Demonstration of a tandem pair of complement protein modules in $GABA_B$ receptor 1a

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Abstract We have subcloned and expressed the N-terminal portion of the recently sequenced metabotropic GABA receptor, $GABA_B$R1a. This region of the receptor contains a complement protein-like amino acid sequence. The purified 140-residue recombinant protein fragment was soluble and stable. Mass spectrometry indicated formation of four disulfide bonds, as expected if two complement protein modules (CPs, also known as SCRs, Sushi domains) are formed. The circular dichroism spectrum was unusual and characteristic of CPs. Differential scanning calorimetry demonstrated a melting point (64°C), and total enthalpy commensurate with two fully folded domains. We thus conclude that the 1a subtype of the $GABA_B$ receptor, but not the 1b subtype, contains a pair of CPs and we present a three-dimensional model of this region.

Key words: SCR; Sushi; $GABA_B$ receptor; Epilepsy; Drug target

1. Introduction

The $GABA_B$ receptor ($GABA_B$r) has seven trans-membrane helices [1], is guanine nucleotide binding protein-linked, and is stimulated selectively by baclofen [2]. Activation of $GABA_B$r results in a range of profound and relatively long-term metabolic effects (reviewed in [3]). Baclofen is used to treat spasticity arising from multiple sclerosis or spinal injury and $GABA_B$ agonists may have applications in absence epilepsy, anxiety, depression and cognitive dysfunction [4].

Recently, the amino acid sequences of two subtypes of the rat $GABA_B$r were determined using expression cloning [1]. Subtypes 1a and 1b represent alternatively spliced forms and are identical with the exception of a 130-residue extension at the extracellular N-terminus of $GABA_B$r1a, immediately following the putative signalling sequence. Interest has so far focused on similarities in amino acid sequence between the extracellular regions of $GABA_B$r1 and the metabotropic glutamate receptors and bacterial amino-acid transport proteins. However, significant homology with these proteins does not extend to the N-terminal portion of $GABA_B$r1a. The authors referred to, but did not comment upon a 'weak homology' between $GABA_B$r1a and complement receptor type 1 (CR1) [1]. By considering only the N-terminal extension of subtype 1a, we have noticed a previously unreported and striking homology with the family of proteins known as the regulators of complement activation, of which CR1 is a member (reviewed in [5]). Specifically, residues 11–134 appear to comprise a tandem pair of consensus sequences for the 'CP' module (also called SCR and Sushi domain) (Fig. 1).

The occurrence of CPs is limited mainly but not exclusively to proteins of the complement, immune and clotting systems where they engage in specific protein/protein recognition events. In general, CPs located towards the N-terminus of receptors are directly involved in ligand binding (e.g. [6–9]). The putative CPs at the N-terminus of $GABA_B$r1a could therefore be involved in the recognition of an as yet unidentified ligand. Thus we have set out to provide experimental evidence that this sequence does indeed fold into CP modules.

2. Materials and methods

Based on the published sequence of the rat $GABA_B$R1a gene (accession no. Y10369), we devised a reverse transcriptase-polymerase chain reaction (PCR) strategy to subclone into a *Pichia pastoris* expression vector, the portion of the N-terminal extracellular region of the $GABA_B$r1a gene product that has sequence homology with CPs. This region, in the published sequence, begins with the 11th residue following the putative signal peptide cleavage site and extends for 134 residues (Fig. 1). We designed two oligonucleotides that would allow us to PCR-amplify and subclone the sequence corresponding to residues 8–144 of the rat $GABA_B$r1a gene (where residue 1 is the Gly residue immediately following the proposed signal peptide sequence). The 5′ PCR primer was designed to include an *Eco*RI restriction site. The opposing flanking primer incorporated a *Not*I restriction site following two sequential stop codons. The template DNA used for the PCR amplification was cDNA, prepared from a mixture of poly(A) and total mRNA from adult rat brain, provided by Dr. Diane Lipscombe (Department of Neuroscience, Brown University). The amplification was performed using the Boehringer Mannheim Expand High Fidelity PCR System according to the manufacturer's recommendations. A band of the appropriate mobility was observed following electrophoresis in 1.5% agarose. The resulting PCR product was treated with *Eco*RI and *Not*I. Ligation to pPICZα vector DNA (Invitrogen) similarly restricted with *Eco*RI and *Not*I (provided by Dr. Nick Mullin, University of Edinburgh) was performed under standard conditions.

Top10F′ cells were transformed with the ligation product by electroporation and Zeocin-resistant transformants were selected on low-salt LB plates containing Zeocin according to the Invitrogen protocol. Several transformants were isolated and checked for insert sequence by PCR. The expected vector insert DNA sequence was confirmed in one of the clones by sequencing both strands.

