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J Immunol (1995) 154 (9): 4485–4494.

<https://doi.org/10.4049/jimmunol.154.9.4485>

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Identification and Analysis of the Expression of CD8 $\alpha\beta$ and CD8 $\alpha\alpha$ Isoforms in Chickens Reveals a Major TCR- $\gamma\delta$ CD8 $\alpha\beta$ Subset of Intestinal Intraepithelial Lymphocytes¹

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Expression screening has been used to clone cDNAs encoding the α - and β -chains of chicken CD8. Amino acid sequence similarities with the mammalian sequences were about 30%. Many amino acid residues of structural or functional importance were more highly conserved, as were the overall structures of both chains. Like human CD8 α , the chicken α -chain lacked sites for *N*-linked glycosylation, but the β -chain contained three such sites. In COS cells transfected with CD8 β cDNA, surface expression of the β -chain was dependent on co-transfection of the α -chain cDNA, indicating that, as in mammals, chicken CD8 can be expressed as a CD8 $\alpha\alpha$ homodimer or as a CD8 $\alpha\beta$ heterodimer. Immunofluorescence analysis with mAbs that were shown to identify the CD8 α - and CD8 β -chains revealed that the vast majority of the CD8⁺ cells in the thymus, spleen, and blood of adult chickens express both CD8 α - and CD8 β -chains. However, a relatively large proportion of the CD8⁺ TCR- $\gamma\delta$ cells in the spleens of embryos and young chicks express only the α -chain of CD8. Among intestinal epithelial lymphocytes the major CD8⁺ T cell populations present in mice are conserved, but there is a population of TCR- $\gamma\delta$ CD8 $\alpha\beta$ cells that is not found in rodents. This observation is important in interpretation of experiments examining the pathways of development of intestinal intraepithelial lymphocytes in chickens. *The Journal of Immunology*, 1995, 154: 4485–4494.

The CD8 molecule is intimately involved in Ag recognition and signaling by the Ag-specific receptor of MHC class I-restricted T cells. The cytoplasmic domain of CD8 binds the tyrosine protein kinase p56^{lck} whereas the extracellular domain binds to a site on the nonpolymorphic $\alpha 3$ domain of class I MHC molecules

that are recognized by the TCR. As well as bringing p56^{lck} into the TCR complex to contribute to signaling function, the CD8 interaction with MHC increases the adhesion of the T cell to its target cell and decreases the threshold for T cell activation by increasing the overall avidity of TCR-MHC-peptide binding (1), or by other means (2).

In most T cells that express CD8, the molecule exists on the surface as a heterodimer of 32- to 34-kDa α - and β -chains (3, 4), each consisting of a single Ig-like domain connected to the transmembrane region by a flexible hinge carrying *O*-linked glycosylation (3, 5, 6). The CD8 α -chain carries the binding sites for both MHC class I (7–9) and p56^{lck} (10). Comparable binding sites have not been identified in the β -chain. In mammals, a homodimeric form consisting of two CD8 α -chains is expressed, in the absence of β -chain, by a small population of peripheral $\gamma\delta$ T cells (11), a large proportion of IELs⁵ (12), NK cells (11), some dendritic cells (13, 14), and

Received for publication October 14, 1994. Accepted for publication January 20, 1995.

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¹ Parts of this work were supported from the following sources: U.S. Public Health Service Grants AI30879, CA13148, and AR03555; U.S. Department of Energy Grant DE-FG-0692ER1467; and Medical Research Council of Canada Grant MT10040. M.J.H.R. is a Senior Chercheur-boursier of the Fonds de la Recherche en Santé de Québec. E.P. is the recipient of a Fonds de la Formation de Chercheurs et l'aide à la Recherche scholarship.

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⁵ Abbreviations used in this paper: IEL, intraepithelial lymphocyte; RIR, Rhode Island Red.

other activated T cells (15, 16). Because CD8 $\alpha\alpha$ homodimers are capable of fulfilling the role of the molecule in T cell activation (17, 18), the function of the β -chain in the $\alpha\beta$ heterodimer is unclear. It has been suggested that it may modify the effective threshold level of TCR ligation required for T cell activation and selection (19, 20), or it may alter the specificity of binding to class I MHC molecules (21). Homodimeric CD8 $\alpha\alpha$ may be able to cross-link MHC molecules on the presenting cell and thereby deliver a qualitatively distinct signal to that cell through the MHC molecule (22, 23). In this case the heterodimer may differ in not being able to provide this signal.

A role for the CD8 β -chain in thymic selection has recently been described (24), which is consistent with the expression of CD8 $\alpha\beta$ heterodimers by essentially all CD8 $^{+}$ thymocytes. In the intestinal epithelium, however, there are populations of both CD8 $^{+}$ TCR- $\alpha\beta$ and TCR- $\gamma\delta$ cells that express only CD8 $\alpha\alpha$ homodimers. An increasing body of evidence implies that these cells develop and are selected extrathymically (12, 25–28). In the chicken, however, existing evidence suggests that all intestinal epithelial lymphocytes may be thymus dependent (29, 30). Definition of the expression of CD8 α - and β -chains on chicken T cells is therefore essential to further examine the apparent differences in pathways of development in rodents and birds.

