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Notch Signaling Suppresses IgH Gene Expression in Chicken B Cells: Implication in Spatially Restricted Expression of Serrate2/Notch1 in the Bursa of Fabricius

Toshifumi Morimura,* Seiji Miyatani,† Daisuke Kitamura,* and Ryo Goitsuka2*‡

The bursa of Fabricius is a central organ for chicken B cell development and provides an essential microenvironment for expansion of the B cell pool and for generation of a diversified B cell repertoire. We report here that genes encoding the Notch family of transmembrane proteins, key regulators of cell fate determination in development, are differentially expressed in the bursa of Fabricius: Notch1 is expressed in medullary B cells located close to the basement membrane-associated epithelium (BMAE). In contrast, a Notch ligand, Serrate2, is expressed exclusively in the BMAE, which surrounds bursal medulla. A basic helix-loop-helix-type transcription factor, Hairy1, a downstream target of Notch signaling, is expressed in the bursa coordinately with Notch1 and Serrate2 and an immature B cell line, TLT1, which expresses both Notch1 and Serrate2. Furthermore, stable expression of a constitutively active form of chicken Notch1 or Notch2 in a B cell line results in a down-regulation of surface IgM expression, which is accompanied by the reduction of IgH gene transcripts. Transient reporter assay with the human IgH gene intronic enhancer reveals that an active form of Notch1 inhibits the IgH enhancer activity in chicken B cells, suggesting that Notch-mediated signals suppress the IgH gene expression via influencing the IgH intronic enhancer. These findings raise the possibility that the local activation of Notch1 in a subset of B cells by Serrate2 expressed in BMAE may influence the cell fate decision that is involved in B cell differentiation and selection inside the bursa. The Journal of Immunology, 2001, 166: 3277–3283.

In mice and humans, B cells are generated continuously throughout life with ongoing rearrangement of Ig genes in the bone marrow. By contrast, in chickens, B cell generation and Ig diversification are restricted to a relatively short period of life and to a specialized lymphoid organ, the bursa of Fabricius (reviewed in Refs. 1 and 2). The bursa develops from a dorsal appendage of the cloaca as an endodermal bud surrounded by mesenchymal tissue, which begins around embryonic day 4. The commitment to B cell lineage and Ig rearrangement occur in embryonic hemopoietic cells outside the bursa (3–5), and these B lineage-committed cells colonize the bursa between days 8 and 14 (2–3 cells per follicle; Refs. 6 and 7). In the bursa, these cells expand extensively, diversify their Ig genes by gene conversion machinery (8–10), and form ~10^4 discrete lymphoid follicles. Each follicle has a central medullary region containing B cells, dendritic cells, and epithelial cells, which is surrounded by a layer of basement membrane-associated epithelium (BMAE).3 The cortical region of the bursa begins to develop outside the BMAE after hatching and contains a rapidly dividing B cell population. Emigration of B cells from the bursa to the peripheral circulation starts a few days before hatching and continues until bursal involution at ~6 mo of age. Subsequently, the peripheral B cell pool is thought to be maintained by self-renewal of long-lived bursa-derived B cells. Therefore, in chickens, the entire B cell compartment originates from <10^5 precursors that colonized the embryonic bursa.

To gain an insight into the molecular mechanisms regulating B cell homeostasis and diversification in the bursa, we focused our attention on members of the Notch family of transmembrane receptor proteins because Notch plays a critical role in various cell fate decisions during development, including lymphopoiesis (11, 12). In the hemo-lymphopoietic system, two Notch functions have been postulated, namely binary cell fate decision and maintenance of cells in an undifferentiated state. Notch-mediated binary cell fate decision has been suggested by experiments involving transgenic expression of an activated form of Notch1 in mice, which resulted in a biased CD4 vs CD8 lineage fate (13) as well as αβ vs γδ T cell fate (14). Recent reconstitution experiments that used bone marrow cells infected with retroviruses encoding an active form of Notch1 demonstrated that Notch1 also influences B vs T cell lineage commitment (15). A role of Notch1 in maintaining a progenitor pool was suggested by its expression in CD34+ human hemopoietic stem cells (16) and by in vitro experiments demonstrating that Notch signaling can prevent or delay myeloid cell differentiation (17–21).