Midi-preps were prepared of the shuttle vector containing the insert and 20 μg of the resulting DNA was linearised with *Sac*I. Transformation into *P. pastoris* strain KM71 (Mut^S) was accomplished by electroporationn. Candidate Zeocin-resistant transformants were purified by re-streaking onto Zeocin-containing plates, then tested for protein expression. Single colonies were used to inoculate 20 ml buffered minimal medium with glycerol as sole carbon source (BMG) in a 50-ml tube (see the Invitrogen Expression manual). After overnight growth in a 30°C shaking incubator, cells were harvested by centrifugation and resuspended in 4 ml of the induction medium containing

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methanol (0.5% final concentration) in place of glycerol as sole carbon source (BMM). The induced cultures were incubated for two days in a 30°C shaking incubator and additional methanol was added after 24 h to maintain a final concentration of 0.5%. Subsequently, the supernatant was collected and the protein in the supernatant precipitated with 10% trichloroacetic acid (TCA; final concentration), pelleted by centrifugation and washed twice with a 1:1 mixture of ethanol/ether. The final protein pellet was analysed by SDS-PAGE for protein expression. Of twenty isolates tested, all expressed a protein of the appropriate molecular weight not found in control transformed cultures or in control untransformed cultures. One isolate, designated K8, exhibited a much higher level of protein production than the remaining transformants.

Larger cultures of K8 were prepared and the culture medium from these growths was used after 2–3 days of methanol induction to prepare protein samples on a larger scale. As a first step in purification, the culture medium was concentrated ~20-fold by ultrafiltration (Amicon YM3000). The resulting concentrate contains protein at a concentration of ~3 mg/ml (Bradford assay). N-terminal sequencing was performed on a protein sample that was TCA-precipitated from an ultrafiltration concentrate and subjected to SDS-PAGE. The SDS-PAGE-fractionated protein was transferred from the gel to a PVDF membrane by electrophoresis and stained with Coomassie Blue. Following air-drying, the section of membrane containing the major band of stained protein was excised.

For further purification, samples of protein concentrate were applied to an analytical C18 RP-HPLC column and eluted with a gradient of acetonitrile (containing 0.1% trifluoracetic acid). Most of the protein eluted at an acetonitrile concentration of ~46%. The eluate containing the major fraction was collected and solvent was removed by evaporation.

N-terminal sequencing was conducted by Dr Andrew Cronshaw, Department of Biochemistry, University of Edinburgh. Electrospray ion mass spectrometry (MS) was kindly performed on a Micromass Platform II instrument by Joanne O'Leary, Department of Chemistry, University of Edinburgh. Measurements of circular dichroism (CD) were made using a Jasco-600 spectropolarimeter (Japan Spectroscopic Co., Tokyo) with cylindrical quartz cell of pathlength 0.5 cm at the BBSRC-funded centre for CD at the University of Stirling with the kind assistance of Dr Sharon Kelly and Professor Nick Price. The protein concentration was 0.03 mM. Differential scanning calorimetry (DSC) studies were conducted on an MC-2 (Microcal Inc., Northampton, MA, USA), at the BBSRC-funded UK Microcalorimetry Facility in the Department of Chemistry, University of Glasgow with the kind assistance of Professor Alan Cooper. The cell volume was 1.5 ml, rate of heating was 1°C min^{-1}, and excess pressure was kept equal to 80 bar. The protein concentration was 0.1 mM. The partial molar heat capacity and melting curve were analysed using standard procedures [10]. The data were processed using the software ORIGIN 2 (Microcal Inc.). Three-dimensional molecular models were constructed within the program MODELLER [11] using homology to the known single and double CP-module structures.

