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IMMUNOLOGICAL AND PARTIAL SEQUENCE IDENTITY OF MOUSE BM180 WITH WHEAT α–GLIADIN

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BM180, a novel 180-kDa basement membrane protein enriched in guanidine-HCl extracts of lacrimal and parotid exocrine secretory glands, was immunopurified using the secretion inhibitory monoclonal antibody 3E12. The N-terminal amino acid sequence was found to be VRVPVPQLQPQNP. An identical sequence comprises the N-terminus of the wheat storage protein α-gliadin. The presence of a gliadin-like protein in basement membranes was confirmed using a monoclonal and several polyclonal anti-gliadin antibodies, the former of which detected a 180-kDa protein in basement membrane blots. A full-length α-gliadin cDNA was found to hybridize at high stringency with mouse and human genomic DNA; and in lacrimal gland Northern blots with a 2.3-kb message. Since BM180 appears to be required for stimulus-secretion coupling by lacrimal acinar cells, circulating anti-α-gliadin antibodies associated with Sjögren's syndrome ('Dry Eye') and more commonly in Coeliac disease, may be secretion inhibitory.

Basement membrane is a thin adhesive extracellular matrix which influences cell behavior. A functional screen of basement membrane was used to search for activity(s) possibly required for the developmental appearance of stimulus-secretion coupling by lacrimal acinar cells (1). An active lower molecular weight peak was pursued by preparation of monoclonal antibodies one of which partially inhibited stimulated secretion, and in Western blots detected a 180 kDa protein (designated 'BM180') which localized to the periacinar basement membrane (1).

Here we present N-terminal sequence data for BM180 which, together with use of a monoclonal and several polyclonal antibodies, suggest that BM180 is a mammalian form of the gliadin family of wheat proteins. These data are of potential interest to the molecular understanding of Sjögren's syndrome and Coeliac disease in which circulating anti-α-gliadin antibodies are present. (2).

MATERIALS AND METHODS

Substrates and antibodies: Unfractionated gliadin was purchased from Fluka (Ronkonkoma NY). BMS was prepared by EDTA extraction of EHS tumor matrix (3). Preparation of rat anti-

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Abbreviations: BMS, basement membrane substrate; mab, monoclonal antibody.

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mouse BM180 mab ('3E12'; ref 1); and of rabbit anti-α-, -β-, -γ-, and -ω-gliadin antisera (4) was as previously described. Mouse anti-α-gliadin monoclonal antibodies KG9 (IgM; antigen is amino acids 206 - 217 (5)), WB8 (IgG1) and WC2 (IgM; antigen for both is amino acids 3 - 56 (6)) were previously prepared and characterized for their reactivity with α-gliadin.

Enzyme-linked immunoassay and Western blotting: ELISA's were performed as described (7). Blots were carried out using a PBS, 0.1% Tween block, KG9 supernatant diluted 1/50 - 1/500 in PBS, 0.1% Tween, 1% BSA, and a secondary peroxidase-conjugated donkey anti-mouse antibody diluted 1/1000 in PBS, 0.1% Tween, 1% normal goat serum. BMS for blotting and immunopurification was precleared of endogenous mouse IgG by preincubation with protein G columns using a bridging goat anti-mouse antibody.

Immunopurification: Precleared BMS in 0.05 M Tris containing 0.15 M NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, pH 8.0, was incubated overnight (4°C) with columns prepared by coupling purified 3E12 mab or serum-free concentrated 3E12 culture supernatant with AminoLink or Affi-Gel Hz (BioRad, Hercules CA), respectively. Following extensive PBS washing, elution was with triethylamine. Eluant was neutralized, and either applied directly to an ELISA plate (detection with KG9 mab), or both concentrated and PBS washed in a Centricon 10. Concentrated eluant was examined by silver staining SDS-PAGE gels. For N-terminal sequencing, the BM180 band was cut from a SDS PAGE gel containing a lower molecular weight BMS peak ('peak 2'; ref 1) run without prior DTT reduction; the sample was sequenced in an ABI 470a (University of Virginia Biomolecular Research Facility; Applied Biosystems; Foster City CA) using a membrane cartridge block.

