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Conservation of biological properties of the CD40 ligand, CD154 in a non-mammalian vertebrate

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Abstract

Signals delivered by the CD40 ligand, CD154, have crucial roles in immune responses in mammals, being required for development of germinal centres, maturation of T-dependent antibody responses, and generation of B-cell memory. To determine whether these functions were conserved in a non-mammalian species, a putative chicken CD154 cDNA was used to make an oligomeric fusion protein, and to raise monoclonal antibodies. The antibodies detected surface expression on activated T-cells. The fusion protein detected expression of a receptor on B-cells, thrombocytes and macrophages. Biological effects of the fusion protein included induction of NO synthesis in a macrophage cell line, enhancement of splenic B-cell survival, and induction of apoptosis in a bursal lymphoma cell line. These observations demonstrated substantial functional equivalence with mammalian CD154 and thus provided evidence for the early evolutionary emergence of the set of functions associated with this molecule, and its central role in the vertebrate immune system.

Keywords: CD154; CD40 ligand; CD40; Fusion protein; TNFR; Evolution; Chicken

Abbreviations: CHO, Chinese hamster ovary cells; ConA, concanavalin A; DNA, deoxyribonucleic acid; ELISA, enzyme linked immunosorbent assay; EST, expressed sequence tag; FITC, fluorescein isothiocyanate; HPLC, high pressure liquid chromatography; IgG, immunoglobulin G; MS, mass spectroscopy; NO, nitric oxide; PBL, peripheral blood leukocytes; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PMA, phorbol myristate acetate; PVC, polyvinylchloride; R-PE, R-phycoerythrin; RPMI, Roswell Park Memorial Institute; TNF, Tumour necrosis factor; TNFR, TNF receptor.

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1. Introduction

Several of the 18 known members of the TNF superfamily [1] have important functions in the development and regulation of the immune system and its responses. These functions are essentially determined by the signalling properties and expression profiles of their receptors, members of the TNFR superfamily [2]. TNF family members share a low degree of sequence similarity, with the majority of conserved residues involved in maintaining the structure of the C terminal TNF domain [3]. The majority of these proteins are type II transmembrane proteins, although one is secreted [4], and several others may be cleaved from the membrane by proteases and released as soluble proteins [2].

The TNF superfamily member CD154 (CD40L) has several essential functions in the regulation of immune responses in mammals [5], as clearly evidenced by the severe consequences of inactivating mutations in X-linked hyper IgM syndrome [6]. It is required for the formation of germinal centres, and thus for affinity maturation of antibodies and the generation of class-switched memory B-cells in T-cell dependent humoral responses [7]. It can also provide signals for the maturation of dendritic cells [8], thus playing an important role in the initiation of T-cell responses. As well as B-cells and other antigen-presenting cells, a variety of non-immune cells also express CD40, the cognate receptor for CD154, and can respond to a CD154 stimulus by secreting pro-inflammatory cytokines [9,10]. In contrast to the wide tissue and cell distribution of CD40, CD154 was initially reported to be expressed by activated T-cells [11,12], although expression has now been shown on several other cell types [5].

Despite basic similarities, there are some important differences between the immune systems of mammals and birds. These may imply differences in the set of molecules involved in regulation of the immune system and their functions. For example, the TNF family member lymphotoxin-α is essential, in mice, for the development of classical lymph nodes, which are absent in chickens [13]. Other examples are provided by the smaller family of Ig constant regions in the chicken [14], the chicken MHC [15], chemokines and their receptors [16] (N. Bumstead personal communication).

Having identified a possible orthologue of CD154 in chickens, on the basis of sequence analysis, we needed to investigate its functional properties in order to determine whether function was also conserved. A number of different engineered soluble CD154 proteins have been used in studies of the functions of this molecule [17–19], which have shown that the protein is active only in the form of a trimer, formed by non-covalent interactions between the TNF domains of the monomer, and that higher degrees of oligomerisation may be required for some signalling functions [20]. We prepared a soluble fusion protein consisting of the extra-cellular domain of mouse CD8α joined to the extra-cellular domain of the putative chicken CD154. This molecule, analogous to similar fusions with mammalian CD154 that have biological activity, bound to a chicken CD40-Ig fusion protein. It was used to study the functions of the chicken CD154-like molecule, to determine whether some of the functional properties of the molecule were conserved, and thus whether the role of this molecule was established before the divergence of birds and mammals.

2. Methods

2.1. Reagents

A cDNA library was prepared from purified splenocytes of a normal juvenile RPRL Line 0 inbred White Leghorn bird, in the mammalian cell expression vector pcInX as described elsewhere [21,22]. A cDNA clone containing the coding sequence for mouse CD8α [23] was a kind gift from Dr R. Zamoyska (NIMR, London, UK). The plasmid vector pcIpac was constructed from pcIneo (Promega, Madison, WI) by replacement of the *Bam*HI–*Sma*I fragment carrying the neomycin resistance gene with the *Bam*HI–*Pvu*II fragment of pPur (Clontech, Palo Alto, CA) carrying puromycin resistance. Fusion proteins expressing the murine CD8α extra-cellular domain, or that of a chicken CD40-like molecule (AJ293700), fused to human Ig heavy chain C2 and C3 domains were made from COS cells transfected with a plasmid constructed essentially as described elsewhere [21]. The YTS169.4.2.1 hybridoma, expressing a rat monoclonal antibody recognising mouse CD8α was obtained

from the European Collection of Cell Cultures (ECACC, Porton Down, UK). Hybridoma cultures were grown in Technomouse (Integra Biosciences, Chur, Switzerland) cultures with Ig-depleted foetal calf serum, and the antibodies were purified using a HiTrap proteinG column (Amersham Biosciences, Little Chalfont, UK). Oligonucleotides were obtained from MWG (Ebersberg, Germany) or InVitrogen (Paisley, UK). The anti-chicken monoclonal antibodies AV7 (CD28), AV20 (Bu-1), KuL01 (monocyte-macrophage marker) and 11C3 (thrombocyte marker), all IgG1, have been described elsewhere [24–27]. Anti-bovine IgG1 isotope control antibodies, CC29 [28] and CC32 [29], and IgG2a isotype control antibodies, CC63 [30] and CC101 [31] were from the Institute for Animal Health.

