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Generation of a novel *Cr2* gene allele by homologous recombination that abrogates production of Cr2 but is sufficient for expression of Cr1

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ABSTRACT

The enhancing effects of the complement system for humoral immunity have primarily focused upon the recognition of complement-bound foreign antigens by a co-receptor complex of the antigen-specific B cell receptor (BCR) and complement receptor 2 (Cr2). *In vivo* experiments using *Cr2* gene deficient mice (which lack the expression of both the Cr1 and Cr2 proteins) do demonstrate depressed humoral responses to immunization but cannot be used to define specific contributions of the singular Cr1 or Cr2 proteins on B cell functions. To study the effect of a Cr2 deficiency in a Cr1 sufficient environment we created a mouse line in which the alternative splice site required for the expression of the Cr2 isoform was removed. This mouse line, *Cr2KO*, still expressed Cr1 on B cells but was deficient for the full length Cr2 protein. Surprisingly a new alternative splice within the *Cr2* gene created a truncated product that encoded a novel protein termed iCr2 that was expressed on the surface of the cells. The *Cr2KO* mouse thus provides a new model system for the analysis of Cr1 and Cr2 functions in the immune response of the mouse.

Introduction

The enhancing effects of complement for humoral immunity have been apparent since the foundational studies of Pepys (1974), and the identification of the enhancement of B cell activation through complement receptor (CR) 2 binding of complement bound antigen (Carter et al., 1988). This well accepted model of CR2 function relies initially on activation of the complement system by one of the three pathways: classical, alternative, or mannose-binding lectin. All three pathways lead to cleavage and deposition of complement component 3 (C3) fragments on antigen. The iC3b and C3d(g) fragments maintain a covalent bond with the antigen and act to direct the antigen to Cr2/CR2 via the iC3b/C3d(g) binding site on the proteins (Molina et al., 1995; Pramoonjago et al., 1993). (Cr1/Cr2 versus CR1/CR2 will be used here to clearly differentiate the mouse from the human proteins, respectively). *In vitro* experiments extensively defined the enhancement of B cell responses by CR2 (Carter et al., 1988; Matsumoto et al., 1991, 1993) and the overlapping binding, co-association and co-activation characteristics for Cr2 (Krop et al., 1996; Molina et al., 1994).

Cr2 null mouse lines (*Cr1/2KO*) have further supported the hypotheses that *Cr2* gene products expressed by B cells and follicular dendritic cells (FDC) are critical for the detection of antigen and the generation of optimal antibody responses (Haas et al., 2002; Molina et al., 1996). The mouse *Cr2* gene, unlike the human, creates both the Cr1 and Cr2 proteins from a single gene. The same signal sequence – encoding exon is alternatively spliced to either the domain encoding the first short consensus repeat (SCR) domain of the gene (thus generating the 190,000 Da Cr1 protein) or the exon encoding the seventh and eighth SCR (see Fig. 1a) domains of the *Cr2* gene, which represent the most N-terminal coding sequences of the mature Cr2 protein (145,000 Da). The sequence of the Cr2 protein is thus fully included within that of the Cr1 protein. Recently we have found that this alternative splicing pattern is unique to B cells in that murine FDCs express only Cr1 protein from *Cr2* gene transcripts (Donius et al., 2013).

The extensive research on the *Cr1/2KO* mice however has not fully discriminated the roles of the Cr1 and Cr2 proteins. The 6 N-terminal SCR domains of the Cr1 protein (which are not included in the Cr2 protein) can act as a co-factor in the regulation of C3 convertase stability and function (Molina et al., 1994), and recent studies have suggested that Cr1 may be important in regulating complement activation in the immune microenvironment (Jacobson et al., 2008; Seregin et al., 2009). To define the functions of the Cr1 protein we have recently created and described

Abbreviations: Cr1/CR1, complement receptor 1; Cr2/CR2, complement receptor 2; C3, complement component 3; BCR, B cell receptor; s, follicular dendritic cell; SCR, short consensus repeat; PCR, polymerase chain reaction; TK, thymidine kinase; WT, wild type; SS, signal sequence; iCr2, incomplete Cr2; IC, isotype control; gMFI, geometric mean fluorescent intensity.

