

Evidence That HLA-G is the Functional Homolog of Mouse Qa-2, the *Ped* Gene Product

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ABSTRACT: Qa-2, a murine class Ib major histocompatibility complex (MHC) molecule, is a possible functional homolog of human leukocyte antigen G (HLA-G). Both molecules have been implicated in immunoregulation and embryonic development and both occur in membrane-bound and soluble isoforms that arise by alternative splicing. Soluble splice variants have been implicated in the reproductive functions of HLA-G. While soluble variants of Qa-2 have been previously detected in T lymphocytes, we now demonstrate the presence of mRNA for one of the two known soluble forms of Qa-2 in eight-cell embryos and in blastocysts. Qa-2 is glycosylphosphatidylinositol (GPI) linked in the outer leaflet of the cell membrane and is found in lipid raft microdomains where other raft-associated proteins transduce signals into the cell. In contrast, HLA-G has a truncated six amino acid

cytoplasmic tail. By fluorescence co-localization in JEG-3 cells, using fluorescent cholera toxin β subunit (a lipid raft marker) and anti-HLA-G antibody, we have demonstrated that membrane-bound HLA-G also localizes to lipid rafts, consistent with functional homology between the two molecules. Finally, our experiments in which we have purified Qa-2 and transferred it via a process known as protein painting to Qa-2 negative cells represent a model for potential therapy involving HLA-G. *Human Immunology* 64, 999–1004 (2003). © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Inc.

KEYWORDS: Qa-2; HLA-G; preimplantation embryos; *Ped* gene

ABBREVIATIONS

CT β cholera toxin β subunit

GPI glycosylphosphatidylinositol

INTRODUCTION

Qa-2, like human leukocyte antigen G (HLA-G), is a nonclassical class Ib major histocompatibility complex (MHC) protein and is the product of the preimplantation embryo development (*Ped*) gene. Embryonic expression of Qa-2 provides a range of advantageous phenotypes, including faster preimplantation cleavage rate, better survival to term, and higher birth and weaning weights compared with embryos lacking the gene [1]. During clinical assisted reproductive procedures, faster cleaving embryos are associated with more successful pregnancy

outcomes [2]. This phenomenon suggests the existence of a *Ped*-like gene in humans. Orthologous relationships of MHC class I genes have not been found among different mammalian orders. However convergent evolution can result in nonorthologous genes acquiring similar functions, as is seen in the case of *HLA-E* and *Qa-1* [3]. The many similarities of HLA-G and Qa-2 are summarized in Table 1 and reviewed in Warner and Brenner [1]. *HLA-G* is, therefore, an excellent candidate for the human homolog of the *Ped* gene.

Alternative RNA splicing of Qa-2 results in several products, including membrane-bound and soluble isoforms as depicted in Figure 1A [4]. The first purpose of this study is to determine whether or not the two soluble isoforms of Qa-2 are present in preimplantation mouse embryos.

Neither membrane-bound Qa-2 nor membrane-

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TABLE 1 Comparison of HLA-G and Qa-2, the *Ped* gene product [1, 16, 18]

Feature	HLA-G	Qa-2
MHC class 1b molecule, binding nonapeptide	Yes	Yes
Membrane-bound and soluble forms	Yes	Yes
Expression by preimplantation embryos	Yes	Yes
Increases preimplantation growth rate	Yes	Yes
Enhances fetal survival	Yes	Yes
Increases birth weight	Unknown	Yes
Increases weaning weight	Unknown	Yes
Short tail	Yes	Yes
GPI linkage of short tail to membrane	No	Yes
Increased cell proliferation with cross-linking	Unknown	Yes
Acts as a signal transduction molecule	Unknown	Probable

Abbreviations: GPI = glycosylphosphatidylinositol; HLA = human leukocyte antigen; MHC = major histocompatibility complex.

bound HLA-G contains a cytoplasmic domain capable of transducing signals from the cell surface to the nucleus to influence the cleavage rate of embryos. Full-length Qa-2 is glycosylphosphatidylinositol (GPI) linked in the cell membrane [5], while HLA-G has a truncated six amino

acid cytoplasmic tail [6]. GPI-linked proteins are targeted to lipid raft microdomains in the cell, where they potentially interact with raft-associated signaling proteins. The second goal of the present research is to establish whether HLA-G can also localize to lipid raft microdomains in the cell membrane, thus reinforcing the potential functional homology of Qa-2 and HLA-G.