2.2. Animals

RPRL Line 0 chickens were maintained in the Poultry Production Unit of the Institute for Animal Health, and were used at between 6 and 10 weeks of age. All procedures involving animals were conducted in accordance with the applicable regulations of the UK Home Office.

2.3. Cell cultures

Cell cultures were maintained in 5% CO_2, at 37 °C for mammalian cells, or at 41 °C for chicken cells. COS-7 [32] cells were grown and transfected as described elsewhere [33]. CHO cells [34] were grown in Ham's F12 medium (InVitrogen) with 10% FCS. HD11, a virally transformed chicken macrophage cell line [35], was cultured in RPMI 1640 medium (InVitrogen), 7% FCS, 3% chicken serum (Sigma, Poole, UK). DT40 and DT95 bursal lymphoma lines [36] were cultured in RPMI 1640, 10% tryptose phosphate broth, 7% FCS serum, 3% chicken serum, 50 μM β-mercaptoethanol (InVitrogen). To prepare chicken splenocytes, spleen cells were suspended in RPMI 1640, centrifuged at $35\times g$ for 10 min at 4 °C, decanted from pelleted erythrocytes, pelleted by centrifugation at $400\times g$ for 5 min at 4 °C, and resuspended in the culture medium, RPMI 1640, 5% FCS. Activated splenocytes were obtained by layering spleen cells on histopaque 1077 (Sigma), centrifugation at $2600\times g$ for 15 min at 4 °C, washing, and culture in RPMI containing 5% FCS, 25 nM PMA and 1.25 μg/ml ionomycin (both from Calbiochem, Nottingham, UK) for 24 h.

2.4. Full-length coding sequence of a possible CD154 (CD40L)

The sequence of EST AI982044 was used to design PCR primers (CTGATGAAGGGACTTGAC; TCTACAGCTTGAACATGC) to amplify an internal fragment from cDNA libraries in the form of purified plasmid DNA. A PCR product with the predicted sequence was obtained from the RPRL line 0 spleen cDNA library. Library inserts were excised with *Xho*I and ligated to *Xho*I adapters containing a single nucleotide mismatch (TCGAGGGAATCTTGATATCGAATTCCTGC + GCAGGAATTCGATATCAAGCTTCCC). An adapter primer ending at the mismatch (GCAGGAATTCGATATCAAGC) was used with each of the internal primers to amplify the ends of cDNA inserts from the library. Products were cloned into pBluescript II SK(+) (Stratagene, La Jolla, CA) using the adapter *Xho*I site and a *Sst*I site within the EST sequence. DNA sequencing reactions (Amersham) were analysed using ABI Prism 373 or 377 automated sequencers. Sequence data was analysed using STADEN [37] and GCG (Wisconsin Package Version 10.2, Genetics Computer Group, Madison, WI). Multiple clones of PCR product were sequenced from each amplification to obtain the consensus sequence and to identify clones free from PCR errors. The consensus sequence obtained was submitted to the EMBL database (Acc. No. AJ243435). A full length coding sequence was assembled by inserting *Kpn*I–*Sst*I and *Sst*I–*Xho*I fragments, from the 5′ and 3′ extended clones, respectively, into *Kpn*I–*Xho*I cut pcIpac to yield the clone pcCD154.

2.5. Fusion proteins

The sequence encoding the extra-cellular domain of the TNF encoded in pcCD154 was amplified with a vector primer and the primer GGACTTCGCCATGGATAAGATGGAAGAGGTG. The sequence encoding the signal peptide and extra-cellular domain of mouse CD8α (encoding residues 1–192 of the precursor peptide sequence; Swissprot: P01731) was

amplified from a cDNA clone [23] with the primers GACCTAAGCTTCTTGCTGG, (5′ of the coding sequence introducing a *Hin*dIII site) and ATCTTATCCATGGCGAAGTCCAATCCGGTCC. The fragments were joined by annealing and extension of the complementary 20 nucleotides at the 5′ ends of the two internal primers, followed by PCR amplification with the external primers, and ligated into pBluescript between *Hin*dIII and *Apa*I sites. After verification of the sequence in one clone, the insert was excised with *Xba*I and *Kpn*I and inserted into *Nhe*I–*Kpn*I cut pcIpac. COS cells transfected with the resulting plasmid, named pm8c154 (m8 indicating the N-terminal mouse CD8 domain and c154 the fused C-terminal chicken CD154 domain of the encoded fusion protein), were incubated with serum-free medium from 24 to 72 h after transfection to produce medium containing fusion protein. CHO cells were transfected with pm8c154 using Effectene (Qiagen, Crawley, UK) and plated in culture dishes in which transfected cells were selected by addition of 20 μg/ml puromycin (Affiniti Research Products, Exeter, UK) after 24 h. Surviving colonies were cloned by limiting dilution in the presence of 15 μg/ml puromycin.

A mutant fusion protein carrying the single amino acid substitution (R213M in the full-length cDNA sequence, Uniprot accession number Q9I8D8) was produced by PCR amplification of overlapping segments representing the two ends of the pm8c154 insert with appropriate vector primers and internal primers which introduced the desired mutation and an *Ase*I site, without any other changes to the encoded sequence (forward: CCGGCTATTAATGATGGGACTTGACACGCACAG and reverse: CCCTTCATTAATAGCCGGTCCTCTTCCATGGGGAG; mismatches underlined). The two fragments, digested with *Ase*I and *Spe*I or *Not*I, were inserted in a single ligation into pcIpac cut with *Nhe*I and *Not*I. A clone, pm8c154x1, was selected after sequence verification of the TNF domain.

