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The DC-SIGN of Zebrafish: Insights into the Existence of a CD209 Homologue in a Lower Vertebrate and Its Involvement in Adaptive Immunity

Ai-fu Lin,* Li-xin Xiang,* Qu-long Wang,* Wei-ren Dong,* Yong-feng Gong,* and Jian-zhong Shao2*

Dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN; designated as CD209) family members are homologous, type II membrane-associated, C-type lectins that function as cell adhesion molecules and pathogen receptors involved in innate and adaptive immunity (1–3). Currently, three family members, DC-SIGN/CD209, L-SIGN/CD209L, and CD209L2, have been identified from human and nonhuman primates (4–6). DC-SIGN/CD209 was first discovered as a human placental protein capable of binding HIV type 1 (HIV-1) gp120 and was later identified from human monocyte-derived dendritic cells (DCs) (5, 7). As an important surface adhesion molecule, DC-SIGN/CD209 has been demonstrated to play a variety of roles in innate and adaptive immunity, such as establishing interactions between APCs and resting T cells and participating in the activation of T and B lymphocytes and the aggregation and migration of APC, as well as inflammatory responses (8–12). It also acts as a pattern recognition receptor similar to the TLR, sharing a common profile of evolutionary constraints and facilitating the recognition of a wide range of pathogens, including viruses (such as HIV-1, SIV, and the hepatitis B and C viruses), bacteria, and parasites (10, 13–15). The latter may have significant implications for developing new ways of preventing pathogen infection (16). However, although numerous, the studies on DC-SIGN/CD209 have tended to be confined to a few mammals (11, 17–19), and the precise functional roles of this molecule remain elusive. For example, it is still controversial whether DC-SIGN/ICAM-3 interactions are required to establish lymphocyte activation and whether DC-SIGN/CD209 is DC specific, as previously implied (9, 20–22). To explore the functional characterization of the DC-SIGN/CD209 molecule in more depth, and especially to probe the evolutionary history of this family, it is essential that different research models, including those of lower vertebrates, also be integral participants.

Therefore, in the present paper we describe the identification and biological characterization of the DC-SIGN/CD209 homologue from a nonmammalian vertebrate species, the zebrafish (Danio rerio), a model organism representing lower vertebrates. This molecule is shown to closely associate with various APCs, including macrophages, B lymphocytes, and a possible DC-like...
The DC-SIGN OF ZEBRAFISH

Aeromonas hydrophila. Furthermore, the expression of this molecule could be up-regulated by exogenous Ags (KLH or BSA) and IL-4, a typical Th2 cytokine that is crucial for the development of adaptive humoral immunity. These observations provide the first evidence that a functional DC-SIGN/CD209 homologue exists in a lower vertebrate and strongly suggest that this molecule is essential for establishing the T cell activation crucial for the initiation of adaptive immunity to foreign Ags.

From an evolutionary standpoint, it seems reasonable to suggest that the intervention of DC-SIGN/CD209 molecules in adaptive immunity may be their primitive/basic function (at least) during early vertebrate evolution. The precise DC-SIGN/CD209-mediated cell-to-cell interactions or signaling mechanisms that underlie the adaptive immunity of lower vertebrates may have therefore become established ~450 million years ago in teleosts, wherein a number of adaptive immunity-related cells and molecules such as macrophages, T and B lymphocytes, IgGs, TCRs, BCRs, CD3, CD4, CD8, and MHCs originated (23–25).

Nonetheless, the exact molecular and cellular mechanisms that underlie teleost adaptive immunity remain to be elucidated. Given the important roles played by DC-SIGN/CD209 on DC and other APC surfaces, the identification of this molecule in zebrafish will enrich the knowledge of fish immunology. In addition, it will also contribute to a better cross-species understanding of the evolutionary history of the DC-SIGN/CD209 family and DC/APC-mediated adaptive immunity, and even to the overall mechanism of HIV infection. The latter will provide an opportunity to develop a novel fish model for clinical investigations or medical applications of DC-SIGN/CD209-based therapies.

Materials and Methods

Experimental fish

Zebrafish, 1 year old of both sexes weighing 0.5–1.0 g with body lengths of 1–2 cm, were maintained in recirculating water at 26°C. All fish were held in the laboratory for at least 2 wk before use in experiments for acclimatization and evaluation of overall fish health. Only healthy fish, as determined by general appearance and level of activity, were used for studies.

Sequence retrieval

The genome and expressed sequence tag (EST) databases maintained by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/) were used to predict the DC-SIGN/CD209 homologues in fish and other lower vertebrates, including Xenopus tropicalis and chicken, using the primate and mammalian DC-SIGN/CD209 sequences as queries. After sequences similar to DC-SIGN/CD209 were obtained by BLAST (basic local alignment search tool), they were further characterized using DNA and protein sequence analysis software with the methods that we have described previously (26–28). Genomic structures and the organizations of fish DC-SIGN/CD209 genes were predicted from genomic sequences using cDNA or ESTs available from each species.

Molecular cloning

Fish were sacrificed following anesthesia and total RNA was isolated from the whole fish using a TRIzol reagent (Invitrogen) treated with RNase-free DNase I (Qiagen). RNA concentrations were measured using a spectrophotometer and integrity was ensured by analysis on a 1.5% (w/v) agarose gel. cDNA was synthesized from 2.5 μg of total RNA by reverse transcription into first single-stranded cDNA using a 3′-Full RACE core set (TaKaRa). The cDNAs of DC-SIGN/CD209 were generated by RT-PCR with the primers shown in Table I. The 5′-Full RACE core set and 3′-Full RACE core set (TaKaRa) were used to obtain

