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Alternative splicing of CD200 is regulated by an exonic splicing enhancer and SF2/ASF

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ABSTRACT

CD200, a type I membrane glycoprotein, plays an important role in prevention of inflammatory disorders, graft rejection, autoimmune diseases and spontaneous fetal loss. It also regulates tumor immunity. A truncated CD200 ($CD200_{tr}$) resulting from alternative splicing has been identified and characterized as a functional antagonist to full-length CD200. Thus, it is important to explore the mechanism(s) controlling alternative splicing of CD200. In this study, we identified an exonic splicing enhancer (ESE) located in exon 2, which is a putative binding site for a splicing regulatory protein SF2/ASF. Deletion or mutation of the ESE site decreased expression of the full-length CD200. Direct binding of SF2/ASF to the ESE site was confirmed by RNA electrophoretic mobility shift assay (EMSA). Knockdown of expression of SF2/ASF resulted in the same splicing pattern as seen after deletion or mutation of the ESE, whereas overexpression of SF2/ASF increased expression of the full-length CD200. *In vivo* studies showed that viral infection reversed the alternative splicing pattern of CD200 with increased expression of SF2/ASF and the full-length CD200. Taken together, our data suggest for the first time that SF2/ASF regulates the function of CD200 by controlling CD200 alternative splicing, through direct binding to an ESE located in exon 2 of CD200.

INTRODUCTION

CD200 is a type 1 membrane glycoprotein, delivering immunoregulatory signals through binding to its receptors (CD200Rs) (1–4). It is present on neurons, B cells, activated T cells, thymocytes, dendritic cells and endothelium in mice, rats and human (5,6). A large and growing body of studies demonstrates that expression level of CD200 regulates graft survival (7–9), susceptibility to autoimmune diseases (10–12), fetal loss (13), inflammation/infection (14) and tumor immunity (15–18).

Alternative splicing is a major mechanism for regulating biological systems, producing multiple messenger RNA (mRNA) and protein isoforms. Some of these isoforms have distinct or even opposing functions (19). Many genes in the immune system have been found to be alternatively spliced (20–22) and a growing number of human diseases are associated with aberrant splicing of the genes (23–25). However, few studies to date have identified the mechanisms that regulate alternative splicing in the immune system. While CD200 exists as a single copy gene, data from Borriello *et al*. (26), confirmed by our experiments (27), have reported that a splice variant of CD200 exists. Although exon 2 deletion of CD200 caused by alternative splicing results in a frame shift and premature translational termination, we noted the existence of a downstream ATG start codon in a perfect Kozak context (27). When the first start codon is followed shortly by a terminator codon and creates a small open reading frame (ORF; 5′-mini-cistron), the 40S ribosomal subunit remains bound to the mRNA, resumes scanning, and potentially reinitiates at the next ATG codon downstream (28). It is known that the NH2-terminal region of CD200 is important for its biological interaction with CD200Rs (29,30), and translation from the second ATG start codon would produce a truncated form of CD200 ($CD200_{tr}$) lacking the NH2-terminal 43 amino acids which includes regions important for the interaction with CD200Rs. Indeed, our previous studies have shown that expressed $CD200_{tr}$ is a functional antagonist to CD200 (27).

Exons often contain specific short oligonucleotide sequences that affect their ability to be spliced. Exonic splicing enhancers (ESEs) within exons promote splicing of the corresponding exons and subsequent exon inclusion mediated by splicing regulatory proteins. The best-studied family of splicing regulatory proteins are Serine/Arginine-rich proteins (SR proteins), which include the proteins SF2/ASF, SC35, SRp20, SRp30c and many others (31,32). It has become clear that many exons

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contain ESE elements that bind to specific members of the SR family (25), leading to exon inclusion.

