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# Antibody reactivity profiles following immunization with diverse peptides of the PERB11 (MIC) family

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**SUMMARY**

PERB11 (MIC) is a gene family possessing multiple copies located within the MHC. Structurally, PERB11 is related to the MHC class I, neonatal IgG Fc receptor (FcRn) and Zn-$\alpha_2$-glycoprotein molecules. The MHC class I family is complex in terms of its genomic arrangement, expression and function, and available evidence suggests that the PERB11 family may be similarly complex. We have adopted an approach to study the expression of such complex gene families by immunizing with multiple peptides and by screening the resulting antibodies against a large range of tissues. The amino acid sequences of PERB11.1 and PERB11.2 as well as those of other related molecules were analysed and compared. Peptides were chosen for immunization based upon (i) loop formation within the equivalent known structure of the MHC class I molecules; (ii) immunogenicity by computer analysis; and (iii) evolutionary relationships. Antibodies in serum from immunized rabbits bound to three out of six peptides used for immunization. ELISA and immunoprecipitation demonstrated binding both to the peptides and to the PERB11.2 recombinant protein. By immunofluorescent staining of various tissues of several species, the three antisera generated overlapping profiles of activity. These included reactions with kidney, small and large intestine, oesophagus, testis, ovary and human neutrophils. This is the first description of antibodies induced by the PERB11 peptides. The extreme complexity of these profiles requires further investigation, but may be explained in terms of antibodies against diverse products of the PERB11 gene family and/or related molecules.

**Keywords** PERB11 (MIC) MHC genes anti-peptide antibodies HLA class I like molecules immunofluorescence

## INTRODUCTION

The MHC contains many multicopy gene families which remain to be characterized in full [1,2]. The HLA class I genes can be considered a prototype for studies of other gene families. The number of class I genes is still unknown, as new members are still being discovered. Some are expressed but others are pseudo genes or merely gene fragments [3,4]. Although less well characterized, the PERB11 (MIC) family appears to be similar in these respects [5,6] and also in the fact that there are gene copies physically associated with HLA class I genes [7]. The situation is further complicated by the fact that HLA class I and PERB11 genes can be highly polymorphic [6].

In developing a strategy for the study of the expression of emerging complex gene families, we chose to immunize with diverse peptides, some of which appeared likely to be characteristic of the whole family. We argued that antibodies induced by these peptides would cross-react to a sufficient degree to detect members of the family yet to be sequenced and characterized. Whilst it is appreciated that some motifs may not be specific for a particular family, we hoped that the peptide selected would result in antibodies which would include most members of the family. Our strategy therefore included screening on a very wide range of different tissues. For this purpose we selected immunofluorescence and we included a variety of different preparative procedures. We also

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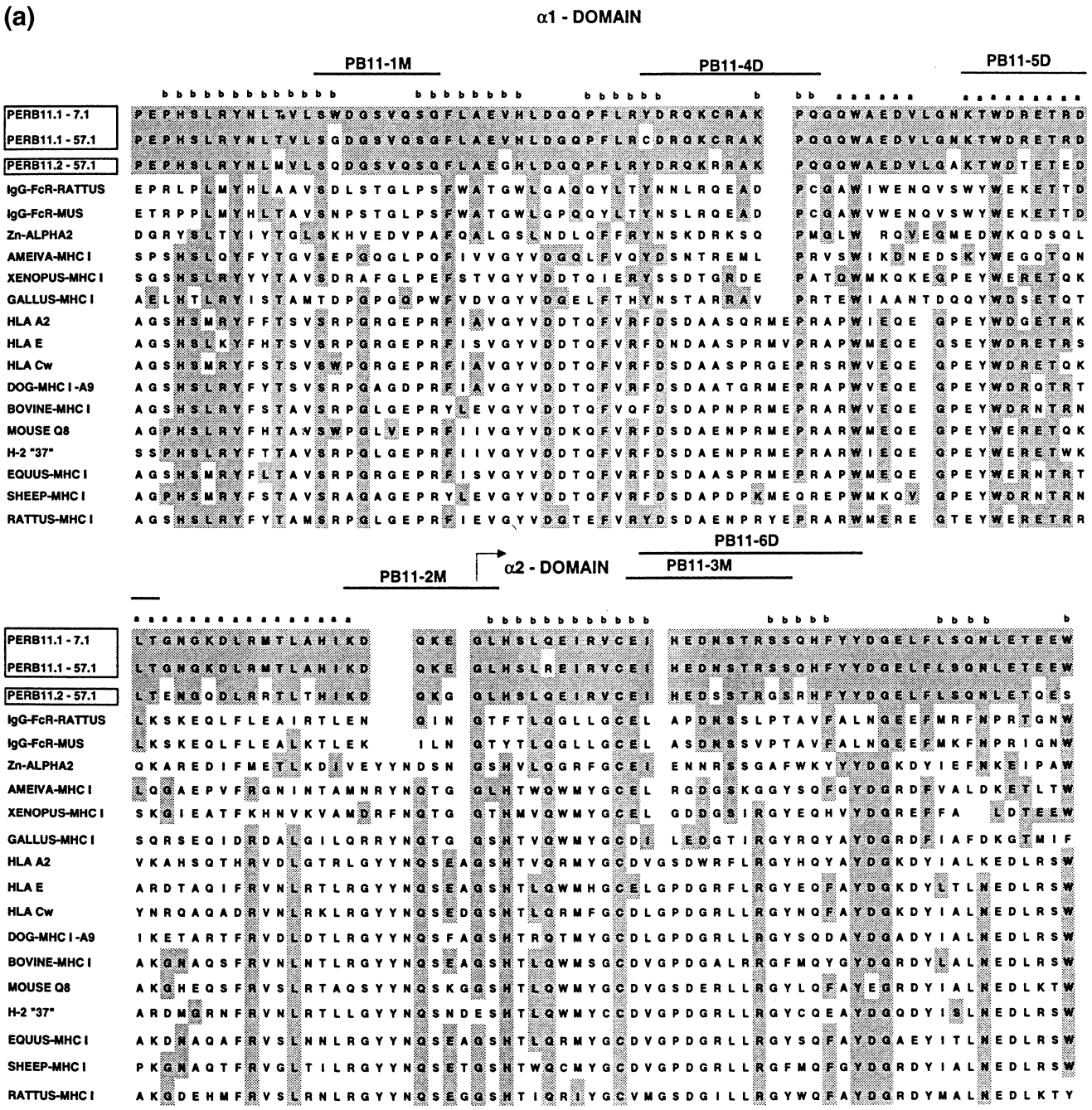