Southern and Northern analysis: Genomic mouse and human DNA were isolated from F9 teratocarcinoma and HT1080 cells, respectively, following standard protocols (8). Genomic wheat (triticum uartu) DNA was supplied by Dr. T. Okita (Washington State University, Pullman WA). *Bam* HI/*Hind* III digested genomic DNA's (10 μg/lane) were separated on 0.7% agarose gels, transferred to nitrocellulose, and immobilized. Blots were prehybridized at 42°C for 4 hr in 5 x Denhardt's, 5 x SSPE (1 x SSPE = 150 mM NaCl, 10 mM sodium phosphate, 1mM EDTA; pH 7.4), 30% formamide, 0.1% SDS, and 200 μg/ml salmon sperm DNA. Hybridization (42°C for 24 - 48 hr) was carried out in the same solution containing in addition [^{32}P]α-gliadin cDNA. Probe was prepared using glass wool purified insert (full length α-gliadin cDNA 212 [1.1 kb; ref 9), [^{32}P]dCTP (3000 Ci/mmol; ICN, Irvine CA) and the random priming method. Unincorporated labeled nucleotide was removed using a nick spin column (Pharmacia, Piscataway NJ). Following hybridization, blots were washed in 2 x SSC, 0.1% SDS at 65°C, or in 0.1 x SSC, 0.1% SDS at 42°C (1 x SSC = 150 mM NaCl, 15 mM sodium citrate; pH 7.4), and then exposed to x-ray film. Independent experiments were carried out using genomic mouse (kidney) and human (placenta) DNA purchased from Clontech (Palo Alto, CA) with similar results. For Northern analysis, poly A+ RNA was extracted from freshly isolated rat and mouse lacrimal acinar cells, and various other mouse organs, or human HT1080 cells, using an mRNA purification kit (Pharmacia, Piscataway NJ). Wheat poly A+ RNA was provided by Dr. T. Okita. RNA (9 μg/lane) was separated on 0.8% agarose gels and transferred to nitrocellulose. Hybridization and washing were as above.

RESULTS

The BM180 band cut from a gel of lower molecular weight BMS material was subjected to microsequencing analysis revealing a 13 amino acid N-terminal sequence with identity to the N-terminus of the wheat storage protein α-gliadin (Fig. 1). Recently, the cytoplasmic Ca^{+2} binding protein calreticulin was found to contain sequence similarity to amino acids 21 - 30 of α-gliadin, an

	1	20
α-Gliadin	VRVPVPQLQPQNPSQQQPQE	
BM180	VRVPVPQLQPQNP	

Fig. 1. N-terminal sequence of the secreted form of α-gliadin (Triticum aestivum), as compared to BM180 sequence obtained by Edman degradation.

observation leading to the suggestion that calreticulin (62 kDa without reduction) was an autoantigen in Coeliac disease (10). Coeliac disease (gluten intolerance) is a common autoimmune disease characterized by the presence of circulating anti-α-gliadin antibodies (2), the latter of which have also been reported in Sjögren's syndrome (11) whose ocular component is insufficient lacrimal gland secretion.

We tested various anti-gliadin antisera and mab's for cross-reactivity with BMS. Anti-α-gliadin antiserum cross-reacted strongly (Fig. 2a, inset), whereas anti-β- (Fig. 2a, inset), anti-γ-, and anti-ω-gliadin (not shown) antisera displayed lower titer. Of three anti-α-gliadin mab's, KG9 but not WB8 or WC2 cross-reacted with BMS (Fig. 2a). Comparative titer on gliadin was at least 50 fold higher for all the antibodies. 3E12 mab displayed cross-reactivity with gliadin (titer approximately 25 fold lower than for BMS; not shown) and could be inhibited from binding to BMS by soluble gliadin (IC_{50} of 37 µg/well vs. 1.5 µg/well for BMS; cytochrome C used as an internal negative control at a three-fold molar excess to gliadin had no effect).