Since CD200 is involved in many diseases and its splice variant $CD200_{tr}$ is an antagonist to CD200, identification of the mechanism controlling the relative expression levels of CD200 versus $CD200_{tr}$ may provide insight into novel strategies for treatment of clinical disorders. In the present study, we have explored the mechanism controlling CD200 alternative splicing and show that SF2/ASF regulates CD200 alternative splicing through its direct binding to an ESE site in exon 2 of this gene. The level of SF2/ASF determines the alternative splicing patterns in different tissues or cells. Interestingly, in a mouse model of viral infection, we detected for the first time that the normal splicing pattern of CD200 was reversed in the lung tissue of A/J mice infected with mouse hepatitis virus strain I (MHV-1), following an increase in expression of SF2/ASF in this MHV-1 susceptible mouse strain.

MATERIALS AND METHODS

Cells and reagents

All human cell lines were obtained from American Type Culture Collection. Human B cell lines Daudi, Raji and TEM were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS). The human neuronal cell lines SK-N and HCN-1A were cultured with 10% FBS in α-MEM media (Invitrogen).

Total RNAs from different human tissues were purchased from Clontech. A human BAC clone containing the whole human CD200 gene and a pcDNA3.2 expression vector containing SF2/ASF were obtained from The Center for Applied Genomics (Hospital for Sick Children, Toronto). Taq DNA polymerase, T4 DNA ligase and all restriction endonucleases were purchased from New England Biolabs. Random Primers, Superscript Reverse Transcriptase II, Elongase Enzyme, pcDNA3.0 expression vector and all competent cells were purchased from Invitrogen. EndoFree Plasmid purification Maxi Kit and QIAEX II Gel Extraction Kit were ordered from QIAGEN. A purified SF2/ASF recombinant protein was kindly provided by Dr. Blencowe (University of Toronto). Anti-human and mouse SF2/ASF antibody was obtained from Santa Cruz Biotechnology. Anti-human and mouse β-actin antibody was purchased from BD Biosciences. Small-interfering RNA (siRNA) including SF2/ASF siRNA and a 'scrambled' siRNA were synthesized by Eurogentec. RNA oligonucleotides were synthesized by DNA and RNA Synthesis Center at Hospital for Sick Children (Toronto). All the primers used for polymerase chain reactions (PCRs), real-time PCRs and mutations were synthesized by Invitrogen.

Mice and viral infection

Female A/J and C57BL/6J mice, 6–8 weeks of age were purchased from Jackson laboratories. The mice were maintained in microisolator cages, housed in the animal facility at The Toronto Hospital Research Institute, University of Toronto, and fed standard lab chow diet and water *ad libitum*. All protocols were approved by the animal Welfare Committee. Parental virus Mouse Hepatitis Virus strain 1 (MHV1) was ordered from the American Type Culture Collection. As previously described (33), MHV1 infection was carried out in a viral isolation room. A/J and C57BL/6J mice were anesthetized by intraperitoneal injection with 0.2 ml 10% pentobarbital diluted in normal saline. Mice were left untreated or received 5000 plaque forming unit (PFU) of MHV1 intranasally. Mice were sacrificed 12, 36 and 96 h postinfection and lung tissue was collected.

RNA isolation and regular or real-time reverse transcriptase-PCR

Total RNA was isolated from human B cell lines (Daudi, Raji, TEM), human neuronal cell lines (SK-N, HCN-1A) and mouse lung tissue using TRIzol reagent. Five micrograms of total RNA from human tissues (brain, heart, skeletal muscle, colon, liver, thymus, kidney, intestine, lung, placenta and spleen), or human B cell lines (Daudi, Raji, TEM) and human neuronal cell lines (SK-N, HCN-1A), or mouse lung tissue was treated with DNase I and reverse transcribed in the presence of 250 ng of Random Primers, 1× PCR Buffer, 10 mM dNTPs and 200 U of SuperScript II reverse transcriptase (RT; Invitrogen) in a final reaction volume of 20 µl. Reactions were carried out at 25°C for 10 min, 42°C for 50 min, followed by a 15-min step at 70°C to denature the enzyme.

