Biochem. J. (1990) 272, 265–268 (Printed in Great Britain)

The membrane domain of the human erythrocyte anion transport protein

Epitope mapping of a monoclonal antibody defines the location of a cytoplasmic loop near the *C*-terminus of the protein

Shane D. WAINWRIGHT, William J. MAWBY and Michael J. A. TANNER*
Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

We have used synthetic peptides to study the location of the amino acid sequences in the human erythrocyte anion transport protein (band 3) which are recognized by four murine monoclonal antibodies, BRIC 130, 132, 154 and 155. These antibodies are known to react with epitopes in the protein which are on the cytoplasmic side of the membrane. The results suggest that the amino acid residues important for the reaction of BRIC 130 and BRIC 154/155 are located within amino acids 899–908 and 895–901 respectively in the cytoplasmic tail of the protein. The BRIC 132 epitope is located within amino acid residues 813–824. This is part of a surface loop in the protein which probably extends from residue 814 to residue 832 and is located on the cytoplasmic side of the membrane. These results provide direct evidence for the topographical location of a sequence in a poorly understood region of the protein.

INTRODUCTION

The erythrocyte anion transport protein [band 3; see Jennings (1989) for a recent review] carries out the rapid one-for-one exchange of anions across the erythrocyte membrane. This abundant 95 kDa integral membrane glycoprotein has two structurally and functionally distinct domains. The 40 kDa *N*-terminal domain is entirely within the erythrocyte cytoplasm and provides a membrane anchorage site for the erythrocyte skeleton and for several other proteins which include glycolytic enzymes. The *C*-terminal 55 kDa domain is membrane-associated and carries out the anion exchange function of the protein. cDNA cloning and sequencing studies have yielded the complete amino acid sequence of the erythrocyte anion transporter from mouse (Kopito & Lodish, 1985), human (Tanner *et al.*, 1988) and chicken sources (Cox & Lazarides, 1988; Kim *et al.*, 1988).

The mechanism of anion transport in erythrocytes has been extensively studied. The many studies on the binding sites of anion transport inhibitors and on the chemical and enzymic modification of the protein to study its structure and topology have also been reviewed (Jennings, 1985, 1989; Jay & Cantley, 1986; Passow, 1986; Bjerrum, 1989; Tanner, 1989). Knowledge of the topography of the protein will be helpful towards providing a structural framework for understanding the mechanism of anion transport. A basic requirement for any topographical model is the definition of the membrane-spanning regions and the location of the surface loops in the protein. Different authors have suggested that there may be 10 (Jay & Cantley, 1986), 12 (Kopito & Lodish, 1985) or 14 (Passow, 1986; Bjerrum, 1989; Tanner, 1989) membrane-crossing regions. This is largely because of the difficulty of obtaining an unambiguous interpretation of the hydropathy analysis of the sequence of the protein and the incomplete experimental data on the topography. Although there is direct experimental evidence defining the topological location of several points in the protein sequence, there is still uncertainty about the detailed topology in some areas of the protein, particularly towards the *C*-terminus.

We recently described the preparation of several monoclonal antibodies which are reactive with the membrane domain of the human erythrocyte anion transporter and mapped the regions of the anion transporter which are reactive with the antibodies (Wainwright *et al.*, 1989). In this paper we describe experiments using synthetic peptides which have allowed us to examine the epitopes for the antibodies in more detail. The results are consistent with our earlier mapping studies and also allow us to show that a region near the *C*-terminus which had a previously uncertain topological location forms part of a cytoplasmic loop of the protein.

MATERIALS AND METHODS

Protein molecular mass standards and BSA were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Horseradish peroxidase-conjugated anti-mouse immunoglobulins were from Bio-Rad, St. Albans, Herts., U.K. Phosphate-buffered saline (PBS) contained 9 parts of 0.15 M-NaCl and 1 part of 310 mosM sodium phosphate buffer, pH 7.4, prepared as described by Dodge *et al.* (1963).

Synthesis of peptides

Peptides were synthesized using the fMoc [*N*-(9-fluorenylmethoxycarbonyl)] protocol (Atherton *et al.*, 1981) employing a continuous flow solid-phase method with polyamide–kieselguhr resin (Dryland & Sheppard, 1986) on a Milligen 9050 Pep-Synthesiser. The peptides were linked to BSA using glutaraldehyde or *m*-maleimidobenzoyl-*N*-hydroxysulphosuccinimide ester (sulpho-MBS; Pierce & Warriner Ltd., Chester, Cheshire, U.K.).