In the present report, we demonstrate that Notch and one of its ligands, Serrate2, are coordinately expressed in the different compartments of the bursa of Fabricius. We also demonstrate that Notch signaling inhibits IgH gene expression in the chicken B cells, which suggests the potential involvement of Notch-mediated signaling in B cell differentiation in the bursa.
Materials and Methods

Animals and cell lines

The congenic White Leghorn chicken line, H-B15, was provided from National Institute of Animal Health (Tsukuba, Japan). The avian leukemia virus-transformed B cell lines TLTI, DT40, and 249L4 and the Marek’s disease virus-induced T cell lines RP1, JP2, and MSB1 were cultured in a humidified atmosphere at 40°C and 5% CO2 in RPMI 1640 medium supplemented with 10% FCS and antibiotics. The human B cell line, Ramos, was maintained in the same medium described above at 37°C.

Cloning of chicken Notch1, Notch2, Serrate2, and Hairy1 cDNAs

Partial cDNA fragments of chicken Notch1 and Notch2 genes were amplified by PCR by using a single-stranded bursa cDNA as a template. The oligonucleotide primers used for PCR amplification were as follows: Notch1- and Notch2-specific 5′ primers (5′-GGAGATTCTTGTGGATGCCGGTCACACACCTACTCAACTG-3′ and 5′-GGAGATCTAAGTGGTGACGACCTGCTGAC-3′) and 3′ degenerate oligonucleotide primer (5′-GGATCCCGGAGGARCTNGACCAYTGRTCNGG-3′), which corresponds to the conserved sequence (PDQWSRSS) in the cytoplasmic domain of rat Notch1 and Notch2. The amplified PCR products were subcloned into pBluescript plasmids and subjected to sequencing by the dyeoxy-chain termination method with an automatic DNA sequencer (Applied Biosystems, Foster City, CA). A 500-bp cDNA fragment identical with the reported chicken Serrate2 cDNA (22) was used to screen a bursa cDNA library constructed in AZAP-XR (Strategene, La Jolla, CA) and isolated a 2.5-kb cDNA fragment encoding chicken Serrate2. An entire coding region of chicken Hairy1 cDNA (23) was also isolated from the bursal cDNA library by PCR with a pair of primers (5′-CGAATTCCGGGAGGARCTNGACCAYTGRTCNGG-3′ and 5′-CCGGATCCTGCGTGGATGGCATCAAC-3′) which correspond to the IgH genes.

Whole-mount in situ hybridization analysis

Tissues were dissected from chicks just after the hatching, and fixed overnight at 4°C with 4% paraformaldehyde in PBS. Digoxigenin-labeled sense and antisense riboprobes were prepared from linearized plasmids by digoxigenin RNA labeling kit (Boehringer Mannheim, Mannheim, Germany) by using T3 and T7 RNA polymerases, respectively. Tissues were treated with 10 µg/ml proteinase K and then fixed with 0.2% glutaraldehyde and 4% paraformaldehyde in PBS for 20 min at room temperature.

Expression plasmids and generation of stable transfectants

The cDNAs encoding the intracellular domain of chicken Notch1 and Notch2 (NotchIC) (29), which correspond to amino acids 1748–2142 and 1785–2143 of rat Notch1 and Notch2, respectively, were ligated in pAT7neo expression vector carrying the chicken β-actin promoter and the double T7 epitope tag at the N-terminal end to create pAT7neo-Notch1IC and pAT7neo-Notch2IC (24). 249L4 cells stably expressing T7-tagged chicken Notch1IC or Notch2IC were generated by electroporation with 30 µg of linearized pAT7neo-NotchIC under the same condition described below and subsequent selection in the presence of 1 mg/ml G418. Drug-resistant clones expressing T7-tagged NotchIC were verified by Western blotting with anti-T7 Ab (Novagen, Madison, WI). Surface expression of IgM and MHC class I and II on these transfectants were analyzed by FACSsort (Becton Dickinson, Mountain View, CA) with anti-chicken IgM and MHC class I and class II Abs (25, 26), which were kindly provided by Dr. C. H. Chen (University of Alabama, Birmingham, AL). FITC-labeled rabbit anti-mouse IgG Ab (Southern Biotechnology Associates) was used as a secondary Ab.