Abs have been produced recognizing a disulphide-linked dimer on the surface of chicken T cells that resembles CD8 in size, expression during ontogeny, tissue distribution (31), and binding to an associated kinase (32, 33). This 63-kDa molecule is separated into unresolved 34-kDa subunits under reducing conditions, leaving uncertainty as to whether chickens might express only a homodimeric form of CD8 (31). If mammalian cells expressing CD8 $\alpha\beta$ and those expressing exclusively the $\alpha\alpha$ homodimer have distinct and important functions, we would expect these differences to be conserved in avian species. We have now cloned cDNAs encoding chicken homologues of both CD8 α and CD8 β . We report here that both homodimeric and heterodimeric forms of CD8 exist in the chicken. The pattern of expression of the $\alpha\alpha$ and $\alpha\beta$ isoforms in different lymphoid tissues is generally conserved in chickens and mice, but an additional major population of TCR- $\gamma\delta$ cells expressing CD8 $\alpha\beta$ is found in the intestinal epithelium of chickens.

Materials and Methods

Animals and cells

Chickens of the inbred RPL lines 0 and 7 and Rhode Island Red (RIR) chickens were produced and maintained at IAH, Compton, United Kingdom. SC strain chickens were obtained from Hy-Line International, Dallas Center, IA. Peripheral blood leukocytes were isolated by low speed centrifugation (60 \times 20 min) of heparinized blood samples. Single-cell suspensions of thymus and spleen were purified by passage through fine mesh screens followed by centrifugation over Ficoll-Hypaque density gradients. For intestinal epithelial lymphocyte preparation, small intestine free of the lumen contents was turned inside out and washed in cold

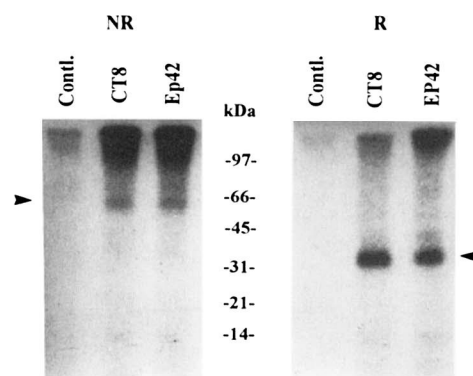


FIGURE 1. Immunoprecipitation of lymphocyte surface-labeled molecules with CT8 and EP42 Abs. Lysates of ^{125}I -labeled lymphocytes were precipitated with control mAb, CT8, or EP42 and analyzed by electrophoresis through 10% acrylamide SDS gels followed by autoradiography. The control sample (Contl.) contains material immunoprecipitated with an irrelevant, isotype-matched control mAb.

PBS. After washing, the intestine was cut into segments about 1 cm long and incubated with 0.1 mM EDTA and 0.1 mM DTT with stirring, at 37°C for 30 min. After the supernatant was passed through a glass wool column, cells were separated by Ficoll density gradient centrifugation.

Abs

Hybridoma clones producing the Abs EP42 (IgG2a) and EP72 (IgG2b) were obtained after fusion of SP2/0 cells with spleen cells from a BALB/c mouse that had been immunized with chicken splenocytes (34). Hybridomas producing the Abs AV12 (IgG1), AV13 (IgG1), and AV14 (IgG2b) were obtained by fusion of NS1 cells with spleen cells of BALB/c mice that had first been twice immunized with PBL from RIR chickens and then immunized with RIR chicken thymocytes. Other Abs have been described elsewhere; CT3 (35), CT4, CT8 (31), TCR1 (36), and TCR3 (37, 38); recognize, respectively, CD3, CD4, CD8, TCR- $\gamma\delta$, and V β 2 $^{+}$ TCR- $\alpha\beta$. The Ab TCR2 (38, 39), with V β 1 $^{+}$ specificity, was the kind gift of Drs. J. Cihak and U. Lösch (Institute for Animal Physiology, University of Munich, Munich, Germany). All Abs were IgG1 except where indicated. AV14 and CT8 stained similar cell populations. AV14 blocked the binding of CT8 to chicken lymphocytes and was considered therefore to recognize the same molecule. FITC and phycoerythrin conjugation of Abs was conducted by Southern Biotechnology Associates (Birmingham, AL).

cDNA cloning and analysis

A library of cDNA from Con A-stimulated spleen cells of a Line 7 chicken, in the vector pCDM8 (40) has been described (41). A second library was prepared from Line 0 whole thymus mRNA in the same way. Transfection of COS-7 cells with the libraries and screening of transfected cells with Abs was conducted exactly as described by Horst et al. (42). Following the recovery of plasmids from a single positive COS-7 cell, successively smaller pools of plasmids were screened until a single colony was identified that gave positive staining of transfected COS-7 cells. The cDNA inserts were subcloned into M13 vectors and sequenced by using dideoxy chain termination (Sequenase v 2; USB, Cleveland, OH). Sequence data were analyzed with the Wisconsin Package software (Genetics Computer Group, Madison, WI) (43). Amino acid sequence similarities are expressed as the numbers of identical residues divided by the total number of aligned residues, excluding gaps. The sequences have been deposited in the EMBL database with accession numbers Z22726 (pAV14, CD8 α) and Z26484 (pEP42, CD8 β). The amino acid sequence alignments shown in Figures 1 and 2 were produced by manual adjustment of alignments given by the outputs of the GAP program by using various parameters. The positions of gaps were adjusted to maximize