Immunoblotting

BSA–peptide conjugates (containing 2.5 μg of BSA) were separated by SDS/PAGE on gels containing 8% acrylamide using the method of Laemmli (1970). Immunoblots of the gels (Towbin *et al.*, 1979) were done using Immobilon (Millipore,

Abbreviation used: PBS, phosphate-buffered saline.
* To whom correspondence should be addressed.

Bedford, MA, U.S.A.) as the transfer membrane, and the membrane was blocked with PBS containing 5% skimmed milk powder and 0.2% Tween 20 and washed using PBS containing 0.2% Tween 20. Reaction with the primary antibody and the peroxidase-conjugated second antibody was done as described by Wainwright *et al.* (1989).

Enzyme-linked immunoassay

The BSA–peptide conjugate was dissolved in 0.05 M-Na_2CO_3 and portions containing 1 μg of BSA were added to each well of a 96-well Falcon Microtest III plate (Becton Dickinson Labware, Oxnard, CA, U.S.A.). The plates were left at 4 °C overnight, washed three times with PBS containing 0.05% Tween 20 and blocked by treatment with PBS containing 20% skimmed milk powder and 0.05% Tween 20 for 1 h at 37 °C. A 2-fold serial dilution of the antibody was added to one row of wells and the plate was incubated at 4 °C for 1 h. The plate was washed three times with PBS containing 0.02% Tween 20 and then incubated for 2 h at 4 °C with horseradish peroxidase-conjugated anti-mouse immunoglobulins (1:1000 dilution). After washing three times with PBS containing 0.05% Tween 20 and twice with water, the plate was developed in the dark with *o*-phenylenediamine (0.4 mg/ml) in 0.2 M-Na_2HPO_4 titrated with 0.1 M-citric acid to pH 5.4, containing 0.1 μl of 30% (v/v) H_2O_2/ml. After 15 min the reaction was stopped by the addition of 1 vol. of 4 M-H_2SO_4, and the absorbance of each well was read at 492 nm using a microtitre plate reader (Titretek Multiscan Plus).

RESULTS AND DISCUSSION

We previously examined the regions in the membrane domain of the human erythrocyte anion transporter which bind the mouse monoclonal antibodies BRIC 130, BRIC 132, BRIC 154 and BRIC 155 by studying the reactivity of fragments of the protein generated by proteolytic cleavage and carboxypeptidase Y treatment (Wainwright *et al.*, 1989). In the present study we attempted to define more closely the epitopes in the protein which bound the antibodies by examining the reactivity of synthetic peptides based on the amino acid sequence of the human protein (Tanner *et al.*, 1988).

Sequences reactive with BRIC 130, BRIC 154 and BRIC 155

Our earlier studies showed that the sequences reactive with these three antibodies were degraded by carboxypeptidase Y treatment of the intact protein or fragments of the protein containing the *C*-terminus (Wainwright *et al.*, 1989). The epitopes lie in the cytoplasmically located *C*-terminal tail of the protein, and study of the time course of digestion by carboxypeptidase Y suggested that the epitopes for all of these antibodies were destroyed by the stage when the carboxypeptidase had removed about 1.5 kDa from the *C*-terminus of the anion transporter. The epitope for BRIC 130 was located closer to the *C*-terminus than the epitopes for the other antibodies.

We therefore prepared a series of overlapping synthetic peptides covering the 27 residues of the human anion transporter from amino acid 885 to the *C*-terminus. The amino acid sequences of the peptides and their designations are shown in Fig. 1. The peptides were coupled to BSA and the reactivity of the peptide conjugates with the antibodies was tested by immunoblotting of the conjugates separated by SDS/PAGE.

