The expression pattern of the Qa-2 antigen in mouse preimplantation embryos and its correlation with the Ped gene phenotype

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The Qa-2 antigen, the product of the Ped (Preimplantation embryo development) gene, is a glycosyl-phosphatidylinositol-linked cell surface protein encoded in the Q region of the mouse major histocompatibility complex (MHC). Ped fast (Qa-2+) mouse strains have significantly higher preimplantation embryo cleavage rates both in vivo and in vitro than Ped slow (Qa-2–) mice. In this study, we determined whether the Ped fast phenotype of blastocysts is due to an increased number of blastomeres in the trophectoderm (TE), the inner cell mass (ICM), or both. We also analysed the Ped gene expression pattern, both at the mRNA and at the protein level, in these lineages. Blastocysts were collected from the congenic mouse strains B6.K2 (Qa-2+) and B6.K1 (Qa-2–). We performed reverse transcription–polymerase chain reaction (PCR) and Immuno-PCR and found that the Ped gene is expressed at the mRNA and protein level in whole embryos and in isolated ICM cells. Lastly, we differentially stained embryos from these strains and found that B6.K2 blastocysts had significantly higher cell numbers (P < 0.05) in both the ICM and in the TE than B6.K1 blastocysts. These results suggest that Qa-2 expression in both the TE and the ICM of blastocysts directly contributes to the Ped phenotype.

Key words: blastocyst/ICM/Immuno-PCR/mouse/Ped gene
1997), our group has shown that the Ped gene product, Qa-2, is expressed on the surface of mouse blastocysts. Since blastocyst stage embryos consist of two distinct cell lineages, the outer trophectoderm (TE) epithelium and the inner cell mass (ICM), it is important to determine the expression pattern of the Ped gene, its protein product, and its phenotype in both of these lineages. We therefore performed both RT–PCR and the newly developed technique of I-PCR on isolated ICM from Ped fast and Ped slow mouse strains. We also differentially stained blastocysts from the two strains in order to quantify the effect of the Ped gene on the number of cells in both the TE and ICM. For these studies, we used the inbred congenic mouse strains B6.K2 (Qa-2+) and B6.K1 (Qa-2−), which are genetically identical except for the Q region of the MHC, in order to eliminate background gene effects. Our results show that Ped fast blastocysts contain a significantly higher number of cells in both the ICM and the TE than Ped slow blastocysts, and that this phenotype is directly related to the expression of the Qa-2 antigen in both lineages.

**Materials and methods**

**Mouse strains**

The inbred congenic mouse strains B6.K2 (Qa-2+, Ped fast) and B6.K1 (Qa-2−, Ped slow), originally obtained from L.Flaherty, were bred in our laboratory. The mice were housed in an AAALAC (American Association for the Accreditation of Laboratory Animal Care)-approved day–night cycled room (lights on 0400–1800 h eastern standard time) with food and water ad libitum.

Female mice were superovulated with 5 IU of equine chorionic gonadotrophin (eCG; Sigma, St Louis, MO, USA), injected i.p. at the 11th hour of the light cycle, followed 48 h later by 10 IU of human chorionic gonadotrophin (HCG; Sigma). One female was placed with a single male immediately following HCG injection and the 11th hour of the light cycle, followed 48 h later by 10 IU of gonadotrophin (eCG; Sigma, St Louis, MO, USA), injected i.p. at standard time) with food and water ad libitum.

Embryo collection

Mice were killed by cervical dislocation and embryos were flushed from the excised oviducts using a 30 gauge needle attached to a 1 ml syringe. About 0.2 ml of Whitten–Biggers (WB) medium was used to flush each oviduct, and morphologically healthy embryos were pooled and kept under gas in a watch glass containing 0.5 ml of WB.