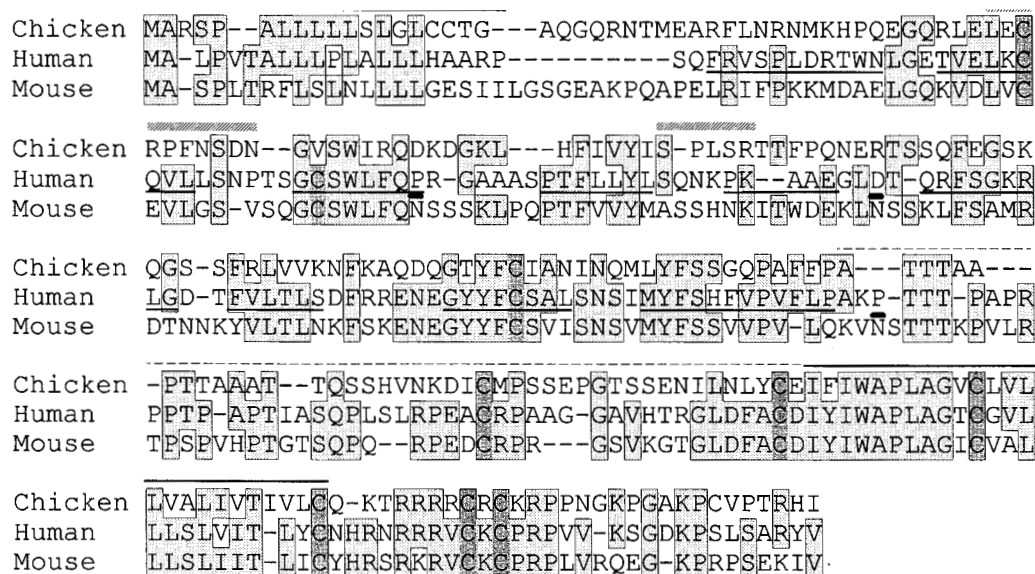


FIGURE 2. Alignment of amino acid sequences of chicken, human, and mouse CD8 α -chain precursors. Gaps have been introduced to maximize biologically significant alignments. The location of the signal peptide cleaved from the mature human molecule is shown by a solid line above the sequences, as is the transmembrane domain. Residues shared by at least two sequences are inside lightly shaded boxes. Shared cysteine residues are heavily shaded. Short black bars above individual sequences indicate the presence of potential sites for N-linked glycosylation. The dotted line above the sequences shows the proline/serine/threonine-rich membrane proximal domain that is heavily O-glycosylated in the mammalian molecules. The line beneath the sequences shows the location of the tyrosine kinase binding sites in the cytoplasmic domain. The locations of the β -sheets of the Ig fold in the human CD8 α sequence (22) are shown by underlining of the human sequence. Shaded bars above the sequences show the regions reproduced in Figure 10, which correspond approximately to the CDR1 and CDR2 loops and include residues involved in binding of human CD8 to class I MHC molecules.

similarity and maintain alignment of cysteine residues. As far as possible, gaps have been confined to regions between β sheets identified in the human sequence, except in the case of the CDR2 region of the α -chains, where the alignment of Leahy, Axel, and Hendrickson (22) has been maintained. Large gaps between the putative or known signal peptides were introduced to separate these regions despite substantial differences from the computer-generated alignments. Messenger RNA for Northern blots was prepared by the method of Chomczynski and Sacchi (44), as modified by a commercial supplier (Biotex Laboratories, Houston, TX), followed by purification of mRNA using oligo(dT) latex beads (Qiagen, Hilden, Germany). Northern blots of mRNA resolved in agarose gels containing formaldehyde were conducted by standard methods (45).

Cell surface labeling and immunoprecipitation

Viable thymocytes (5×10^7) were surface labeled with Na¹²⁵I by the lactoperoxidase method and lysed with 1% Nonidet P-40 in 0.05 M Tris-HCl, pH 7.5, containing protease inhibitors at 4°C for 1 h. Immunoprecipitation was performed by the solid phase immunoprecipitation technique in 96-well microtiter plates as described (46). Immunoabsorbed molecules were eluted from the plate by incubation with 1% SDS (non-reducing conditions) or with 1% SDS and 5% 2-ME (reducing conditions). The samples were analyzed by electrophoresis in 10% polyacrylamide gels by using the SDS-PAGE system of Laemmli (47). To remove N-linked oligosaccharides, the immunoabsorbed molecules recovered from the 96-well microtiter plates under reducing conditions were treated with 5000 U of peptide:N-glycosidase F (New England Biolabs, Beverly, MA) at 37°C for 1 h.

Immunofluorescence

Cells (10^6) were incubated with specific mAb or isotype-matched control mAb, washed, and then stained with FITC-conjugated goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL). For

two-color analyses, indirect staining with mAb and FITC-conjugated goat anti-mouse Ig was followed by direct staining with phycoerythrin-conjugated mAb. Normal mouse serum was used to block the residual binding sites on anti-mouse Ig. Fluorescently labeled cells were analyzed on a FACScan instrument (Becton Dickinson, Mountain View, CA) as described (36).