### Table I. Oligonucleotide primers used for amplifying cDNAs and gene expression analysis

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5′→3′)</th>
<th>Use</th>
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<tbody>
<tr>
<td>CD209-F1</td>
<td>TACTACCTTAAAGTGACCTGAAAAG</td>
<td>3′-RACE, gene expression</td>
</tr>
<tr>
<td>CD209-F2</td>
<td>GGGACAAGATGAGCTGCTGTCTCAG</td>
<td>3′-RACE, gene expression</td>
</tr>
<tr>
<td>CD209-F3</td>
<td>TTTGTGCTATGCGGGGACAGAAGAC</td>
<td>3′-RACE</td>
</tr>
<tr>
<td>CD209-F4</td>
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<td>3′-RACE</td>
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<td>3′-RACE</td>
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<tr>
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<tr>
<td>3′-Adaptor</td>
<td>CTCGGTATTAGTACTAGCACCACACAG</td>
<td>3′-RACE</td>
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<tr>
<td>3′-RACE core set</td>
<td>Gene expression</td>
<td></td>
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<td>CD209-R1</td>
<td>AACAAATCTGCTACTGTTTACA</td>
<td>5′-RACE, gene expression</td>
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<td>CD209-R2</td>
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<td>5′-RACE, gene expression</td>
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<td>CD209-R2</td>
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<tr>
<td>CD154-F</td>
<td>CCTCCTGTAGTGCTTGATCGTTTAC</td>
<td>Real-time RT-PCR</td>
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<td>CD154-R</td>
<td>TCTAAACACTCTGGTACGAGTCC</td>
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<tr>
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<td>GCCTCGCCCACTACGATGATGCC</td>
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</tr>
<tr>
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<td>CCCTCGAAGAAGAACTCAGAGAAGAC</td>
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<td>Gene expression</td>
</tr>
<tr>
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<td>GCCAAGTACCAACAACCT</td>
<td>Gene expression</td>
</tr>
<tr>
<td>CSF1R-R</td>
<td>GCCAAGTACCAACAACCT</td>
<td>Gene expression</td>
</tr>
<tr>
<td>CD154-F</td>
<td>CD154-R</td>
<td>Lck-F</td>
</tr>
<tr>
<td>CD209-expF</td>
<td>CD209-expR</td>
<td>CD154-F</td>
</tr>
</tbody>
</table>

a F, Forward primer; R, reverse primer.
5'- and 3'-unknown regions. Finally, full-length cDNA sequences containing the 5'-untranslated region (UTR) and 3'-UTR were assembled. PCR products were loaded on a 1.2% (w/v) agarose gel and visualized by staining in 0.1 mg/ml ethidium bromide. All PCR products were purified using a gel extraction kit (Qiagen) and then ligated into the pUCm-T vector. The recombinant DNA was transformed into competent Escherichia coli TOP10 cells (Invitrogen). Plasmid DNA was purified using a plasmid Miniprep method (Qiagen) and sequenced on MegaBace 1000 DNA sequencer (GE Healthcare) using DYEnamic ET dye terminator cycle sequencing kit (GE Healthcare).

Sequence analysis
Potential functional motifs were searched at the PROSITE database (www.expasy.ch/prosite/). Protein sequence alignments were generated using the ClustalW program (version 1.83). Phylogenies of protein sequences were estimated with MEGA 3.0 using parsimony and a neighbor-joining method. Gene organizations (intron/exon boundaries) were elucidated by comparing fish DC-SIGN/Cd209 cDNAs with genome sequences, and figures were drawn by using GeneMapper 2.5. The EST sequences were used to help predict the structure of DC-SIGN/Cd209 genes.

Tissue distributions
To determine the distribution pattern of the DC-SIGN/Cd209 transcripts, selected tissues including kidney, spleen, liver, intestine, gill, skin, brain, heart, muscle, and gonad in healthy fish were carefully removed, flash frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted as described above and then reverse transcribed using a RNA PCR kit (avian myeloblastosis virus), version 3.0 set (TaKaRa). A nested-PCR was used to amplify DC-SIGN/Cd209 transcripts with the following program: 94°C for 30s, 55°C for 30s, and 72°C for 30s for 27 cycles using the forward (F) and reverse (R) primers Cd209f1/Cd209r1 and then 94°C for 30s, 55°C for 30s, and 72°C for 30s for 38 cycles using primers Cd209f2/Cd209r2 (shown in Table I). In parallel, β-actin (as a standard) was amplified at 94°C for 30s, 55°C for 30s, and 72°C for 30s for 28 cycles.

Soluble DC-SIGN/Cd209 production
The sequence encoding the extracellular domains of DC-SIGN/Cd209 was amplified with primers containing an EcoR1 site added on the 5'-end and an XhoI site on the 3'-end, as shown in Table I. The resulting PCR product was digested and subcloned into vector pET22b. A single colony of E. coli Rossetta (DE3) pLYsS harboring the expression plasmids was inoculated into 100 ml of Luria-Bertani medium containing chloramphenicol (100 mg/ml) and kanamycin (25 mg/ml), incubated by shaking at 37°C until the OD 600 value reached 0.6, and isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM. The culture was continued for 6 h. The expression level of the protein was assessed by analysis of total proteins on a final concentration of 1 mmol/L. The culture was continued for 6 h. The recombinant protein was purified by using Ni-NTA agarose affinity chromatography according to the manufacturer’s instructions (Qiagen). The expression level of the protein was assessed by analysis of total proteins on a final concentration of 1 mmol/L. The culture was continued for 6 h. The recombinant protein was purified by using Ni-NTA agarose affinity chromatography according to the manufacturer’s instructions (Qiagen).

Polyclonal Ab preparation
The male New Zealand White rabbits, 6 wk old and weighing ~3 kg, were immunized with 100 μg of the purified recombinant DC-SIGN/Cd209 protein in complete Freund’s adjuvant eight times at biweekly intervals. In parallel, 6-wk-old male BALB/c mice were immunized with 20 μg of purified recombinant protein in complete Freund’s adjuvant, eight times at biweekly intervals. One week after the final immunization, rabbits and mice were bled when Ab titeres above 1/10,000 as determined by microplate-based ELISA using recombinant protein adsorbed to the solid phase. The Ab was affinity purified into IgG by a protein A-agarose column and affinity-purified rabbit IgG microbeads; Miltenyi Biotec) following the manufacturer’s protocol.

