New Approach for M-Cell-Specific Molecules Screening by Comprehensive Transcriptome Analysis

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New Approach for M-Cell-Specific Molecules Screening by Comprehensive Transcriptome Analysis

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Abstract

A minor population of M cells within the follicle-associated epithelium (FAE) of intestinal Peyer's patches (PPs) serves as a major portal for entry of exogenous antigens. Characterization of the mammalian M cells, including identification of M-cell surface molecules used for bacterial uptake, has been hampered by their relative rarity. In contrast, M cells constitute virtually all of the FAE cells in the avian bursa of Fabricius. We therefore performed comparative gene expression profiling of chicken and murine FAE to identify commonly expressed genes by M cells in both species. The comprehensive transcriptome analysis revealed that 28 genes were commonly up-regulated in FAE from both species. *In situ* hybridization revealed that annexin A10 (*Anxa10*) mRNA was scattered in FAE, and co-localized with *Ulex europaeus* agglutinin-1 binding to M cells. Whole-mount immunostaining also revealed that cellular prion protein (PrP^C) was expressed on the luminal side of the apical plasma membrane of M cells, and co-localized with grycoprotein 2 that recognizes only M cells in murine PP. Our findings identify new M-cell-specific molecules through using comprehensive transcriptome analysis. These conserved molecules in M cells of mice and chickens may play essential roles in M-cell function and/or differentiation.

Key words: M cell; the bursa of Fabricius; annexin A10; cellular prion protein

1. Introduction

The mucosal surface of the gastrointestinal tract is continuously exposed to enormous numbers of commensal microorganisms and occasionally to pathogens. In this context, the gut-associated lymphoid tissue (GALT), such as Peyer's patches (PPs) and appendix, serve as sentinel for recognition and initiation of the immune response to intestinal microbes.[1] One of the unique features of the GALT is to sample luminal antigens directly across the mucosal epithelium for the purpose of immunosurveillance. The luminal side of the GALT lymphoid follicles is covered by follicle-associated epithelium (FAE), within which are M cells that actively transport luminal macromolecules through transepithelial membrane trafficking, a process referred to as transcytosis.[2,3]

The apical plasma membrane of M cells is specialized for the uptake of antigen, featuring reduced glycocalyx and lack of hydrolytic enzymes. In addition, the basal plasma membrane of M cells often is deeply invaginated to form a so-called 'M-cell pocket' where dendritic cells (DCs) and lymphocytes migrate. This reduces the migratory distance between the apical and basal plasma membranes for antigen transport. Furthermore, reduction in the number of lysosomes as well as the activity of lysosomal hydrolases has

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been reported for M cells. These observations are consistent with the notion that M cells transport luminal antigens intact to the GALT lymphoid follicles. The delivered antigens are then captured by immature DCs residing in the subepithelial dome region beneath the FAE, and the antigen-primed DCs undergo maturation and migrate in the T-cell area of the GALT to present antigens to T cells, leading ultimately to the production of IgA antibodies by B cells.[1] Antigen delivery through M cells is thus critical for induction of mucosal immune responses for host defense. Paradoxically, the M-cell-dependent antigen uptake can be exploited by diverse pathogenic microbes, including *Salmonella* spp. and *Yersinia enterocolitica*, as a portal for entry into the host.[4,5,6]

Despite the importance of M cells in mucosal immune response as well as microbial infection, there are only a few M-cell-specific molecules so far been identified. Elucidation of M-cell-specific molecules is needed for better understanding of M cells, such as the molecular mechanisms for antigen transcytosis and invasion of microorganisms, which may lead to the development of new methods for drug delivery, vaccination as well as prevention of infectious diseases. In an attempt to identify M-cell-specific molecules, we established a method to separate murine FAE and villous epithelium (VE) for subsequent comparative transcriptome analysis.[7] However, it is still difficult to identify M-cell-specific molecules, especially those expressed at low levels, because the M cells constitute only ∼5−10% of the FAE cells.[8]

The bursa of Fabricius (BF), a central lymphoid organ in birds, is essential for the diversification of the B-cell repertoire. BF contains 8000−12 000 B-cell follicles, the luminal surface for which is covered almost exclusively by M cells.[2,9] The bursal FAE is thus a convenient source of highly enriched M cells for the purpose of identifying M-cell-specific molecules, including the posited receptors for antigen uptake and transcytosis.