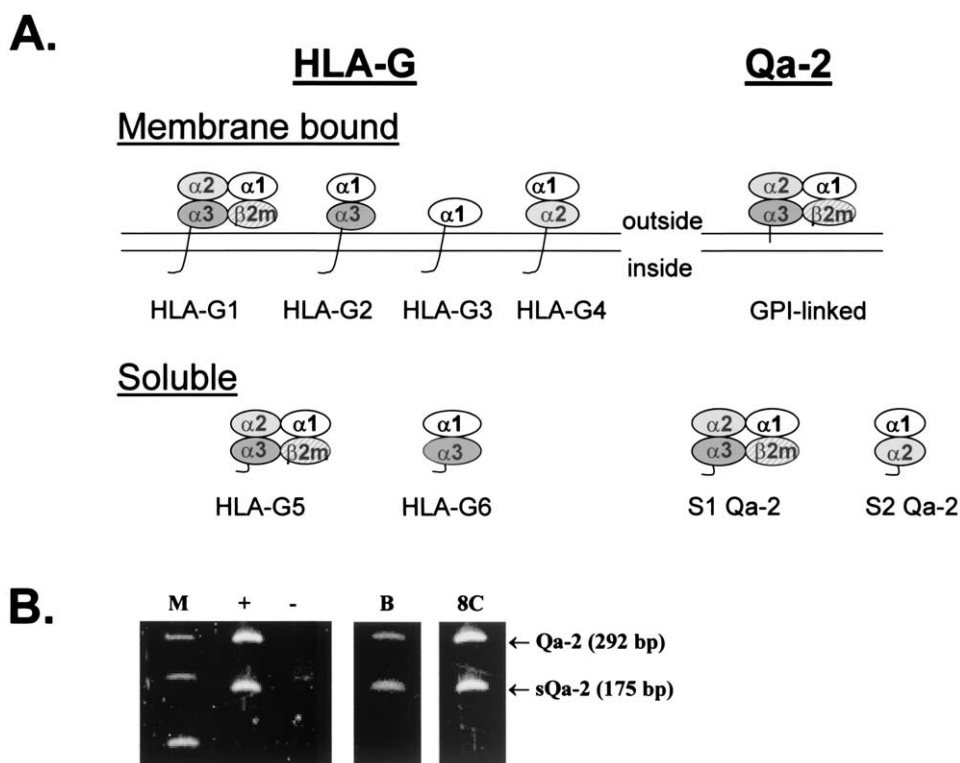
Finally, GPI-linkage of proteins affords a unique opportunity for cell engineering. When cells are incubated with isolated GPI-linked proteins, the proteins spontaneously insert into the cell membrane ("protein painting") [7]. Previously we demonstrated that Qa-2 negative lymphocytes and preimplantation embryos could be painted with crude lysates from Qa-2 positive spleen cells [8], with Qa-2 retaining its function after transfer. It may be beneficial to paint embryos with HLA-G engineered to have a GPI linkage. Thus, the third goal of the present work is to continue the study of protein painting with Qa-2 as a model of a potential application of functional homology between Qa-2 and HLA-G.

MATERIALS AND METHODS

Mice

CBA/CaJ (Qa-2-negative) and C57BL/6J (Qa-2-positive) mice (The Jackson Laboratory, Bar Harbor, ME, USA) and congenic B6.K1 (Qa-2-negative) and B6.K2 (Qa-2-positive) mice (from L. Flaherty), were bred in North-

FIGURE 1 (A) Membrane bound and soluble isoforms of human leukocyte antigen G (HLA-G) and Qa-2. The membrane bound isoforms of HLA-G and Qa-2 have a 6-amino acid cytosolic tail and a glycosylphosphatidylinositol (GPI) tail in the outer leaflet of the membrane, respectively. The figure is derived from mRNA and protein data [4, 16]. The S1 Qa-2 isoform is structurally similar to the soluble HLA-G5 isoform, while S2 Qa-2 would correspond with a soluble HLA-G4 isoform. (B) Expression of Qa-2 mRNA in preimplantation embryos (M = 100 bp ladder; + = C57BL/6J; - = CBA/CaJ; 8C = 8-cell embryos, B = blastocysts). The positive and negative controls are from activated T cells, prepared as described in the text. GPI-linked Qa-2 appears at 292 bp while S1 Qa-2 appears at 175 bp.



eastern University's AAALAC-accredited animal care facility. All use and care of the mice followed NIH guidelines.