3. Results and discussion

Fig. 1 shows the sequences 11–76 and 77–134 of $GABA_BR1a$ aligned with the third and fourth CPs from Vaccinia virus complement control protein (VCP) and CPs 15 and 16 from human factor H. Structures of both these pairs have been solved [12,13]. Also included are a pair of CP-like sequences found in the mouse brain protein SEZ-6 [14]. A schematic summary of secondary structure is shown along with the consensus sequence. The overall sequence identity between VCP module 3, a typical CP of known three-dimensional structure, and $GABA_BR1a$~11–76 is 15% while sequence similarity is 23%. Equivalent values for $GABA_BR1a$~77–134 are 22% and 37%, respectively. The first sequence is characterised by an insertion between putative strands two and three. This is called the hypervariable loop and is frequently a site for insertions; however, the $GABA_BR1a$~11–76 sequence carries a longer insertion at this position than has been observed in any other known CP-like sequence. The second sequence is unusual (but not exceptional) in having no prolines between putative strands one and two. In a multiple sequence alignment involving CP sequences from >50 distinct proteins (Barlow and Coulson, unpublished data), the second $GABA_Br$ CP-like sequence clusters with the 6th, 13th, 20th and 27th CP of CR1 and the 4th CP of CR2 while the first CP-like sequence was an outlier. The two $GABA_Br$ CP-like sequences are joined by a linking sequence of four amino acid residues which is typical for tandemly arranged CPs. Thus, the sequence of this region is suggestive of the presence of CPs in $GABA_Br1a$. However, neither of the sequences is typical – therefore it was necessary to obtain experimental proof that the CP fold is indeed adopted by expressing and analysing the relevant fragment.

The N-terminal sequence of the recombinantly expressed protein was determined to be EAEFATSEG. This corresponds to the expected sequence of ATSEG preceded by four extra residues. The EF sequence is encoded by the *Eco*RI site that was engineered into the 5′-end of the initial PCR product. The N-terminal EA sequence is derived from the pPICZα vector and is due to incomplete signal cleavage. The molecular weight of the resulting protein was determined by MS. The major protein in the sample had a mass of 15 628.7 Da – consistent with the expected size of the target

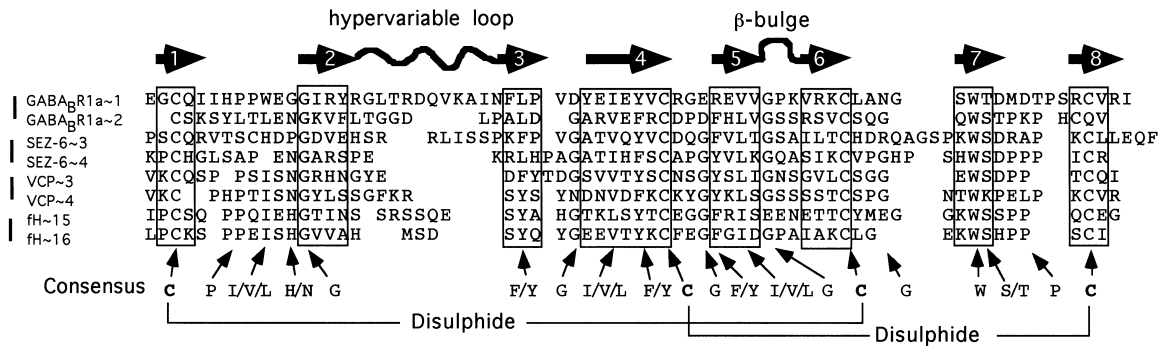

Fig. 1. Primary and secondary structure. The two putative CP sequences from $GABA_BR1a$ are shown aligned with a pair of CP-like sequences found in SEZ-6. Also included in the alignment are the 3rd and 4th CPs from Vaccinia complement control protein (VCP) and the 15th and 16th from human factor H. Solution structures of both these pairs have been solved by NMR. A schematic summary of secondary structure (numbered arrows and boxes indicate approximate extent of β-strands) as determined (for VCP3 and 4 and for fH15 and 16), is shown along with the consensus sequence (defined as residues which occur at a particular position in more than 80 out of a sample of 140 CP-module sequences).

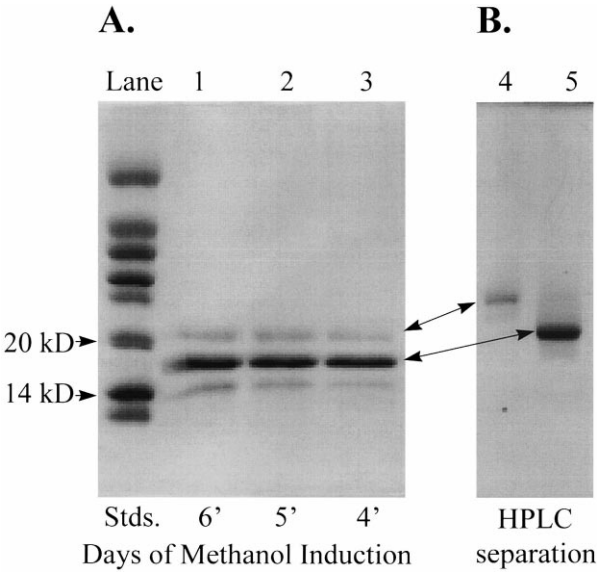

Fig. 2. Purification of $GABA_Br1a$ N-terminal fragment. A: The results of polyacrylamide gel electrophoresis (15% acrylamide) run under denaturing and reducing conditions and stained with Coomassie Blue. Lanes 1–3: 20 μl loaded derives from 500 μl of cell media following TCA precipitation, 6, 5 and 4 days post-induction. B: Lanes 4 and 5: two peaks from a reverse phase HPLC (C18) run illustrating resolution of larger, glycosylated (lane 4) from non-glycosylated protein (lane 5).