For regular PCR, 2 µl of first strand complementary DNA (cDNA) was amplified in a 50-µl reactions in the presence of 1× PCR buffer, 1.5 mM $MgCl_2$, 2.5 µM of dNTPs, 5 U of *Taq* DNA Polymerase (New England Biolab). A first cycle of 5 min at 94°C was followed by 30 cycles of 30 s at 94°C, 30 s at a different annealing temperature (based on different primer pairs), and 1 min at 72°C. The final extension step was at 72°C for 15 min. For real-time PCR, first strain cDNA was diluted 1:20 and quantified using an ABI 7900HT Sequence Detection System (Applied Biosystems). The sequences of the primers used for regular and real-time PCR were indicated in Table 1.

The endogenous human CD200 primer pairs for regular PCR were also used to construct an amplicon-containing plasmid (endogenous) for a standard curve. An exogenous amplicon-containing plasmid (exogenous) for a standard curve was constructed using the primers shown in Table 1. Samples were tested in triplicate using 4 µl of first strand cDNA in a 20 µl total volume with 1× universal master mix (Applied Biosystems). The results were normalized to that of the housekeeping gene GAPDH and HPRT. The copy number of transcripts was determined by comparison with a calibration curve of known amounts of amplicon-containing plasmid.

Control reactions were performed for the specificity of the real-time PCR primers. A DNA fragment, containing either exon 1, exon 2 and exon 3 or only exon 1 and exon 3, was gel purified and subcloned into pcDNA 3.0 between NotI and XhoI sites. The CD200-bearing plasmids were then linearized by XhoI. *In vitro* transcription was carried out using TranscriptAid T7 High Yield

Table 1. The oligonucleotides used in this study

Primers for regular PCR	
Human CD200	
sense (exon 1)	5′-AGCAAGGATGGAGAGGCTG-3′
antisense (exon 3)	5′-GGTATTGAAGAGACACATG-3′
Murine CD200	
sense (exon 1)	5′-GCAAGGATGGGCAGTCTG-3′
antisense (exon 3)	5′-CATGGGCTTTGCTGTAAG-3′
Primers for real-time PCR (the location of the numbered primers was shown in Figure 3A)	
Endogenous human full-length CD200	
(1) sense (exon 2)	5′-CAGCCTGGTTTGGGTCATG-3′
(2) antisense (exon 3)	5′-GCAGAGAGCATTTTAAGGAAGCA-3′
Endogenous human truncated CD200	
(3) sense (the end of exon 1 directly linked to the beginning of exon 3)	5′-GATGGAGAGGCTGTGCAAGTG-3′
(4) antisense (exon 3)	5′-GCAGAGAGCATTTTAAGGAAGCA-3′
Exogenous human full-length CD200	
(5) sense (5′-UTR of pcDNA 3.0 vector)	5′-TCTGCAGATATCCATCACACTG-3′
(6) antisense (exon 2)	5′-CCCAAACCAGGCTGTAGGTA-3′
Exogenous human truncated CD200	
(7) sense (5′-UTR of pcDNA 3.0 vector)	5′-GTAACGGCCGCCAGTGT-3′
(8) antisense (end of exon 3 directly linked to exon 1)	5′-CACTTGCACAGCCTCTCCAT-3′
Exogenous human total CD200	
(9) sense (exon 3)	5′-GGCCTGCCTCACCGTCTAT-3′
(10)antisense (pcDNA3.0 vector downstream of Xho 1)	5′-ATCAGCGAGCTCTAGCATTTAGG-3′
Murine full-length CD200	
sense (exon 2)	5′-GGGCATAGCAGCAGTAGCG-3′
antisense (exon 3)	5′-TGTGCAGCGCCTTTCTTTC-3′
Murine truncated CD200	
sense (exon 1 directly linked to exon 3)	5′-GATGGGCAGTCTGTGGAAGTG-3′
antisense (exon 3)	5′-GAGAACATCGTAAGGATGCAGTTG-3′
Primers for an exogenous amplicon-containing plasmid construct	
sense (5′UTR of pcDNA 3.0)	5′-AGTGTGCTGGAATTCTGCAG-3′
antisense (exon 3)	5′-ATGTCACAATGAGGGCTTCC-3′
Primers for alternative splicing minigene construct	
sense (underlined is Not I site)	5′-CTATGCGGCCGCATGGAGAGGCTGGTGAGCGGGGG-3′
antisense (underlined is Sal I site)	5′-CTATGTCGACCATAGACGGTGAGGCAGGCCGTTCC-3′
Primers for mutation (the mutated region was underlined)	
sense	5′-GCTTTCTGTCTTCAGGTGACGTACGGCCCTTCTCTCATCT GTC-3′
antisense	5′-GACAGATGAGAGAAGGGCACGTACGTCACCTGAAGACAG AAAGC-3′
Primers for deletion	
sense	5′-GCTTTCTGTCTTCAGGTGAGCCCTTCTCTCATCTGTC-3′
antisense	5′-GACAGATGAGAGAAGGGCATCACCTGAAGACAGAAAGC-3′