Reverse-Transcriptase–Polymerase Chain Reaction

RNA was isolated from C57BL/6J eight-cell stage embryos, blastocysts, and Con A-activated lymphocytes using the Stratagene (Cedar Creek, TX, USA) Micro RNA Isolation Kit. cDNAs were synthesized from the isolated RNAs under standard conditions using 100 units RT-MuLV reverse transcriptase, 2.5 μ M random hexamers, and 6 μ l RNA in a 40 μ l reaction. Hemi-nested polymerase chain reaction (PCR) was performed using the published protocol and primers of Ulker *et al.* [9]. The second round PCR used the same upper primer and a new lower primer (5' GAGCCCATAGGCTCAGGGGA) designed in our laboratory and 4 μ l first round PCR reaction for template.

Lipid Raft Colocalization

JEG-3 cells (HLA-G-expressing choriocarcinoma) and EL-4 cells (Qa-2-positive lymphoma), labeled with Hoechst 33342 (Molecular Probes, Eugene, OR, USA), were incubated at 37 °C with anti-HLA-G (clone MEM/G-9, Serotec, Raleigh, NC, USA) or anti-Qa-2 (clone 69H1-9-9, eBioscience, San Diego, CA, USA) as the primary antibody. Lipid rafts were labeled by adding Alexa Fluor594-labelled cholera toxin β subunit (CT β ; Molecular Probes) with the primary antibodies. The primary antibodies were crosslinked using Alexa Fluor488-goat anti-mouse IgG at 37 °C. Raft patching was enhanced by treatment with unlabeled goat anti-CT β (Calbiochem, San Diego, CA, USA). Fluorescence microscopy images were collected with a Zeiss Axioplan2 epifluorescence microscope and processed using Metamorph software (Universal Imaging Corporation, Downingtown, PA, USA).

Protein Painting With Immunoaffinity-Isolated Qa-2

Qa-2 was extracted from C57BL/6J splenic mononuclear cells in 0.1% NP-40 and 0.1% saponin (from *Quilaja* bark; Sigma, St. Louis, MO, USA; 2×10^9 cells/60 ml), clarified by ultracentrifugation and concentrated by centrifugal ultrafiltration (10 kD-cutoff units; Vivaspin, Carlsbad, CA, USA). The extract was applied to an immunoaffinity matrix made with anti-Qa-2 (ascites from clone Qa.m2; Cedarlane, Hornby, Ontario) and immobilized protein A (Pierce, Rockford, IL, USA). Qa-2 was eluted with 0.5% sodium deoxycholate/0.65M NaCl, pH 7.8. The eluate was positive for Qa-2 by ELISA and produced a single 40 kD SDS-PAGE electrophoresis band. Aliquots of purified Qa-2 were processed in Vivaspin units, reducing detergent concentration to \leq

1×10^{-4} % and exchanging the extraction buffer for Dulbecco's Modified Minimal Essential Medium (GIBCO, Grand Island, NY, USA) and 0.1% gelatin (Sigma). Splenic mononuclear cells from Qa-2 negative mice (CBA/CaJ or B6.K1) were incubated in Qa-2 for 2 hours at 37 °C. Painting of extensively washed cells was demonstrated by immunostaining (anti-Qa-2 1-1-2-biotin; BDPharmingen; and streptavidin-TriColor; Caltag, South San Francisco, CA, USA) and flow cytometry.

RESULTS

Detection of Soluble Splice Variants of Qa-2 in Preimplantation Embryos

Hemi-nested PCR yielded no bands in the Con A-activated CBA/CaJ lymphocyte sample, but two bands were observed in the C57BL/6J sample (Figure 1B, left panel). The upper 292-bp band represents the longer GPI-linked Qa-2 mRNA splice variant, and the lower 175-bp band represents the shorter S1 Qa-2 mRNA splice variant. The blastocyst (Figure 1B, middle panel), and 8-cell (Figure 1B, right panel) samples yielded two bands, demonstrating the presence of both GPI-linked and S1 Qa-2 mRNA in preimplantation embryos.

Lipid Raft Colocalization

CT β conjugated with Alexa Fluor594 was used to label rafts in EL-4 cells. As expected [10], we confirmed that GPI-linked Qa-2 localizes in lipid rafts; cross-linked Qa-2 was found to co-localize with rafts in EL-4 cells in the capping pattern characteristic of activated lymphocytes (Figure 2 [a–e]). HLA-G colocalization with CT β -labeled lipid rafts was also seen in JEG-3 cells (Figure 2 [f–j]), although HLA-G expression was heterogeneous and co-localization was seen in most, but not all, cells.