Ag-mediated DC-SIGN up-regulation
To evaluate whether DC-SIGN/Cd209 was involved in immune responses to exogenous Ags, an Ag-mediated DC-SIGN/Cd209 up-regulation analysis of DC-SIGN/Cd209 was performed at both the mRNA and the protein level. After anesthetization with 0.02% tricaine, the fish were i.m. injected with two thymus-dependent Ags, BSA (Sigma-Aldrich) and KLH (Sigma-Aldrich), at a dosage of 10 μg per fish, and one bacterial pathogen, A. hydrophila, was i.p. injected at a dosage of 1 × 10⁸ CFU per fish for 2 days. For mRNA analysis the DC-SIGN/Cd209 transcripts were analyzed by RT-PCR in selected tissues, including kidney, spleen, intestine, and gill, using the protocols described above. The PCR program was the same as that described above. For protein analysis, tissues including kidney, spleen, liver, intestine, gill, skin, brain, heart, muscle, and gonad (ovary) were collected and homogenized on ice in a lysis buffer containing 50 mMol/L Tris (pH 8.0), 150 mMol/L NaCl, 100 μMol/ml PMSF, 1g/ml aprotinin, and 1% Triton X-100. The lysates were centrifuged and protein levels in the supernatants were estimated by a protein assay kit (Sigma-Aldrich). Equal aliquots of the protein extract were mixed with loading buffer, boiled for 10 min, separated by electrophoresis with a 10% SDS-polyacrylamide gel, and then transferred onto a nitrocellulose membrane for Western blot analysis. The membrane was blocked in TBS (500 mMol NaCl in 20 mM Tris (pH 7.5)) containing 2% BSA and 0.05% Tween 20 and then incubated with anti-DC-SIGN antibodies, the membrane was incubated with a HRP-conjugated secondary Ab and visualized with an enhanced chemiluminescence detection system and x-ray film. In addition, kinetic expression of the DC-SIGN/Cd209 protein was detected by ELISA from 1 to 7 days after treating the fish with KLH or A. hydrophila.

Flow cytometric analysis
To further analyze the regulation of DC-SIGN/Cd209 expression, fish were injected i.p. with 0.1, 1, 5, or 10 μg of recombinant soluble zebrafish IL-4 (produced by our laboratory), 10 μl of PBS (negative control), or 1 μg of IL-4 plus 1, 10, or 100 ng of LPS (5555.B E. coli; Sigma-Aldrich). Then, after 5 days of stimulation, whole blood cell suspensions were collected with a heparinized capillary tube. The IL-4-elevated, CD4+ cells and IL-4 plus LPS-elevated CD209+ cells were examined by flow cytometric analysis. Briefly, cells collected from the IL-4- and IL-4 plus LPS-injected groups were washed with PBS and then the leukocytes were separated by Ficoll-Hypaque centrifugation (1.077 g/ml). After blocking with 5% normal goat serum, the cells were incubated with affinity-purified rabbit anti-CD209 IgG. For control analysis, normal rabbit IgG was used instead of primary Ab. Then, the samples were incubated with secondary FITC-labeled anti-rabbit IgG (1/400; Chemicon). After washing, cell fluorescence signals were determined immediately using an argon laser-equipped FACSscan flow cytometer (BD Biosciences) with emissions at 485 nm. At least 10,000 events were collected. The data were analyzed using CellQuest software (BD Biosciences).

In vivo blockade assay
To examine whether DC-SIGN/Cd209 was involved in T cell activation and subsequent Ab production, an in vivo DC-SIGN/Cd209 blockade assay was designed. After anesthetization with 0.02% tricaine, the fish were i.m. injected with affinity-purified rabbit anti-CD209 IgG2 IgG2 at doses of 10 μg per fish, together with immunization using 10 μg of KLH per fish. In a parallel experiment, the same dosage of rabbit IgG was used as a negative control after immunization with KLH for 2 days. After 3–4 days, the activation of T cells in response to KLH was determined as up-regulated expression of T cell marker genes, including CD154 and T cell-specific tyrosine kinase (Lck), by real-time PCR (29–32). The primers used in real-time PCR are shown in Table I. After 21 days, the serum samples were collected and the production of IgM in response to KLH was measured by ELISA using anti-IgM produced by our laboratory (data not shown).

Immunofluorescence staining
After 3 days of KLH stimulation, whole blood cell suspensions were collected with a heparinized capillary tube. Leukocytes were separated by Ficol-Hypaque centrifugation (1.077 g/ml), fixed with cold methanol, and incubated with primary Abs, including mouse anti-zebrafish CD209, rabbit anti-zebrafish IgM, rabbit anti-zebrafish CD80, and rabbit anti-zebrafish CD83 (all of these Abs were prepared in our laboratory). For control staining, normal rabbit IgG and mouse IgG were used in place of primary Abs. The secondary Abs, including tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse-IgG and FITC-conjugated goat anti-rabbit-IgG (Santa Cruz Biotechnology) were used according to the manufacturer’s instructions. Samples were photomicrographed under a confocal laser-scanning microscope (LSM 510; Carl Zeiss).

Magnetic cell sorting and gene expression analysis
To further examine the type of cell expressing the DC-SIGN/Cd209 molecule, the cells were sorted out by magnetic beads (goat anti-rabbit IgG microbeads; Miltenyi Biotec) following the manufacturer’s protocol and identified by their gene expression profiles. Briefly, cells isolated from the whole blood cell suspensions were washed with PBS and then

The Journal of Immunology
incubated with mouse anti-CD209 IgG in MACS buffer for 15 min at 11°C. For control analysis, normal mouse IgG was used in place of primary Ab. After washing with MACS buffer, the samples were incubated with goat anti-rabbit IgG microbeads for 15 min at 11°C and then the cells were washed with MACS buffer and sorted using a MACS separator (Miltenyi Biotec). Before gene expression analysis of the sorted cells, flow cytometric analysis was performed to detect the purity of CD209 cells followed by the method described above. The gene expression profile was analyzed by RT-PCR, which included T cell marker genes (TCRα, TCRβ, and Lck) (24), a B cell marker gene (membrane IgM) (24), the universal markers of monocyte/macrophage lineage (CSF-1R and FcγRII) in mammals and teleosts (24), and two general DC markers (CD80 and CD83) (33, 34).