2.6. Fusion protein purification

Twenty milligrams of purified YTS169 anti-mouse CD8α antibody was coupled to a 5 ml NHS-activated HiTrap column (Amersham) as specified by the manufacturer. CHO cell culture supernatants of 4E7-E31 or 9B1, clones transfected with active and control fusion protein plasmids, produced either in roller bottles or 'Miniperm' vessels (Vivascience, Hannover, Germany) were loaded onto the YTS169 column, washed with 10 column volumes of PBS and eluted with 0.1 M glycine/HCl pH 2.7, which was immediately neutralised with 0.1 volumes of 1 M Tris/HCl pH 9. After dialysis against PBS, the A_{280} of the protein solution was measured and the concentration estimated by using a molar extinction coefficient of $37350\ M^{-1}\ cm^{-1}$, calculated from the predicted amino acid composition [38] and a M_r estimate of 55 kDa for the monomeric glycoprotein.

Purified fusion proteins were directly conjugated with Alexa-Fluor 488 fluorochrome using the Alexa-Fluor 488 monoclonal antibody labelling kit as described in the manufacturer's protocols (Molecular Probes, Leiden, Netherlands). Calculations based on the A_{280} and A_{494} indicated that the average number of molecules of fluorochrome incorporated per molecule of fusion protein were 2.7 and 2.9 for m8c154 and m8c154x1, respectively.

2.7. Biochemical analyses

Affinity purified fusion proteins were analysed by size exclusion chromatography using a Smart system (Amersham) with a 3.2 mm × 30 cm Superose12 column in PBS at a flow rate of 40 μl/min, monitoring eluting protein by absorbance at 280, 230 and 214 nm. Both the affinity purified proteins and eluted column fractions were analysed by PAGE [39].

2.8. Mass spectrometry

A solution of m8c154 (100 μl at 1 mg/ml), was dialysed into 2 M urea, 100 mM ammonium bicarbonate, pH 7.8. Approximately 5% (w/w) trypsin was added and the solution was incubated overnight at 37 °C. The resulting tryptic peptides were analysed by online capillary HPLC-mass spectrometry. The capillary column was 180 μm i.d. and was packed with Hichrom C18 media (3.5 μm bead size, 150 Å pore size). The eluent from the capillary column was passed, via a uv detector, to a Quattro II tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a commercial Z-spray ionisation source operating in continuous flow

nanospray mode. The mass spectrometer was scanned from *m/z* 350–2100 with a scan time of 4.5 s. To generate sequence ions from tryptic peptides, these were desalted by use of a ZipTip (Millipore, Watford, UK), loaded into a platinum-coated, pulled borosilicate glass capillary, and sprayed directly into the mass spectrometer at a flow rate of around 20 nl/min. Collision energies between 15 and 30 V were used.

2.9. Monoclonal antibodies and ELISA

Female BalB/c mice were injected in each quadriceps with 50 μl 0.25% bupivicaine (Sigma) in PBS and 1 day later with 50 μl 1 mg/ml pcCD154 in PBS at the same sites. This was repeated at 30 days. Tail blood taken at 37 days after primary immunisation was used in an ELISA of reactivity with protein in transfected COS cell supernatants. Seventy days after the initial injection, a final boost was given by intraperitoneal inoculation with 0.5 ml of the COS cell supernatant containing m8c154 fusion protein. Four days later mice were killed and spleens removed for fusion with NS0 cells. Hybridoma supernatants were screened by ELISA with m8c154 fusion protein and with control murine CD8-human Ig fusion protein.

ELISA was used to detect and measure fusion proteins and anti-CD154 antibodies, and to test the binding of fusion proteins to chicken CD40-human Ig. The chicken CD40-Ig fusion protein was produced from a chicken CD40 cDNA (accession number AJ293700) using standard methods. For antibody and fusion protein assays, purified YTS169, 50 μl at 1 μg/ml in 50 mM sodium bicarbonate buffer pH 9.6, was used to coat wells of 96-well PVC plates (Falcon 353912, Becton Dickinson, Oxford, UK) for 16–24 h at 4 °C, and wells were successively incubated for 30 min each, at room temperature, with 50 μl each of 1%BSA in PBS, fusion protein, anti-chicken CD154 antibody, HRP conjugated goat anti-mouse-Ig (Absorbed with Rat Ig, Serotec, Oxford, UK), and TMB substrate (Sigma). For receptor-binding activity, wells were coated with goat anti-human-Ig (Southern Biotechnology Associates, Birmingham, AL), at 1 μg/ml in bicarbonate buffer as above. Wells were then incubated with COS cell supernatants containing chicken CD40-human Ig fusion protein followed, in succession, by COS cell supernatants containing the m8c154 or m8c154x1 fusion proteins, YTS169, HRP conjugated goat anti-rat Ig (Southern Biotechnology Associates) and substrate. Wells were washed three times with PBS, 0.05% Tween 20 between all incubations. Colour development was stopped by addition of 50 μl of 2 M sulphuric acid, and absorbance measured at 450 nm. For estimation of relative concentrations, serial two-fold dilutions of either fusion protein or anti-CD154 antibody were used with a saturating concentration of the other component.

2.10. Flow cytometry

Cells were incubated with the appropriate dilution of monoclonal antibody, followed by R-PE conjugated goat anti-mouse Ig (Southern Biotechnology Associates). Binding of fusion protein to cells was detected either by using Alexa-Fluor 488 labelled fusion protein, or by using unlabelled fusion protein followed by R-PE conjugated YTS169 monoclonal antibody (Serotec). Flow cytometry was performed using a Becton Dickinson FACSCalibur and data were analysed using WinMDI version 2.8 software (http://facs.scripps.edu/software.html).

2.11. Biological activities of fusion proteins

Nitrite accumulation in supernatants of HD11 cells, cultured at 4×10^5 cells per well of a 96 well plate, was measured using Greiss reagent as described [40]. The DNA contents of ethanol-permeablised DT40 cells was measured by staining with propidium iodide [41]. B cell survival in culture was measured by staining cells with the monoclonal antibody AV20, followed by FITC conjugated goat-anti-mouse Ig (Southern Biotechnology Associates). Live cells were identified by exclusion of propidium iodide. Flow cytometry and analysis was carried out as above.