**Fig. 1.** The PERB11 peptides used for antibody production. (a) Amino acid sequences of the $\alpha$-1 and part of the $\alpha$-2 domains of PERB11 and PERB11-related molecules were aligned. These include two alleles of PERB11.1 derived from the 7.1 (PERB11.1–7.1) and 57.1 ancestral haplotypes (PERB11.1–57.1), PERB11.2 from 57.1 ancestral haplotype (PERB11.2–57.1), neonatal IgG Fc receptor from mouse (IgG-FcR-MUS) and rat (IgG-FcR-RATTUS), Zn-$\alpha_2$-glycoprotein (Zn-ALPHA2) and MHC class I from various species including mammalian (HLA-A2, HLA-E, HLA-Cw, DOG-MHCI-A9, BOVINE-MHC I, MOUSE-Q8, H-2 ''37'' EQUSS-MHCI, SHEEP-MHC I and RATTUS-MHC I) and non-mammalian molecules (AMEIVA-MHC I, XENOPUS-MHC I and GALLUS-MHC I). Blanks in the sequences indicate insertions introduced to make the best alignment. Residues identical to those of PERB11.1 derived from the 7.1 ancestral haplotype are shaded. 'b' and 'a' above the sequences represent the regions equivalent to a $\beta$-pleated sheet and an $\alpha$ helix revealed by the crystallography study of the HLA-A2 molecule [16]. Heavy lines above the sequences indicate the peptide sequences chosen for synthesis and immunization of rabbits. It can be seen that some of these peptide sequences are quite specific to the gene family, such as PERB11-4D. In contrast, PB11-5D shares some residues with other related molecules. (b) (See next page.) Plot of predicted characteristics of the PERB11.1 polypeptide, residues 1–128, encoded by the 7.1 ancestral haplotype. The amino acid sequence of PERB11.1 is shown on the top. The predicted properties of the corresponding sequence are shown underneath. The information was used for the selection of peptides for immunization.

 

included tissues at different stages of development and of differing phylogeny.

## MATERIALS AND METHODS

*Peptide syntheses*

Two systems were used to produce the PERB11 peptides. The multiple antigen peptide (MAP) system developed by Posnett *et al.* [8] was used to produce PERB11-1M, -2M and -3M, kindly provided by Dr Posnett (Basel Institute for Immunology, Basel, Switzerland). Each peptide antigen consists of a branching lysine core with eight copies of the peptide linked to the core by the COOH-terminal of the amino acid. The complex has a high immunogenicity without the need to conjugate to a protein carrier and yields a good response to the linked peptide in animals injected [8]. The other three peptides, PERB11-4D, -5D and -6D (purchased from Chiron Mimotopes Pty Ltd, Clayton, Victoria, Australia) were synthesized and conjugated to diphtheria toxoid. The six peptides were used to immunize rabbits as described below.

*Immunization procedure*

For PERB11-4D, -5D and -6D, 1 mg of each peptide–diphtheria conjugate was dissolved separately in 400 $\mu$l of sterile distilled water. Four hundred microlitres of each peptide solution were added to 400 $\mu$l of Titremax adjuvant and emulsified by sonication for 90 s on ice. Forty microlitres of each emulsion were injected intramuscularly into both hind legs of rabbits. Rabbit sera were collected between 9 and 14 weeks post-immunization. One microgram of each PERB11-1M, -2M and -3M peptide was used to make 50% emulsions with Freund's complete adjuvant (FCA) and injected subcutaneously into three rabbits with one peptide per rabbit. The same emulsions were prepared and injected weekly for another 3 weeks before collection of rabbit sera on week 4. All sera were tested for activities against the corresponding peptides using the indirect ELISA technique.