Direct counting of blastomeres

In order to quantify the overall number of blastomeres per embryo, blastocysts from each mouse strain were stained by a cell-spread method first described by Tarkowski (1966). Embryos were washed in Ringer’s solution three times, followed by three washes in 0.8% sodium citrate to allow the blastomeres to swell. The embryos were then placed onto a glass microscope slide. They were fixed by mouth-pipetting a small volume of 3:1 (v/v) of methanol:acetic acid onto the slide three times, letting the fixative dry in between. At this point, the slide was placed into a beaker containing a 1:20 dilution of Giemsa stain (Sigma) for 5–15 min, rinsed with distilled water, air-dried, and examined with a Zeiss phase contrast microscope at ×400. Giemsa stains nuclei dark blue, and the number of nuclei counted per embryo was used as a measure of the number of cells per embryo. Data were analysed by Student’s t-test.

**Immunosurgery for isolation of ICM**

Immunosurgery was performed on blastocysts to isolate the ICM (Soler and Knowles, 1975). Briefly, the embryos were incubated in acid Tyrode’s (pH 2.5) for 3–5 min to dissolve the zona pellucidae. They were washed immediately in WB. The embryos were then incubated in heat-inactivated rabbit anti-mouse lymphocyte serum (diluted 1:3 in WB) for 1 h at 37°C, 7% CO2. After washing 3–5 times in WB, the antibody-labelled TE cells were lysed after a 45 min incubation in guinea-pig complement (Gibco BRL, Gaithersburg, MD, USA) (diluted 1:5 in WB), at 37°C, 7% CO2. The lysed TE cells were removed by pipetting up and down and the isolated ICM were washed twice in WB.

**RT–PCR analysis of Ped gene mRNA expression**

We have determined by analysis of mRNA transcripts that the gene which encodes the Ped fast phenotype, Q9, is transcribed in B6.K2 whole embryos (Wu et al., 1998). In this study, we analysed mRNA transcripts from B6.K2 ICM with the same Q9 gene primers. Total RNA was isolated from ICM using a kit from Stratagene (La Jolla, CA, USA). Blastocyst embryos were collected and ICM were isolated as described above. The ICM were transferred into a 0.5 ml PCR tube in a small volume of WB and 100 µl of denaturing solution; 0.72 µl of β-mercaptoethanol was immediately added and the tube was vortexed on high speed for 10 s. To each sample tube, 10 µl of 2 M sodium acetate (pH 4.0), 100 µl of water-saturated acid phenol, and 30 µl of chloroform–isoamyl alcohol were added. The tubes were vortexed again and microfuged at maximum speed for 5 min. The upper phase from each sample, containing RNA, was transferred to a clean tube, and 1 µl of glycoen carrier and 100 µl of isopropanol were added. The samples were then microfuged at high speed for 45 min at room temperature, and the supernatant was discarded. The RNA pellet was washed with 200 µl of 75% ethanol:25% nuclease-free water. The wash was removed and the pellets were dried for 5–10 min under vacuum. All cDNA reagents were obtained from Promega (Madison, WI, USA). 7.25 µl of RNase-free water, 0.2 µl of 0.1 M DTT, 1 µl of 50 µM random hexamers, and 0.05 µl of 20 U/µl RNAsin were added to each RNA pellet, and 20 µl of autoclaved mineral oil was added to the surface of the solution. The RNA was denatured at 70°C for 5 min, the random hexamers were annealed at 30°C for 5 min, and then 4 µl of 25 mM MgCl2, 2 µl of 10X PCR buffer, 4 µl of a 10 mM dNTP stock, 1 µl of 20 U/µl of RNAsin, and 0.5 µl of 200 U/µl Moloney Murine Leukaemia Virus reverse transcriptase were added to each tube. The tubes were incubated at 37°C for 180 min for the production of cDNA, and heated to 99°C for 5 min to denature the reverse transcriptase. To increase the sensitivity and specificity of amplification of the cDNA, two rounds of PCR were carried out using hemi-nested primers designed to amplify a sequence of 380 bp from Q9 transcripts. For the first PCR reaction, 4 µl of a 10 mM stock of dNTP, 1 µl of each 10 µM primer (upper primer: 5’ GCCCAACACTGCTGCAATAAT 3’, lower primer: 5’ ATCTCCCCCATCTAGGGTA 3’), 2.5 µl of 10X PCR buffer, 1.1 µl of 50 mM MgCl2, and 15.4 µl of distilled water were added to individual HotStart 100 (Molecular Bio-Products, San Diego, CA, USA) PCR tubes. These tubes contain a bead of wax, which was melted at 80°C for 5 min, followed by cooling to room temperature for 1 min. Next, 4 µl of cDNA was added to the specific tube, along with 18.2 µl of distilled water, 2.5 µl of 10X buffer, and 0.25 µl of 5 U/µl Taq polymerase. PCR was carried out under the following conditions: one cycle at 96°C for 1 min, two cycles at 96°C for 1 min for denaturation, 58°C for 1 min for annealing, and 72°C for 1 min for extension, 18 cycles at 94°C for 1 min, 57°C for 1 min, and 72°C for 45 s, 20 cycles at 94°C for 1 min, 57°C for 30 s, and 72°C for 45 s, and one cycle for 5 min at 72°C for final extension. For the
second round of PCR, 15.1 µl water, 2.5 µl of 10X buffer, 4 µl of the 10 mM mix of dNTP, 1.4 µl of 50 mM MgCl₂, and 1 µl of each 10 µM primer were added to clean HotStart 100 PCR tubes. (The PCR is hemi-nested in that the same lower primer is used in both PCR rounds. The upper primer in the second round is 5’ TGGTATTGCAGAGAAAGACCA 3’.) The wax was melted as described above, and then 17.25 µl of water, 2.5 µl of 10X PCR buffer, 0.25 µl of 5 U/µl Taq polymerase, and 5 µl of the first PCR reaction were added to the tubes. The second round of PCR was carried out using the same conditions described above. The PCR products were analysed on a 6% polyacrylamide mini-gel and stained with ethidium bromide for 5 min.