Evaluation of the involvement of DC-SIGN/CD209 in Ag uptake

To evaluate whether zebrafish DC-SIGN/CD209 could act as an attachment molecule that would contribute to the uptake of Ags, an in vivo FITC-conjugated KLH (FITC-KLH; Sigma-Aldrich) uptake inhibition assay was performed by blockade of this molecule using an anti-CD209 Ab. For this, fish were preinjected with anti-CD209 (10 μg/fish) two times at an interval of 12 h. Twenty-four hours after the first injection, fish were inoculated i.p. with FITC-KLH (10 μg/fish). In two parallel experiments, the same dosage of rabbit IgG and PBS was used as a negative control, then these fish were injected with FITC-KLH (10 μg/fish). The leukocytes were separated at various time points, and the CD209+ cells were sorted out using the methods described above. Flow cytometric analysis of FITC-positive cells (FITC+ cells) in the CD209+ population was performed, followed by determination of the purity of CD209+ cells as described above. To provide further confirmation that CD209 and MHC-II colocalized on APC, a CD209 and MHC-II (hallmark of APC) double immunofluorescence staining analysis was performed in FITC+ cells.

Immunoprotection assay

To give further evidence that DC-SIGN/CD209 participates in immune responses, an immunoprotection inhibition assay was performed. After anesthesia, as described previously, the fish were divided into two groups. One group was i.p. immunized with a bacterial vaccine derived from 0.5% formaldehyde-inactivated A. hydrophila (1 × 10⁵ CFU/fish), a pathogen of infectious sepsis in a number of fish (35, 36). A rabbit Ab (IgG) was used as a negative control. Another group was immunized with the same bacterial vaccine at the same dosage accompanied by the in vivo blockage of DC-SIGN/CD209, followed by the method as described above. After 21 days, both immunized groups and one unimmunized control group were challenged with the live A. hydrophila (1 × 10⁵ CFU/fish). Mortality of each group was recorded over 1 wk and statistics of survival were analyzed.

Statistical analysis

Statistical evaluation of differences between means of experimental groups was done by analysis of variance and multiple Student t tests. A value of p < 0.05 was considered significant. The sample number for each group

FIGURE 1. A, Comparative gene location map of DC-SIGN/CD209 in zebrafish and human. Numbers below the gene names indicate gene sizes. The contig harboring the zebrafish DC-SIGN/CD209 gene shows conserved linkage of three human Chr.19 genes. B, Genomic structures of DC-SIGN/CD209 gene family compared with those of human, chimpanzee (Chimp), mouse, dog, and teleosts. The rectangles represent the exons whereas the lines between them indicate the introns. The sizes of exons are indicated above the exons, and the sizes of introns are indicated below the exons. UTRs are shown as white boxes. C, Schematic overview of the structure of CD209 family from higher vertebrates to lower vertebrates. All DC-SIGN/CD209 homologues are transmembrane receptors. Within the CRD structures, four amino acids (Glu347, Asn349, Glu354, and Asn365 in the human CD209 amino acid sequence and Glu305, Asn307, Glu313, and Ala324 in zebrafish) that interact with Ca²⁺ are relatively conserved. In contrast to DC-SIGN and L-SIGN, which contain neck regions consisting of tandem repeats, there are no such domains in other CD209 genes. Several internalization motifs are found within the cytoplasmic tail. The dileucine (LL) motif, the tyrosine-based motif, and the triacidic cluster are thought to be involved in the internalization of DC-SIGN. Tetraodon CD209 lacks the internalization motifs, indicating that it may have another specific mechanism for internalization. The accession nos. are noted in the legend of Fig. 2.
FIGURE 2. A, Full-length nucleotide sequence of zebrafish DC-SIGN/CD209 with the deduced amino acid sequence below. The transmembrane domain is shown in dark gray. The C-type lectin in the N-terminal intracellular domain is boxed and shown in gray. The EPN motif is underlined. The potential N-glycosylation site is boxed. The asterisk represents the stop codon, and the polyadenylation signal (AATAAA) is denoted by italics and boldface.

B, Phylogenetic tree showing the relationship between zebrafish CD209 amino acid sequence and the other species CD209 family. This unrooted phylogenetic tree was constructed by the neighbor-joining method, based on the amino acid alignment (ClustalW) of protein sequences (excluding the neck repeat region). Bootstrap values were calculated from 2000 repetitions.

C and D, CD209 protein alignment. Amino acid sequences were aligned using ClustalX. C, Multiple alignment of zebrafish CD209 with the other homologues. D, Multiple alignment of zebrafish CD209 with the predicted amino acid sequence of three teleost fish CD209. Residues shaded in black are completely conserved across all species aligned, and residues shaded in gray are similar with respect to side chains. The dashes in the amino acid sequences indicate gaps introduced to maximize alignment. NCBI accession numbers of CD209 are as follows: human (Homo) CD209, AF290886 (www.ncbi.nlm.nih.gov/nuccore/13383467); Homo CD209L, BC038851 (www.ncbi.nlm.nih.gov/nuccore/24416562); chimpanzee (chimp) CD209, AY078913 (www.ncbi.nlm.nih.gov/nuccore/27356929); chimp CD209L, AH011538 (www.ncbi.nlm.nih.gov/nuccore/27356846); bornean orangutan (born) CD209, AY078905; and mouse CD209g (www.ncbi.nlm.nih.gov/nuccore/11929292).
The cytoplasmic membrane, and an N-terminal intracellular domain, a (calcium-dependent) lectins. It contains a C-terminal extracellular domain that anchors the protein onto the poly(A) tail in the 3'-UTR (Fig. 2A). In addition, there is an orthodox polyadenylation signal (AATAAA) at 91 bp downstream of the translation termination codon and 20 bp upstream from the poly(A) tail in the 3'-UTR (Fig. 2A).