*Production of the recombinant PERB11.2 protein*

A PERB11.2 clone, HSB2G6, in the 410 expression vector (Immunex Research and Development Corp. Pty Ltd, Seattle, WA) was cloned and isolated. The clone was used to transfect the CV1 cell line using the DEAE method. The transfectants were

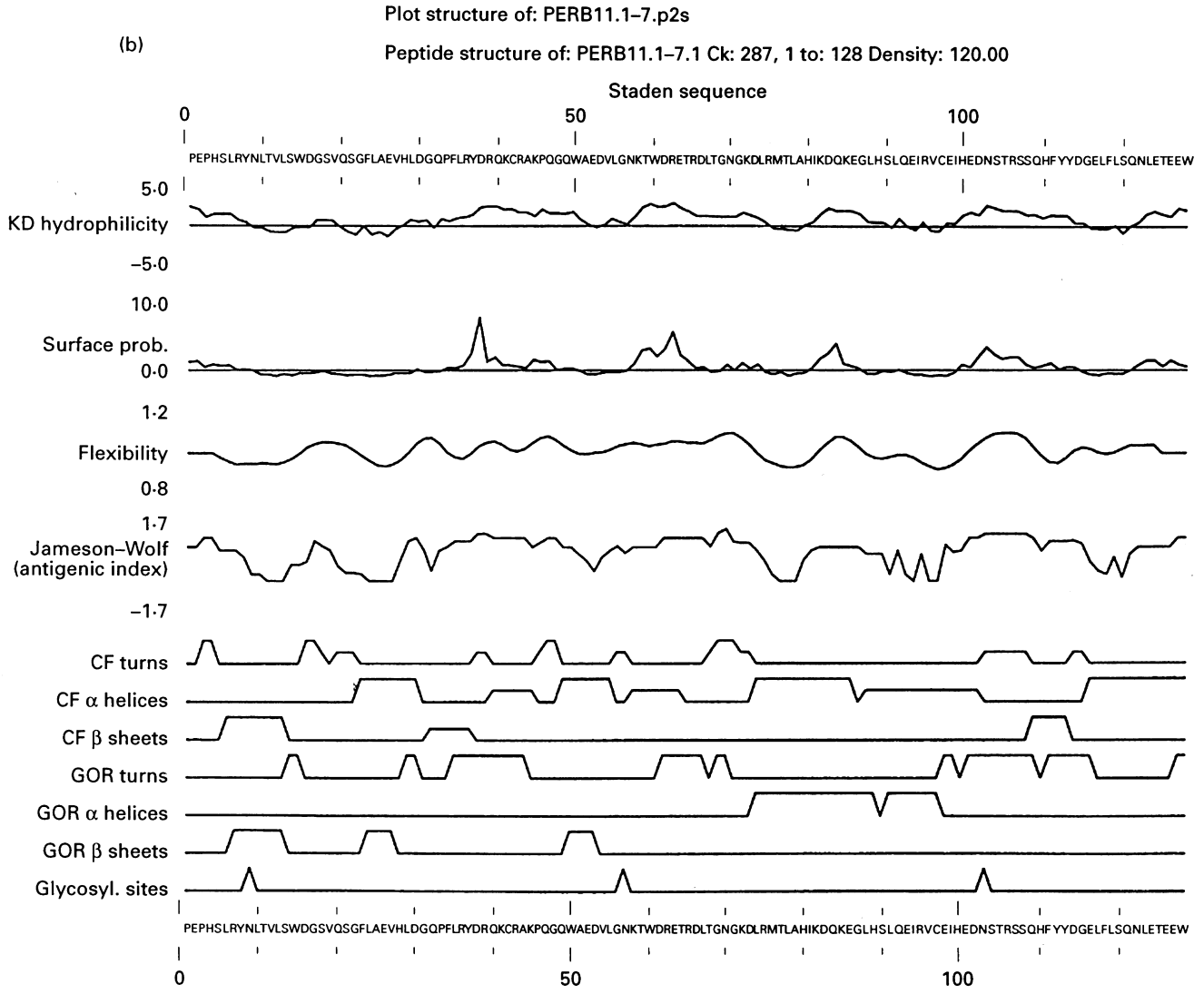


**Fig. 1.** (See previous page for caption.)

grown in the presence of $^{35}$S-methionine and $^{35}$S-cysteine. The transfectants were extracted on ice for 2 min using lysing buffer (EDTA, PMSF, leupeptin, pepstatin A and NP-40 in phosphate buffer). The total extraction of the transfectants derived from a 6-mm dish was used for immunoprecipitation with each antiserum.

*Determination of anti-peptide activities using ELISA*

Peptides produced by the MAP system were used to coat ELISA plates directly (Nunc, Naperville, IL; high absorption for ELISA). Carbonate buffer (100 $\mu$l; pH 9·6) containing 1 $\mu$g of peptide PERB11-1M, -2M or -3M was added to each well and incubated at 4°C overnight or 37°C for 2 h. Biotinylated PERB11-4D, -5D and -6D were purchased from Chiron Mimotopes Pty Ltd. These peptides were bound to the ELISA plate via strepavidin.

