244.
[14] Cherubini, E., Ben Ari, Y., Gho, M., Bidard, J. and Lazdunski, M. (1987) Nature 328, 70–73.
[15] Rehm, H. and Betz, H. (1982) J. Biol. Chem. 257, 10015–10022.
[16] Rehm, H. and Betz, H. (1984) J. Biol. Chem. 259, 6865–6869.
[17] Taylor, J., Bidard, J. and Lazdunski, M. (1984) J. Biol. Chem. 259, 13957–13967.
[18] Bidard, J., Mourre, C. and Lazdunski, M. (1987) Biochem. Biophys. Res. Commun. 143, 383–389.
[19] Rehm, H., Bidard, J., Schweitz, H. and Lazdunski, M. (1988) Biochemistry 27, 1827–1832.
[20] Schmidt, R., Betz, H. and Rehm, H. (1988) Biochemistry 27, 963–967.
[21] Rehm, H., Pelzer, S., Cochet, C., Chambaz, E., Trautwein, W., Pelzer, D. and Lazdunski, M. (1989) Biochemistry, submitted.
[22] Rehm, H. and Lazdunski, M. (1988) Proc. Natl. Acad. Sci. USA 85, 4919–4923.

[23] Rehm, H. and Lazdunski, M. (1988) Biochem. Biophys. Res. Commun. 153, 231–240.
[24] Rehm, H. and Betz, H. (1983) EMBO J. 2, 1119–1122.
[25] Rehm, H. (1989) FEBS Lett., in press.
[26] Parcej, D. and Dolly, J. (1989) Biochem J. 257, 899–903.
[27] Harvey, A. and Karlsson, E. (1980) Naunyn-Schmiedebergs Arch. Pharmacol. 312, 1–6.
[28] Liu, F., Zinnecker, M., Hamaoka, T. and Katz, D. (1979) Biochemistry 18, 690–697.
[29] Wessel, D. and Fluegge, U. (1984) Anal. Biochem. 138, 141–143.
[30] Laemmli, U.K. (1970) Nature 227, 680–685.
[31] Merril, C., Goldman, D., Edman, S. and Ebert M. (1981) Science 211, 1437–1439.
[32] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[33] Stuehmer, W., Stocker, M., Sakmann, B., Seeburg, P., Baumann, A., Grupe, A. and Pongs, O. (1988) FEBS Lett. 242, 199–206.