3. Results

3.1. A possible chicken CD154 (CD40L) sequence

The EST AI982044, obtained from a library from ConA activated T-cells [42], encoded an amino acid sequence similar to CD154. The full-length coding sequence corresponding to this EST was obtained from

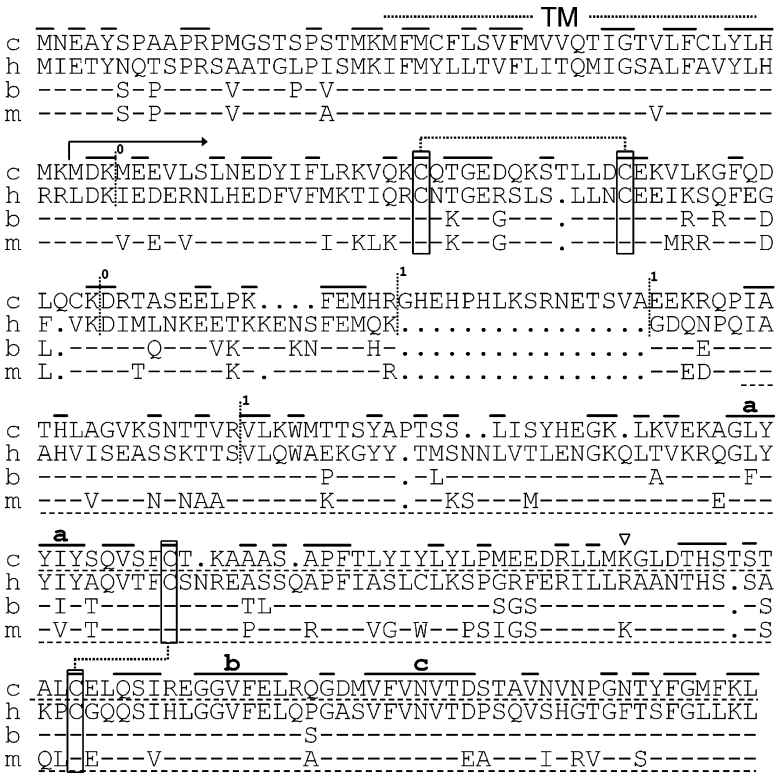

Fig. 1. Alignment of putative chicken CD154 amino acid sequence from pcCD154 (c) with human (h), murine (m) and bovine (b) CD154 sequences. (Swissprot accession numbers h:P29965, m:P27548, b:P51749). Identities in chicken and human sequences are indicated by bars above. Residues in the murine and bovine sequences identical to those in the human sequence are replaced by dashes. Gaps introduced for the purposes of alignment are shown by dots. The transmembrane region (TM) is indicated. The sequence present in the EST AI982044 is shown by a dashed line beneath the chicken sequence. The dashed line below the murine sequence indicates the TNF domain. The large arrow shows the start of the segment of the sequence included in the fusion protein m8c154. The inverted triangle points to the lysine residue changed to methionine in the mutant control fusion protein m8c154x1. Boxed residues are cysteines conserved in all the sequences, and the dotted lies joining them show disulphide bonds confirmed as present in the purified fusion protein. Vertical dotted lines show the boundaries of exons, each numbered to indicate the phase of the splice junctions in the codon for the following amino acid residue.

a spleen cDNA library using primers internal to the EST. The peptide sequence encoded by the combined consensus sequence of PCR products is shown, aligned with mammalian CD154 sequences, in Fig. 1. Comparisons of the whole encoded sequence with those of other TNF superfamily members revealed higher similarity with CD154 than other family members, but on its own this was not enough to identify the molecule as the chicken CD154 orthologue. In the alignments shown, the chicken sequence has 44–49% similarity with the mammalian sequences, the most similar of which is the murine, while the most dissimilar of the mammalian sequences share 77% of residues. Apart from the transmembrane region, the highest similarities are in the TNF domain. Of the three patches of six or more amino acids identical in all four species, two are aligned with beta strands that form inter-monomer contacts in the mouse CD154 trimer (Fig. 1a and b; [43]). The third is unlikely to be involved either in trimerisation or receptor binding, as it contains a conserved N-linked glycosylation signal amongst hydrophobic residues that form the interior of a depression on the outside of mouse CD154. Thus conservation of sequence between mammals and chickens is dominated by the requirements of structure rather than of receptor-binding function.

Exons encoding the majority of the chicken CD154 cDNA sequence were found in at least six exons

within two adjacent contigs in the preliminary assembly of the chicken genome sequence (http://www.nhgri.nih.gov/11510730), although the 5′ end of the C-terminal TNF domain-encoding exon was missing from the genome sequence. In the first draft assembly of the genome (http://www.ensembl.org/Gallus_gallus/, version 22.1.1), the splice acceptor site for the last intron is located in a gap, and consequenlty the gene (ENSGALG00000006415) is annotated according to a cDNA carrying a non-splice deletion, which lacks most of the TNF domain. No other genes with greater sequence similarity to mammalian CD154 than to other members of the TNF superfamily are present in the sequence assembly. All available intron–exon boundaries of the chicken CD154 gene had normal splice donor and acceptor sites, and all were in the same phase as the equivalent exons of mouse and human CD154 genes. As shown in Fig. 1, the chicken gene contains an additional exon, and thus a longer region separating the TNF domain from the transmembrane region. The extra sequence is hydrophilic and contains one potential site for N-linked glycosylation. It is therefore likely to be part of a longer flexible stem than those present in the mammalian proteins.