The deduced amino acid sequence of zebrafish DC-SIGN/CD209 is shown in Table II. The DC-SIGN OF ZEBRAFISH was >10 fish of equal mean body weight. Data points represent the means of three independent experiments.

**Results**

**Characterization of zebrafish DC-SIGN/CD209**

Based on the chromosome synteny between zebrafish and humans, genes in the vicinity of the DC-SIGN/CD209 locus on a fish chromosome were overall coincident with human genes, although a translocation between ZNF358 and LRRC8E genes was seen (Fig. 1A). Thus, the DC-SIGN/CD209 homologue may exist in fish, and it maps to a similar chromosomal region known in humans. By comparison with human DC-SIGN/CD209 genes (NP_066978), the open reading frame and exon/intron boundaries of the presumptive zebrafish DC-SIGN/CD209 were obtained using GeneScan (Applied Biosystems) (Fig. 1B). The results showed that the zebrafish DC-SIGN/CD209-encoding gene is located on chromosome 4 within a 30-kb segment and comprises eight exons and seven introns, and this organization pattern seems to be conserved from fish to mammals throughout vertebrate evolution (Fig. 1B). By PCR amplification, we successfully cloned the full-length fragment of the open reading frame encoding a polypeptide of 343 amino acids with a predicted molecular mass of 35.95 kDa (Fig. 2A). In addition, using the open reading frame and exon/intron boundaries of the presumptive zebrafish DC-SIGN/CD209, we performed to evaluate the phylogenetic relationships between human, chimp, bonobo, dog, mouse, chicken, and zebrafish. Two CD-SIGN/CD209 homologues were identified.

**Alignment and phylogenetic analysis**

Multiple alignment of DC-SIGN/CD209 family members was performed to evaluate the phylogenetic relationships between (Fig. 1, B and C). The extracellular domain has 221 aa and contains a carbohydrate recognition domain (CRD) that can recognize certain carbohydrate-containing Ags. Within the CRD, one highly conserved EPN motif essential for recognizing mannose-containing motifs and four relatively conserved amino acids (Glu347, Asn354, Glu354, and Asn365) in humans and Glu305, Asn307, Glu313, and Ala324 in fish that interact with Ca2+ were identified.

In contrast to human DC-SIGN/CD209 and L-SIGN/CD209L, which contain tandem repeats in the neck regions, there were no such repeat structures found in fish or other vertebrate DC-SIGN/CD209 molecules, except in mouse CD209b, which contains four repeats, and CD209c, which contains two repeats. The intracellular domain contains 96 aa. Several conserved internalization motifs are found within the cytoplasmic tail, including a dileucine (LL) motif, a tyrosine-based motif, and a triacidic cluster, which are thought to be involved in the internalization and signal transduction of DC-SIGN/CD209 (Fig. 1C). Moreover, the CD-SIGN/CD209 homologues were also identified from other fish species, including medaka (Oryzias latipes), green spotted pufferfish (Tetraodon nigroviridis), and rainbow trout (Oncorhynchus mykiss), and similar structural characterizations were seen among fish CD-SIGN/CD209 homologues. Interestingly, the Tetraodon DC-SIGN/CD209 molecule was found to totally lack the internalization motifs, indicating a possible molecular and functional diversity in DC-SIGN/CD209 molecules from fish. A schematic overview of the structural features of DC-SIGN/CD209 family, ranging from fish to humans, is shown in Fig. 1C.

**Table II. Percentages (%) of amino acid sequence identity for DC-SIGN/CD209 (signr/209) genes**

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<th>X. tropicalis</th>
<th>Medaka 209</th>
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* The accession numbers are noted in the legend of Fig. 2. Chimp, Chimpanzee; Signr, DC-SIGN; 209, CD209; 209L, 209L1, 209L2; 209L1; 209L2, CD209L2.
zebrafish and other species (Fig. 2, C and D). The zebrafish DC-SIGN/CD209 has a stretch similar to that seen in different species. The important functional amino acid residues and domains in the molecules, such as the LL motif, the tyrosine-based motif, the triacidic cluster, the CRD structure, and the Ca\(^{2+}\)-binding amino acids Glu, Asn, Glu, and Asn/Ala in CRD, are well conserved from human to fish, although the sequence identities among the family members were only 17.5—32.4% (Table II). Interestingly, the extracellular neck regions that contain seven or eight complete tandem repeats were only seen in human and nonhuman primate DC-SIGN/CD209 and L-SIGN/CD209L molecules. Thus, the appearance of tandem repeats in the neck regions may give human and nonhuman primate DC-SIGN/CD209s distinct functions, such as interaction with HIV and SIV, and this might partially explain the reason why HIV or SIV only infects humans and primates.

For phylogenetic analysis, a total of 25 DC-SIGN/CD209 family members from 11 representative vertebrates (Fig. 2B), including human (Homo sapiens), chimpanzee (Pan troglodytes), Bornean orangutan (Pongo pygmaeus), bonobo (Pan paniscus), lar gibbon (Hylobates lar), nomascus (Hylobates leucogenys), siamang (Hylobates syndactylus), mouse (Mus musculus), dog (Canis lupus), chicken (Gallus domesticus), X. tropicalis (Xenopus laevis), medaka (O. latipes), and zebrafish (D. rerio) were identified. The phylogenetic analysis showed that DC-SIGN/CD209 in teleost is classified in a solitary cluster and shows a genetic relationship to chicken, X. tropicalis, mammalian, and primate CD209 homologues.

Preparation of recombinant DC-SIGN/CD209 and Ab

The extracellular region of zebrafish DC-SIGN/CD209 was expressed in E. coli as a soluble protein and was cleanly purified by using Ni-NTA agarose affinity chromatography as examined by SDS-PAGE (data not shown). The Ab for DC-SIGN/CD209 (anti-CD209) was affinity purified from the immunized rabbit serum into the IgG isotype by a protein A-agarose column and nitrocellulose membrane immunosorbent protocols. ELISA and Western blot analyses showed that the purified Ab had an average titer above 1/10,000 and showed specificity for DC-SIGN/CD209, because no cross-reactions between anti-CD209 and other proteins from tissue extracts were observed (Fig. 3B). The other Abs used in these experiments, including anti-zebrafish 80, 83, IgM, and MHC-II, were prepared using similar protocols.