BRIC 130 reacted strongly with HB3-1, rather more weakly with HB3-1B and showed traces of reactivity with HB3-1A (Fig. 2*a*). Under these conditions no reactivity was observed with unconjugated BSA or with the other peptides in Fig. 1. (These include HB3-1C, which represents the *C*-terminal 5 amino acids, and the HB3-3 series of peptides, which represent the amino acid sequence 813–838.) The reactivity of BRIC 130 with HB3-1 and HB3-1B was confirmed by enzyme-linked immunoassay of the BSA–peptide conjugates immobilized on microtitre plates.

```
          885       890                 900                 911
HB3-1                          F D E E E G R D E Y D E V A M P V
HB3-1A                         F D E E E G R
HB3-1B                                     D E Y D E V A M P V
HB3-1C                                           V A M P V
HB3-2   C L D A D D A K A T F D E E E G
HB3-2A            A K A T F D E E E G

          813       820                 830                 838
HB3-3   F K P P K Y H P D V P Y V K R V K T W R M H L F T G
HB3-3A                                 K T W R M H L F T G
HB3-3B                       Y V K R V K T W R M H L F T G
```

Fig. 1. Sequences of synthetic peptides used to study antibody reactivity

The peptides were prepared as described in the Materials and methods section. The numbers show their location in the sequence of the human erythrocyte anion transport protein (Tanner *et al.*, 1988).

BRIC 155 reacted strongly with HB3-1 and showed reactivity towards HB3-2 and HB3-2A and traces of reactivity with HB3-1A when the antibody was used at a high concentration (Fig. 2*a*). Under these conditions the antibody also showed traces of non-specific reaction with BSA and the other peptide conjugates, although this was significantly weaker than the reaction with HB3-2 and HB3-2A. Strong reaction with HB3-1 remained when more dilute antibody was used, and there was no evidence of non-specific reaction with BSA or the other peptide conjugates. BRIC 154 showed a similar spectrum of reactivity with the peptides, consistent with our previous evidence that BRIC 154 and BRIC 155 bind at closely similar epitopes, although they are different IgG subtypes (Wainwright *et al.*, 1989).

BRIC 130 reacts strongly with HB3-1, which corresponds to amino acids 895–911 of the human anion transporter. The weak reaction obtained with HB3-1B (residues 902–911) and the lack of reaction with HB3-2 and HB3-2A (which span residues 885–900), suggest that the region from around residue 900 towards the *C*-terminus is involved in interaction with this antibody. The lack of reactivity with the *C*-terminal pentapeptide HB3-1C (residues 907–911) may be a consequence of the small size of this peptide, since we have shown that the BRIC 130 epitope is extremely sensitive to carboxypeptidase Y digestion of the anion transporter (Wainwright *et al.*, 1989), suggesting that it extends close to the *C*-terminus of the protein. BRIC 130 does not react with the murine anion transporter (Wainwright *et al.*, 1989), and the differences in sequence between the mouse and human anion transport proteins in the region encompassed by HB3-1 are that the mouse protein contains Asn, Leu and Pro substituted at residues corresponding to 899, 901 and 908 respectively of the human sequence (Kopito & Lodish, 1985). Taken together, the results suggest that these residues, which are important in determining the BRIC 130 epitope, are located in the region between amino acids 899 and 908.

The BRIC 154/155 epitopes are located further from the *C*-terminus of the anion transporter than the BRIC 130 epitope, since they are more resistant to carboxypeptidase Y treatment of the protein (Wainwright *et al.*, 1989). BRIC 154 and BRIC 155 both react with the murine protein, although BRIC 155 shows a weaker reaction. This is consistent with the strong reaction of the antibodies with HB3-1 and the lack of reaction with the peptides corresponding to residues 902–911. The very weak reaction with HB3-1A, which comprises the *N*-terminal portion of HB3-1 (residues 895–901), may be due to the small size of this hexapaptide. The results suggest that the highly charged cluster with an overall strongly acidic character represented by residues 895–901 is important in the epitopes for BRIC 154 and BRIC