3.2. Construction of plasmids encoding mouse CD8-chicken CD154 fusion proteins

To investigate the functional consequences of signalling by the putative chicken CD154 molecule we produced a fusion protein analogous to the CD8-CD154 construct used in studies of mammalian CD154 [18], which was shown to form both trimers and hexamers in solution [20]. A full-length coding sequence was reconstructed in the expression vector pcIpac to yield the plasmid pcCD154, and a chimaeric sequence with the mouse CD8α extra-cellular region fused to the extra-cellular region of the putative chicken CD154 sequence (Fig. 1) was introduced into the same vector to produce the plasmid pm8c154. We use the naming scheme with ‘m8’ to indicate the N-terminal mouse CD8a domain and ‘c154’ to denote the C-terminal chicken CD154 domain, for the fusion proteins, with the prefix p to indicate a plasmid encoding the protein. The ideal control would be a protein of near identical structure with a specific defect in receptor binding. Therefore we modelled the chicken TNF domain by homology with human CD154, and identified a lysine residue equivalent to arginine 217 in the human protein (Fig. 1), whose replacement by methionine abolishes activity in mammalian CD154 but maintains trimerisation [44]. A mutant control protein, m8c154x1 (x1 indicating the mutation), was produced in which this lysine was likewise replaced by methionine.

3.3. Monoclonal antibodies recognising chicken CD154 fusion proteins

A large number of hybridomas were obtained after DNA immunisation of mice with pm8c154 and boosting with the m8c154 fusion protein. Most of these secreted antibodies that bound to the m8c154 fusion protein, but not to a control CD8Ig fusion protein. Six of these, named AV71–AV76, were twice cloned by limiting dilution. The fusion protein m8c154, present in the supernatant of COS cells transfected with pm8c154, bound both the monoclonal antibody AV71 and chicken CD40-Ig fusion protein, while the control m8c154x1 fusion protein was recognised by AV71, but did not bind chicken CD40-Ig (Fig. 2).

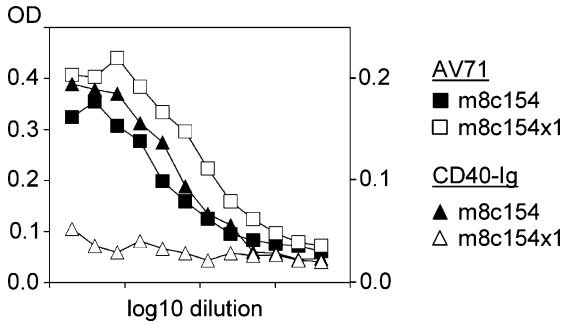

Fig. 2. Binding of anti-chicken CD154 mAb AV71, and of chicken CD40-human Ig fusion proteins to m8c154 and m8c154x1 fusion proteins in transfected COS cell supernatants. Binding to CD40-Ig fusion protein (triangles, left hand scale) was measured by capture with CD40-Ig bound to anti-Human Ig, and detection with YTS169 mAb. Binding to AV71 mAb (squares, right hand scale) was detected by capture with YTS169 and detection with AV71. Two-fold dilutions of the same transfected COS cell supernatants containing m8c154 (solid symbols) or m8c154x1 (open symbols) were used in both assays at the same time.

3.4. Expression of CD154 (CD40L) on activated chicken T-cells

Spleen cells were activated with PMA and ionomycin for 24 h before staining with the antibodies AV71–76 or isotype matched control antibodies. The population of blasting cells, detected by high forward scatter, contained 60% $CD8^+$ and 10% $CD4^+$ cells. Five of the monoclonal anti-chicken CD154 antibodies detected low levels of expression on the surface of these activated cells (Fig. 3), while they did not detect expression on the freshly isolated or control cells (not shown). We did not discern differences in the staining of $CD8^+$ or $CD4^+$ cells in double-staining experiments (not shown).

3.5. Biochemical properties of chicken CD154 fusion proteins

To produce large amounts of recombinant fusion protein, CHO cells were transfected with the plasmids pm8c154 or pm8c154x1. Cells expressing high levels of either fusion protein were obtained from several thousand initial colonies, cloned by limiting dilution and screened by ELISA of culture supernatants. Both fusion proteins were purified from CHO cell supernatants by affinity chromatography using immobilised YTS169, anti-mouse CD8α antibody. Analysed by PAGE, the affinity purified m8c154 protein appeared to consist of a single component with an apparent M_r of 55 kDa (Fig. 4C). A second, minor, component with an apparent M_r of 52 kDa was present in independent preparations of the m8c154x1 protein preparation (Fig. 4D).

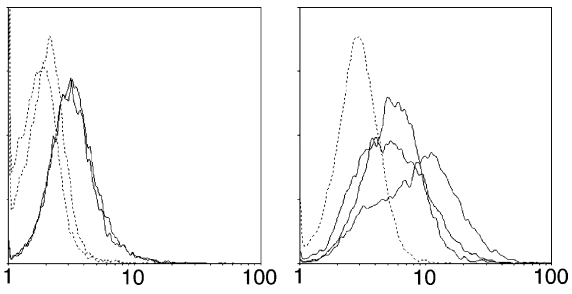

Fig. 3. Expression of m8c154 binding activity on activated chicken T-cells. Splenocytes cultured for 25 h in the presence of 25 μg/ml Phorbol Myristate Acetate (PMA) and 1.25 μg/ml Ionomycin were stained with either: (A) IgG1 antibodies AV71 or AV74 (solid lines) or isotype-matched controls CC32 or CC29 (dotted lines); or (B) IgG2a antibodies AV72, AV73 or AV75 (solid lines) or isotype matched control CC8. The histograms show fluorescence of blasting cells gated for high forward scatter.

To compare the oligomerisation state of the two proteins, both were analysed by size exclusion chromatography (Fig. 4A and B). Peaks representing approximate sizes of 50, 150 and 300 kDa were evident in these separations, as well as a variable amount of larger excluded material. No components resolvable from the fusion protein were detected in any of these fractions by PAGE. Therefore we assume that these components represent a small amount of monomer, in the shoulder at 50 kDa, trimer, hexamer, and larger aggregates of the fusion protein. The oligomerisation state of the two preparations was similar, with similar ratios of material in the trimer and hexamer peaks, although there was more aggregated material in the control protein preparation. The monomer was more evident in the product from another CHO cell clone expressing 1/100 the level of m8c154, perhaps as the result of lower intra-cellular concentration (not shown).