Northern blot analysis

Poly(A)+ RNA and total cellular RNA samples were extracted from cell lines using a FAST-TRACK mRNA isolation kit (Invitrogen, San Diego, CA) and TRIzol (Life Technologies, Rockville, MD), respectively. The resultant RNAs were electrophoresed in 1.0 M formaldehyde/1% agarose gel and then transferred to nylon membranes. The blot was hybridized with 32P-labeled chicken Notch1, Notch2, Serrate2, and Hairy1 cDNA probes, as described previously (27). Transcripts of chicken IgL and IgH genes were also analyzed by using the following DNA probes: EcoRI-Sal genomic fragment containing the entire coding sequence of the IgL constant region (10), which was provided by Dr. W. T. McCormack (University of Florida, Gainesville, FL) and a partial IgH cDNA fragment corresponding to the Igα constant region (28). The equivalent loading of the RNA samples were confirmed by probing with chicken β-actin cDNA probe.

Reporter assay

The luciferase reporter plasmids driven by conalbumin promoter with or without the MluI-Hpal fragment of the human IgH gene intrinsic enhancer pEcoa-Luc and pcoa-Luc were provided by Dr. T. Watanabe (Kyusyu University, Fukuoka, Japan) (29). Cell lines were cotransfected with 10 µg of a luciferase reporter plasmid and 1 µg of a pGAL plasmid (a gift of Dr. T. Yagi, National Institute for Physiological Science, Okazaki, Japan), in combination with 10 µg of pAT7neo-Notch1IC or pAT7neo-Notch2IC, in serum-free RPMI 1640 at a density of 106 cells/400 µl per cuvette with a Gene Pulser (Bio-Rad) set at 250 V and 975 µF. After electroporation, the cells were transferred to complete RPMI 1640 and incubated at 40°C for 48 h, and luciferase activity was determined in cell extracts according to the instructions of the luciferase assay kit (Promega, Madison, WI). β-galactosidase assay was conducted by using a Galact-light kit (Tropix, Bedford, MA) to normalize the transfection efficiency between the samples. Light emission was measured in a Lumat LB9501 luminometer (Berthold, Wildbad, Germany).

Results and Discussion

Identification of Notch and Serrate mRNA expression in the bursa of Fabricius

To examine whether Notch receptors and their ligands are expressed in the bursa, we conducted RT-PCR with bursal cDNA by using a pair of degenerate primers corresponding to the conserved sequences of Notch family members. This screening yielded several cDNA clones potentially encoding Notch family proteins (data not shown). Subsequently, we isolated the longer cDNA clones by RT-PCR with specific primers corresponding to the sequences obtained from the initial screening or by the phage library screening. Sequence comparison of these cDNA clones with known Notch hybridization. Sections were first incubated overnight at 4°C with a rabbit anti-pancycokeratin Ab (Boehringer Mannheim), rinsed, and incubated with a HRP-labeled anti-rabbit IgG Ab (Southern Biotechnology Associates, Birmingham, AL) followed by biotinylated tyramide. The reactions were detected with fluorescein-labeled streptavidin. The sections were examined under a laser scanning confocal microscope (Bio-Rad, Hercules, CA).

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Immunohistochemical analysis

The tyramide-based signal amplification method (TSA-indirect kit; NEN, Boston, MA) was used for immunohistochemical analysis after the in situ
family genes revealed that the chicken homologues of Serrate2 (22) and Notch1 (30) and the previously unidentified Notch2 were expressed in the bursa. The chicken Notch2 showed ~57% and 79% identities to the corresponding regions of chicken Notch1 (30) and rat Notch2 (31), respectively (Fig. 1). The extracellular region of chicken Notch2, including the LIN12/Notch repeat, is more homologous to rat Notch2 than to chicken Notch1. The intracellular region of chicken Notch2 contains the RAM domain, which interacts with the RBP-Jκ transcription factor (32, 33), and six highly conserved ankyrin/cdc10 repeats, which are found in all Notch proteins. The former is conserved only between chicken and rat Notch2, whereas the latter is conserved among these three proteins.