*Immunoprecipitation*

The radioactively labelled cell lysate described above (200 $\mu$l) was incubated with 10 $\mu$l each of the three antisera at 37°C for 2 h. Protein-A and protein-G Sepharose (2–5 $\mu$g; Phamacia, Uppsala, Sweden) were added to each reaction and incubated at 4°C overnight. Then, the reactions were spun at 12 000 ***g*** for 1 min and washed three times in 1% NP-40 PBS. The precipitations were electrophoresed through 8–16% SDS-gradient polyacrylamide gel at 35 mA for 45 min. The gel was fixed and exposed to an autoradiographic film at –70°C for 18 h.

*Indirect immunofluorescence of various tissues in rodents, monkeys and humans*

Rats, mice and guinea pigs were obtained from the Animal House of the Research Centre at Royal Perth Hospital. The animals were dissected, and tissues were combined into composite blocks and frozen in liquid nitrogen within 1 h of animal death using Tissue Tek OCT embedding medium (Miles Inc., Pymble, Australia). The tissues used were stomach with oesophagus, kidney, liver, heart, ovary, testis, skin (tail), intestine, brain, adrenal, thymus, spleen, salivary gland, and skeletal muscle. In addition a single block of the torso of a mouse fetus was prepared. Human skin, kidney and pancreas, as well as monkey kidney and oesophagus (INOVA Diagnostics, San Diego, CA) were also examined.

Cryosections of 4 $\mu$m thickness were air-dried and covered with sera from rabbits immunized with PB11-2M, -4D and -5D. After incubation, the sections were washed, then stained with FITC-conjugated sheep anti-rabbit immunoglobulin, $F(ab')_2$ fragment, affinity-purified (Silenus Labs, Hawthorn, Australia). The sera were diluted 1:10 and the conjugate 1:40, in PBS pH 7·6. After the second incubation, slides were washed three times, then mounted in glycerol-based semipermanent medium pH 9·1 (Immunoconcepts, Sacramento, CA) and read blind by at least two independent readers on an immunofluorescence microscope. A conjugate control section (FITC sheep anti-rabbit immunoglobulin only) was included on each run.

In addition, rabbit sera were tested on ethanol-fixed human neutrophils. Neutrophils from a normal human donor (blood group O, positive) were sprayed onto glass slides using a cytocentrifuge

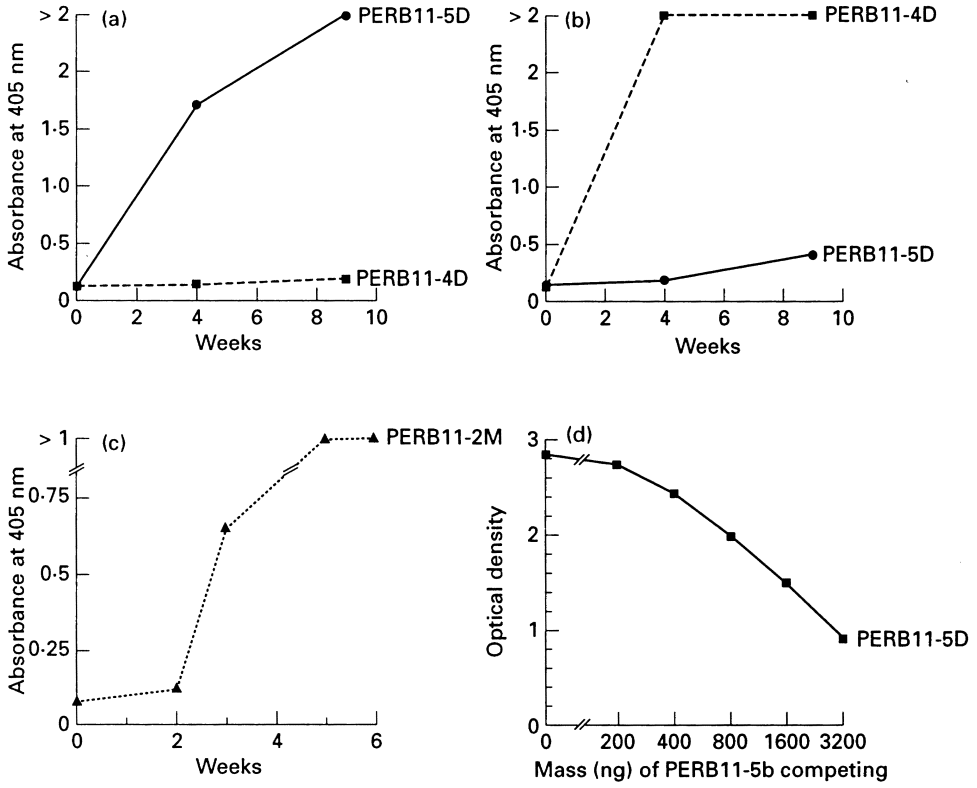


**Fig. 2.** Binding and specificity of binding, of antibodies in immune serum from rabbits to PERB11 peptides, demonstrated by ELISA. (a) Immunized with PERB11-5D, tested against PERB11-5D and PERB11-4D. (b) Immunized with PERB11-4D, tested against PERB11-5D and PERB11-4D. (c) Immunized with PERB11-2M, tested against PERB11-2M. (d) Inhibition of binding of antibodies in rabbit anti-PERB11-5D serum, tested at 1:80 000 dilution, by addition of soluble PERB11-5D in the amounts shown and incubation for 90 min before assay by ELISA against PERB11-5D.