**Immunop-PCR detection of Qa-2 antigen**
Recently, we described a novel technique for the detection of MHC Class Ia molecules, (D³), on the surface of a single, intact blastocyst (McElhinny and Warner, 1997). In the present study, we extended this technique to analyse the MHC Class Ib molecule, Qa-2, on B6.K2 blastocysts and isolated ICM. Briefly, whole embryos or isolated ICM were incubated for 1 h at 4°C in anti-Qa-2 antibody [protein A purified 1-12-1 monoclonal antibody (Sharrow et al., 1989)] at a concentration of 0.5 mg/ml in PBS + 0.01% sodium azide + 1% BSA (PBSAZ). After removal of the zona pellucida, the embryos were washed five times in PBSAZ + 0.05% Tween-20 followed by five washes in PBSAZ, changing the pipette with each set of washes. They were then incubated in 1 X 10⁻¹⁷ M bispecific chimeric protein–DNA complex (Sano et al., 1992) for 45 min. (The chimeric protein contains a protein A domain, which recognizes the Fc portion of the first antibody, and a streptavidin domain, which binds a biotinylated DNA fragment.) After a final extensive set of washes, the embryos or ICM were placed into thin-walled 0.5 ml tubes, and PCR was carried out as previously described using primers that amplify 769 bp of the biotinylated DNA fragment (McElhinny and Warner, 1997). The PCR products were run on a 6% polyacrylamide gel and stained with ethidium bromide.

**Differential staining of the TE and ICM**
To differentially stain the ICM and the TE of blastocysts, we used a protocol modified from Handyside and Hunter (1984) and Papaioannou and Ebert (1988). B6.K2 and B6.K1 blastocysts that had been collected 89 h post-HCG into WB under gas were subjected to immunosurgery as described above, except that two dyes were added with the guinea-pig complement. Propidium iodide (final concentration of 5 µg/ml) stains dead cells but is excluded from viable cells. Bisbenzamide (final concentration of 5 µg/ml) stains both live and dead cells. The combined staining of these two dyes renders the lysed TE cells pink and the intact ICM cells blue under UV light. The embryos were washed twice in WB after the final incubation and mounted in as small a volume as possible (~5 µl) of WB onto a microscope slide and squashed gently with a glass coverslip. They were immediately examined under UV light using an Olympus microscope at ×200, and the number of nuclei of each colour were counted. Data were analysed by Student’s t-test.