We also attempted to examine the expression of other Notch ligands, Delta1 and Serrate1, by RT-PCR with primers based on the reported sequences of chicken Delta1 (34) and Serrate1 (35). We detected Serrate1, but not Delta1 expression in the bursa (data not shown).

Localization of Notch- and Serrate2-expressing cells in the bursa of Fabricius

To explore the role of Notch and their ligands in chicken B cell development, we first examined spatial expression patterns of the corresponding genes by whole-mount in situ hybridization. Expression of Serrate2 gene was observed in the outermost zone surrounding each follicle and was absent from the central zone of the follicle (Fig. 2A). Similarly, a ring-shaped pattern of Notch1 mRNA expression was detected in each follicle (Fig. 2B). However, the areas that were positive for Notch1 expression did not overlap with the areas positive for Serrate2 expression (Fig. 2A and B). Notch 2 was very weakly expressed in some follicles in the bursa (Fig. 2C). Serrate1 was weakly expressed in a similar pattern to Serrate2 (data not shown).

To further characterize the expression patterns of Notch1 and Serrate2, the samples after whole-mount in situ hybridization were sectioned and carefully examined by microscopy. Serrate2 transcripts were localized in the outermost cell layer of the bursal follicles, which anatomically corresponds to the BMAE at the corticomedullary junction (Fig. 2G). By contrast, Notch1 transcripts were diffusely distributed in the margin of the medulla, possibly in a subset of medullary B cells (Fig. 2H). To identify the cell type expressing Serrate2, we subjected these tissue sections to immunohistochemical staining with anti-cytokeratin Ab, which reacts with bursal epithelium, including BMAE (36). As expected, the Serrate2-positive cell layer surrounding the B cell follicle was stained by the Ab, indicating that Serrate2 is expressed in BMAE (Fig. 3). These findings indicate that Notch and their ligands are coordinately expressed in the different compartments of the bursa of Fabricius.

Expression of Notch and Serrate2 in chicken B and T cell lines

We next examined the expression of Notch1, Notch2, and Serrate2 in chicken B and T cell lines by Northern blotting. All cell lines expressed both Notch1 and Notch2 at different levels (Fig. 4A). Serrate2 transcripts were detected in two immature B cell lines: DT40, in which gene conversion of the variable region of IgL chain locus occurs continuously (37, 38), and TLT1, which has a similar surface phenotype with bursal B cells (our unpublished data and Ref. 39). The amount of Serrate2 mRNA in TLT1 cells was at least 10 times higher than that in DT40 cell, as deduced from the intensity of hybridization signals. No Serrate2 mRNA was detectable in a mature B cell line, 249L4, or in Marek’s disease virus-transformed T cell lines (RP1, JP2, MSB1). The expression of Serrate2 in B cell lines is apparently inconsistent with the histological observation that Serrate2 is expressed in BMAE but not in B cells. With regard to this, we were able to detect the expression of Serrate2 as well as Notch1 and Notch2 in sorted surface IgM-positive bursal B cells by RT-PCR (data not shown). Thus, some B cells in the bursa probably express low levels of...
Serrate2 below the detection limit of whole-mount in situ hybridization. However, there remains a possibility that Serrate2 expression may have been artificially induced by the avian leukosis virus-mediated transformation in the process generating these cell lines. Hairy1 expression in an immature B cell line expressing both Notch1 and Serrate2 and in the bursa