In this study, we attempted to identify M-cell specific molecules, by taking advantage of transcriptome analysis of the avian bursal FAE that is composed almost entirely of the M cells. The data obtained from chicken FAE were then paired with data obtained from murine PP FAE for comparative transcriptome analysis. M-cell-specific expression of the candidate genes was examined by quantitative PCR (Q-PCR), *in situ* hybridization (ISH) and immunostaining.

2. Materials and methods

2.1. Animals
Nineteen-day-old line-M chickens were purchased from the Nippon Institute Biological Science. BALB/c mice were purchased from CLEA Japan and were maintained under specific pathogen-free condition in the vivarium of RIKEN Research Center for Allergy and Immunology and Yokohama City University until use in experiments. All animal experiments were approved by the Animal Research Committee of RIKEN Yokohama Research Institute and Yokohama City University.

2.2. Preparation of bursal FAE and interfollicular epithelium
The BF was dissected from the cloaca and soaked in Hank's Buffered Salt Solution (HBSS) (GIBCO) containing black ink for 5 min at room temperature in order to visualize the FAE. After incubation, the tissue was treated with 40 mM EDTA/25 mM Hepes (pH 7.4) in HBSS for 5 min at room temperature to collect bursal FAE, majority of which is liberated from interfollicular epithelium (IFE) in this condition; to recover pure bursal IFE, the tissue was treaded in the same solution for 30 min at 37°C to completely remove FAE. Bursal FAE and IFE were collected with 29G needle under stereomicroscope monitoring as described previously.[7]

2.3. Gene expression analysis
Chicken genome-wide gene expression was examined by using GeneChip Chicken Genome Array (Affymetrix) containing oligonucleotide probe sets for 32 733 transcripts and expressed sequence tags. Total RNA was extracted from FAE and IFE using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Double-stranded cDNA was synthesized from 0.1 μg of total RNA by two-cycle target labeling according to the manufacturer's instructions. The cDNA was subjected to *in vitro* transcription in the presence of biotinylated nucleotide triphosphates. The biotinylated cRNA was hybridized with a probe array for 16 h at 45°C. The hybridization was performed with pooled samples of FAE or IFE from a chicken. The hybridized products were stained with streptavidin−phycoerythrin, and then scanned with a Hewlett-Packard Gene Array Scanner. The fluorescence intensity of each probe was quantified using GeneChip Analysis Suite 5.0 software (Affymetrix). The level of gene expression was determined as the average difference using the GeneChip software. Data analysis was further performed with Genespring software version 7.0 (Silicon Genetics). Measurement values <0.01 were set at 0.01, and per-chip normalization was performed to the median of all measurements. Finally, two filtering were performed; one was to filter on expression level select raw from 100 to 61 022, and the other on flags that presence or marginal in FAE and IFE.

Expression data were considered significant when they differed by at least 2-fold between FAE and IFE. All microarray data have been deposited at GEO under accession number GSE 16081.

2.4. Database analysis

The expression of candidates for M-cell-specific molecules in various tissue and immune cells was analyzed with Reference Database for Immune Cells (RefDIC: http://refdic.rcai.riken.jp).[10] GOstat (http://gostat.wehi.e.du.au/) was used to find functional annotation or Gene-Ontology (GO) groups which are highly represented in the selected sets of genes.[11]

2.5. Q-PCR analysis

Total RNA was extracted from FAE and VE of mouse PP using RNeasy Kit (Qiagen) and was reverse-transcribed using ReverTra Ace-α (TOYOBO). Q-PCR was performed to quantify mRNA expression levels using the SYBR Premix Ex Taq and the Thermal Cycler Dice Real Time System (TAKARA). A specific primer set for each gene was as follows: *Anxa10* 5′-AACTTGGTCCAGGCAAACAGG-3′ (forward) and 5′-ACGAACAATAGCAACCAGCAGC-3′ (reverse), *Prnp* 5′-GGTCCCTTTGATGGAGTCTGTC-3′ (forward) and 5′-TGTGGATGCTCTAGCTATCCCA-3′ (reverse).