(Shandon Elliott, London,UK). The slides were air-dried and then fixed in cold ethanol for 10 min. Rabbit sera were tested by indirect immunofluorescence and read as described above, except the FITC conjugate was diluted at 1:800.

## RESULTS

*Peptides selected for immunization*

Putative amino acid sequences of PERB11.1 derived from 7.1 (HLA-Cw7, B7, TNF L, BAT3 L, BfS, C4A3, C4B1, DRB1*1501, DQB1*0602) and 57.1 (HLA-Cw6, B57, TNF L, BAT3 L, BfS, C4A6 C4B1, DRB1*0700, DQB1*03032) as well as PERB11.2 derived from the 57.1 MHC ancestral haplotypes were compared with those of MHC class I of various species, IgG FcRn and Zn-$\alpha_2$-glycoprotein (Fig. 1.). Based upon structural comparison, sequence similarity and immunogenicity, six peptides were chosen for synthesis. Three peptides, PERB11-1M, PERB11-2M and PERB11-3M were chosen based upon the equivalent structure to the MHC class I molecule. These sequences are likely to be outwardly exposing linear epitopes, have no conformational hindrance, and may be forming turns connecting different parts of the primary structure ($\beta$-sheet or $\alpha$-helix) of the molecule. These peptides are located in the $\alpha$-1 and $\alpha$-2 domains of PERB11 (Fig. 1) and were produced using the MAP system as described in Materials and Methods. PERB11-4D, -5D and -6D were chosen based upon computer analysis of the PERB11.1 sequence for immunogenicity and surface probability (Fig. 1). These three

**Table 1.** Tissue binding of rabbit antibodies raised against PERB11 peptides, demonstrated by indirect immunofluorescence

| | Peptide used for immunization | | | |
|---|---|---|---|---|
| Binding | PERB11-2M | PERB11-4D | PERB11-5D | Fig. |
| Cells in glomeruli | | | | |
| Rat kidney | | | + | 3 |
| Mouse kidney | | | | |
| Guinea pig kidney | N/A | | | |
| Primate kidney | | | + | |
| Apical cytoplasm of mucosal cells | | | | |
| Rat small intestine | + | | ++ | |
| Mouse small intestine | + | | ++ | |
| Guinea pig small intestine | | | | + |
| Apical cytoplasm of mucosal cells | | | | |
| Rat large intestine | | | + | 4 |
| Mouse large intestine | + | | ++ | |
| Guinea pig large intestine | N/A | | + | |
| Cytoplasm of basal layer(s) of epithelium | | | | |
| Rat oesophagus | + | + | ++ | 5 |
| Mouse oesophagus | ++ | | ++ | |
| Guinea pig oesophagus | ++ | | + | |
| Primate oesophagus | + | | ++ | |
| Mouse embryo skin | | | ++ | |
| Dots in spermatids in semeniferous tubule | | | | |
| Rat testis | | ++ | + | 6 |
| Guinea pig testis | | ++ | + | |
| Primate testis | | + | + | |
| Rods in lumen of semeniferous tubule | | | | |
| Rat testis | | + | + | |
| Guinea pig testis | | ++ | + | |
| Primate testis | | + | + | |
| Cytoplasm of oocytes | | | | |
| Rat ovary | | + | + | |
| Guinea pig ovary | | | + | |
| Primate ovary | | + | + | |
| Liver | | | | |
| Rat liver | | + | ++ | |
| Human liver | | + | ++ | |
| Cytoplasm of some neutrophils | | | | |
| Human neutrophils | N/A | + | ++ | 7 |

N/A, Data not available.

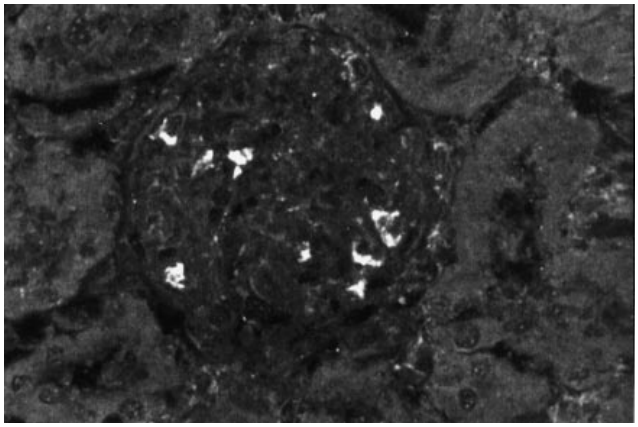

**Fig. 3.** Rat kidney glomerulus, × 420, confocal microscopy. Indirect immunofluorescence with serum from rabbit immunized with PERB11-5D showing speckles within the glomerulus. The cytoplasm of cells that may be endothelial in origin is staining. Other structures within the glomerulus are negative.