To address whether the interaction of Notch with Serrate2 in the cell line and the bursa could activate Notch receptor-mediated signaling pathway, we examined the expression of chicken Hairy1 gene, one of the downstream targets of Notch signaling, which belongs to Hairy and Enhancer of Split family of the repressor-type basic helix-loop-helix genes (40–42). Hairy1 mRNA was readily detectable in TLT1 cells, but not in other cell lines, including DT40 cells (Fig. 4A). Whole-mount in situ hybridization analysis revealed a ring-shaped pattern of Hairy1 expression in each follicle of the bursa (Fig. 4B). This correlative expression pattern of Hairy1 with Notch1 and Serrate2 in TLT1 cells as well as in the bursa suggests that Serrate2-mediated Notch1 signal transduction is active in bursal B cells. This is also supported by our findings that the introduction of a constitutively active form of chicken Notch1 or Notch2 into Hairy1-negative 249L4 cells induced an endogenous Hairy1 expression, as described below. However, we could not rule out the possibility of Notch-independent basal transcription of Hairy1 gene. The lack of endogenous Hairy1 expression in DT40 cells might be attributable to a low level of Serrate2 expression, which may be insufficient to trigger Notch-mediated signaling, or to the presence of intracellular proteins that inhibit Notch-mediated signalings, such as Numb (43) and sel10 (44).

FIGURE 2. Whole-mount in situ hybridization analysis of Notch1, Notch2, and Serrate2 gene expression in the bursa of Fabricius. Tissues were taken from chicks at the day of hatching and hybridized with either antisense (A–C) or sense (D–F) riboprobes for Serrate2 (A and D), Notch1 (B and E), or Notch2 (C and F). The positive hybridization signals are visible as dark blue staining. The scale bar indicates 200 μm. The tissue sections after the whole-mount in situ hybridization with antisense riboprobes for Serrate2 (G; magnification: ×200) and Notch1 (H; ×200) were also shown with a schematic diagram of the histological architecture of the bursa (I). The Notch1-positive medullary region (m) and the BMAE (bmae) are indicated by arrows and arrowheads, respectively. The area between the dotted line and the BMAE in the diagram corresponds to the cortical region (c), which is not well developed at this stage of the bursa. ICT, Interfollicular connective tissue; fe, follicular epithelium.

FIGURE 3. Serrate2 is expressed in BMAE cells in the bursa of Fabricius. Tissue sections after the whole-mount in situ hybridization with Serrate2 riboprobe were stained with anticytokeratin Ab as described in Materials and Methods. Shown are Serrate2 expression (A) and cytokeratin expression (B) in the same section (×200). Anticytokeratin Ab staining are observed in Serrate2-positive cells (indicated by arrow heads) as well as follicular epithelial cells. m, Medulla; ICT, interfollicular connective tissue; fe, follicular epithelium.
A constitutively active form of chicken Notch suppresses surface IgM expression in B cells by inhibiting the IgH gene intronic enhancer activity

To understand the meaning of the specific expression pattern and possible interaction of Notch and Serrate2 in the bursa, we examined the function of Notch in the 249L4 B cell line by stably expressing Notch1IC or Notch2IC, constitutively active forms of Notch. Endogenous Hairy1 expression was undetectable in parental and mock-transfected clones, while Hairy1 mRNA was induced in transfectants expressing Notch1IC or Notch2IC (Fig. 5A), indicating that chicken NotchIC is indeed active in signal transduction in 249L4 cells. Notably, we found that the level of surface IgM expressed on Notch1IC or Notch2IC transfectants was significantly lower than that on parental and mock-transfected controls (Fig. 5A). The other surface molecules expressed on 249L4 cells, including MHC class I and class II, were not altered (Fig. 5A and data not shown). Furthermore, in transfectants expressing Notch1IC or Notch2IC, the level of IgH mRNA was markedly reduced, compared with parental and mock-transfected controls, whereas there was no significant difference in the IgL mRNA levels between mock- and Notch1IC- or Notch2IC-transfected clones (Fig. 5B). These results indicate that Notch signalingselectively down-regulates the expression of the IgH gene in chicken B cells.

Transcription of IgH gene is regulated by at least two cis elements: promoter 5' of variable regions and an enhancer within the J-C intron, and multiple transcription factors, such as E2A, Oct1/2, and Pu.1, interacting with these cis-acting elements (reviewed in Ref. 45). Therefore, we examined the effect of Notch1IC on IgH gene enhancer by using the reporter luciferase plasmid containing the human IgH gene intronic enhancer region (29). As shown in Fig. 6A, the reporter expression was strongly elevated by the presence of the human IgH gene enhancer in DT40 and 249L4 cells, as well as in a human Ramos B cell line, indicating the presence of active transcription factors interacting with the IgH gene enhancer in chicken B cells. We then transiently expressed Notch1IC in these chicken B cell lines and analyzed the IgH gene enhancer activity. Expression of chicken Notch1IC inhibited the IgH gene enhancer activity to 66% and 28% of the levels in mock-transfected DT40 and 249L4 cells, respectively (Fig. 6B). This suggests that the down-regulation of IgH gene expression results from Notch1IC-mediated inhibition of the IgH gene enhancer activity.