2.6. In situ *hybridization*

A 470 bp fragment of *Anxa10* cDNA was amplified from FAE-derived cDNA by PCR using the specific primers: 5′-ATAGGATCCTAACACAACGCAGCAATG-3′ (forward) and 5′-TTACTCGAGGATCATCTGCATCA TGGTTTT-3′ (reverse). The PCR product was digested with *Bam*HI and *Xho*I, and subcloned into pcDNA3 (Invitrogen) and was used for generation of non-targeted RNA probes as a control probes or antisense RNA probes. Paraffin-embedded PP sections of mice were obtained from Genostaff. ISH was done by Genostaff. In brief, the sections were hybridized with digoxigenin labeled RNA probes at 60°C for 16 h. The bound label was detected using NBT-BCIP, an alkaline phosphate color substrate. After ISH, sections were treated with 3% hydrogen peroxide in PBS for 15 min followed by Protein Block (Dako) for 10 min. They were incubated with 0.25 mg/ml Biotinylated *Ulex europaeus* agglutinin-1 (UEA-1) (Vector), at 4°C overnight. After washing with PBS, the sections reacted with peroxidase conjugated streptavidin (Nichirei) for 5 min. Peroxidase activity was visualized by diaminobenzidine. The control probe treated sections were counterstained with Kernechtrot (MUTO PURE CHEMICALS).

2.7. Whole-mount immunostaining

PPs were excised from the small intestine, fixed with Cytofix/Cytoperm (BD Biosciences) for 1 h at 4°C and then incubated with 10 μg/ml anti-CD16/32 mAb (93; eBioscience)/0.1% saponin/0.5% BSA in PBS to block non-specific Fc binding. The whole-mount specimens were then stained with 2 μg/ml mouse PrP mAb (SAF-32; Cayman Chemical) or an isotype-matched control IgG (BD Biosciences) overnight at 4°C, followed by 10 μg/ml Alexa Fluor 488-conjugated anti-mouse IgG Ab (Molecular Probes). The specimens were further stained with 20 μg/ml Rhodamine-conjugated UEA-1 (Vector Laboratories) or with a glycoprotein 2 (GP2) mAb (2F11C3) followed by Alexa Fluor 555-conjugated anti-rat IgG (Invitrogen). The specimens were analyzed with a DM-IRE2 confocal laser scanning microscope and Leica confocal software (Leica Microsystems).

2.8. Statistics

Statistical analysis was performed with the Mann–Whitney U-test. Differences were considered as significant at $P < 0.05$.