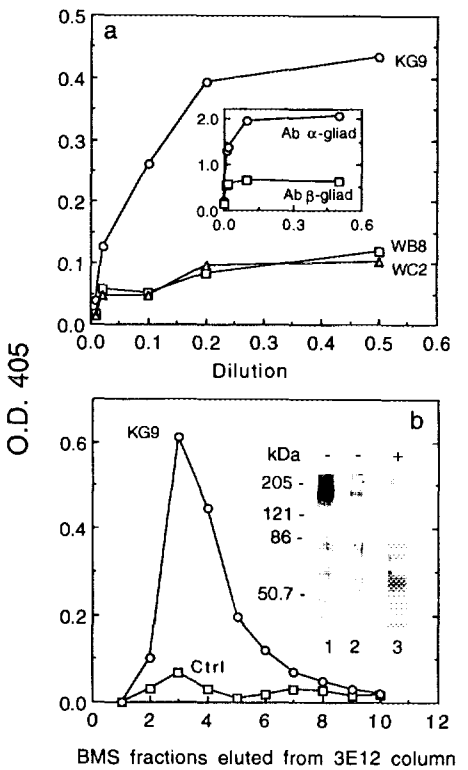

Fig. 2. (a) ELISA dilution series of anti-α-gliadin mab's on BMS-coated wells. Inset, dilution series of anti-α-gliadin and anti-β-gliadin antisera. 0.1 = 1/10 dilution. (b) ELISA detection of BM180 eluate from a 3E12 mab column using KG9 mab diluted 1/50. Inset, silver stained SDS PAGE gel (8%) of eluate run without or with prior DTT reduction. Lanes 1 and 2 are from two different columns. Lanes 2 and 3 contain the same eluate in equal loads. Contaminating column rat IgG is the thin band above the 205-kDa marker nonreduced and the lightly stained material below the 60-kDa BM180 band reduced.

To ask whether KG9 mab detects BM180, two sets of experiments were performed. KG9 mab was first tested for reactivity with immunopurified BM180, the latter of which (Fig. 2b, inset) was prepared by incubating BMS with 3E12 mab columns followed by extensive (100 column volume) PBS washing and triethylamine elution. KG9 mab specifically detected the eluted material (Fig. 2b) and showed no reaction with the last 0.5 ml of the PBS wash, nor with triethylamine alone (not shown). A similar result was obtained using anti-α-gliadin antiserum (not shown). We next incubated KG9 mab with BMS blots, and found that it detected a single non-reduced 180 kDa protein (Fig. 3, lane 1), an interaction which was lost upon reduction (not shown). Native wheat α-gliadin, by comparison, has a molecular weight of 32 - 36 kDa (12).

Are gliadin-like genes present in the mammalian genome, and if so, are they expressed? A full length (1.1 kb) α-gliadin cDNA probe was hybridized with Southern blots containing *Bam* HI/*Hind* III fragmented wheat and mouse genomic DNA (Fig. 3, lanes 2, 3), or wheat, mouse and human genomic DNA (Fig. 3, lanes 4-6). Washing at moderately high stringency (2 x SSC, 65°C) revealed five wheat and eight mouse bands consistent with the existence of several genes (Fig. 3, lanes 2, 3; in wheat >100 genes/genome exist differing in single base substitutions, duplications and deletions; ref 9). Similar results were obtained at higher stringency (0.1 x SSC, 42°C; Fig. 3, lanes 4 - 6) for wheat, mouse and human DNA. The same probe detected one or two mRNA species in Northern blots (Fig. 4) of poly A+ RNA from various mouse organs (Fig. 4a). An mRNA species of 2.3 kb was apparent in freshly isolated mouse and rat lacrimal acinar cells (Fig. 4b; non-wheat signal lost at 0.1 x SSC, 50°C), in keeping with a 60 kDa protein. An additional 5.2 kb message becomes prominent in poly A+ RNA from whole mouse lacrimal gland (not shown). These differ from calreticulin for which mRNA of 1.9 kb is observed (13).