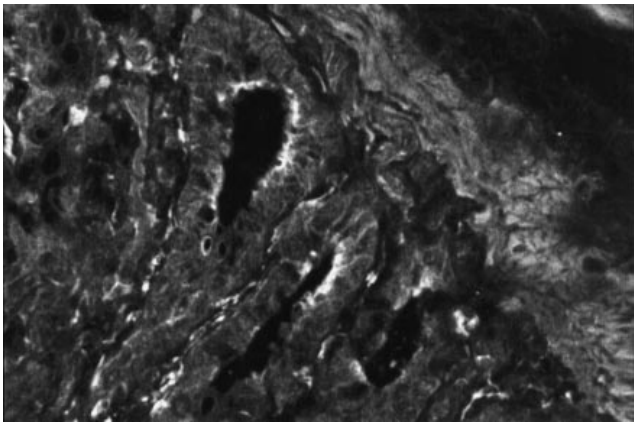

**Fig. 4.** Rat large intestine, × 140, confocal microscopy. Indirect immunofluorescence with serum from a rabbit immunized with PERB11-5D showing cytoplasmic staining of mucosal lining cells with highlighting of the apical portion of many cells. The smooth muscle layer of the intestine is at the top right.

peptides were synthesized and conjugated to the diphtheria toxoid. According to the sequence alignment, antibodies induced by PERB11-4D should be more specific to the gene family. In contrast, antibodies induced by PERB11-5D should reveal cross-reactive activities to other related molecules.

Only sera derived from the rabbits immunized with PERB11-2M, -4D and -5D contained activities corresponding to the injected peptides as shown by the indirect ELISA technique (Fig. 2). These antisera were chosen for further characterization by immunoprecipitation with the PERB11.2 recombinant protein and by the indirect immunofluorescent technique on various mammalian tissues.

*Tissue binding of three rabbit antisera induced by the PERB11 peptides, demonstrated by indirect immunofluorescence*

The immunofluorescence patterns were surprising in their strength, diversity and uniqueness. Some of the more remarkable features are summarized in Table 1. In developing this summary, we chose to eliminate all but the clearest patterns. None of the features included was seen in preimmune sera of rabbits that did not produce antipeptide antibodies detectable by ELISA. Details of the patterns of binding include the following.

*Kidney*. Antibodies in the PERB11-5D immune serum bound to all glomeruli of the rat kidney (Fig. 3). The pattern consisted of specks that were $\approx$ 10 $\mu$m in diameter and numbered $\approx$ 7–10 per

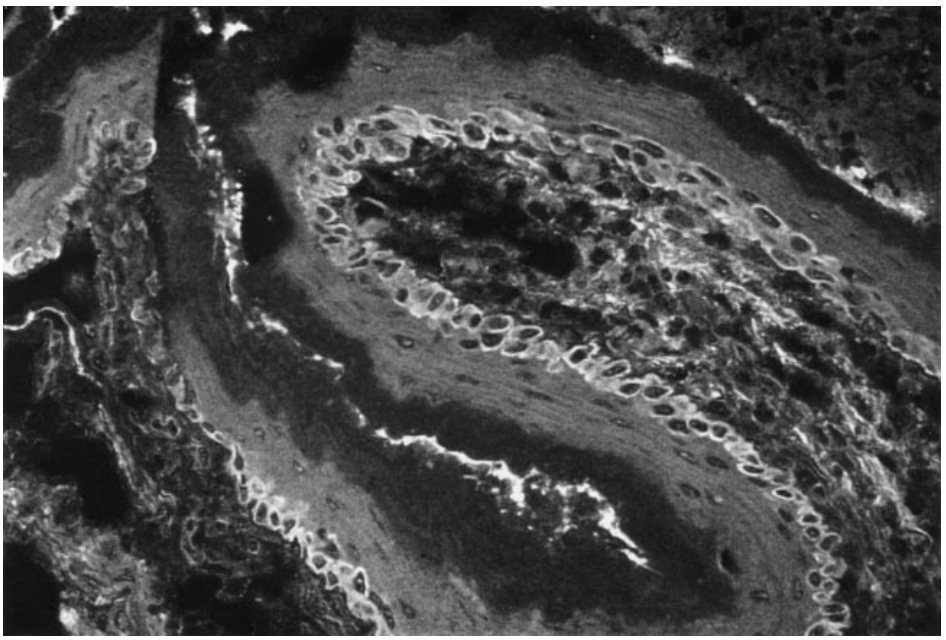

**Fig. 5.** Rat oesophagus, confocal microscopy. Indirect immunofluorescence with serum from a rabbit immunized with PERB11-5D. One to two layers of the basal epithelial cells show bright staining of the cytoplasm. The remainder of the epithelial cells are clearly negative, as are most cells in the sub epithelial layer.

glomerulus. They appeared to be cytoplasmic in distribution and may be within endothelial cells. The same staining pattern of the PERB11-5D serum was also found when the serum was tested on a human kidney section, but a weaker activity was detected with the PERB11-2M serum. The PERB11-4D serum showed a negative staining of glomeruli but positive cytoplasmic staining of tubules. The pattern seen in primate kidney glomeruli with PERB11-5D was similar to that found in the rat kidney, but more extensive.