3. Result and discussion

3.1. Comparative transcriptome analysis of chicken and murine FAE

We separated the bursal FAE and the IFE surrounding the FAE in the chicken BF by modifying the dissection method used to separate mammalian FAE cells.[7] After soaking the BF in HBSS containing black ink, the FAE was easily distinguished as black areas under a stereomicroscope (Fig. 1A, upper left). From these specimens, we separated the bursal FAE and the surrounding IFE (Fig. 1A, lower left and right, respectively). Total RNA prepared from these samples was used for microarray analysis. The scattered plots of the normalized gene expression profile for bursal FAE versus IFE revealed that the expression levels were within 2-fold for the majority of genes (Fig. 1B), whereas the expression of 682 annotated genes was up-regulated at least 2-fold in bursal FAE compared with IFE and the expression of 260 annotated genes was down-regulated in bursal FAE by at least 2-fold. To characterize these genes, we classified them using GOstat. Most of the top 10 GO categories for the up-regulated genes were converged into 'transmembrane transporter activity' or 'ATPase activity' (Table 1). In contrast, there was no characteristic enrichment of GO categories in the down-regulated genes. Given that BF M cells vigorously take up and transport exogenous antigens, it is conceivable that a group of genes responsible for membrane transport using ATP as a driving force may be constitutively
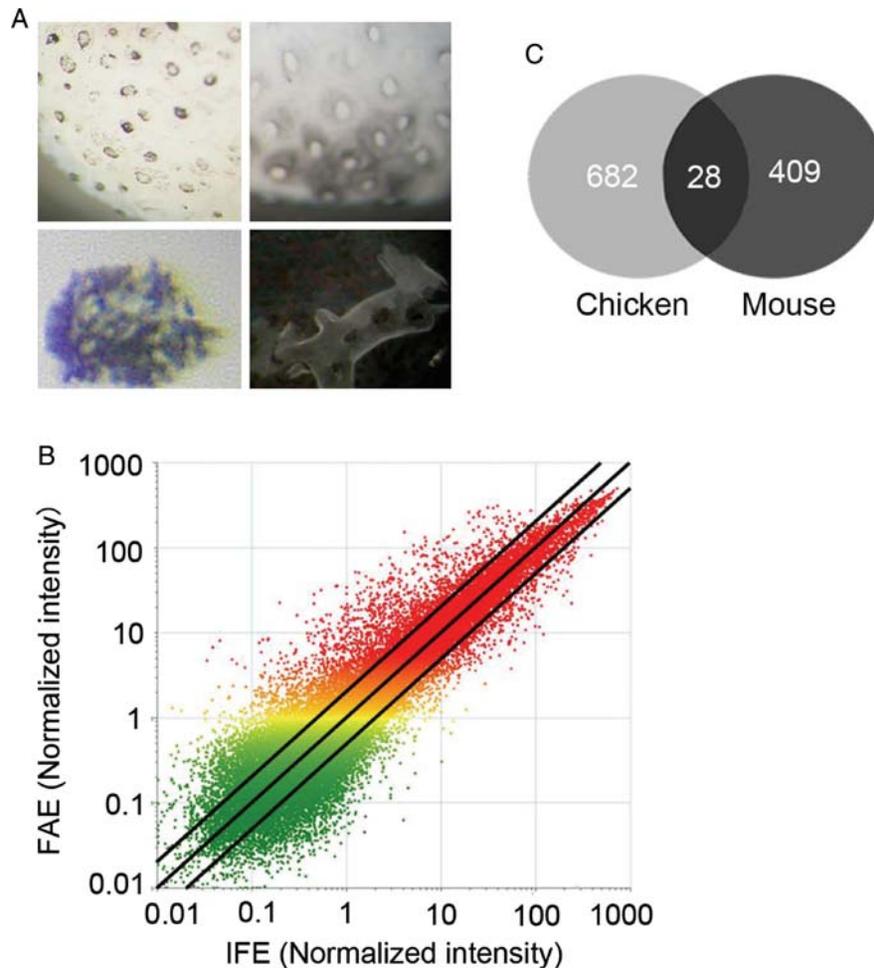
Figure 1. Comparative transcriptome analysis. (A) Dissection of bursal FAE and IFE. Upper left, BF was soaked in HBSS containing black ink and observed by stereomicroscopy. FAEs taking up the ink dye are easily distinguished as black spots. Upper right, BF after collecting FAEs. The FAE removal sites appear as white spots. Lower panels show an isolated bursal FAE (left) and bursal IFE (right). (B) Comparison of gene expression profiling between FAE and IFE. Normalized data obtained by microarray analysis and applied filtration on flags are illustrated by a scatter plot of gene expression. The upper and lower lines indicate the threshold levels of 2-fold change. (C) Comparative transcriptome analysis of chicken and murine FAEs. Annotated genes up-regulated 2-fold or more in FAE from both species are shown in a Venn diagram.

up-regulated in FAE. Thus, the feature of the enriched GO categories for the up-regulated genes in bursal FAE could reflect the specialization of M cells.