In order to confirm the primary sequence of the fusion proteins, and the single amino acid replacement in m8c154x1 the affinity purified fusion proteins were digested with trypsin and resulting peptides analysed by capillary HPLC-MS. Various peptides, covering the majority of the sequence of the proteins, were identified based on their mass. The predicted N-terminus of the murine CD8α moiety was confirmed. Digestion of m8c154 liberated a peptide of mass 2433.65 Da corresponding to residues 308–328 (theoretical mass 2433.19 Da) and resulting from cleavage after Lys331. In m8c154x1, this lysine residue has been mutated to a methionine residue, preventing tryptic cleavage. This was confirmed by the lack of a peptide of this mass and the presence instead of a peptide of mass 2893.80 Da corresponding to residues 308–332 (theoretical mass 2893.44 Da). A number of peptides containing disulphide linkages were identified by comparison of peptide profiles before and after reduction with DTT. Specifically, we found disulphide bonds linking cysteines 187–200, and 305–344. The identities of these peptides were confirmed by collision activation experiments and analysis of the resulting fragment ions. Their positions are shown in Fig. 1. A number of

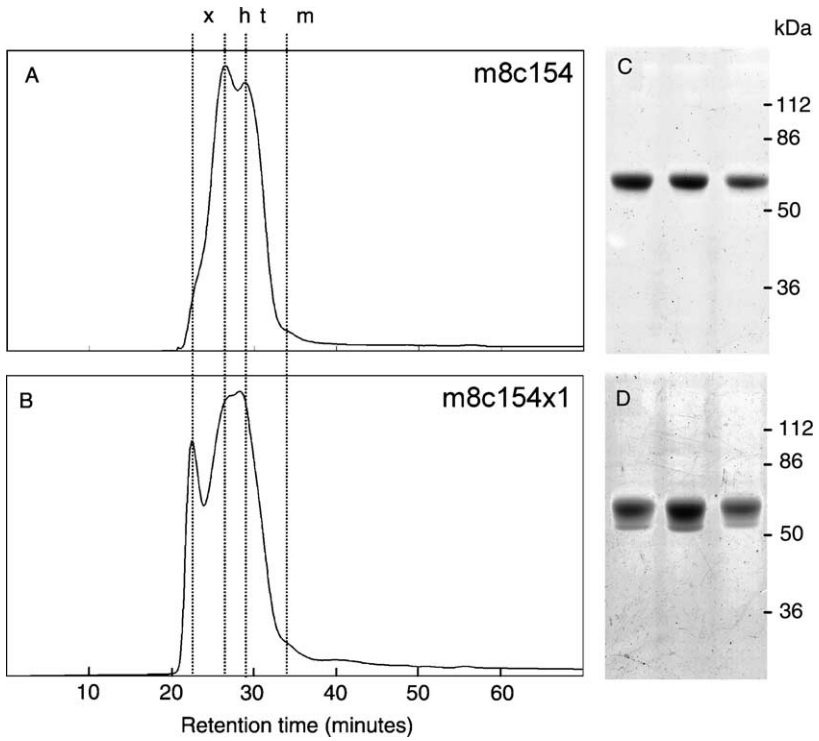

Fig. 4. Biochemical analysis of affinity-purified fusion proteins. (A, B) Normalised traces of the A_{230} of eluate from the Superose12 column as a function of time, shown in minutes on the horizontal axis, for m8c154 (A) and m8c154x1 (B). Vertical lines indicate the positions of peaks interpreted as representing monomer (m), trimer (t), hexamer (h) and larger aggregates excluded from the column matrix (x). (C, D) Photographs of coomassie-blue stained PAGE analysis of the products of three separate batches of each affinity purified fusion protein m8c154 (C) and m8c154x1 (D). The migration of standards with the indicated M_rs in the same gel are shown to the right.

peptides that gave fragment ions consistent with glycosylation were also identified.

3.6. Expression of a receptor for CD154 on chicken cells

Expression of receptors binding the m8c154 protein was detected by flow cytometry of cells from various tissues double-stained with fluorescent fusion protein or control fusion protein and with cell-lineage-specific antibodies. Specific binding of m8c154, but not the mutant control, was observed with all $AV20^+$ B-cells from bursa, spleen and PBL, at increasing levels in this series of tissues (Fig. 5A). In PBL, T-cells, recognised by AV7, did not bind the fusion protein, but all monocytes or macrophages, recognised by KUL01, did express high levels of receptor (Fig. 5B). Thrombocytes, detected by 11C3 binding, appeared to bind the fusion protein, albeit at much lower levels than the other positive cell populations (Fig. 5B). This low level expression on thrombocytes was confirmed by observing the stronger staining with fusion protein and YTS169 (not shown). Specific staining with the normal fusion protein was also seen on cell lines representing macrophages (HD11) and both immature B-cells (DT40) and more mature B-cells (DT95), but not on the T-cell line HP9 (Fig. 5C).

3.7. Biological activities of chicken CD154 fusion protein

We examined the activity of m8c154 in three in-vitro assays. CD40 signalling to mammalian macrophages causes the production of NO [45]. To assay for this response to m8c154, accumulation of