Tissue distribution and Ag-mediated up-regulation of DC-SIGN

The tissue distribution of DC-SIGN/CD209 mRNA and the changes of expression levels after in vivo stimulation with bacterial and thymus-dependent Ags were analyzed by semiquantitative RT-PCR using specific primers derived from different exons (Table I). The results showed that, in the unstimulated group, the DC-SIGN/CD209 mRNA was strongly expressed in the ovary and detectably expressed in the other tissues examined, including spleen, gill, kidney, intestine, muscle, brain, and heart. The strong expression of DC-SIGN/CD209 in the ovary is the same situation as that seen in humans. In contrast to the unstimulated controls, levels of the DC-SIGN/CD209 transcript in most immune-related tissues, such as spleen, kidney, intestine, muscle, brain, and heart, were significantly (\(p < 0.05\)) up-regulated after A. hydrophila, BSA, and KLH stimulation (Fig. 3A). From Western blot analysis, the protein levels were in agreement (Fig. 3B). These observations showed that the expression of zebrafish DC-SIGN/CD209 could be up-regulated by different Ags in immune-related tissues, which indicated that this molecule may be involved in the initiation of immune responses.
To obtain more evidence, kinetic expressions of the zebrafish DC-SIGN/CD209 protein in the kidney (also called kidney marrow, which corresponds to the marrow in higher vertebrates) and gill (a thymus coupling tissue) in response to Ag stimulations were examined by ELISA. As shown in Fig. 4, the maximum expression of DC-SIGN/CD209 was observed after 2 days in the gill and 3 days in the kidney after the administration of KLH or 3 days in gill and 4 days in the kidney in the A. hydrophila treatment group. This seems earlier than that of CD154, a marker of T cell activation (data not shown), suggesting that T cell activation might be a subsequent event followed by the activation of DC-SIGN/CD209. Among the three Ags that showed elevated kinetics, similar profiles were seen for BSA- and KLH-elevated kinetics, whereas A. hydrophila-elevated kinetics exhibited a different profile. This difference suggested that different Ags may have different stimulatory intensity and that even different mechanisms underlying different Ags may be involved. As a complicated bacterial Ag, the cocktail effect of various components harbored on the A. hydrophila surface also should not be ignored. In fact, the observation that DC-SIGN/CD209 on the DC/APC surface contributes to T cell activation by presenting Ags or by establishing interactions between DC/APC and T cells has also been noted in humans (11, 37). Thus, whether zebrafish DC-SIGN/CD209 plays a similar role will be interesting to elucidate.

Up-regulation of DC-SIGN/CD209 by IL-4

Several previous studies using mammalian models have suggested that the expression of DC-SIGN/CD209 is tightly regulated by the interplay between Th1 and Th2 cytokines. In these cases, IL-4 directly participates in DC development/activation by up-regulation of CD209, whereas LPS might indirectly inhibit this process via induction of Th1 cytokines, such as IFN-γ and TNF-α (7, 38). Therefore, we investigated whether these regulatory interactions might also exist in zebrafish. Flow cytometric analysis showed that the expression of DC-SIGN/CD209 on leukocyte cellular surfaces was dramatically \( p < 0.05 \) up-regulated by in vivo inoculation of the fish with rIL-4. This effect could be significantly \( p < 0.05 \) down-regulated by LPS in an analogous manner to that seen in higher vertebrates (Fig. 5). In light of this observation, we suggest that the up-regulated expression of zebrafish DC-SIGN/CD209 by exogenous Ags (KLH, BSA, or A. hydrophila) might be an early event involving a possible DC-like development/activation that is

**FIGURE 4.** Kinetic expression of zebrafish CD209 in two selected tissues (gill and kidney). **Top,** Kinetic expression of zebrafish CD209 7 days after treatment with KLH (10 μg/fish). **Bottom,** Kinetic expression of zebrafish CD209 7 days after treatment with A. hydrophila \( A.h; 1 \times 10^8 \) CFU/fish). Both graphs represent the means of results obtained from 10 fish.

**FIGURE 5.** A, Flow cytometric analysis of CD209-positive cells in the control group, IL-4-injected group, and IL-4 plus LPS-injected group. B, Statistical analysis of the percentage of CD209-positive cells after stimulation. C, Statistical analysis of the increased expression of CD209 in the geometric mean of the fluorescence intensity after stimulation. The results showed that the expression of DC-SIGN/CD209 was up-regulated by IL-4 and that this effect could be significantly down-regulated by LPS. Significance in comparison with the normalized sample is denoted with an asterisk (*). Bars represent SE. Each result was obtained from 60 fish. Data represent results obtained from at least three independent experiments for each panel.
crucial for the development of adaptive immunity. This event may also be regulated by the induction of Th2 cytokine production, for which IL-4 at least appeared to be essential. In support of this, we have been able to show that KLH can induce the secretion of IL-4 (data not shown).

Inhibition of T cell activation by DC-SIGN/CD209 blockade

To determine whether zebrafish DC-SIGN/CD209 plays a role in T cell activation, a DC-SIGN/CD209 blockade assay was conducted to evaluate whether T cell activation can be inhibited by the blockade of this molecule. The expression of CD154 and T cell-specific tyrosine kinase (Lck), two typical markers of T cell activation in fish, were examined (29–32). The DC-SIGN/CD209 was blocked by in vivo inoculation of anti-CD209, followed by the stimulation of KLH. The expression of CD154 and Lck were examined on days 3 and 4, which is the peak time of CD154 and Lck expression in response to KLH (data not shown). As shown in Fig. 6, the expression of CD154 and Lck in the KLH-stimulated group were dramatically up-regulated in tissues of gill, spleen, and kidney compared with the unstimulated control group. However, significant \( p < 0.05 \) down-regulated expressions of CD154 and Lck were seen in the DC-SIGN/CD209 blockade groups. This observation showed that blockade of DC-SIGN/CD209 resulted in the inhibition of T cell activation, suggesting the possibility that a DC-SIGN/CD209-mediated T cell activation mechanism may exist in fish.