3.2. Identification of candidates M-cell-specific molecules

We have previously identified genes specifically up-regulated in FAE compared with VE in mice.[7] To identify M-cell-specific molecules conserved in chickens and mice, we compared the genes up-regulated in the FAE of chickens and mice. As shown in Fig. 1C, 28 genes were up-regulated in both species (Supplementary Table S1). These genes were classified mainly as cytoskeleton genes (*Actn1*, *Evl*, *Lcp1*, *Rac2* and *Vim*), nucleic acid binding genes (*Cugbp2*, *Fli1*, *Poll*, *Pou2af1* and *Slbp*), receptor activity genes (*CD44*, *Cxcr4*, *Tnfrsf19*, *Tnfrsf1b*, *Traf3* and *Tyro3*)

and others. We next utilized a public database RefDIC that contains microarray analysis data of different tissues and immune cells, which enabled us to examine tissue- and cell-wide expression of the target genes. Since immune cells often reside in the M-cell pockets, it could be possible that expression of immune cell-specific genes is falsely detected in our FAE preparation. Therefore, we chose genes specifically expressed in FAE but not in VE, with relatively low expression levels in immune cells (Fig. 2). Ten genes (*Anxa10*, *Clu*, *Gpr137b*, *Poll*, *Prnp*, *Sacs*, *Sgsh*, *Slc16a3*, *Tnfrsf19* and *Tyro3*) were selected using these criteria, whereas others were not (Supplementary Fig. S1). Among these 10 genes, the expression levels of three genes, *Anxa10*, *Clu* and *Prnp*, were more than 2-fold higher in FAE than in VE regardless of housing condition of mice, germfree or conventional, in RefDIC.[10] Since our Q-PCR analysis

Table 1. The top 10 GO categories for the genes up-regulated in FAE

GO number	GO category	Observed transcripts	Total transcripts	P-value
0015399	Primary active transmembrane transporter activity	8	153	0.0717
0015405	P–P-bond-hydrolysis-driven transmembrane transporter activity	8	153	0.0717
0042625	ATPase activity, coupled to transmembrane movement of ions	8	111	0.0717
0042626	ATPase activity, coupled to transmembrane movement of substances	8	146	0.0717
0043492	ATPase activity, coupled to movement of substances	8	147	0.0717
0019829	Cation-transporting ATPase activity	5	54	0.0717
0016820	Hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances	8	151	0.0717
0003676	Nucleic acid binding	17	2762	0.0717
0019887	Protein kinase regulator activity	4	28	0.0717
0019207	Kinase regulator activity	4	37	0.0911

Genes up-regulated more than twice in the FAE compared with the IFE were analyzed with GOstat (http://gostat.wehie.du.au/). The table summarizes the top 10 GO categories according to the P-value.

confirmed the up-regulation of *Anxa10* and *Prnp* in FAE compared with VE (Fig. 3), we decided to further analyze these two molecules to determine whether they are M-cell-specific. Concerning *Clu*, recent studies have reported that this gene, which encodes clusterin, is expressed in entire FAE, among which a particularly high expression was observed in M cells for mice and humans,[12,13] in keeping with our finding.

3.3. Expression of Anxa10 mRNA in murine PP M cells

Having identified *Anxa10* and *Prnp* by our comparative transcriptome analysis as candidate for M-cell-specific genes in mice, we examined their expression pattern in FAE. Because antibodies specific for annexin A10 encoded by *Anxa10* were not available, we examined the cellular expression of *Anxa10* by ISH. A positive signal for *Anxa10* mRNA was observed in some of the cells in mouse FAE (Fig. 4A and B), whereas no signals were observed with a control probe (Fig. 4C). The *Anxa10* signal co-localized with UEA-1 that recognizes M cells in FAE (Fig. 4B). The result suggests that *Anxa10* mRNA is expressed specifically in M cells in the murine gut epithelium.

Owing to the property to bind to phospholipids in a Ca^{2+}-dependent manner, annexin family proteins are involved in various membrane-related functions.[14,15] Although annexin A10 is implicated in regulating hepatocyte differentiation[16] and pituitary hormone synthesis and/or secretion,[17] the molecular basis for its function remains unclear.