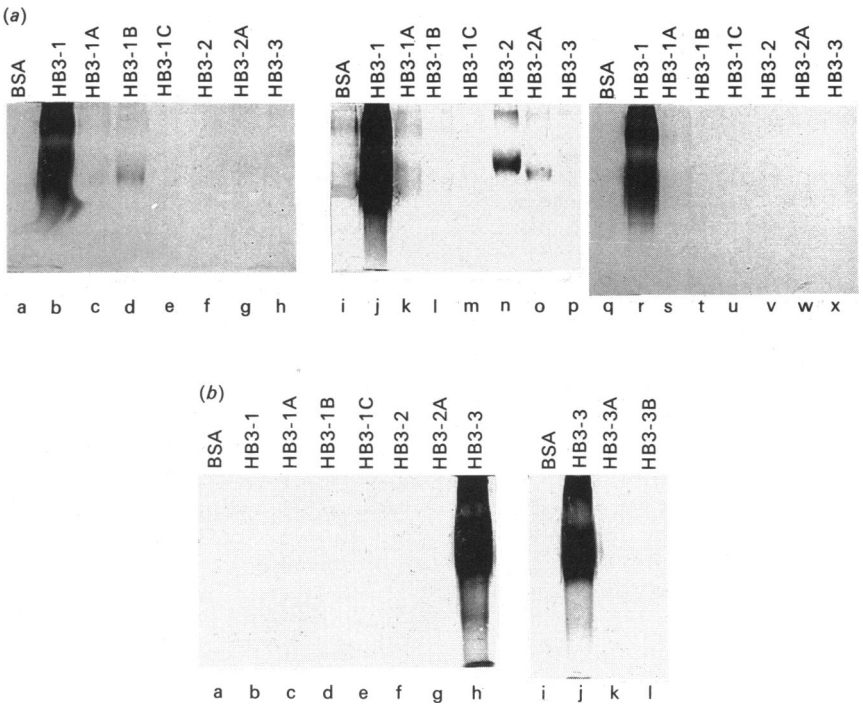

Fig. 2. Immunoblots of BSA-conjugated peptides probed with monoclonal antibodies

(*a*) Immunoblots probed with BRIC 130 and BRIC 155. BSA and BSA-conjugated peptides were separated by SDS/PAGE using 8% polyacrylamide gels. The gels were transferred to Immobilon and probed with BRIC 130 (lanes a–h), neat culture supernatant of BRIC 155 (lanes i–p) or a 1:1000 dilution of the culture supernatant of BRIC 155 (lanes q–x). Tracks a, i and q contained 2.5 μg of unconjugated BSA as a control; tracks b, j and r contained 2.5 μg of BSA-conjugated HB3-1; tracks c, k and s contained 2.5 μg of BSA-conjugated HB3-1A; tracks d, l and t contained 2.5 μg of BSA-conjugated HB3-1B; tracks e, m and u contained 2.5 μg of BSA-conjugated HB3-1C; tracks f, n and v contained 2.5 μg of BSA-conjugated HB3-2; tracks g, o and w contained 2.5 μg of BSA-conjugated HB3-2A; tracks h, p and x contained 2.5 μg of BSA-conjugated HB3-3. (*b*) Immunoblots probed with BRIC 132. BSA and BSA-conjugated peptides were separated by SDS/PAGE using 8% polyacrylamide gels. The gels were transferred to Immobilon and probed with BRIC 132. Tracks a and i contained 2.5 μg of BSA; track b contained 2.5 μg of BSA-conjugated HB3-1; track c contained 2.5 μg of BSA-conjugated HB3-1A; track d contained 2.5 μg of BSA-conjugated HB3-1B; track e contained 2.5 μg of BSA-conjugated HB3-1C; track f contained 2.5 μg of BSA-conjugated HB3-2; track g contained 2.5 μg of BSA-conjugated HB3-2A; tracks h and j contained 2.5 μg of BSA-conjugated HB3-3; track k contained 2.5 μg of BSA-conjugated HB3-3A; track l contained 2.5 μg of BSA-conjugated HB3-3B.

155. The reactivity (albeit weak) of the antibodies with HB3-2 and HB3-2A suggests that there may be a contribution from the sequence on the *N*-terminal side of residue 895 to the structure recognized by the antibody in the intact protein. Although the linear peptide sequences defined in this study probably provide the major sites of binding affinity and specificity for the antibodies, other regions might also contribute to the binding of the antibody to the intact protein.

Sequences reactive with BRIC 132

BRIC 132 was found to react strongly with HB3-3, which corresponds to residues 813–838 of the protein (Fig. 2*b*). No reactivity was observed with any of the peptides corresponding to the *C*-terminus of the protein (HB3-1 and HB3-2 or their subfragments), nor was any reactivity observed with the fragments of HB3-3 (HB3-3A and HB3-3B), which span residues 824–838 of the protein. The residues important for the reactivity of BRIC 132 therefore appear to lie in the proline-rich region at the *N*-terminus of HB3-3, which corresponds to residues 813–824 and has an overall basic charge. The BRIC 132 epitope has been shown to be completely resistant to digestion of erythrocyte membranes by carboxypeptidase Y, suggesting that the epitope is located on an internal loop in the protein where it is protected from exopeptidase digestion because the polypeptide enters the membrane on the *C*-terminal side of the epitope. The location of the epitope within amino acid residues 813–824 is consistent with this result.