Inhibition of Ab production by DC-SIGN/CD209 blockade

To further address the role that DC-SIGN/CD209 plays in T cell activation and adaptive humoral immunity, an Ab (IgM) production inhibition assay was conducted after a blockade of DC-SIGN/CD209. As shown in Fig. 7, in the DC-SIGN/CD209 blockade group the titers of the Ab were significantly reduced \( p < 0.01 \) compared with those of the KLH-stimulated group, supporting the observation that DC-SIGN/CD209 participates in T cell-mediated Ab production.

Association of DC-SIGN/CD209 with APCs

To investigate whether there is an association of zebrafish DC-SIGN/CD209 with APCs that is similar to that seen in mammals, a number of double immunofluorescence staining experiments were performed. The results showed that a number of peripheral leukocytes were IgM and CD209 double positive (IgM^+ CD209^+). The CD209^+ cells were also shown to be positive for MHC-II (the overall marker of APC) (Fig. 9). Providing further evidence that DC-SIGN/CD209 had associated with APCs. These CD209^+ cells were then sorted out from the total peripheral blood leukocytes by magnetic beads. Over 95% of the sorted cells were CD209^+ according to FACS analysis.
would be similar to the distribution of mammalian DC-SIGN/CD209. However, these DC-like cells need to be studied further.

**Evaluation of DC-SIGN/CD209 in the uptake of KLH Ag**

Although it apparently can act as an adhesive molecule on the surfaces of APCs, a necessary function for the activation of T cells, it is not clear whether DC-SIGN/DC209 can act as a grabbing molecule that would participate in the uptake of Ags. For this purpose, an in vivo phagocytic inhibition assay was performed by blocking of DC-SIGN/DC209 using a specific Ab. The fish were injected with FITC-KLH for 0.5, 1, and 2 h after an anti-CD209 IgG blockade, which allowed sufficient time for phagocytosis by leukocytes. The leukocytes were then collected, and the CD209+ cells were sorted out from these cells. The phagocytic effect of CD209+ cells was determined by immunofluorescence staining and flow cytometric analysis. A considerable number of CD209+ cells exhibited a positive FITC-KLH phagocytic signal (FITC+). These FITC+ cells were also found to be colabeled with anti-MHC-II (a vital marker of APCs), which provided further support for CD209+ cells behaving like APCs (Fig. 9). Flow cytometric analysis showed that the percentage of FITC+ cells in the CD209+ cell population isolated from the nontreated control groups was as high as 15.87 ± 1.53% (Fig. 9B). However, the percentages of FITC+ cells in CD209+ cells isolated from the blockaded groups were not significantly (p > 0.05) decreased (Fig. 9B). Similar results were also seen when the geometric mean of the fluorescence intensity was analyzed (Fig. 9). These observations indicated that a blockade of DC-SIGN/CD209 on APC surfaces had no effect on the cellular capture of KLH, suggesting that the DC-SIGN/CD209 molecule does not participate substantially in the uptake of protein Ags. A direct Ag binding ELISA also showed no dose-dependent interactions between DC-SIGN/CD209 and KLH (or BSA) (data not shown).

Considering the fact that zebrafish DC-SIGN/CD209 did not contribute to KLH-elevated T cell activation and immune responses, it seems reasonable to suggest that this molecule does not act directly as an Ag-grabbing molecule to capture more protein Ags. Therefore, other mechanisms that underlie DC-SIGN/CD209 involvement must be postulated, such as DC/APC aggression and migration or DC-SIGN/ICAM-3 mediated DC/APC-T cell interactions, which still remain to be further elucidated.

**Immunoprotection is inhibited by DC-SIGN/CD209 blockade**

In an attempt to further verify that DC-SIGN/CD209 is involved in immune response, we devised an immunoprotection inhibitory assay by using a bacterial vaccine (inactivated *A. hydrophila* vaccine). As shown in Fig. 10, in the unimmunized negative control group, which did not receive the vaccination, only 10% of the fish survived when challenged with *A. hydrophila*. However, in the immunized positive control group, which received vaccination, 90% of the fish survived when challenged with the same pathogen, indicating that the fish were well protected after vaccination. In the DC-SIGN/CD209 blockade group, which received vaccination and the DC-SIGN/CD209 Ab, the survival rate was significantly decreased from 90 to 60%, showing that the immunoprotection effect was significantly inhibited by DC-SIGN/CD209 blockade. The results supported the idea that zebrafish DC-SIGN/CD209 plays an important role in the immune process after vaccination.

**Discussion**

Because CD209 was first discovered in humans as a placental protein capable of binding HIV-1 gp120 (5), CD209 homologues have also been characterized from other species, including nonhuman...
primates such as rhesus monkeys (*Macaca mulatta*), pigtailed macaques (*Macaca nemestrina*), and chimpanzees (*P. troglodytes*), and other mammals such as the mouse. However, little is known about its occurrence in lower vertebrates. In an attempt to map the evolutionary history of the CD209 gene family throughout vertebrate evolution, we described the identification and functional characterization of this family member in fish and its partial characterization in *X. tropicalis* and chicken. This is the first description of this molecule in lower vertebrates. A number of conserved structural lines among DC-SIGN/CD209 molecules of fish and other species (similar chromosomal synteny, location, and exon-intron organizations of CD209 genes, similar protein domains such as the CRD structure conserved in the C-type lectin family, the Ca2+ binding essential amino acids Glu, Asn, Glu and Asn/Ala conserved in CRD, and the LL and tyrosine-based motifs as well as the triacidic cluster in intracellular tail) supported the conclusion that cloned zebrafish DC-SIGN/CD209 is a homologue of the human CD209 family.