$GABA_BR1a$-derived fragment, i.e. a 140 amino acid protein with the sequence EAEFATSEGCQIIHPPWEGGIRYRGL-TRDQVKAINFLPVDYEIEYVCRGEREVVGPKVRKCL-ANGSWTDMDTPSRCVRICSKSYLTLENGKVFLTGGD-LPALDGARVEFRCDPDFHLVG SSRSVCSQGQWSTPK-PHCQVN.

If all eight cysteines were in the reduced form, the expected mass for this polypeptide sequence would be 15 636.7 (average molecular weight). The observed mass of 15 628.7 corresponds to the expected mass if all 8 cysteines are in the disulphide form, as would be expected if two CPs are formed. In addition to the major secreted protein, a minor protein of apparent mass ~20 kDa is also secreted into the medium (Fig. 2).

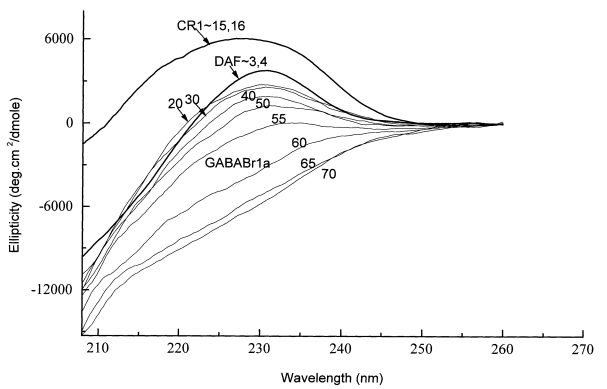

Fig. 3. CD spectra of CR1~15,16 at pH 6.0 and 20°C; DAF~3,4 under the same conditions; and the $GABA_Br1a$ N-terminal fragment at pH 8.0 over a range of temperatures from 20 to 70°C as indicated by the numbers on the plot. The protein concentration was 0.03 mM and the buffer was 25 mM sodium phosphate for all experiments.

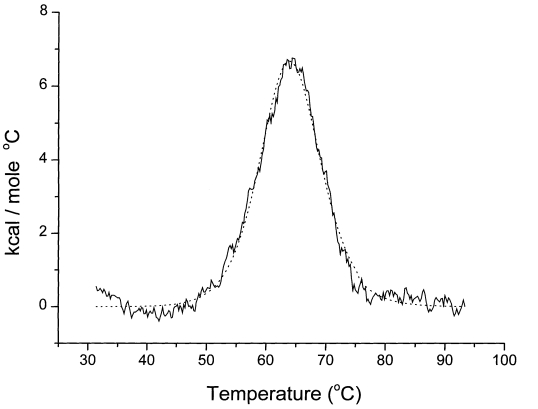

Fig. 4. Temperature dependence of partial molar heat capacity of $GABA_Br1a$ N-terminal fragment at pH 8.0, 25 mM sodium phosphate, the protein concentration was 0.1 mM. Experimental curves are shown by the solid line, and the fitted curve (using ORIGIN) by a dotted line.

This second protein remains a minor component after 2–3 days of induction. It was found to have the same N-terminal sequence as the major fraction. The higher mass is presumably due to glycosylation as there is one N-linked glycosylation consensus Asn residue within the subcloned region. Treatment of the mixture with Endo-H resulted in loss of this band (not shown). Some other variants observed by MS following longer induction periods (5–6 days) include proteins lacking either the first 2, 4, or 6 N-terminal residues of the major protein species described above.

Fig. 3 shows CD spectra collected on a sample of the $GABA_Br1a$ fragment. Also shown are CD spectra of 120-residue fragments of: complement control receptor type 1 (CR1) encompassing the 15th and 16th CP modules, provided by Dr. M. Krych and Professor J. Atkinson of the Washington University School of Medicine, St. Louis, MO; and decay accelerating factor (DAF) modules 3 and 4 (supplied by R. Powell, University of Reading). The profiles at 20°C are highly unusual in that they display a large positive ellipticity in the range 220–260 nm. However, they are very similar to one another. Also shown in Fig. 3 are a series of CD spectra for the $GABA_Br1a$ fragment collected over a range of temperatures (20–70°C). The positive maximum decreases as the temperature is raised, and the spectra become characteristic of random coil above 60–65°C. Other CP-module pairs display a roughly similar behaviour, according to CD, upon denaturation by temperature or guanidine HCl. It was also observed (not shown) that disruption of the disulphide bonds causes loss of the positive maximum. Thus, these plots provide very compelling evidence for the existence of folded CP-modules in the $GABA_Br1a$ fragment.