Transcription Kit (Fermantas Inc.) following the manufacturer's instruction. Transcribed RNA was treated with DNaseI to remove template DNA and purified by phenol:choloroform extraction and ethanol precipitation. First strand cDNA was then synthesized and real time PCR was performed. The primer pairs used for real-time PCR are shown in Figure 3A and Table 1.

Preparation of an alternative splicing construct

A human BAC clone containing the whole human CD200 gene was used as a template for long-distance PCR to obtain a region bearing exon 1, intron 1, exon 2, intron 2 and exon 3 of the human CD200. Two mixtures were prepared: mix 1 (20 μl) contained 0.1 μg of DNA template, 0.5 mM dNTP mix and 0.5 μm of sense and antisense primers; mix 2 (30 μl) included elongase enzyme mix and 1× long-distance PCR buffer A and B provided by the manufacturer (the ratio of buffer A and B is 1:4). The sense primer started with the NotI cleavage site and the antisense primer with the SalI site. The sequences of the primers were shown in Table 1. Mix 1 and mix 2 were combined on ice and subject to PCR under the following condition: 94°C for 1 min followed by three cycles at 94°C for 30 s, 59°C for 30 s, 69°C for 20 min, and then 29 cycles of 94°C for 30 s, 69°C for 20 min. The final extension was 69°C for 15 min. The 12-kb CD200 fragment was displayed on 0.7% TAE-agarose gel and purified using QIAEX II Agarose Gel Extraction Kit following the manufacturer's instruction. For more efficient elution of the large size DNA, the final incubation time was extended to 30 min at 60°C. The gel-purified DNA fragment was verified by restriction enzyme digestion with BamHI, BglII, EcoRI and HindIII, respectively, and DNA sequencing. For ligation to pcDNA 3.0 expression vector, the CD200 fragment was digested with NotI and SalI. Meanwhile, pcDNA 3.0 expression vector was digested with NotI and XhoI. Afterwards, pcDNA 3.0 vector was further dephosphorylated to remove the 5′ phosphoryl group, preventing the vector from self-ligation. The enzyme-treated CD200 fragment and pcDNA 3.0 were ligated, at a molar ratio of 3:1, using

400 U of T4 DNA ligase in the presence of 1× T4 ligase buffer in a 20-μl reaction at 16°C overnight.

Transformation and clone screening

Ligation products containing the alternative splicing construct were transformed into DH 10β *Escherichia coli* cells by electroporation using a Cell-Porator Electroporation System (Life Technologies) at 401 V, 330 μF capacitance, low Ω and 4 kΩ (for Booster). The cells were plated onto LB/ampicillin plates and incubated at 37°C overnight. Twenty isolated clones were randomly picked. Only one clone showed a DNA supercoil band with much larger size than that of the vector clone on the gel. This clone was further characterized by the combination of restriction enzyme digestion and sequence analysis.