It has been shown that BRIC 132 reacts with the protein on the cytoplasmic side of the membrane (Wainwright *et al.*, 1989), and therefore part of the sequence Phe-813–Tyr-824 must lie on this side of the membrane. This is part of a very basic and polar sequence which extends from Lys-814 to Arg-832, which is therefore likely to form a surface loop in the protein which is located in the cytoplasm. Direct evidence on the topographical location of this portion of the protein sequence has previously been lacking, although we have suggested that tyrosine residues in the *C*-terminal region of the protein which are susceptible to lactoperoxidase radioiodination from the cytoplasmic side of the membrane originate from this sequence (Tanner, 1989). Since the *C*-terminus of the protein is known to be located on the cytoplasmic side of the membrane (Lieberman *et al.*, 1987; Lieberman & Reithmeier, 1988; Wainwright *et al.*, 1989), the polypeptide chain must cross the membrane at least twice between the BRIC 132 epitope and the *C*-terminus. Lys-851 is found between the two likely membrane-crossing segments in this region and would therefore be expected to be extracellular. There is evidence that this is the case, since this lysine residue is labelled by pyridoxal phosphate treatment and is also a likely site of covalent reaction with 4,4′-di-isothiocyanatodihydrostilbene-2,2′-disulphonate (H_2DIDS) at an alkaline pH. (Jennings *et al.*,

1986; Kawano *et al.*, 1988; Jennings, 1989; Tanner, 1989). The results in this paper help to build up our knowledge of the structure of the erythrocyte anion transporter by providing direct evidence for the location of a region of the protein for which there is little topographical information.

This work was supported in part by a grant from the Wellcome Trust. W.J.M. is supported by the Central Blood Laboratory Authority.

REFERENCES

Atherton, E., Logan, C. J. & Sheppard, R. C. (1981) J. Chem. Soc. Perkin Trans. 1 538–546
Bjerrum, P. J. (1989) Methods Enzymol. **173**, 466–493
Cox, J. V. & Lazarides, E. (1988) Mol. Cell. Biol. **8**, 1327–1335
Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) Arch. Biochem. Biophys. **100**, 119–130
Dryland, A. & Sheppard, R. C. (1986) J. Chem. Soc. Perkin Trans. 1 125–137
Jay, D. & Cantley, L. (1986) Annu. Rev. Biochem. **55**, 511–538
Jennings, M. L. (1985) Annu. Rev. Physiol. **47**, 519–533
Jennings, M. L. (1989) Annu. Rev. Biochem. **18**, 397–430
Jennings, M. L., Anderson, M. P. & Monaghan, R. (1986) J. Biol. Chem. **261**, 9002–9010
Kawano, Y., Okubo, K., Tokunaga, F., Miyata, T., Iwanaga, S. & Hamasaki, N. (1988) J. Biol. Chem. **263**, 8232–8238
Kim, H. C., Yew, N. S., Ansorge, W., Voss, H., Schwager, C., Vennstrom, B., Zenke, M. & Engel, J. D. (1988) Mol. Cell. Biol. **8**, 4416–4424
Kopito, R. R. & Lodish, H. F. (1985) Nature (London) **316**, 234–238
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lieberman, D. M. & Reithmeier, R. A. F. (1988) J. Biol. Chem. **263**, 10022–10028
Lieberman, D. M., Nattriss, M. & Reithmeier, R. A. F. (1987) Biochim. Biophys. Acta **903**, 37–47
Passow, H. (1986) Rev. Physiol. Biochem. Pharmacol. **103**, 62–203
Tanner, M. J. A. (1989) Methods Enzymol. **173**, 423–431
Tanner, M. J. A., Martin, P. G. & High, S. (1988) Biochem. J. **256**, 703–712
Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
Wainwright, S. D., Tanner, M. J. A., Martin, G. E. M., Yendle, J. E. & Holmes, C. (1989) Biochem. J. **258**, 211–220

Received 28 August 1990/17 September 1990; accepted 18 September 1990