Fig. 4 shows the profile obtained from a DSC study. The value of C_p for the folded protein was 0.3 cal $(g\ °C)^{-1}$ which falls in the range of folded globular proteins, 0.28 ± 0.55 cal $(g\ °C)^{-1}$ [10]. The protein aggregated immediately after melting and therefore values of ΔC_p were not calculated [15]. The melting temperature is relatively high (64°C), and is consistent with a protein that has a stable and well-defined tertiary structure. This observation is consistent with the melting profiles obtained by CD in Fig. 3. The value of calorimetric enthalpy (90 kcal/mol) is consistent with values obtained for other globular proteins of a similar size. It is somewhat lower

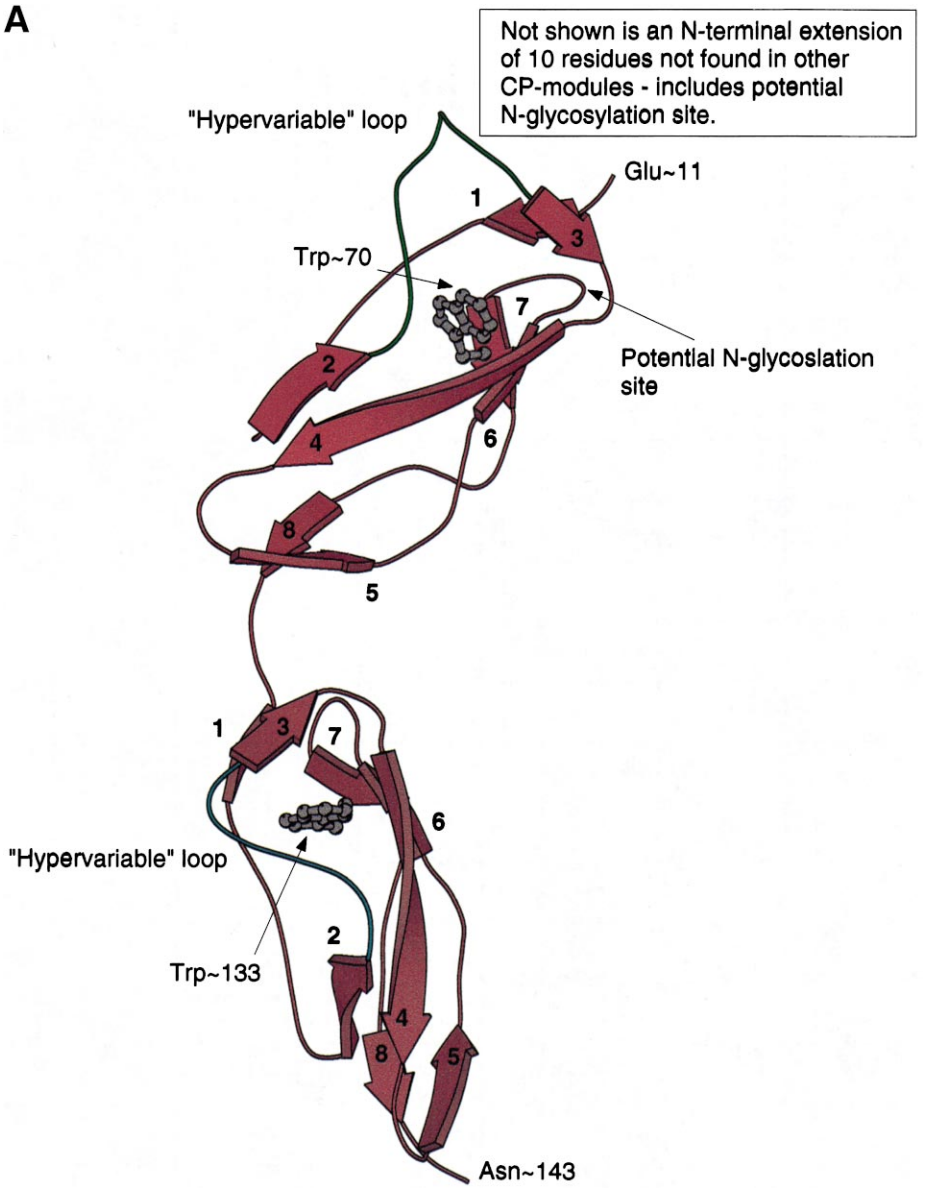

Fig. 5. Model of the N-terminus of $GABA_BR1a$. A: MOLSCRIPT [20] representation. The invariant Trp residues are shown, potential *N*-glycosylation sites and the hypervariable loops are indicated. Strands are numbered as in Fig. 1. B: Accessible surface calculation and imaging (same orientation as the previous diagram) done using GRASP [21]. The hypervariable loops are coloured purple in the first module and green in the second module.