Results

Identification of a possible CD8 β -chain

The chicken homologue of mammalian CD8 has been identified by mAbs (31, 48). The chicken molecule is similar to mammalian CD8 in terms of m.w., distribution and ontogeny of expression (31, 49), and binding of *tkl*, the avian homologue of p56^{lck} (32, 33). However, the presence of $\alpha\alpha$ and $\alpha\beta$ isoforms, known to exist in mammals, has not been demonstrated in chickens. A new Ab, EP42, recognized a molecule with a distribution on T cell populations very similar but not coincident with that of other CD8 Abs. It did not stain all cell lines stained by the existing anti-CD8 Abs (not shown). EP42 and the prototypic anti-CD8 Ab CT8 precipitated molecules from surface-iodinated lymphocytes that, apart from complexed or aggregated material at the top of the gels, comigrated, whether analyzed under reducing or nonreducing conditions (Fig. 1). In addition, EP42 and CT8 co-precipitated

the *tkl* CD8-associated kinase (data not shown). This suggested that the 63-kDa CD8 molecule observed under non-reducing conditions might consist of a heterodimer of antigenically distinct subunits that comigrate after reduction, and either that CD8 α - and β -chains were recognized by CT8 and EP42 respectively, or that EP42 recognized only the heterodimer.

Sequence of CD8 α -chain cDNA

A clone, pAV14, was obtained by screening a library of cDNA from Con A-stimulated splenocytes from a Line 7 chicken with the anti-CD8 Ab AV14. The insert was 1381 bp and contained a 705-residue open reading frame starting with an ATG codon at the fifth nucleotide. Searches of a protein sequence database with the encoded amino acid sequence yielded the highest similarities with mammalian CD8 α , confirming that the cloned cDNA encoded an avian homologue of CD8 α . CD8 mAbs AV12, AV13, AV14, CT8, and EP72 all specifically stained COS cells transfected with pAV14, but EP42 did not (not shown). The sequence between nucleotides 1049 and 1160, in the 3'-untranslated region, was identifiable as a member of the CR1 repetitive sequence family (50) in the reverse orientation to the sense strand of the insert. The insert did not include a poly(A) tail.

The peptide sequence encoded within pAV14 is shown aligned with human and mouse CD8 α in Figure 2. The N-terminus of the mature protein is uncertain because the algorithm of Heijne (51) predicts four highly likely cleavage sites removing signal peptides of 18, 19, 21, or 22 amino acids. The predicted molecular mass of the mature polypeptide could then be between 24.3 and 23.9 kDa. In the alignments shown, similarity of the mature chicken polypeptide with the human sequence is 37% and with the mouse sequence, 32%. As in the human-mouse comparison, the extracellular domains are much less conserved than the transmembrane and cytoplasmic regions. Cysteine residues delimiting the single Ig superfamily V-like domain are conserved in the chicken molecule, but an internal cysteine, present in both mouse and human sequences, which forms an unusual intradomain disulphide bond in murine CD8 α (52), is not conserved in the chicken molecule. Two cysteine residues in the membrane-proximal region that may be involved in formation of $\alpha\alpha$ and $\alpha\beta$ dimers are conserved, as are the two cysteine residues in the transmembrane region. In the cytoplasmic domain, a very basic region containing two cysteine residues, which is responsible for binding p56^{lck} in mammals (53), is also found in the chicken molecule, although the sequence is not identical. Chicken CD8 has been shown to bind the avian equivalent tyrosine kinase *tkl* (32, 33), which contains a binding site very similar to that of mammalian p56^{lck}, except for the exchange of histidine and asparagine on either side of one of two cysteine residues (33).

Sequence of CD8 β -chain cDNA

The Ab EP42 was used to screen a library of cDNA from the thymus of a line 0 chicken. Because surface expression of the CD8 β -chain in mammals depends upon co-expression of the α -chain, in the initial screening the library was co-transfected into COS cells with the CD8 α clone pAV14, to guard against the possibility that the cytoplasmic β -chain might be unstable, or that EP42 might recognize only the $\alpha\beta$ heterodimer. The purified cDNA clone obtained, pEP42, did produce detectable but weak cytoplasmic staining of fixed transfected COS cells with EP42, showing that the Ab recognized the β -chain in isolation, but surface staining revealed by flow cytometry was dependent on co-transfection with pAV14 (not shown). Thus, at least in transfected COS cells, surface expression of the chicken CD8 β -chain is dependent on expression of the α -chain, as was found with mouse CD8 (54).

The sequence of the cDNA insert of pEP42 was 1327 nucleotides, containing an open reading frame of 621 nucleotides from the first in-frame initiation codon. The highest similarities found by searching databases with the encoded 207-residue peptide were with mammalian CD8 β (Fig. 3). Similarities with the human and mouse sequences aligned as shown are 34.6% and 32%, respectively. Two cysteine residues forming the Ig-like domain, and two near the membrane, responsible for dimerization, are conserved in the chicken sequence, but a single cysteine residue in the transmembrane region of both mammalian molecules is absent in the chicken. All three molecules have a short positively charged cytoplasmic domain. Comparison of the amino acid sequences of the Ig-like domains of the chicken α - and β -chains revealed slightly higher similarity, 26%, than between the equivalent comparisons of either mouse or human sequences, 18 and 22%, respectively. All these are lower than the similarities between chicken and mammalian sequences for either chain, indicating that α - and β -chains diverged before humans and chickens. The 5'-untranslated region of the cDNA consisting of 400 nucleotides was very much longer than those of human or mouse CD8 β mRNAs (55, 56). The 305-nucleotide 3'-untranslated region was followed by a poly(A) tail.