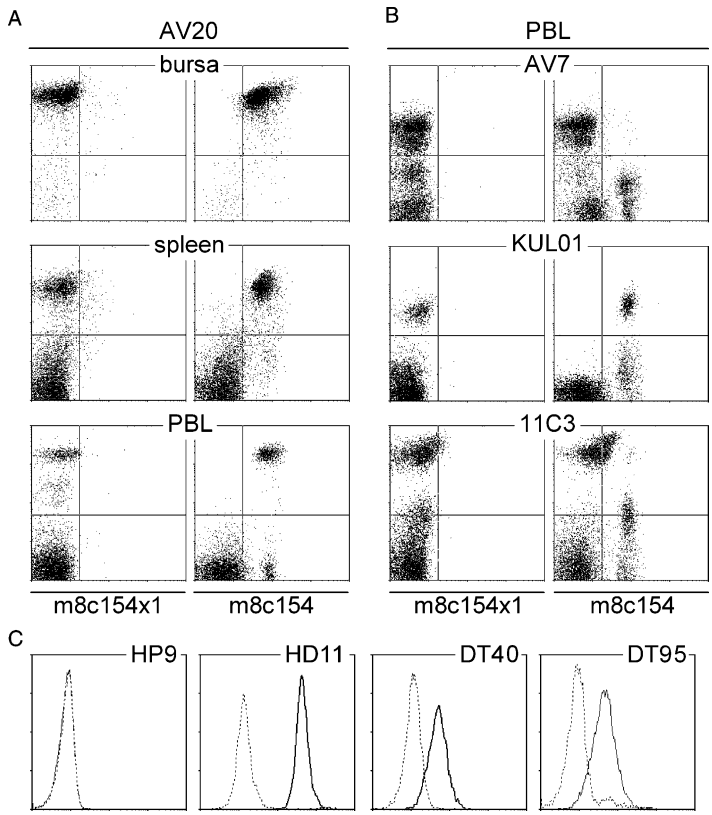

Fig. 5. Expression of m8c154-binding receptors on cells from bursa, spleen, PBL and cell lines. (A) Expression on B-cells. Each pair of dot-plots shows staining with Alexa-Fluor 488-labelled control mutant fusion protein m8c154x1 (left) or m8c154 (right) on the horizontal axis, and with AV20 on the vertical axis. (B) Double staining of PBL with specific markers for T-cells (AV7), monocytes/macrophages (KUL01) or thrombocytes (11C3) on the vertical axis, and with mutant and normal fusion proteins on the horizontal axis. (C) Distributions of fluorescence intensities of cell lines HP9, HD11, DT40 and DT95 stained with Alexa-Fluor 488-labelled control m8c154x1 (dotted line) or m8c154 (solid line).

nitrite in the supernatants of the HD11 chicken macrophage cell line cultured for 48 h in the presence of varying concentrations of m8c154 or m8c154x1 was measured (Fig. 6A). The m8c154 protein was active in inducing NO production in this cell line, with half-maximal activity at a concentration of approximately 0.3 μg/ml and maximal activity at concentrations above 3 μg/ml. The control m8c154x1 fusion protein did not induce detectable NO production at any concentration up to 40 μg/ml. The possibility that the control fusion protein might contain an inhibitory component was excluded by the observation that activity of m8c154 over this range of concentrations was not inhibited by the inclusion of 20 μg/ml of the control fusion protein (not shown).

The outcomes of CD40 signalling to mammalian B-cells depends on the developmental stage and other intra- and extra-cellular factors. In most cases it leads to increased survival and proliferation [46], but it may also lead to apoptosis, especially at high levels receptor cross-linking [47]. To observe the effects of the m8c154 fusion protein on the survival of mature B-cells, splenocytes were cultured with either the active or control fusion protein (Fig. 6B). The proportion of surviving B-cells, identified by surface staining with Bu-1 antibody AV20, was measured by exclusion of propidium iodide. The proportion of B-cells in the surviving cell population after 48 or 120 h was unaffected by the presence of the control m8c154x1 fusion protein over the whole range of concentrations tested. At concentrations of the active m8c154 fusion protein lower than 1 μg/ml, the proportion of surviving B-cells at both 48 and 120 h increased

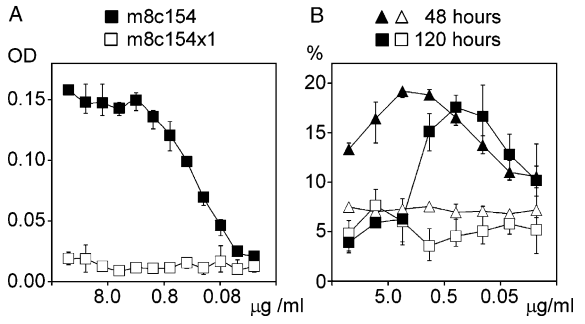

Fig. 6. Biological activity of m8c154. (A) Activation of HD11 cells by m8c154 fusion protein. The vertical axis shows the colour development in the NO production assay from supernatants of HD11 cultures incubated with serial two-fold dilutions of affinity-purified m8c154 fusion protein (solid squares) or control m8c154x1 (open squares). (B) Enhanced survival of spleen B-cells in culture. The vertical axis shows the percentage of live cells that are stained with AV20 mAb in splenocytes cultured for 48 h (triangles) or 120 h (squares), in the presence of three-fold dilutions of affinity-purified fusion proteins (solid m8c154, open m8c154x1). Bars show the range for triplicate samples. The horizontal axes are labelled with fusion protein concentration (µg/ml) estimated from the A_{280}.

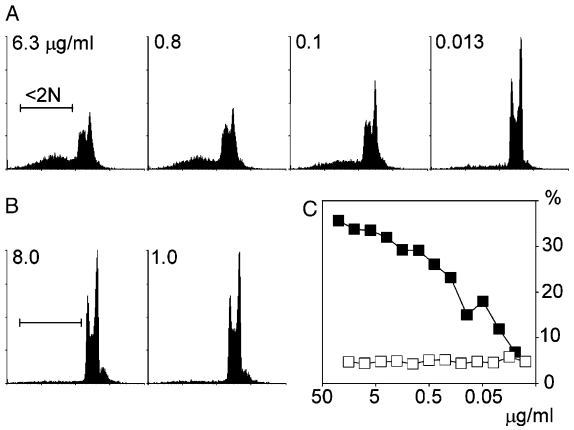

Fig. 7. Biological activity of m8c154: induction of apoptosis in DT40 cells. Distributions of propidium iodide fluorescence in fixed DT40 cells cultured for 24 h in the presence of serial two-fold dilutions of m8c154 fusion protein, or control m8c154x1, were determined by flow cytometry. Example histograms are shown, with the fusion protein concentrations indicated (µg/ml), from the m8c154 series (A) and the m8c154x1 series (B). Horizontal axes show fluorescence intensity on a logarithmic scale covering four $\log_{10}$. Bars indicate regions used to estimate the percentage of counted events representing cells with lower than 2N DNA content. The graph (C) shows these percentages on the vertical axis, with the fusion protein concentrations on the horizontal axis (µg/ml).

in a dose-dependent manner. With increasing concentrations above 1 µg/ml, the enhanced survival was reduced (48 h), or abolished (120 h). Similar experiments with bursal B-cells (not shown) did not reveal reduction in the rapid apoptosis of these cells after removal from the bursa [48].