**Results**
We first confirmed the Ped phenotypes of the B6.K1 and B6.K2 mouse strains used in this study. We collected embryos from both strains at 89 h post-HCG and performed a cell-spread method (Tarkowski, 1966) to quantify the total number of cells per embryo. As expected, the B6.K2 embryos had a significantly higher (P < 0.05) mean number of cells per embryo than the B6.K1 embryos (experiment 1 in Table I). This confirmed results from our previous studies that showed that the Qa-2+ B6.K2 embryos are Ped fast, while the Qa-2– B6.K1 embryos are Ped slow (Warner et al., 1991).

Previously, our laboratory has shown by RT–PCR analysis that the Ped gene is transcribed in Ped fast blastocyst stage embryos (Jin et al., 1992). In B6.K2 mice, the Ped gene is encoded by Q9 (Wu et al., 1998). Here, we have extended these studies in order to analyse the transcription of the Ped gene in ICM isolated from B6.K2 blastocysts. We were able to detect Q9 transcripts in ICM from the Ped fast, but not the Ped slow, blastocysts after semi-nested PCR (Figure 1). Lane 3 contains the PCR product (380 bp) from ten B6.K2 ICM.

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*Table I. Quantitation of blastomeres in B6.K2 (Qa-2+) and B6.K1 (Qa-2–) embryos*²

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mouse strain</th>
<th>Material analysed</th>
<th>n</th>
<th>Cell number</th>
<th>SEM</th>
<th>P²</th>
</tr>
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<tbody>
<tr>
<td>1 (direct staining)</td>
<td>B6.K1</td>
<td>Whole embryo</td>
<td>27</td>
<td>19.7 ± 1.6</td>
<td>P &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6.K2</td>
<td>Whole embryo</td>
<td>33</td>
<td>30.5 ± 1.3</td>
<td>P &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>2 (differential staining)</td>
<td>B6.K1</td>
<td>Whole embryo</td>
<td>28</td>
<td>40.2 ± 11.1</td>
<td>P &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6.K2</td>
<td>Whole embryo</td>
<td>30</td>
<td>49.0 ± 8.9</td>
<td>P &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6.K1</td>
<td>TE</td>
<td>30.6 ± 8.4</td>
<td>P &lt; 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6.K2</td>
<td>TE</td>
<td>3.6 ± 7.1</td>
<td>P &lt; 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B6.K1</td>
<td>ICM</td>
<td>9.6 ± 4.2</td>
<td>P &lt; 0.05</td>
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</tr>
<tr>
<td></td>
<td>B6.K2</td>
<td>ICM</td>
<td>12.5 ± 4.0</td>
<td>P &lt; 0.05</td>
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</table>

*Embryos were collected after superovulation at 89 h post-human chorionic gonadotrophin injection.

²Probability that B6.K2 values are higher than B6.K1 values as determined by Student's t-test.

TE = trophectoderm; ICM = inner cell mass.