Mutation and deletion of the ESE site in exon 2 of CD200

An ESE site was identified in exon 2 of the human CD200 using computational methods RESCUE-ESE (34) and ESEfinder (35). To mutate the ESE site, site-directed mutagenesis was employed using QuickChange II XL site-directed mutagenesis kit from Stratagene. Two mutagenic primers were synthesized, in which the ESE site was replaced by a BsiWI site or deleted, and purified by polyacrylamide gel electrophoresis (PAGE). The sequences of the primers used are shown in Table 1 (the mutated region was underlined). The mutagenesis reaction was carried out in 50 μl total volume with 40 ng of template DNA, 125 ng of each primer and 2.5 U PfuUltra high-fidelity (HF) DNA polymerase and 3 μl of QuickSolution reagent provided by Stratagene. The cycling conditions included a 1-min initial denaturation at 95°C, 18 cycles with 50 s denaturation at 95°C, 50 s annealing at 58°C and 40 min extension at 68°C, and a final extension of 7 min at 68°C. The product was then subjected to digestion with 10 U of DpnI for 2 h at 37°C, selectively removing the parental, methylated, and nonmutated strands. Four microliters of DpnI-treated DNA was then transformed into XL10-Gold Ultracompetent cells. Cells were plated and incubated for selection of ampicillin-resistant clones. Ten isolated ampicillin-resistant clones were picked at random and their mutated or deleted regions were characterized by DNA sequencing.

Transient transfection

The B cell line Daudi was washed and resuspended in 1× Hanks Balanced Salt Solution (HBSS) to a cell density of 2×10^7 cells/ml. The neuronal cell line SK-N was trypsinized and resuspended in 1× phosphate-buffered saline (PBS) with 2% FBS at a density of 10^7 cells/ml. Thee-hundred microliters of the Daudi cells or 500 μl of the SK-N cells were transfected with 10 μg of the alternative splicing minigene construct, the minigene construct with the ESE site deleted or mutated, the minigene construct plus SF2/ASF expression vector, or the ESE deleted construct plus SF2/ASF expression vector. Electroporation was performed with square waves of 700 V, 99 μs pulse length for four pulses for Daudi and square waves of 200 V, 70 ms pulse length for one pulse for SK-N using T820 ElectroSquarePorator (BTX). Both Daudi and SK-N cells were cultured in 5 ml of pre-warmed complete medium for 48 h before harvesting.

RNA gel mobility shift assay

The RNA oligonucleotides used for gel mobility shift assay were as follows:

CD200 exon 2 with the wild-type ESE, 5′-GUGAUCAG GAUGCCCUUCUC-3′;
CD200 exon 2 with the mutated ESE, 5′-GUGACGUAC GUGCCCUUCUC-3′;

The RNA gel mobility shift assay was carried out as previously described (36). The RNA oligonucleotides were 5′-end labeled with γ-^{32}P-ATP (Perkin Elmer) using KinaseMax kit from Applied Biosystems following the manufacturer's instruction. Unincorporated nucleotides were removed by using G-25 Sephadex Columns. Fifteen femtomoles of radiolabeled RNA oligonucleotides were mixed with 4 pmol of SF2/ASF recombinant protein in a 20-μl binding reaction containing 2 μg yeast tRNA (Applied Biosystems). For competition, 100× cold CD200 exon 2 oligonucleotide was added to the reaction containing the radiolabeled CD200 exon 2 oligonucleotide and SF2/ASF. After incubation for 20 min on ice, the RNA–protein complexes were separated from free RNA by electrophoresis on a 5% native polyacrylamide gel, run at 170 V for 2 h in 0.5% TBE buffer. The gel was then dried and autoradiographed at −80°C with intensifying screen.

RNA interference

SF2/ASF siRNA was designed based on the information described by Cartegni *et al.* (37). A 'scrambled' siRNA, which has no match with any mRNA of the human database, was used as a control. The siRNAs were synthesized by Eurogentec with the following sequences:

SF2/ASF siRNA: 5′-ACGAUUGCCGCAUCUACG U-3′;
Scramble siRNA: 5′-GCCGAUACGUACGCUUAC U-3′.

7.5×10^5 Daudi cells or 5×10^5 SK-N cells were seeded into 12-well plates 24 h before transfection. Two-and-a-half micrograms of siRNA was transfected to Daudi or SK-N cells using Lipofectamine 2000 (Invitrogen) to examine endogenous expression pattern of CD200 following silencing SF2/ASF. Two-and-a-half micrograms of siRNA, together with 10 μg of the alternative splicing construct DNA, was transfected to Daudi or SK-N cells by electroporation to detect exogenous expression pattern of CD200 following silencing SF2/ASF. The cells were harvested 48 h posttransfection. Total RNA and protein were then extracted.