than the calorimetric enthalpy obtained for CR1∼15,16 (120 kcal/mol, data not shown) but much higher than would be expected for a single CP. The enthalpy calculated from the van 't Hoff equation is 67 kcal/mol which is lower than the calorimetric enthalpy. This implies that the protein does not represent a single cooperative system. Rather this situation [16] is consistent with independent, or weakly interdependent, units which have very similar melting temperatures. Therefore, this experiment supports the presence of two similarly-folded modules.

Taken together, the sequence alignment, CD and DSC provide strong support for the hypothesis that this sequence folds into a tandem pair of CPs. Fig. 5 shows the outcome of a homology modelling exercise based on the sequence alignment of Fig. 1. The model was assessed using WHATIF [17] and PROCHECK [18]. For each of the checks performed by WHATIF the quality of the model was comparable to that obtained for the calculated structure of VCP3,4. The overall G factor for the model obtained from PROCHECK, which is a carefully weighted average of all the stereochemical analyses performed by PROCHECK, was −0.46. Structures are considered 'normal' if they have an overall G value greater than −0.50 [18].

Complement protein modules, which are frequently *N*-glycosylated, are globular in structure with a compact hydrophobic core containing side-chains of highly conserved residues, wrapped in β-sheets whose short strands lie approximately parallel with the long axis of the module (reviewed in [19]). Two disulphide linkages stabilise this framework-like structure while loops and turns serve as sites for insertions. A

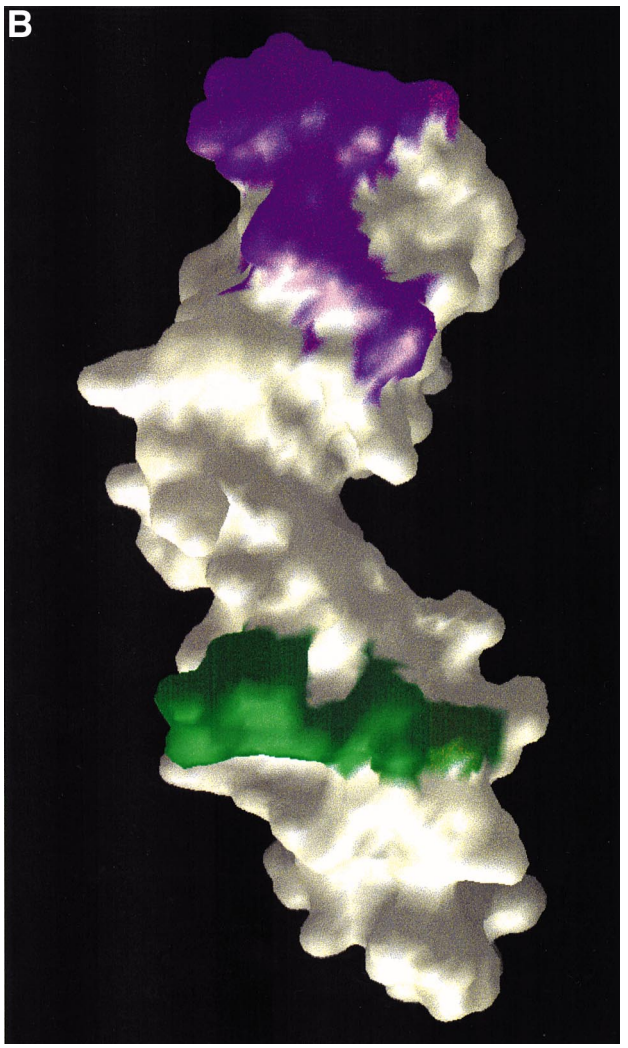

Fig. 5 (*continued*).

prominent loop region of low sequence conservation protrudes laterally to the long axis and is called the hypervariable loop. The N-terminal residue is located at one extremity of the long axis, the C-terminal residue at the other. A MOLSCRIPT [20] representation of the model for $GABA_Br1a$ is shown with strands numbered as in Fig. 1. Also shown is a solvent accessible surface (GRASP [21]). The three-dimensional model reveals a highly extended structure with a large surface area-to-volume ratio. Particularly prominent is the insertion between strands 2 and 3 in the hypervariable region of the first module which results in a large loop close to the N-terminus. This region has been implicated in binding specificity in the case of other CPs. A potential *N*-glycosylation site lies at Asn ~ 7 which is within a 10-residue N-terminal extension (not modelled), and a second *N*-glycosylation site ocurs at Asn ~ 67 which is nearby in the loop between strands 6 and 7. The observed glycosylation of the recombinant $GABA_Br$ fragment is consistent with the surface exposure of this site in the model.