In human and nonhuman primates there are at least three family members named CD209/DC-SIGN, CD209L/L-SIGN, and CD209L2 that have been identified. Overall, these family members have conserved functional domains, except that the tandem repeats in the neck region have been found only in CD209/DC-SIGN and CD209L/L-SIGN molecules. Most of the nonhuman primates tested have all three family members, although CD209L is missing in several species, such as Old World monkeys. Humans have CD209 and CD209L, but lack CD209L2. In nonhuman primates the genes encoding CD209, CD209L, and...
CD209L2 are located on the same chromosome within a 30-kb segment and have similar exon-intron structures, suggesting that they were derived from the duplication of an ancestral precursor gene (39-41). In comparison with primates, the fish CD209 gene is single copy but has similar chromosomal location and organization, thus providing evidence that duplication events have occurred during evolution from fish to human. Structurally, the fish CD209 lacks tandem repeats in the neck region, which is the same situation seen in primate CD209L2 molecules (although the partial CD209L2 has a shorter or interceptive region). This, at least in part, supports the idea that the ancestor of the modern CD209 family was most likely a member that resembled CD209L2 (42).

By characterizing CD209 family members from fish to humans throughout vertebrate evolution, we outlined a hypothetical evolutionary history of the CD209 family. The primitive CD209 might originate from teleosts during early vertebrate evolution, because we have failed in an attempt to search for homologous sequences in the lower species such as lancelet (Branchiostoma floridae), whose genome sequence has been recently identified (43). Although genetic mechanisms such as deletion and mutation have led to the differentiation of CD209 homologues among different species to some extent, there is no remarkable divergence with regard to the basic structure and variety observed among fish, amphibians, avian species, and the mouse until the divergence of CD209, CD209L, and CD209L2 from one another in primates (44). The multigenic nature of the CD209 family in primates, but not in early vertebrates, raises the possibility that the CD209 family was genetically conserved during the long history of vertebrate evolution until recent evolutionary alterations occurred across primate species. A duplication event might have occurred from a common ancestor in anthropoids, yielding CD209L2 and an ancestral CD209 gene. Then, the ancestral CD209 duplicated in the common Old World primate ancestor, which has since undergone evolutionary processes involving the insertion of a neck region ancestral motif and duplications of the ancestral motifs. The inserted ancestral motif increased to seven or eight repeats after duplications to form a complete neck region. This finally gave rise to CD209L and CD209, where the extant CD209L2, which lacks tandem repeats in the neck region, keeps its characterization from the first duplication and became a pseudogene in partial primates (42).

With the appearance of the neck region, the functional behaviors of the CD209 family evolved dramatically. For example, CD209 and CD209L with neck regions are more flexible and tend to form dimers or tetramers that make the molecules stable and effective in binding ICAM-3 or viruses, whereas CD209L2 is substantially less effective in binding ICAM-3 and poorly transmitted HIV-1 and SIV to target cells relative to CD209. Thus, this might be a reasonable explanation of why HIV or SIV can infect humans and primates rather than lower vertebrates, because the CD209 in lower vertebrates lacks the neck repeat regions. The nonhuman primates have retained orthologues of CD209L2 perhaps because of some particularly useful function conferred by this molecule, whereas humans lack CD209L2 because the evolved CD209L shows multiple mutations that may engender new functions to replace the CD209L2, thus allowing the absence of CD209L2.

Functionally, the role that CD209 family members play in adaptive immunity seems to be somewhat puzzling, because controversial conclusions have usually been drawn from different experimental models or occasionally even from the same models. For example, several previous studies have demonstrated that CD209 was an important marker that was expressed on monocyte-derived DCs and participated in the aggregation and migration of DCs and the intercellular adhesion between DCs and other cells (5, 7). It also acted as an Ag-grabbing molecule, internalizing Ags from cell surfaces into lysosomes and improving immune responses by priming naive T cells and promoting T cell proliferation and activation (45, 46). However, it was also reported that most of the DC subsets in humans do not express CD209, whereas macrophages and some other cells do express this C-type lectin (20, 21). In the mouse, it was found that different family members (the IGN orthologues SIGNR1 and SIGNR3) present different cellular distributions and that most do not correlate with DC expression (20, 22, 47). The idea that DC-SIGN/ICAM-3 interactions are required to induce lymphocyte activation is therefore difficult to establish (20). Furthermore, in human monocyte-derived DC and other DC models, DC-SIGN is down-regulated upon DC-activation (7, 38).

Thus, DC-SIGN/CD209 does not seem to be as “DC specific” as was previously believed. These observations also indicate a functional complexity and diversity in the CD209 family. Therefore, further studies using different research models are needed to provide more functional insights into this family. For this purpose it is essential that lower vertebrates also be integral participants and, for this reason, the zebrafish model was used in this study.

Our study showed that zebrafish CD209 may associate with various APCs, including macrophages, B lymphocytes, and a group of CD80+/CD83+CD209+ DC-like cells (which remain to be identified further, much like it does in mammals. It is also essential for the activation of T cells and Ab production in humoral immunity. These characteristics indicate that the regulatory mechanism underlying CD209 involved in the initiation of adaptive immunity has evolved during the early evolution of vertebrates, and zebrafish may prove to be a useful new model for the study of CD209 family. As a C-type lectin, CD209 improves immune responses in mammals in several ways, including pathogen internalization by its recognition of mannosylated Ags, establishment of lymphocyte activation through intercellular adhesion or aggregation, and mediation of cellular migration toward target cells (10, 12, 14, 44, 48). However, the way in which fish CD209 operates still remains to be elucidated. In addition, the identification of zebrafish DC-SIGN/CD209 in present study, together with the studies of other DC-related molecules such as CD80 and CD83 that we have recently isolated from this fish model (data not shown), may help to identify whether DC-like subsets (the existence of these is still completely unclear in lower vertebrates) exist in fish. From this, it might be possible to expose in depth the origin and evolution of DC/APC and its mediated regulatory mechanisms in adaptive immunity.

Disclosures

The authors have no financial conflict of interest.