CD8 α and β transcripts

Whole inserts from the CD8 α and CD8 β clones were used as probes in a Northern blot of mRNA from lymphoid tissues and liver of the same Line 0 chicken (Fig. 4). The CD8 α probe detected a major transcript of 1.5 kb and a minor transcript of 2.6 kb, both in the T cell-rich tissues only. On close inspection of other blots, it was clear that the 1.5-kb transcript was a doublet. Blots probed with only the coding sequence revealed the same pattern of transcripts as the whole insert (not shown). Thus, although the possibility that the 3'-untranslated region in the clone is the result of cloning of disjoint cDNAs cannot be ruled

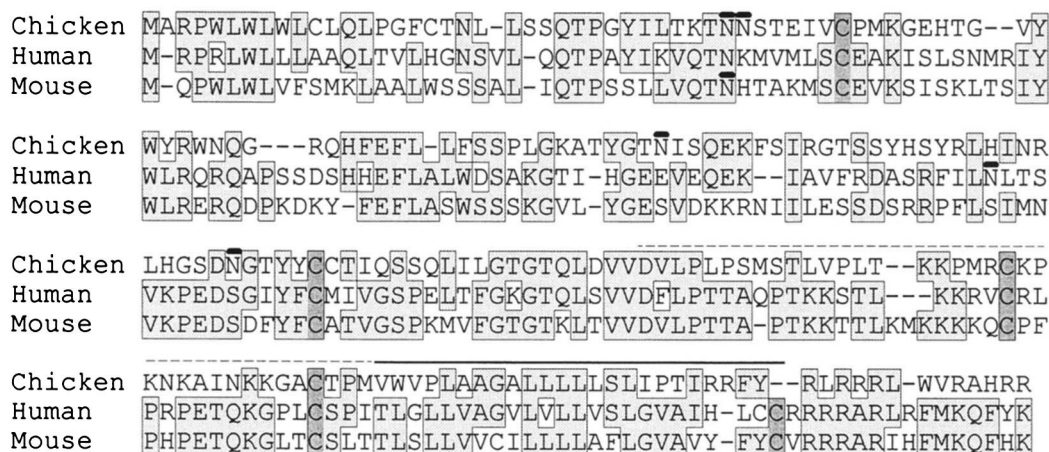


FIGURE 3. Alignment of amino acid sequences of chicken, human, and mouse CD8 β -chain precursors. Annotation is exactly as in Figure 2.

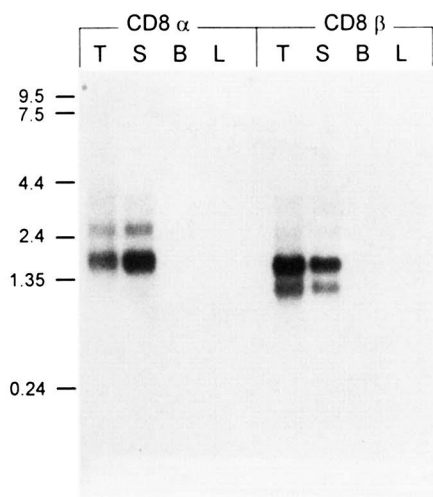


FIGURE 4. Detection of transcripts encoding CD8 α and CD8 β in mRNA from different tissues. Six micrograms of mRNA from whole thymus (T), separated splenocytes (S), whole bursa (B), and whole liver (L) was resolved by electrophoresis in an agarose gel containing formaldehyde and blotted onto a nitrocellulose membrane. Half of the membrane was hybridized with the cDNA insert of either pAV14 (CD8 α) or pEP42 (CD8 β) labeled with 32 P, followed by autoradiography. The positions of ethidium bromide-stained marker RNA molecules after electrophoresis are shown at the left.

out, this cannot be invoked as an explanation for the detection of multiple transcripts. The observed multiplicity could be the result of alternative initiation, splicing, or polyadenylation sites, or the presence of two different alleles in our line 0 chickens, despite their inbred status. Alternative splicing has been extensively documented for both α - and β -chains of CD8 in mammalian species (3, 4, 55, 57, 58).

The CD8 β probe also detected a major and a minor transcript, 1.4 kb and 1.0 kb, respectively, present only in the T cell-rich tissues. The same pattern was obtained with a probe

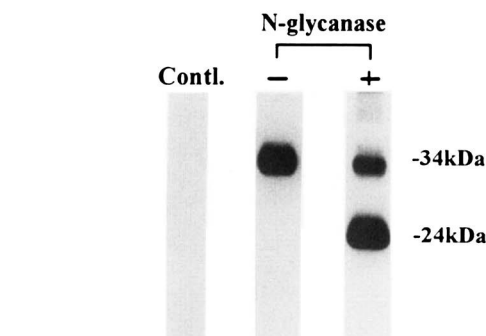


FIGURE 5. N-linked glycosylation of immunoprecipitated chicken CD8. Material immunoprecipitated from 125 I-surface-labeled PBL by the mAb CT8 was reduced with 2-ME, digested with peptide:N-glycosidase F (+) or without the enzyme (-) and then analyzed by electrophoresis in a 10 to 15% acrylamide gradient SDS gel followed by autoradiography. The control sample (Contl.) contains material immunoprecipitated with an irrelevant, isotype-matched control mAb.

carrying only the coding sequence (not shown), indicating that this was present on both transcripts and that they were not the result of a chimeric cDNA cloning. Because the cloned cDNA is longer than the smaller transcript, we assume that it represents the longer transcript. We did not detect hybridization of the smaller transcript to a probe from the 5'-untranslated region of the cloned cDNA (not shown). This would be consistent with the use of alternative transcription initiation sites for the two transcripts.