A range of specific protein/protein recognition events, both at the cell surface and in the serum are mediated by CPs. Amongst the known or suspected ligands for these functionally diverse modules are: key proteins of the complement system; interferon-α and interleukin-15; CD23; CD97; vitamin K-dependent protein S; heparin; viruses including the Epstein-Barr virus, the measles virus, and echovirus; and bacterial proteins (see [13] for references). CP-like sequences have been identified so far in more than 50 distinct proteins from sources such as plasma, the surface of many cell types, the spermatozoon acrosomal matrix (e.g. [22]), the retina [23] and the CNS [14,24–26].

The discovery of CPs in a $GABA_B$ receptor is entirely novel and unexpected. Their location at the N-terminal extremity of the protein indicates that they are probably exposed to the extracellular environment. In general, where CPs occur towards the N-terminus of cell-surface receptors they are involved in ligand recognition rather than playing a purely structural role – this has been shown for the N-terminal two modules of CR2 [6], the N-terminal three modules of CR1 [7], the N-terminal two modules of MCP (for measles virus) [9] and CPs 2, 3 and 4 of DAF [8]. The two CPs of $GABA_Br1a$ are the only features that distinguish it from $GABA_Br1b$ [1]. $GABA_Br1a$ and $GABA_Br1b$ have distinct cellular localisations within the brain suggestive of different physiological roles [27]. It thus appears very likely indeed that the two $GABA_Br1a$ CPs are involved in molecular recognition and furthermore that important physiological functions are mediated via ligation of the CPs. It is unlikely that the ligand is GABA or its analogues since the pharmacological profiles of the two subtypes are nearly identical with regard to established $GABA_Br$ agonists and antagonists [1]. Although the N-terminal regions of the metabotropic glutamate receptors exhibit some similarity to the N-terminus of $GABA_Br1a$ [1], it is not possible that these much shorter regions form CPs.

Precedents do exist for the occurrence of putative CP-modules in CNS proteins. For example, SEZ-6 is a mouse membrane protein of unknown function which contains five CP-like sequences, and whose expression is increased by the action of convulsant drugs [14]. The chondroitin sulfate proteoglycans brevican and neurocan each contain a single CP-like sequence [25,26]. Brevican is found on astrocytes, while neurocan is neurone-specific. Both proteins are involved in development, and neurocan is known to bind to tenascin-C possibly via its CP-like sequence [28]. The Hikaru genki protein is a *Drosophila* protein containing CP-like sequences [26] that is secreted from pre-synaptic terminals into the synaptic cleft at critical stages during development and is thought to be involved in formation of functional neural circuits [29]. It seems, therefore, that putative CNS CPs are involved in binding events critical for both normal and abnormal synaptic function and development. $GABA_BR1a$ is known to have relevance to epilepsy: a decrease in inhibitory neurotransmission levels involving $GABA_Br$ has been reported in animal models of temporal lobe epilepsy [30], while an increased level of $GABA_B$-mediated transmission may be involved in absence seizures [31]; the human gene for $GABA_Br1$ has been mapped to Chr 6p21.3 which is in the vicinity of the locus for juvenile myoclonic epilepsy [32]. Thus the CPs of $GABA_Br1a$ might represent extremely important new drug targets.