Protein Painting

Previously we used crude extracts containing Qa-2 to paint T lymphocytes and preimplantation embryos from Qa-2 negative mice [8]. In Figure 3 we demonstrate that immunoaffinity-purified Qa-2 can be used to change the surface phenotype of Qa-2 negative splenic mononuclear cells to Qa-2 positive. In a separate experiment, cells from the Qa-2-negative B6.K1 congenic strain were also successfully painted. Among B6.K1 cells that double-stained for the B cell marker, CD19, median fluorescent intensity for Qa-2 staining increased from 33.2 for unpainted cells to 58.6 units after painting. Median fluorescence for Qa-2 staining of CD3-positive T cells increased from 9.3 to 12.4 units.

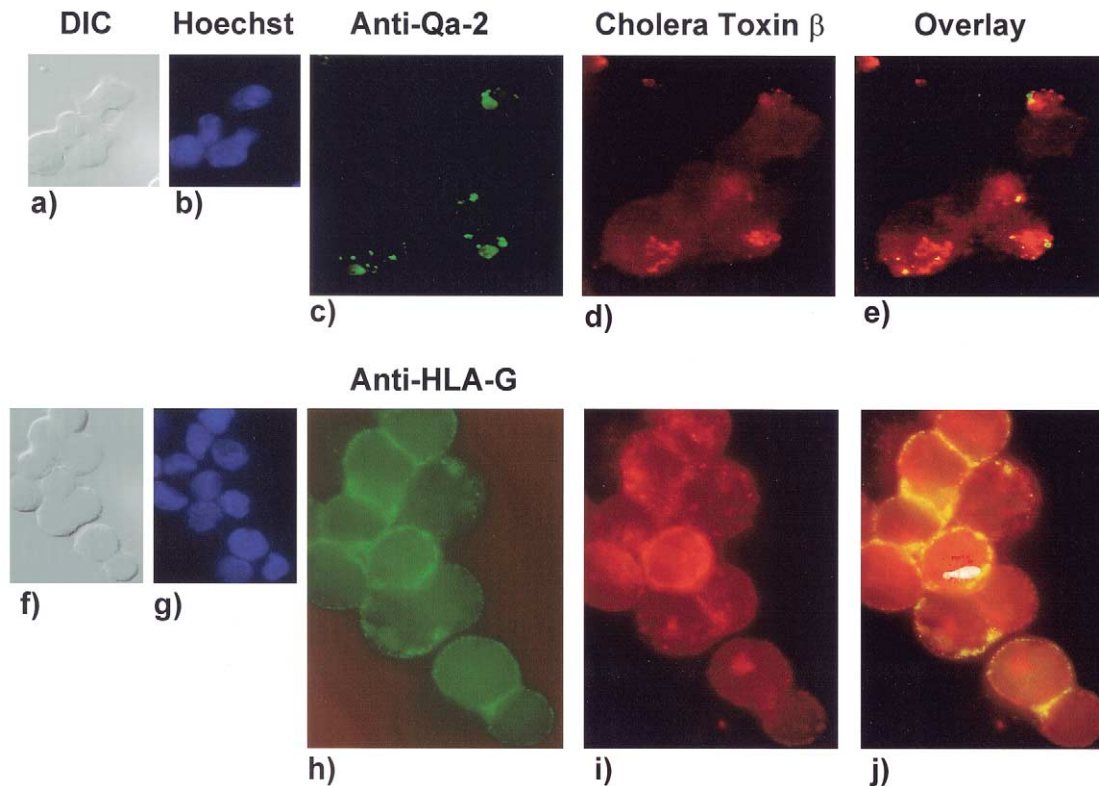


FIGURE 2 Qa-2 and HLA-G co-localize in lipid rafts. EL-4 cells (top row a-e) and JEG-3 cells (bottom row f-j). Differential interference contrast (a and f), Hoechst 33342 nuclear staining (b and g), anti-Qa-2 (c) and anti-HLA-G (h) labeled with Alexa Fluor488 goat anti-mouse IgG, GM1 ganglioside raft marker labeled with cholera toxin subunit β conjugated with Alexa Fluor594 (d) and (i). Overlay images of anti Qa-2 and CT β (e) and HLA-G and CT β (j). Yellow color is indicative of colocalization of Qa-2 or HLA-G and lipid rafts. HLA-G expression in JEG-3 cells was heterogeneous, consistent with previous reports [17].

DISCUSSION

The absence of orthology among class I genes across mammalian orders mandates that functional homology constitute the standard of comparison between mouse and human class I genes [3]. The search for a human *Ped* gene thus requires that the candidates are similar enough in structure to accomplish the same functions, that they are expressed at the appropriate developmental stage, and that they can mediate equivalent inter- and intracellular processes.