*Intestine.* Antibodies in the PERB11-5D immune serum bound to the cytoplasm of mucosal cells with highlighting of the apical portion of each cell in the intestine of the rat, mouse and guinea pig (Fig. 4). This pattern was stronger in the small intestine. Similar, but weaker and less extensive binding of antibodies in the PERB11-2M immune serum was also detected.

*Epithelium.* In the rat, mouse and guinea pig oesophagus, one or two basal layers of epithelial cells showed cytoplasmic staining with all three PERB11 rabbit antisera, which was strongest with PERB11-5D (Fig. 5). The cytoplasm of the other layers of epithelial cells was negative. The same pattern was seen when these sera were tested on a commercial primate oesophagus (INOVA Diagnostics) and human skin. When sera were tested on fetal mouse, this pattern was also seen on the skin, but only with PERB11-5D.

*Gonads.* PERB11-4D and -5D antisera showed unusual patterns in the rat gonads. In rat ovary there was cytoplasmic staining of oocytes. A slightly different cytoplasmic pattern was seen in primate ovary. In rat, guinea pig and primate testis there were small, bright dots in the spermatids in the semeniferous tubules (Fig. 6). This may be similar to a pattern described by Lee *et al.* [9] with respect to mouse nuclear orphan receptor expressed in embryonic and adult testes. This pattern was stronger with the PB11-4D serum, which showed an additional feature of staining of rod-like structures in the lumen of the semeniferous tubules.

*Liver.* In both human and rat liver sections there was binding of the PERB11 anti-5D and anti-4D to the basolateral aspect of the hepatocyte surface, to the bile canaliculi, and to bile ductules.

*Neutrophils.* The pattern seen with PERB11-4D and -5D in human neutrophils was a coarse granular cytoplasmic staining

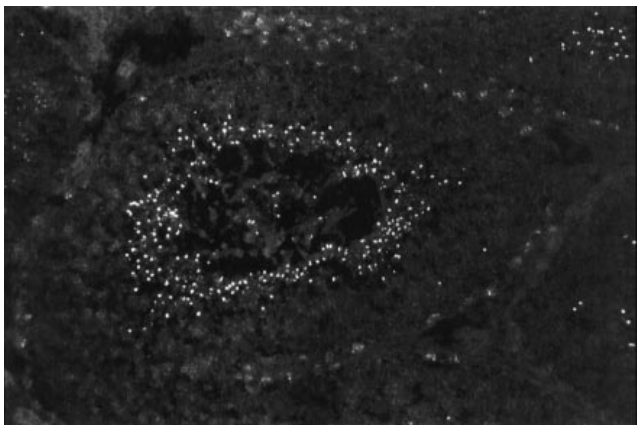

**Fig. 6.** A semeniferous tubule of rat testis, × 140, confocal microscopy. Indirect immunofluorescence with serum from a rabbit immunized with PERB11-4D showing discrete, bright dots concentrated around the lumen. These are localized to the spermatids. A less intense speckled pattern may be seen in some of the cells at the periphery of the tubule. Another collection of bright dots from an adjacent tubule is seen at the top right.

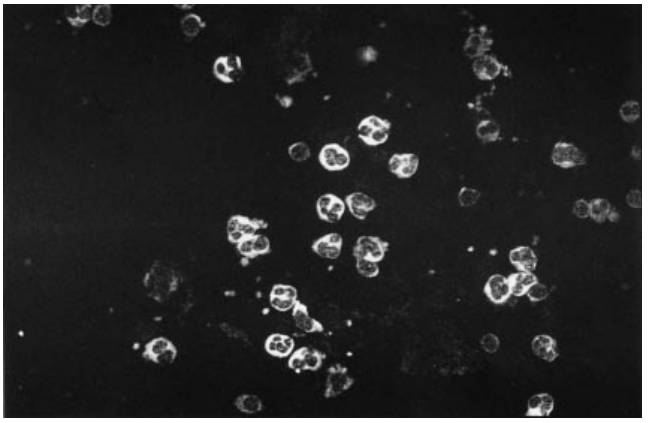

**Fig. 7.** Human neutrophils from a blood group O donor, confocal microscopy. Indirect immunofluorescence with serum from a rabbit immunized with PERB11-5D. This photograph does not represent the overall distribution of staining, since a group of cells with the bright granular pattern was specifically selected. However, negative neutrophils may be seen around the top right quadrant and at the top left. Mononuclear cells are also present in this preparation.

(Fig. 7) which was only present in some 25% of neutrophils. It was unlike the usual patterns attributed to anti-neutrophil cytoplasmic antibody [10], where most or all neutrophils show a cytoplasmic or perinuclear pattern, but similar neutrophil binding has been described by Kain *et al.* [11]. This pattern was stronger with the PERB11-5D serum and was demonstrable with neutrophils from each of five random donors.