Among the family members, annexins A1, A2 and A6 are present on endosomal compartments and are thought to be involved in the endocytic pathway.[14] In particular, annexin A6 has been proposed to be involved in budding of clathrin-coated pit.[18] Annexin A6 arose from the fusion of the genes encoding annexins A5 and A10[15] implicating the involvement of the latter two annexins as well in membrane traffic events in the endosomal pathways. Indeed, the network of annexin A5 reportedly bends the membrane patch into the cell and elicits budding, endocytic vesicle formation and cytoskeleton-dependent trafficking of the endocytic vesicles.[19] Intriguingly, annexin A5 is expressed in M cells and implicated in endocytic transport and membrane scaffolding.[12] Our findings thus suggest that annexin A10 could also be involved in M-cell-specific membrane traffic events such as antigen transcytosis.

3.4. Cellular prion protein (PrP^C) expression on the apical plasma membrane of murine M cells

Prnp encodes cellular prion protein PrP^C, a glycosylphosphatidylinositol (GPI)-anchored membrane protein. We performed whole-mount immunostaining
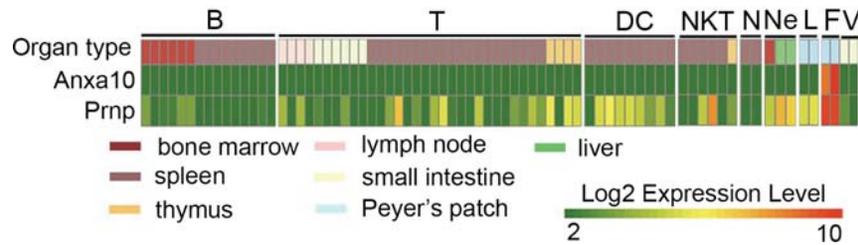
Figure 2. Strong expression of Anxa10 and Prnp mRNA in murine PP FAE among various tissues and cells. Immune cell (B, B cell; T, T cell; DC, dendritic cell; NKT, NK T cell; N, natural killer cell; Ne, neutrophil; L, leukocyte) and intestinal epithelium (F, FAE; V, VE) distribution of the expression of *Anxa10* and *Prnp* analyzed with RefDIC. Normalized data of the expression level of each gene are shown as a gradation from green (low) to red (high). With regard to FAE and VE, two different RNA samples were obtained from germ-free mouse (left column) and conventional mouse (right column) conditioned groups, respectively.
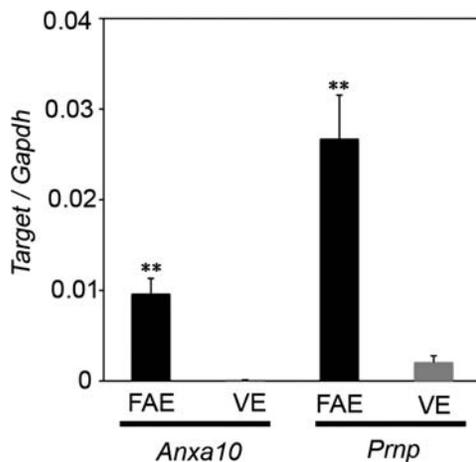

Figure 3. Preferential expression of Anxa10 and Prnp mRNA in murine PP FAE compared with VE. Q-PCR analysis was performed for *Anxa10 Prnp* mRNA expression in murine FAE and VE. The relative expression levels of 'each gene' to *Gapdh* are shown. Values represent the mean ± SD of three samples from different mice. **$P < 0.01$.
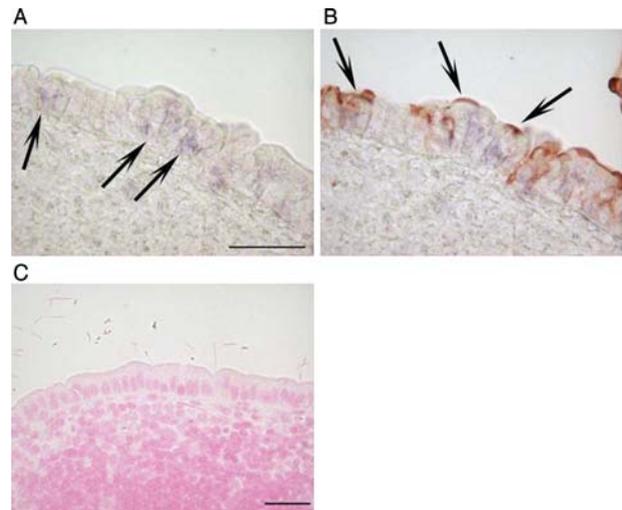