Glycosylation

The protein precipitated by Ab CT8 from a lysate of surface-labeled thymocytes was reduced and digested with peptide:N-glycosidase F. Removal of N-linked sugars resulted in a reduction of relative molecular mass of a more heavily labeled component from 34 to 24 kDa (Fig. 5), whereas a less heavily labeled component was unaffected.

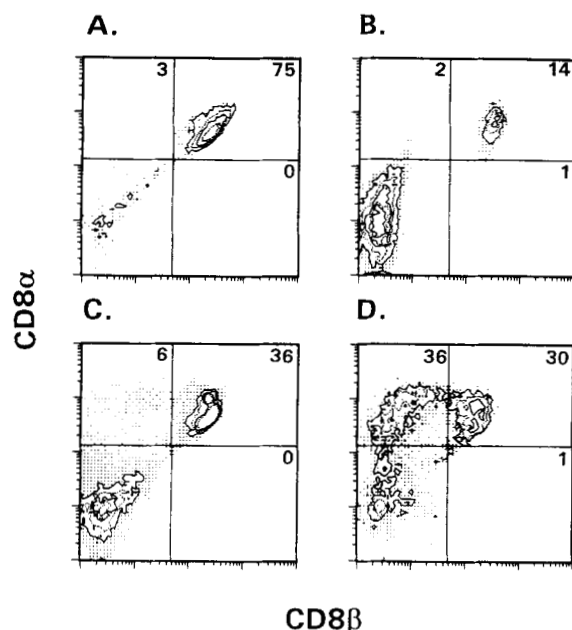


FIGURE 6. Immunofluorescence analysis of the tissue distribution of lymphoid cells bearing CD8 α and CD8 β in thymus (A), blood (B), spleen (C), and intestinal epithelium (D) of an adult chicken, by using the mAbs CT8 (CD8 α) and EP42 (CD8 β).

As in the human, the extracellular domain of chicken CD8 α is devoid of potential *N*-linked glycosylation sites (Fig. 2). This presumably accounts for the unaffected component of the 34-kDa precipitate. The more highly labeled 24-kDa *N*-glycosidase product is presumed to be the chicken CD8 β -chain that has four potential *N*-linked glycosylation sites (Fig. 3) and seven extracellular tyrosine residues, compared with three in CD8 α . This leaves 10 kDa of excess apparent molecular mass for the α -chain over the predicted polypeptide chain (24 kDa) and 3 kDa excess for the β -chain, suggesting that the former is heavily *O*-glycosylated and the latter more lightly. This is consistent with the conservation of a long membrane-proximal region rich in serine, threonine, and proline in the α -chain, and a smaller such region in the β -chain (Figs. 2, 3).

Differential expression of α - and β -chains

Expression of the two CD8 chains in lymphocytes from various adult tissues was examined by two-color flow cytometry (Fig. 6). The majority of thymocytes expressed high levels of both α - and β -chains. No significant number of thymus cells was seen to express the α -chain exclusively, either in embryos or in chickens of all ages (Figs. 6 and 7). Highly correlated levels of expression of the two chains on all T cells in the thymus, even when the levels are low, demonstrate that CD8 α and β are coordinately induced during T cell development in this organ.

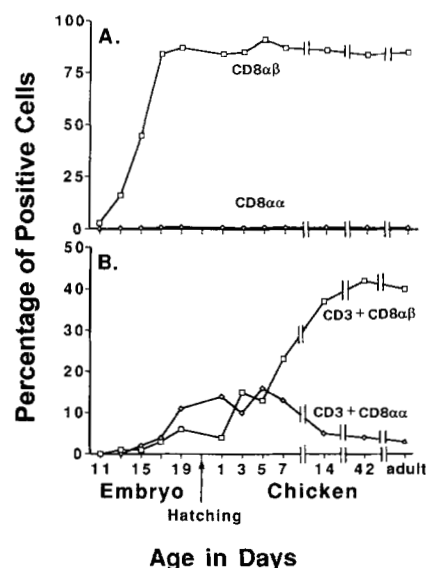


FIGURE 7. Ontogeny of CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ cells in thymus (A) and spleen (B). From the embryonic stages, cells were pooled from at least four embryos. The frequencies of CD8 $\alpha\alpha$ cells were calculated by subtraction of the frequencies of CD8 β ⁺ cells from those of the total CD8 α ⁺ cells. CD3⁻CD8 $\alpha\alpha$, NK cells, and those CD4⁺ cells also expressing CD8 in the spleen, were excluded from the analysis. The data for the adult chicken are averaged from four chickens.

All peripheral blood lymphocytes expressing high levels of CD8 α also expressed CD8 β (Fig. 6B). The 2% population of cells that expressed CD8 at lower levels was later characterized as CD4⁺CD8 α ⁺ β ⁻ cells (not shown). In the adult spleen, the majority of CD8^{high} cells were CD8 $\alpha\beta$ (Fig. 6C). However, a relatively large proportion of splenic T cells in embryos and young chicks expressed only CD8 α (Fig. 7B). Whereas the majority of the CD8⁺ TCR- $\gamma\delta$ cells in the spleens of 5-day-old chicks expressed only CD8 $\alpha\alpha$, in the adult bird nearly all CD8⁺ TCR- $\gamma\delta$ cells expressed CD8 $\alpha\beta$ (Fig. 8). In contrast, the vast majority of TCR- $\alpha\beta$ cells in both embryos and young chicks expressed CD8 $\alpha\beta$ (data not shown).