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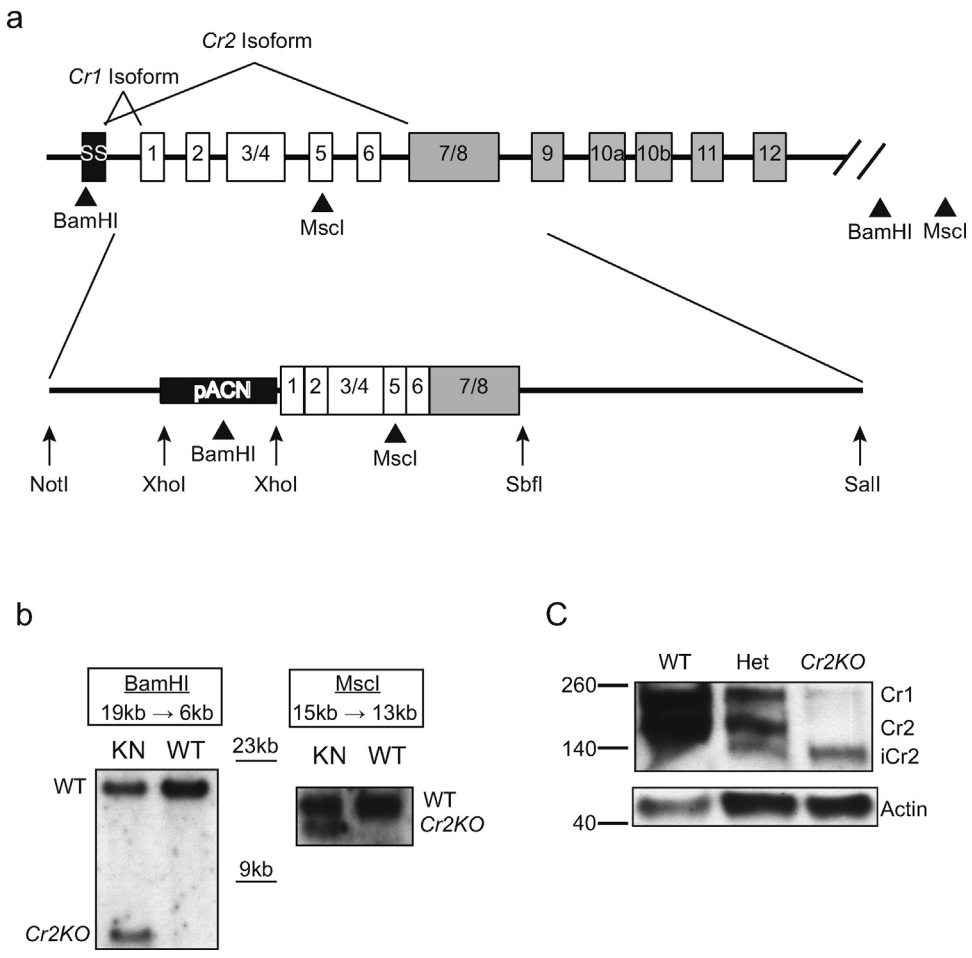

Fig. 1. Schematic diagram and confirmation of *Cr2* alternative splice site disruption. (a) The exons of the *Cr2* gene are shown with numbers defining the number of the short consensus repeats (SCR) encoded within each. The initial exon denoted SS encodes the signal sequence. The *Cr1* isoform is generated via the inclusion of all exons during splicing while the *Cr2* isoform is generated by alternative splicing that excludes the exons encoding SCRs 1–6. The construct was generated from a cDNA version of the *Cr2* gene with the addition of a pACN neomycin selection marker. Arrows represent the approximate location of restriction sites used for cloning and mapping of homologous sequence used for recombination. Triangles represent the approximate location of BamHI and MscI restriction sites utilized to Southern blot for determination of proper homologous recombination. (b) Representative Southern blots are shown for proper 5′ and 3′ homologous recombination. BamHI restriction digestion of wild type (WT) genomic DNA exhibits a 19 kb band detected by a radiolabeled probe. In the *Cr2KO* condition the fragment is reduced to 6 kb. Homologous recombination of the 3′ region was determined by probing for a size reduction from 15 kb to 13 kb. (c) Western blot analysis of *Cr2* gene products from WT and *Cr2KO* mice, as well as mice heterozygous for the *Cr2KO* insertion.

a mouse (*Cr1KO*) that exclusively expresses Cr2 protein on the surface of B cells and lacks expression of either protein on the surface of FDCs.

To define the specific functions of the Cr2 protein in the immune response of the mouse we created an additional mouse line that specifically lacks the Cr2 protein but still produces Cr1. This mouse, dubbed *Cr2KO*, was created by homologous recombination within the *Cr2* gene with a construct that removed the alternative splice junction utilized in B cells to create the Cr2 protein. Analysis of mice generated from this recombination demonstrated expression of the full length Cr1 protein on B cells but the loss of Cr1 expression (and *Cr2* gene expression) on FDCs suggesting the disruption of a FDC-specific transcriptional enhancer site within the *Cr2* gene. Intriguingly, the removal of the native alternative splice site in the *Cr2KO* construct resulted in the utilization of an internal cryptic splice site, generating a truncated *Cr2* gene product, termed iCr2, that lacks the iC3b/C3d(g) binding sites present on the normal Cr2 protein. The *Cr2KO* animals thus represent a novel animal model with which to functionally analyze the role of the Cr1 protein on the surface of B cells in the absence of functional Cr2.

Materials and methods

Generation of the Cr2KO mouse

The pKS+ Bluescript vector was used as a backbone for the creation of the 28 kb construct. Using primers (#4151 5′-GTGGTCCTTATTTCTAGGTCAGTGTAAGTTGCTGC-3′ and #4152 5′-GTTTTAATTTCCTACTTACCACTCTCACAGACTGGCAG-3′) a 1250 bp cDNA version of the exons for SCRs 1–8 of *Cr1* was amplified using the long distance high-fidelity DNA polymerase Platinum *Pfx* (Invitrogen, Carlsbad, CA). The amplicon was additionally modified by PCR utilizing primers with overhang sequences for the restriction fragments XhoI and SbfI, 5′ and 3′, respectively. This new SCRs 1–8 fragment was cloned and ligated via XhoI and SbfI into the mouse *Cr2* gene NotI-XhoI restriction fragment and 3′ to the SbfI-SalI restriction fragment (see Fig. 1a). The XhoI site of the resulting SCR1-8 construct with homology arms was utilized for insertion of the germline self deleting neomycin resistance construct pACN (Bunting et al., 1999). The entire construct was flanked by the TK1TK2 thymidine kinase containing negative selection construct. Electroporation of the *Cr2KO* vector into mouse strain