Figure 4. Specific expression of Anxa10 mRNA by murine M cells. (A) ISH analysis of *Anxa10* mRNA expression detected using specific antisense probe. Arrows indicate the ISH signals for *Anxa10* mRNA. (B) The section after ISH staining (A) was co-staining with UEA-1. *Anxa10* signals were co-localized with UEA-1 (arrows). (C) ISH analysis of murine PP FAE using a control probe. Scale bars: 50 μm.

of PP with a PrP mAb to determine which cell types express PrP^C in mouse FAE. The expression of PrP^C was observed primarily in a subpopulation of cells in the peripheral region of the FAE (Fig. 5A and C); no signal was observed with specimens stained with an isotype-matched IgG control of irrelevant specificity (Fig. 5B and D). Most of the PrP^C-expressing cells were also stained with the UEA-1 lectin (Fig. 5A) and with an mAb to GP2 (Fig. 5C), a specific marker for murine PP M cells,[20] although co-localization of PrP^C with GP2 was apparently less frequent than with UEA-1. This is at least partly because GP2 is not expressed in all M cells but restricted to more matured M cells compared with PrP^C (our unpublished observation). This observation demonstrates that PrP^C is preferentially expressed by M cells in the FAE. In addition, the $X-Z$ sectioning images obtained by confocal microscopic analysis indicated that PrP^C was expressed on the apical plasma membrane of M cells (Fig. 5C), where it observed to be co-localized with GP2, which is

expressed on the apical surface of M cells.[20] These findings suggest that PrP^C molecules are especially abundant on the apical plasma membrane of M cells.