A variable population of CD8^{low} cells, 10 to 70% of the total CD8⁺ cells, was detected in the spleen, blood, and intestinal IEL of about 90% of the chickens examined. These cells were found to be CD4⁺ TCR- $\alpha\beta$ cells that also expressed CD8 α at low levels, apparently distinct from the CD4⁺CD8⁺ double positive cells in the thymus (F. Kong and C. H. Chen, unpublished observations). Cells of similar phenotype have been described in human and mouse (59, 60) and their function is unknown. The embryonic spleen also contains 10 to 40% of CD8 $\alpha\alpha$ cells that are CD3⁻, which are probably NK cells that express cytoplasmic CD3 but lack surface expression of CD3/TCR complex (61, 62) and decrease rapidly to about 5% after hatch (data not shown). In the ontogeny data shown in Figure 7B, both CD4⁺ and CD3⁻ cells were excluded from the analysis of CD8⁺ populations.

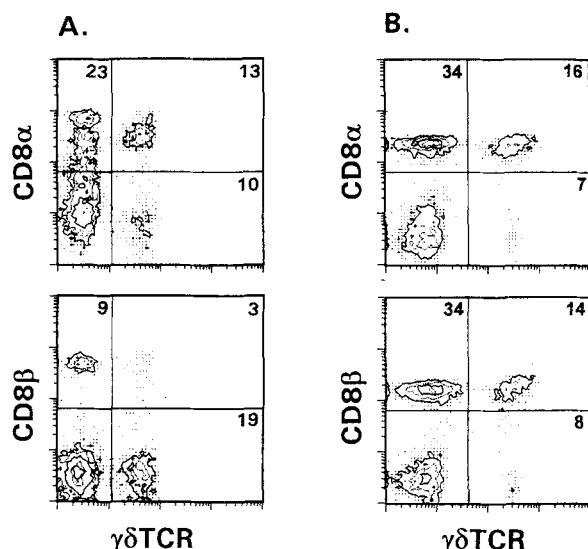


FIGURE 8. Comparison of CD8α and CD8β expression on TCR-γδ spleen cells from 5-day-old chicks (A) and 4-mo-old chickens (B), by using the mAbs CT8 (CD8α), EP42 (CD8β), and TCR1 (TCR-γδ).

The majority of intestinal IELs are CD8⁺ T cells, and a large proportion of them (55%) express CD8α without CD8β (Fig. 6). These include cells expressing both TCR-αβs and TCR-γδs (Fig. 9). Similar proportions of CD8α IELs have been described in the intestines of mammalian species (63). Of the CD8⁺ TCR-αβ cells, 65% express CD8αβ and 35% express CD8αα. Only 60% of the CD8⁺ TCR-γδ cells express the CD8 homodimer, leaving 40% that express CD8αβ. This latter population of TCR-γδ CD8αβ cells is not observed in significant numbers in the intestinal epithelium of rodent species (25–28, 64, 65).

In addition to T cells in the intestine, expression of CD8α alone was detected on the surface of CD8⁺ CD4⁻ γδ T cells in the spleens of chick embryos and of young chicks (Figs. 7 and 8), and on CD4⁺ T cells in blood, spleen, and intestine (data not shown).

Discussion

Our data establish that chicken CD8 T cells, like their mammalian counterparts, can express both homodimeric CD8αα and heterodimeric CD8αβ. Most significant structural features of both α- and β-chains are conserved in the avian molecules, including the presence of a recognizable binding site for an associated tyrosine kinase. Although the precise functional effects of the β-chain are not yet known, demonstration of its conservation in this nonmammalian species, and of its pattern of expression in T cell compartments, indicates that these effects are important.

Giblin et al. have identified residues in human CD8α that are important in binding of the αα homodimer to the α3 exon of class I MHC molecules (23). An essential lysine residue, Lys⁵⁸, in the human CD8α sequence is re-

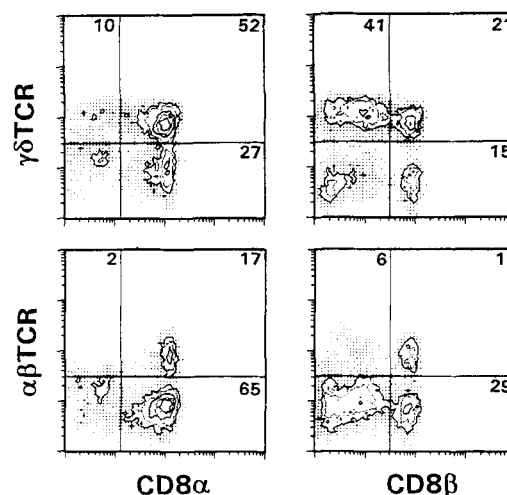


FIGURE 9. Immunofluorescence analysis of the expression of CD8α- and β-chains on TCR-γδ and TCR-αβ cells in intestinal epithelium, by using the mAbs CT8 (CD8α), EP42 (CD8β), TCR1 (TCR-γδ), and TCR2+TCR3 (TCR-αβ).