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We used seven B6.K1 ICM as a negative control, and as expected, no product was detectable after hemi-nested PCR (lane 5) since this mouse strain has a deletion of the Q9 gene. The results from these experiments show that the Ped gene is expressed at the mRNA level in isolated ICM, as well as whole embryos, in B6.K2 mice. In addition to RT–PCR analysis, we determined the Qa-2 antigen expression pattern in ICM isolated from Ped fast blastocysts. We have previously shown by using an ELISA method (Goldbard et al., 1984) that Qa-2 is detectable on the surface of the outer cells in mouse blastocysts, the TE. It was not known, however, whether Qa-2 protein is also expressed on ICM cells. We therefore performed I-PCR on isolated ICM that had been incubated with a monoclonal antibody against Qa-2, followed by incubation with a chimeric protein–DNA complex (Figure 2). The ICM were placed into PCR tubes and the whole complex was subjected to PCR analysis, using primers that recognize a 769 bp fragment of the biotinylated DNA. The presence of a band of 769 bp indicates that the monoclonal antibody has bound to Qa-2, and that the chimeric protein–DNA complex has bound to the monoclonal antibody. Lane 8 is the negative control, which was an ICM from a Ped slow B6.K1 blastocyst. No band is apparent, which shows that any non-specific antibody or chimeric protein binding is undetectable. Lanes 6 and 7 contain single B6.K2 ICM, and lane 4 contains 4 B6.K2 ICM. These reactions are all positive for the 769 bp PCR product, which indicates that the Qa-2 antigen is expressed on the surface of the inner cell mass of B6.K2 blastocysts. Lane 5 contains the PCR reaction from our positive control, two whole B6.K2 blastocysts. Lane 3 is the chimeric protein–DNA complex alone, and lane 1 is a 100 bp ladder used as a molecular weight marker (Gibco BRL). These results show that our I-PCR technique can be extended to detect cell surface antigens on single isolated ICM. Using this new technique, we detected Qa-2 protein on B6.K2 ICM as well as on whole blastocysts.

Since Qa-2 antigen is present on both the ICM and the TE of Ped fast blastocysts, the next set of experiments was designed to determine if this expression pattern correlates with the Ped fast phenotype. We differentially stained embryos collected 89 h post-HCG from B6.K2 and B6.K1 mice in order to quantify the number of blastomeres in the TE and in the ICM of the embryos. The results (experiment 2 in Table I) show that the mean total number of blastomeres per embryo is significantly greater (P < 0.05) in B6.K2 embryos than B6.K1 embryos. It should be noted that the overall total cell numbers obtained from the differential staining procedure (experiment 2) are higher than those obtained from the direct staining method (experiment 1). This is due to the fact that in the Tarkowski direct staining procedure the embryos are collected and immediately fixed for staining, while in the differential staining protocol extra time in culture for incubations and washings causes the embryos to undergo an additional cell division.

After quantifying the number of blastomeres in the ICM and TE from both strains, it was found that Ped fast embryos have significantly higher cell numbers (P < 0.05) in both lineages compared to Ped slow embryos (experiment 2 in Table I). Since the two mouse strains in this study differ genetically only in the Q region of the MHC, Ped gene expression at both the mRNA and protein level directly contributes to the Ped fast phenotype in the ICM and the TE of B6.K2 blastocysts.

**Discussion**

It is well known that cell surface contacts at the 8- and 16-cell stages are crucial for both the cellular differentiation and development of early mammalian embryos (Johnson and Zitomek, 1981, 1983; Collins and Fleming, 1995). Accumulating evidence suggests, however, that there are determinative events that occur even earlier in development which are also vital for differentiation and successful development (reviewed in Edwards and Beard, 1997). For example, the ability to induce the membrane polarity needed for TE and ICM differentiation develops via cell contacts from the 2-cell stage in mouse embryos (Edwards and Beard, 1997). Very early cell interactions also probably influence the embryo in regulating the ratio of inside to outside cells at the 16- to 32-cell stage transition (Fleming, 1987). In addition, an insufficient cell number in the preimplantation stage embryo can jeopardize differentiation of the TE and ICM and subsequent embryo viability (Edwards, 1995). Therefore, a definition and analysis of very early embryonic regulatory genes, including the Ped gene, are required to better understand the origins of the two earliest cell lineages in embryos, as well as mechanisms for successful subsequent development (Edwards and Beard, 1997).