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References

[1] Kaupmann, K., Huggel, K., Heid, J., Flor, P.J., Bischoff, S., Mickel, S.J., McMaster, G., Angst, C., Bittiger, H., Froestl, W. and Bettler, B. (1997) Nature 386, 239–246.
[2] Barnard, E.A., Darlinson, M.G. and Seeburg, P. (1987) Trends Neurosci. 10, 502–509.
[3] Bowery, N.G. (1993) Annu. Rev. Pharmacol. Toxicol. 33, 109–147.
[4] Kerr, D.I. and Ong, J. (1996) Drug Discov. Today 1, 371–380.
[5] Day, A.J., Campbell, R.D. and Reid, K.B.M. (1989) in: Progress in Immunology VII (Melchers, F. et al., Eds.) pp. 209–212, Springer-Verlag, Heidelberg.
[6] Lowell, C.A., Klickstein, L.B., Carter, R.H., Mitchell, J.A., Fearon, D.T. and Ahearn, J.M. (1989) J. Exp. Med. 170, 1931–1943.
[7] Krych, M., Hourcade, D. and Atkinson, J.P. (1991) Proc. Natl. Acad. Sci. USA 88, 4353–4357.
[8] Coyne, K.E., Hall, S.E., Thompson, E.S., Arce, M.A., Kinoshita, T., Fujita, T., Anstee, D.J., Rosse, W. and Lublin, D.M. (1992) J. Immunol. 149, 2906–2913.
[9] Manchester, M., Valsamakis, A., Kaufman, R., Liszewski, M.K., Alvarez, J., Atkinson, J.P., Lublin, D.M. and Oldstone, M.B.A. (1995) Proc. Natl. Acad. Sci. USA 92, 2303–2307.
[10] Privalov, P.L. and Potekhin, S.A. (1986) Methods Enzymol. 131, 4–51.
[11] Sali, A. and Blundell, T.L. (1993) J. Mol. Biol. 234, 779–815.
[12] Barlow, P.N., Steinkasserer, A., Norman, D.G., Kieffer, B., Wiles, A.P., Sim, R.B. and Campbell, I.D. (1993) J. Mol. Biol. 232, 268–284.
[13] Wiles, A.P., Shaw, G., Bright, J., Perczel, A., Campbell, I.D. and Barlow, P.N. (1997) J. Mol. Biol. 272, 253–265.
[14] Shimizunishikawa, K., Kajiwara, K., Kimura, M., Katsuki, M. and Sugaya, E. (1995) Mol. Brain Res. 28, 201–210.
[15] Kirkitadze, M.D., Narizhneva, N.V., Tomashevski, A.Yu., Potekhin, S.A. and Uversky, V.N. (1996) Russ. J. Bioorg. Chem. 22, 353–358.
[16] Privalov, P.L. (1982) Adv. Protein Chem. 35, 1–104.
[17] Vriend, G. and Sander, C. (1993) J. Appl. Crystallogr. 26, 47–60.
[18] Laskowski, R.A., McArthur, M.W., Moss, D.S. and Thornton, J.M. (1993) J. Appl. Crystallogr. 26, 283–291.
[19] Barlow, P.N. and Campbell, I.D. (1994) Methods Enzymol. 239, 464–485.
[20] Kraulis, P.J. (1991) J. Appl. Crystallogr. 24, 946–950.
[21] Nicholls, A., Bharadwaj, R. and Honig, B. (1993) Biophys. J. 64, A166.
[22] Bookbinder, L.H., Cheng, A. and Bleil, J.D. (1995) Science 269, 86–89.
[23] Meindl, A., Carvalho, M.R.S., Herrmann, K., Lorenz, B., Achatz, H., Lorenz, B., Apfelstedtsylla, E., Wittwer, B., Ross, M. and Meitinger, T. (1995) Hum. Mol. Genet. 4, 2339–2346.
[24] Yamada, H., Watanabe, K., Shimonaka, M. and Yamaguchi, Y. (1994) J. Biol. Chem. 269, 10119–10126.
[25] Rauch, U., Karthikeyan, L., Maurel, P., Margolis, R.U. and Margolis, R.K. (1992) J. Biol. Chem. 267, 19536–19547.
[26] Hoshino, M., Matsuzaki, F., Nabeshima, Y. and Hama, C. (1993) Neuron 10, 395–407.
[27] Bischoff, S., Leonhard, S., Reymann, N., Schuler, V., Kaupmann, K. and Bettler, B. (1997) Soc. Neurosci. 23, 954.
[28] Rauch, U., Clement, A., Retzler, C., Frohlich, L., Fassler, R., Gohring, W. and Faissner, A. (1997) J. Biol. Chem. 272, 26905–26912.
[29] Hoshino, M., Suzuki, E., Nabeshima, Y. and Hama, C. (1996) Development 122, 589–597.
[30] Mangan, P.S. and Lothman, E.W. (1996) J. Neurophysiol. 76, 1282–1296.
[31] Vergnes, M., Boehrer, A., Simler, S., Bernasconi, R. and Marescaux, C. (1997) Eur. J. Pharmacol. 332, 245–255.
[32] Kaupmann, K., Mosbacher, J., Schuler, V., Flor, P.J., Froestl, W., Bittiger, H., Sommer, B. and Bettler, B. (1997) Soc. Neurosci. 23, 954.