It has been reported that a constitutively active form of Notch1IC inhibits transcriptional activity of E47, a E2A gene product, which is assessed by transient reporter assay in nonlymphoid cells (15). In addition, mice reconstituted with bone marrow cells with retrovirally introduced constitutively active form of
Notch1 showed a severe block in early B cell development (15), which is very similar to the defects observed in E2A-deficient mice (46, 47). One potential effector molecule in Notch-mediated inhibition of the IgH enhancer activity is Hairy1, because mammalian orthologue, HES1, inhibits neuronal differentiation through forming inactive heterodimers with E47-related activator-type basic helix-loop-helix proteins, including MyoD and Math1 (48). Thus, it may be reasonable to consider that Hairy1 induced by Notch-mediated signaling inhibits a functional homodimerization of E47, leading to suppression of the IgH enhancer activity. Another candidate would be Deltex (49). Deltex is a cytoplasmic protein interacting with the ankyrin repeat region of Notch to positively regulate Notch functions (49). Ordentlich et al, has reported that Deltex is involved in the Notch-mediated inhibition of E47 activity (50). The observed reduction of IgH gene expression in chicken B cells by Notch1C may thus be mediated by either Hairy1 or Deltex or both. This issue is currently under investigation by expressing Hairy1 or Deltex in chicken B cell lines.

Although it is premature to propose the physiological role of Notch in B cell differentiation in the bursa, it is tempting to speculate that Notch might suppress somatic Ig gene conversion in bursal B cells, for which both rearrangement and transcription of the Ig gene are prerequisite (1, 51). If so, Notch may function to maintain a small fraction of B cells as an undiversified progenitor pool in the bursa. We attempted to generate a Notch1C-expressing DT40 cell line to examine the effect of Notch1 signals on the Ig gene conversion machinery. However, we failed to obtain any transfectants, because Notch1IC induced G1 cell-cycle arrest and massive apoptosis in DT40 cells, which we confirmed by the inducible expression system (24). The effect of Notch1 on the Ig gene conversion will be clarified by sequence analysis of Ig genes in Notch1/Hairy1-expressing B cells isolated from the bursa.

Alternatively, Notch1-Serrate2 interaction at the edge of the bursal follicle may regulate cell fate decisions associated with B cell emigration from the bursal follicles to the periphery. It has been estimated that only ~5% of the cells generated in the bursa emigrate to the periphery and the majority of cells die in situ by apoptosis (52, 53). In addition, the loss of surface IgM has been demonstrated to precede the induction of apoptosis in rapidly dividing bursal B cells (54). Based on the findings that Notch1IC induces down-regulation of surface IgM expression in 249L4 cells (this study) and induction of G1 cell-cycle arrest and apoptosis (24), it is possible to speculate that the B cells are arrested in their proliferation by Notch1 signaling and die by apoptosis unless some survival signals are provided. In this way, Notch1 might select functional B cells at the corticomedullary junction for their emigration into the periphery.

The bursa functions as a site for generation of primary B cell repertoire before hatching. However, after hatching, the bursa actively incorporates external Ags from the gut (55). These Ags possibly stimulate bursal B cells and modulate selection and emigration of B cells from the bursa. Further analysis on the expression of Notch and its ligands within the bursa during embryonic and posthatching periods will be required to elucidate a role of Notch signaling in B cell migration, proliferation, and selection in the bursa. In addition, it will be of interest to examine whether Notch and its ligands influence B cell repertoire diversification also in the ileal Peyer’s patches in sheeps and the appendix in rabbits, where somatic Ig hypermutation and gene conversion take place (56–58).

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References