In this study, we used two sensitive techniques to determine the expression pattern of the Ped gene and its protein product, the Qa-2 antigen, in blastocysts from congenic mouse strains. Previous RT–PCR analysis of whole embryos (Jin et al., 1992) and our present study on isolated ICM show that the Ped gene is expressed at the mRNA level in ICM and in intact B6.K2 blastocysts. Determination of the expression of the Ped gene product, the Qa-2 antigen, has been more challenging, however, since MHC Class I proteins are not detectable in preimplantation embryos by conventional immunofluorescence techniques (Roberts et al., 1992). We have had to develop more sensitive antigen detection techniques, such as ELISA (Goldbard et al., 1984) and I-PCR (McElhinny and Warner, 1997) to show that
Qa-2 protein is present on the surface of intact embryos. However, since both of these techniques involve the use of anti-Qa-2 antibodies, which cannot penetrate the tight junctions between neighbouring TE cells, we could not determine whether Qa-2 was also expressed on the surface of ICM blastomeres. In the present study, we have extended our newly developed I-PCR technique to analyse protein expression in isolated ICM acquired from immunosurgery. We found that Qa-2 is expressed on both the TE and the ICM in B6.K2 blastocysts.

Since Qa-2 is expressed in the two distinct cell lineages of Ped fast blastocysts, the next logical step was to determine if its phenotype was maintained in both of these lineages. We used a technique where two different nuclear-staining dyes are incubated with blastocysts during complement-mediated lysis so that the number of nuclei in both lineages could easily be distinguished and quantified. It was found that the Ped fast B6.K2 blastocysts had significantly more total cells per embryo ($P < 0.05$) compared to the Ped slow B6.K1 blastocyst stage embryos, as expected. This difference in cell number was due to a significantly higher number of blastomeres ($P < 0.05$) in both the ICM and in the TE in the Ped fast blastocysts. Therefore, the phenotype of the Ped gene in the ICM and in the TE directly correlates with its expression pattern in whole mouse embryos.

There is evidence for a homologue of the Ped gene in humans, since human preimplantation embryos that develop at a faster rate have a better chance of survival than slow-developing embryos (Bolton et al., 1989; Levy et al., 1991; Warner et al., 1998). It has been suggested that HLA-G may be the human homologue of Qa-2 (Jurisicova et al., 1996), but data from our laboratory show that this is probably not the case (Warner et al., 1998; N.Cao et al., unpublished observations). It is possible that one or more of the other Class Iib genes that map near HLA-G in the human MHC may be the true Ped gene homologue.

Although the exact mechanism of Ped gene action is not known, the results from this study suggest several possible roles for the Ped gene in embryonic development. For example, the higher number of cells in the ICM of Ped fast mice may explain the overall reproductive advantage that Ped fast mouse strains have (Warner et al., 1993), since a critical number of ICM cells are required for implantation and further embryonic development (Tarkowski and Wroblewska, 1967). Since Qa-2 was found in both the ICM and the TE, it is possible that Qa-2 somehow aids in the process of cellular differentiation in preimplantation embryos, which is vital for successful development. Since polarization of membrane components is required for the process of TE and ICM differentiation (reviewed in Watson, 1992) it would be interesting to determine the actual localization pattern of the Qa-2 antigen in the TE and in the ICM. That type of study, however, cannot be done until existing imaging techniques are improved to allow the detection and localization of antigens which are expressed at levels lower than those currently detectable by immunofluorescence.

More studies are needed to determine if there is a ligand or a receptor for Qa-2 that would mediate cell–cell interactions and perhaps generate a signal that could be transmitted into the embryo. Since Qa-2 is a GPI-linked molecule and does not traverse the cell membrane, it is likely that it may be associated with one or more accessory signalling molecules. It is possible that the binding of a receptor molecule to the Qa-2 protein, and its associated nonameric peptide, causes Qa-2 to interact directly or indirectly with one or more accessory proteins, which would transmit a signal to the interior of the cell (Figure 3). Further investigation into the Ped gene mechanism and its signalling pathway, as well as the search for a human homologue, will be valuable to the study of embryonic development and survival.

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Qa-2 expression in mouse blastocysts


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