Both Qa-2 and HLA-G produce soluble isoforms by alternative RNA splicing (Figure 1). Qa-2 has two known soluble isoforms, S1 Qa-2, corresponding structurally with HLA-G5, and S2 Qa-2 lacking the α_3 domain and corresponding with a soluble HLA-G4 molecule. Production of soluble HLA-G by embryos appears

to be a prerequisite for establishing a successful pregnancy [11], whereas the presence of Qa-2 is not mandatory for embryonic survival. Production of soluble Qa-2 isoforms by mouse preimplantation embryos has not previously been assayed. In this study, we have shown that the S1 Qa-2 mRNA transcript is expressed in 8-cell and in blastocyst mouse embryos. The results demonstrated do not preclude the presence of S2 Qa-2 mRNA in preimplantation embryos, because the primers used were targeted to regions of the transcript present only in S1 Qa-2 and the GPI-anchored Qa-2 isoforms. Experiments are currently underway using primers designed to amplify all three known isoforms of Qa-2. The primers have been tested using mouse T cells and the S2 Qa-2 mRNA transcript has been detected in both resting and activated cells (data not shown).

Functional homology between Qa-2 and HLA-G implies that the six amino acid truncated tail of HLA-G is equivalent to the GPI-linkage of Qa-2. Alternative use of GPI-anchored versus short cytoplasmic-tailed protein homologs in rodents and man has been reported for carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), where a short 10–12 amino acid cytoplasmic domain isoform in rodent leukocytes has been functionally replaced by a GPI-linked isoform in the human [12].

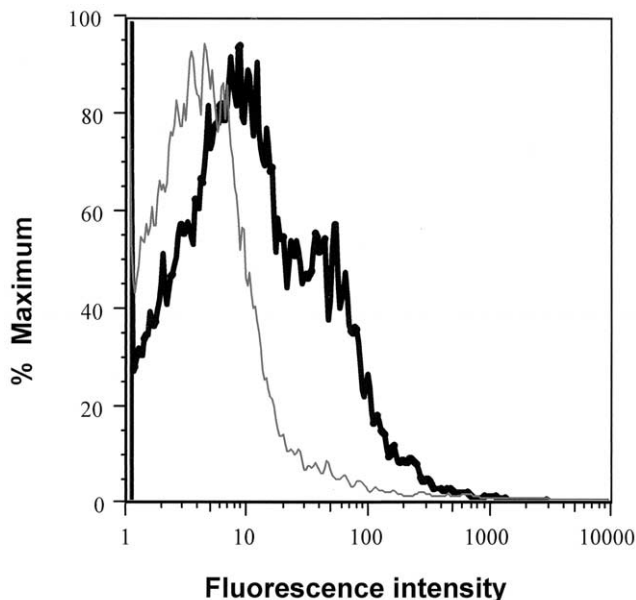


FIGURE 3 Protein painting of Qa-2-negative (CBA/CaJ) splenic mononuclear cells with immunoaffinity-purified Qa-2. Cells were incubated in either medium (pale histogram) or medium containing Qa-2 (dark histogram), washed, and stained with anti-Qa-2-biotin and streptavidin-TriColor.

GPI-linked proteins have been shown to transduce signals when cross-linked on the cell surface [13]. Cross-linking of Qa-2 induces proliferation in murine T-lymphocytes and in embryos [14], a finding consistent with the localization of Qa-2 in lipid rafts in both lymphocytes and preimplantation embryos. We have now demonstrated colocalization of cross-linked Qa-2 with lipid rafts in mouse EL-4 lymphoma cells. Using JEG-3 choriocarcinoma cells, we have also shown that HLA-G can co-localize in lipid rafts in the cell membrane, suggesting that HLA-G, like Qa-2, may have the ability to initiate signal transduction via signaling molecules abundant in lipid rafts.

Finally, the functional homology between Qa-2 and HLA-G may be of more than merely academic interest if experiments modeling potential therapeutic benefits of Qa-2 expression can be extended via this homology to clinical applications. Our laboratory has extensively documented the reproductive advantage possessed by Qa-2 positive (*Ped* gene-positive) mice [1]. Our present results confirm that purified Qa-2 protein can be used to paint a variety of cell types. Also, recombinant class I MHC molecules can be engineered to include GPI tails so that they can be used for painting [15]. Given that painting with Qa-2 increases the rate of embryo cleavage [8], and that rapid cleavage is a trait of the healthiest human embryos observed during assisted reproduction [2], painting with GPI tail-engineered HLA-G may have therapeutic potential.

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