There was no anti-islet cell activity detected when the three sera were tested on a human pancreas substrate.

A rabbit anti-human antibody to Zn-$\alpha_2$-glycoprotein (Nordic Immunology) was also tested on all the tissues listed in Table 1 and showed none of the described patterns.

As summarized in Table 1, the PERB11-5D pattern is the most extensive and includes at least most of the features seen with either of the other two antibodies. It is possible that there are two major components: 'epithelial' (PERB11-2M) binding within the basal layers of the skin and oesophagus and at the apices of intestinal cells, and 'gonadal' (PERB11-4D) binding within the testis and ovary. Two other components contained within the larger PERB11-5D pattern are represented by the neutrophil and glomerular staining.

The specific immunofluorescent patterns observed were unlike any seen, classified and reported previously by this laboratory [12–15] from testing $\approx$ 10 000 human sera per annum over 20 years. These samples are submitted to this Department from throughout Western Australia and beyond for autoantibody screening on rodent tissues, human neutrophils and HEp-2 cells.

*Immunoprecipitation of the PERB11.2 transfectant with the PERB11-2M, -4D and -5D sera*

Immunoprecipitation of the PERB11.2 transfectant with the PERB11-2M, -4D and -5D sera was performed to confirm the activities of these antibodies against the native recombinant molecule. The CV1 cell line transfected with the 410 vector was used as a control. The result of immunoprecipitation of the PERB11-4D serum is presented in Fig. 8. Obviously, the PERB11-4D serum contains antibodies reactive to protein components present in the PERB11.2 transfectant but absent from the

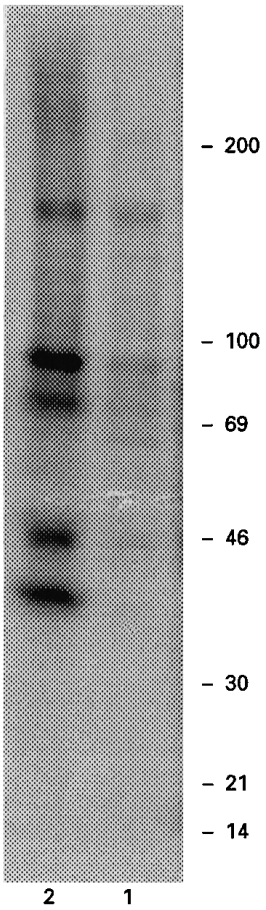


**Fig. 8.** Immunoprecipitation of the PERB11.2 transfectant with the PERB11-4D serum. Lane 1, immunoprecipitation of the cell lysate extracted from the PERB11.2 transfectant with the PERB11-4D serum; lane 2, control immunoprecipitation of PERB11-4D replacing the PERB11.2 lysate with the cell lysate from the vector transfected cells. Molecular weight markers are shown on the right. It can be seen that the PERB11-4D serum contains antibodies reactive to protein components absent from the control transfected cells.

vector control. These include protein species of ≈ 40 kD, 46 kD, 80 kD and 100 kD (Fig. 8). The PERB11-2M and -5D sera contain antibodies reactive to the same species of proteins with an additional protein of ≈ 120 kD (data not shown). The activity contained in PERB11-2M is much weaker than that of PERB11-5D. It should be noted that although antibodies contained in the three antisera are reactive to the same protein components, the relative intensities differ, in keeping with the overlapping immunofluorescence (IF) patterns described above. The strong reactivity of PERB11-5D with the additional protein species (120 kD) may account for the additional IF pattern seen with this serum. According to the molecular weight of the proteins detected, it is possible that the recombinant protein exists in various forms of dimerization or glycosylation.

## DISCUSSION

Initially we were surprised by the extraordinary breadth of reactivity of the antibodies produced by immunization with such peptides. It seems likely that several antibody species were induced by the peptides used for immunization, and quite possible that at least some of these are not directly relevant to PERB11 or related molecules. However, on searching the protein databases, no obvious alternatives were apparent with the possible exception of an activin receptor which does contain a peptide (YDRQECIA-KEE) which resembles 4D (YDRQKCRAKPQ). It would be relevant to test antibodies to other class I like molecules such as FcRn and Zn-$\alpha_2$-glycoprotein but, to date, we have only had the opportunity to test an antiserum to the latter, and results were uniformly negative.

An interesting feature of the results was that the three antisera raised against diverse peptides yeilded related and overlapping patterns of reactivity. The profiles (Table 1) may indicate that, as predicted, PERB11-5D has elicited a broader response that includes reactivity equivalent to those induced by both PERB11-2M and PERB11-4D.

More interesting was the fact that the patterns are unique in our experience. Whilst some components of the various patterns are reminiscent of known antibodies or autoantibodies (see above), such constellations have not been described to our knowledge.

It remains to be determined which particular components are specifically related to individual members of the PERB11 and associated families. However, it is clear that such further characterization should proceed as the proteins become available.

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