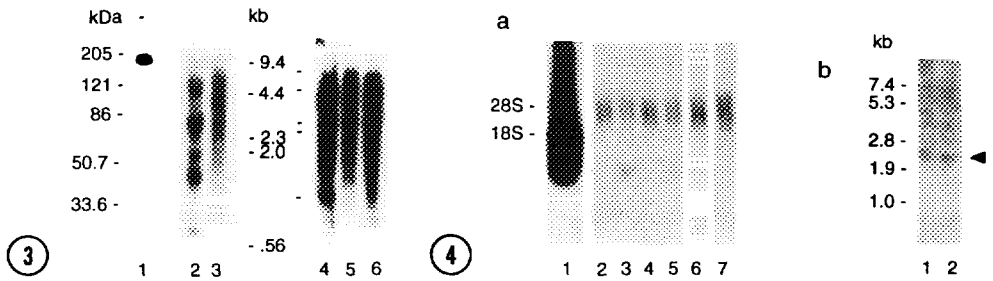

Fig. 3. *Lane 1,* Anti-α-gliadin mab KG9 detects a single 180-kDa band in BMS blots run without reduction. Thin band above 205-kDa marker is BMS-associated mouse IgG which is detected by the secondary anti-mouse antibody. *Lanes 2-6,* hybridization of [^{32}P]α-gliadin cDNA with *Bam* HI/*Hind* III-fragmented wheat (lanes 2, 4), mouse (lanes 3, 5) and human (lane 6) genomic DNA. Wash conditions were 2 x SSC, 65°C (lanes 2, 3) and 0.1 x SSC, 42°C (lanes 4-6). Blot was exposed overnight to x-ray film.

Fig. 4. (a) Hybridization of [^{32}P]labeled wheat α-gliadin cDNA with poly A RNA from wheat (lane 1), mouse kidney, brain, pancreas, lung, small intestine (lanes 2-5, respectively), human HT1080 cells (lane 6). (b) Hybridization of the same probe with poly A RNA from freshly isolated mouse (lane 7) and rat (lane 8) lacrimal acinar cells. Wash conditions were 2 x SSC, 60°C (a) and 0.1 x SSC, 37°C (b); blots were exposed to x-ray film overnight.

DISCUSSION

Here we present evidence suggesting that BM180 is a member of the α-gliadin subfamily of storage proteins common in wheat. Genes encoding such proteins have not to date been investigated in the context of the animal kingdom. A recent search for Coeliac disease autoantigens using an anti-α-gliadin mab column as immunoaborbent and rat enterocyte lysates as substrate led to the immunopurification of the cytoplasmic calcium binding protein calreticulin via a 10-mer sequence with some similarity to amino acids 21 - 30 of α-gliadin (10). Partial sequence homology has been reported between α-gliadin amino acids 211 - 217 and the E1b protein of human adenovirus 12, prior infection with which has been proposed by some (14) but disputed by others (5) to be a precipitating factor in Coeliac disease. Coeliac disease is a gliadin-(gluten) sensitive enteropathy characterized by lowered height of intestinal villi associated with patient sera reactive both with gliadin (2) and basement membrane-like structures (commonly referred to as endomysial or reticular staining; ref 15). Patients suffering from Sjögren's syndrome, an autoimmune disease whose ocular component is deficient lacrimal gland secretion, are also reported to have elevated circulating anti-gliadin antibodies (11). Identification of putative autoantigen(s) has been the focus of a number of groups (10, 16), for which BM180 would appear to be a candidate. Basement membrane components involved in autoimmune disease include collagen IV (α3 chain is the antigen in Goodpasture's syndrome; mutations in α3, α4 or α5 chains trigger Alport's syndrome; ref. 17) and laminin-1 (consequence of Chagas disease; ref. 18).