PrP^C is the causative agent of transmissible spongiform encephalopathy. According to the 'prion hypothesis', the infectious isoform of prion protein, termed PrP^{Sc}, replicates by interacting with PrP^C and mediating its conformational change into PrP^{Sc}.[21] In addition, PrP^C has been shown to bind selectively to PrP^{Sc} *in vitro*.[22,23] Compared with its well-defined pathological significance, the physiological functions of PrP^C remain unclear. PrP^C is highly expressed not only by cells in the central nervous system, but also by follicular DCs, mature myeloid cells and activated T cells. This cellular distribution suggests an immuno-surveillance role for PrP^C.[24] Our data showing that PrP^C is strongly expressed on the apical plasma membrane of M cells (Fig. 5A and C) suggest PrP^C as an
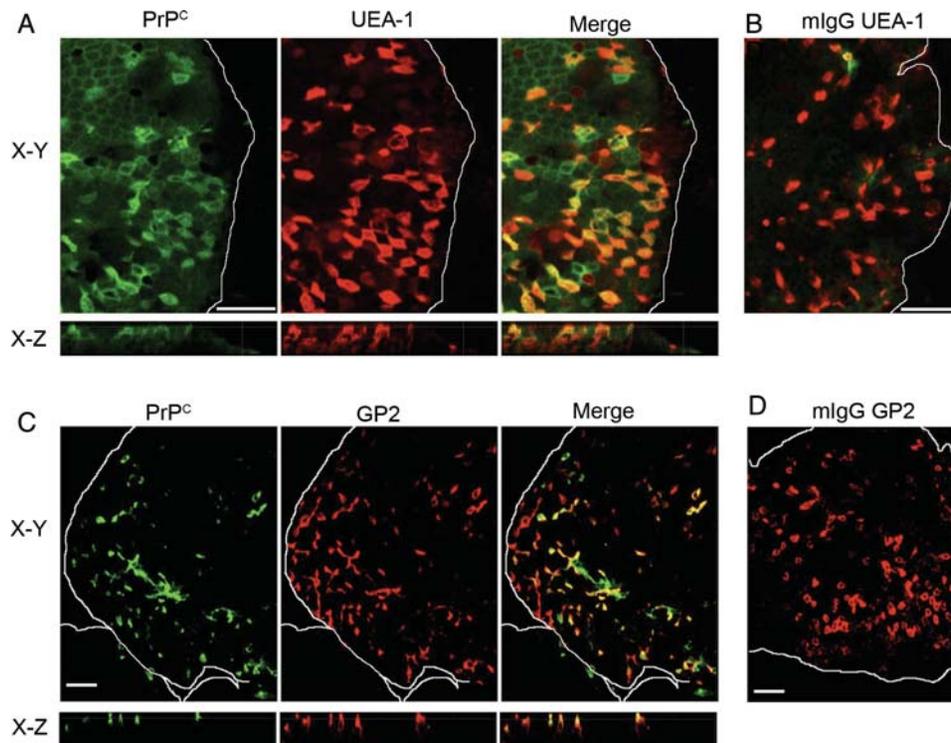
Figure 5. Preferential expression of Cellular prion protein (PrP^C) by murine M cells. Whole-mount specimens of murine PP were stained with a PrP mAb (green), UEA-1 or GP2 (red) and then analyzed by confocal laser-scanning microscopy. (A) The $X-Y$ view from the luminal side stained with PrP^C, UEA-1 and the merged image (PrP^C and UEA-1) is shown. White lines indicate position of FAE in murine PP. (B and D) The $X-Y$ view from the luminal side stained with an isotype-matched control mouse IgG. (C) The $X-Y$ view from the luminal side stained with PrP^C, GP2 and the merged image (PrP^C and GP2) is shown. The $X-Z$ image of FAE sectioning in a vertical direction is displayed below the lower $X-Y$ view (A and C). The upper part of the image is the apical plasma membrane side, and the lower is the basal side. Scale bars: 75 μm.

antigen receptor candidate on M cells. This hypothesis is consistent with the demonstration that exogenously expressed human PrP^C is selectively targeted to the apical plasma membrane in polarized Madin-Darby canine kidney cells and Caco-2 intestinal epithelial cells.[25] Notably, clusterin possesses a chaperone-like function for PrP.[26] Together, the strong expression of both PrP^C and clusterin by M cells[12,13] may suggest a possible role for clusterin in specific membrane transport of PrP^C in M cells.

There is accumulating evidence indicating that diverse infectious agents and their toxins utilize GPI-anchored proteins to gain entry into host cells.[27] For example, CD48 plays an important role in *E. coli* phagocytosis via FimH recognition in macrophages and mast cells.[28] Furthermore, PrP^C on macrophages has been reported to recognize surface-exposed Hsp60 of *Brucella abortus* and to facilitate internalization of the bacteria.[29] By analogy, PrP^C on M cells could also interact with Hsp60 of *B. abortus*. Hsp60 proteins have been recognized as immunodominant antigens of many microbes.[30] Our present results suggest that PrP^C on the apical plasma membrane of M cells is well placed to contribute to mucosal immuno-surveillance through promoting transcytosis of *B. abortus* or other exogenous antigens. Further studies to elucidate PrP^C function on M cells are now underway.

In conclusion, we here demonstrate that comparative transcriptomic analysis of chick bursal FAE, which is almost exclusively composed of M cells,[2] and mouse FAE, which includes M cells as a minority constituents, is a useful approach to the identification of M-cell-specific molecules. In this study, we have focused on the genes that are up-regulated 2-fold or more in FAE versus IFE (for chicken) or VE (for mice). Genes up-regulated 2-fold or more in chick FAE and less than 2-fold in murine FAE could also contain M-cell-specific genes, in view of the fact that M cells constitute 5−10% of the total FAE population in mice.[8] This population of the genes will be a future target in the search for other M-cell-specific genes.

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