The effect of treatment of the bursal lymphoma cell line DT40 with m8c154 and control fusion proteins was investigated. Estimation of the proportion of apoptotic cells, with less than 2 N DNA content, in these cultures revealed a dose-dependent induction of apoptosis without the requirement for BCR signalling (Fig. 7). Half-maximal induction was apparent at a concentration similar to that required for half-maximal induction of NO in HD11 cells, but saturation of the effect was not evident even at much higher concentrations. The control fusion protein had no effect on the level of apoptosis over the range of concentrations tested.

4. Discussion

Sequence similarity of the chicken TNF family molecule described here with mammalian TNF family members suggest that it is the evolutionary orthologue of mammalian CD154. This is further supported by the location of the gene within a region of chicken chromosome 4 having conserved synteny with the location of the CD154 gene on the human X chromosome. This enabled us to ask whether the functional properties of these orthologous genes had been conserved since the divergence of mammals and avian species. Mammalian CD154 was initially described as expressed on activated $CD4^+$ T-cells, but has since been shown to be expressed on other cell types, including a proportion of activated $CD8^+$ T-cells [12]. The chicken molecule was found to be expressed on activated T-cells, but the low intensity of staining by our antibodies prevented us from showing differences between expression on $CD4^+$ and $CD8^+$ subsets. Since the majority of the blasting cell population examined were $CD8^+$ cells, it is likely that a larger proportion of chicken $CD8^+$ cells than mammalian $CD8^+$ cells express this molecule upon activation.

To investigate the distribution of the receptor, and the biological activities of the chicken CD154 molecule, we prepared a soluble multimeric fusion

protein, and a novel control protein with a single amino acid substitution that abolished receptor binding but did not interfere with the oligomerisation. Both fusion proteins accumulated in the supernatants of transfected CHO cells as a mixture, predominantly of trimers and hexamers, when expressed at high levels. Mass spectrometry, used to confirm the structures of the fusion proteins, revealed a disulphide bond, between cysteine residues conserved in all species, in the disordered 'stalk' region of the chicken fusion protein. The presence of this disulphide bond has not been documented in mammalian CD154.

Active and control fluorochrome-labelled m8c154 fusion proteins were used to detect specific binding to chicken B-cells and B-cell lines as well as monocytes/macrophages and a macrophage cell line, but not to T-cells, consistent with binding to a receptor with similar expression profile to mammalian CD40. We also observed a low level of binding to thrombocytes, which in mammals express CD40 constitutively [49] and mobilise intra-cellular CD154 to their surface on activation [50]. Chicken thromobocytes are very different from mammalian platelets in that they are nucleated and have other unusual properties [51,52]. Examination of the effects of CD40 signalling on thrombocytes may reveal special functions in the chicken. We did not detect binding of the chicken CD154 to mammalian B-cells (data not shown).

The biological activities of the m8c154 fusion protein included induction of NO production by macrophages, as with mammalian CD40L [45]. At low concentrations the fusion protein increased the number of surviving B-cells in cultured chicken splenocytes, but this effect was reversed at higher concentrations. Opposing effects of CD40L on B-cell survival at different concentrations have been observed in mammalian cells [47].

There is no equivalent in mouse or human of the bursal stage of B-cell development in chickens, during which proliferating cells diversify their Ig genes by gene conversion [14]. Some immature mammalian B-cells can be maintained in culture by CD40 signalling [53] and immature B cell lines can be protected from apoptosis induced by Ig cross-linking [54]. Bursal B-cells rapidly enter apoptosis when removed from the bursal environment [45]. We did not observe any effect of the m8c154 fusion protein on the survival of bursal B-cells in culture. The immature bursal lymphoma cell line DT40, whose properties resemble those of B-cells at the bursal stage of development [55], can be induced into apoptosis by cross-linking surface IgM [56]. In attempting to determine whether m8c154 could block BCR signalling induced apoptosis in this cell line, we found that the m8c154 fusion protein alone was sufficient to cause an increase in the rate of cell death. The induction of cell death by CD40L in a number of transformed mammalian cells, including lymphoma cells, has been reported [57,58]. Thus the response of DT40 may be equivalent to that of EBV-transformed human B-cells, in which CD40 signalling can induce apoptosis [59].

Many of the extant features of vertebrate immune systems, as most intensively studied in mouse and human, are the products of relatively recent evolution, and alternative choices of the molecular machinery involved in recognisably similar functions may have been taken in even closely related species. Perhaps the most striking example is provided by the inhibitory NK receptors, the Ly49 family in mice and the KIR family in humans, where equivalent functions are carried out by very different protein families [60]. Over the much longer timescale that separates chickens and mammals from their common ancestors, there are likely to be more and greater differences in the functions of recognisably orthologous molecules. In addition to its sequence and the conserved chromosomal location of the chicken CD154 orthologue we have described, its expression on activated T-cells, detection of the receptor on macrophages and B-cells, and comparable activities in functional assays, lead to the conclusion that this molecule is also functionally equivalent to mammalian CD154. Since these functional properties have been conserved since the divergence of mammals and chickens, it is reasonable to conclude that they were already associated with this molecule in the common ancestral immune system.

The functional protein produced in this investigation will have an important role in investigation of the evolution of the function of antigen presenting cells and of the regulation of immunoglobulin class switch recombination.

Acknowledgements

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