Several anti-gliadin antibodies were found to cross-react with BMS, particularly an anti-α-gliadin antiserum and the anti-α-gliadin mab KG9, whose antigen (α-gliadin synthetic peptide comprising amino acids 206 - 217; ref 5) could make the mab capable of detecting adenovirus E1b protein - should it or a similar viral protein be present in BMS. Native E1b protein (54 kDa; ref. 14), however, differs in molecular weight from BM180; a search of the NCBI non-redundant combined protein database failed to detect any other non-gliadin protein with sequence homology to this region. KG9 mab detected a 180 kDa protein in BMS blots and could be used to effectively monitor the elution of BM180 from 3E12 mab immunopurification runs, an observation which when taken together with the nature of the KG9 antigen suggested that BM180's α-gliadin homology extended beyond the sequenced N-terminus. In contradistinction, anti-α-gliadin mab's WB8 and WC2 prepared against an N-terminal α-gliadin tryptic fragment (amino acids 3 - 56; ref. 6) failed to cross-react with BMS, suggesting that α-gliadin amino acids 1 - 13 did not comprise the epitope(s) and that BM180 sequence immediately carboxyl to the sequenced N-terminus shared limited gliadin homology. Using Southern analysis, an α-gliadin cDNA as probe and stringent washing, we determined that several gliadin-like genes exist in mouse and human genomes. Hybridization of the same probe with lacrimal acinar cell poly A+ RNA detected an mRNA species of 2.3 kb, in keeping with a 60 kDa monomer. Also detected was an additional 5.2 kb message in whole lacrimal gland poly A+ RNA. These message sizes differed from calreticulin (1.9 kb; ref 13) whose level of homology (EPPVIQNPEY [calreticulin] vs QVPLVQQQQF [α-gliadin]; ref 10) would appear insufficient to support hybridization under the conditions employed. Further analysis of BM180 awaits cDNA cloning.

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REFERENCES

1. Laurie, G.W., Glass, J.D., Ogle, R.A., Stone, C.M., Sluss, J.R., and Chen, L., submitted.
2. Heine, R.G., and Catto-Smith, A.G. (1993) J. Paediatr. Child Health 29:331-334.
3. Matter, M.L., and Laurie, G.W. (1994) J. Cell Biol. 124:1083-1090.
4. Ciclitira, P.J., Ellis, H.J., Evans, D.J., and Lennox, E.S. (1985) Br. J. Nutr. 53:39-45.
5. Ellis, H.J., Doyle, A.P., Sturgess, R.P., and Ciclitira, P.J. (1992) Gut 33:1504-1507.
6. Ellis, H.J., Doyle, A.P., Wieser, H., Sturgess, R.P., and Ciclitira, P.J. (1993) Scand. J. Gastroenterol. 28:212-216.
7. Engvall, E., and Perlman, P. (1972) J. Immunol. 109:129-135.
8. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
9. Okita, T.W., Cheesbrough, V., and Reeves, C.D. (1985) J. Biol. Chem. 260:8203-8213.
10. Karská, K., Tucková, L., Steiner, L., Tlaskalová-Hogenová, H., and Michalak, M. (1995) Biochem. Biophys. Res. Commun. 209:597-605.
11. Teppo, A.-M., and Maury, C.P.J. (1984) Clin. Exp. Immunol. 57:73-78.
12. Ewart, J.A.D. (1973) J. Sci. Fd. Agric. 24:685-689.
13. Michalak, M., Milner, R.E., Burns, K., and Opas, M. (1992) Biochem. J. 285, 681-692.
14. Lähdeaho, M.-L., Mäki, M., Parkkonen, P., Kantonen, T., and Lehtinen, M. (1992) Acta Virol. 36:524-532.
15. Valeski J.E., Kumar V., Beutner, E.H., Lerner, A., and Chorzelski, T.P. (1990) Int. Arch. Allerg. Appl. Immunol. 93:1-7.
16. Marttinen, A., and Mäki, M. (1993) Ped. Res. 34:420-423.
17. Hudson, B.G., Reeders, S.T., and Tryggvason, K. (1993) J. Biol. Chem. 269:26033-26036.
18. Towbin, H., Rosenfelder, G., Wieslander, J., Avila, J.L., Rojas, M., Szarfman, A., Esser, K., Nowack, H., and Timpl, R. (1987) J. Exp. Med. 166:419-432.