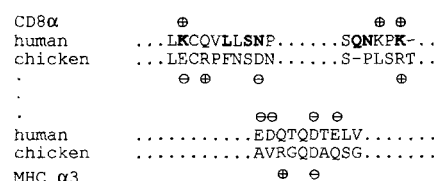


FIGURE 10. Reciprocal changes in charge in the region of interaction of CD8α with the α3 domain of class I MHC molecules in humans and chickens. Residues in bold type are those at which replacement by acidic residues in the human molecule abolished binding (23). Charged amino acids are marked above the human and below the chicken sequences. The positions of the sequences shown in the known or putative mature protein sequences are: human CD8α, 20–29 and 53–58; chicken CD8α, 28–37 and 57–62; chicken MHC (B-F19; Ref. 66), 218–227; and human MHC (HLA-A2; Ref. 67), 222–231.

placed by similarly charged arginine in the chicken, and nearby residues whose replacement by negatively charged residues abolished binding by human CD8, remain neutral. In contrast, three positions surrounding an essential leucine, Leu²⁵, in human CD8α were intolerant of replacement with acidic residues, presumably because of repulsion by the negatively charged loop constituting the binding site on the MHC α3 domain (23). In the chicken α-chain, two of these neutral residues are replaced by acidic amino acids and the leucine is replaced with phenylalanine. The resulting negatively charged site would be expected to repel a negatively charged loop on chicken class I (B-F) molecules. However, although a negatively charged Gln-Asp is conserved in the CD8 binding site of chicken and human class I (66), inspection of the surrounding 10 amino acids shows that the chicken has no overall charge whereas the human has four acidic residues (Fig. 10).

These reciprocal changes in charge in the two molecules are consistent with precise equivalence of the location of binding sites on each molecule in the two species. Other residues of human CD8 α that were essential for binding to MHC included Arg⁴ and Asn⁹⁹, which were conserved in mouse and bovine CD8 (23). Asn⁹⁹ is conserved in our alignment of the chicken sequence and there is an arginine residue shifted by one position from Arg⁴.

Murphy, comparing human and mouse sequences, has proposed that selective pressure resulting from molecular mimicry by parasites offers an explanation for more rapid divergence of exposed extracellular portions of molecules involved in immune responses than of their intracellular portions, or of other molecules (68). Comparisons of the chicken and mammalian CD8 sequences shown here add to a growing number of avian sequences for immunologically significant molecules in which this pattern of diversification is evident across a wider phylogenetic divide. Compensating changes in the CD8 α and MHC binding sites may provide an example of the accelerated co-evolution of ligand-receptor pairs that Murphy proposed as a necessary result of this mechanism (68).

Identification of the individual CD8 chains allowed us to investigate their differential expression by T cells in lymphoid tissues. In the thymus at all ages and in the adult spleen, expression of CD8 isoforms was similar to that observed in rodents. Expression of CD8 $\alpha\alpha$ alone was not observed in the thymus, and in the adult spleen the vast majority of CD3⁺CD8⁺ cells expressed CD8 $\alpha\beta$. However, a significant population of CD8 $\alpha\alpha$ cells were present in the chicken spleen early in ontogeny.

Some uncertainty remains concerning the developmental origin of CD8⁺ intestinal epithelial T cells. Although it is generally accepted that at least a major proportion of mouse CD8 $\alpha\alpha$ IELs originate and are selected extrathymically (12, 25–28), early in ontogeny some may also be derived from the thymus but selected elsewhere (69, 70). Thus the CD8 $\alpha\alpha$ phenotype may be indicative of extrathymic selection and thus be necessarily found in CD8⁺ cells that are entirely thymus independent (28). Conversely, expression of CD8 $\alpha\beta$ would indicate thymic origin and selection. Among rodent intestinal IELs, CD8 $\alpha\beta$ is expressed only by TCR- $\alpha\beta$ T cells and therefore all TCR- $\gamma\delta$ IELs are potentially the products of extrathymic selection. In contrast, we have described an additional large population of TCR- $\gamma\delta$ CD8 $\alpha\beta$ cells in the chicken intestinal epithelium, a population also found among intestinal IELs of cattle (K. R. Parsons and C. J. Howard, personal communication). This means that in chickens the TCR- $\gamma\delta$ CD8⁺ phenotype in IELs is not synonymous with the TCR- $\gamma\delta$ CD8 $\alpha\alpha$ phenotype as it is in mice. Experiments in chickens have indicated that intestinal TCR- $\gamma\delta$ IELs in this species are of thymic origin (29, 30). However, in these experiments it was not possible to distinguish TCR- $\gamma\delta$ cells expressing CD8 $\alpha\alpha$ from those expressing CD8 $\alpha\beta$, which we have now shown to be present.

It will be important to determine whether the thymus-derived TCR- $\gamma\delta$ IELs in these experiments included the CD8 $\alpha\alpha$ populations, which are subject to extrathymic selection in mice. A possible simple explanation for thymus dependence of all chicken IELs, that they might lack the CD8 $\alpha\alpha$ populations in mice, is excluded by the demonstration here of both TCR- $\alpha\beta$ CD8 $\alpha\alpha$ and TCR- $\gamma\delta$ CD8 $\alpha\alpha$ populations.

Conservation of the CD8 $\alpha\alpha$ TCR- $\alpha\beta$ and TCR- $\gamma\delta$ populations in both chicken and mouse intestinal IELs points to an important and distinct functional role for these cells. The presence in chickens of the additional TCR- $\gamma\delta$ CD8 $\alpha\beta$ population, which is not present in rodents but may be important in other mammalian species, presents the opportunity for investigation of the properties of these cells in a more experimentally accessible species.

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