Western blot

Nuclear extracts from Daudi and SK-N cells were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (38) from Pierce Biotechnology following the

manufacturer's instruction. Western blotting was performed using 20 μg of nuclear extracts. After separation on a 10% SDS-PAGE gel, the proteins were transferred to a nitrocellulose membrane and probed with anti- human SF2/ASF antibody [1:200 dilution, goat polyclonal immunoglobulin G (IgG; Santa Cruz Biotechnology] followed by washing in 2% milk-PBS Tween. The membrane was then incubated with Donkey anti-goat IgG (1:5000 dilution; horseradish peroxidase- conjugated (BD Biosciences) and followed by washing again. Substrates, luminal and enhancer were added onto the membrane and incubated for 1 min. The membrane was exposed to Kodak XAR-5 film with intensifying screens for 5 min. Anti-human β-actin antibody (1:6000 dilution, goat monoclonal IgG; BD Biosciences) was used as loading controls. The exposure time for β-actin was 20 s.

Statistical analysis

Statistical significance was calculated with one-way analysis of variance (ANOVA) followed by Tukey tests. *P*-values ≤ 0.05 were considered significant and shown in the figures.

RESULTS

The existence of discrete CD200 splice variants is cell and tissue specific

Human CD200 splice variants were examined in human tissues, B cells and neuronal cells. Total RNAs from different human tissues or human B cell and neuronal cell lines were used for RT-PCR using a sense primer located in exon 1 of human CD200 and an antisense primer in exon 3. As shown in Figure 1A and B, two transcripts were detected in all the human tissues, B cell lines (Daudi, Raji and TEM) and neuronal cell lines (SK-N and HCN-1A). The larger transcript was by far the dominant one seen in the brain and neuronal cell lines. Accordingly, for subsequent experiments, the B cell line Daudi and neuronal cell line SK-N were used as representatives of the two different splicing pattern of CD200. The only tissue not expressing CD200 was human skeletal muscle. The two transcripts were purified from the agarose gel and sequenced. It was confirmed that the larger one represented an exon 2 inclusion, whereas the smaller one represented an exon 2 exclusion ($CD200_{tr}$).

An ESE for binding of SF2/ASF was present in exon 2 of CD200

Since alternatively spliced exons often contain ESEs for binding of splicing regulators that determine the fate of the exon (exon inclusion or exclusion), we wondered whether ESEs for binding of splicing regulatory proteins existed in exon 2 of CD200. For this purpose, both RESCUE-ESE (34) and ESEfinder (35) were used to search for ESEs in the exon 2 of CD200. Only one ESE was identified in exon 2 by both RESCUE-ESE and ESEfinder. The ESE existed in exon 2 of CD200 in human, mouse and rat, with the sequence TCAGGA (Figure 2A). The identified ESE represents a known binding site for a splicing regulatory protein SF2/ASF, a member of the SR protein family (35).

Exogenous expression of CD200/$CD200_{tr}$ shared the similar pattern with the corresponding endogenous one

To gain insight into the role of the ESE in exon 2 of CD200, we generated an alternative splicing minigene construct containing the genomic region from exon 1 to exon 3 of the human CD200 (Figure 2B). A 12-kb fragment bearing this genomic region was characterized by sequencing and restriction enzyme digestion, and ligated to a pcDNA 3.0 expression vector. The construct was transfected independently to human B cell line Daudi and neuronal cell line SK-N. After 48 h, RNA was extracted from each cell population for detection of the exogenous expression of splicing pattern of CD200. RNA was also isolated from nontransfected Daudi and SK-N cells for detection of the endogenously expressed splicing pattern. To measure quantitatively the expression levels of the two splice variants, real-time RT-PCR was performed using the primer pairs located in different regions (Figure 3A). The specificity of the primers for amplification of full-length and truncated CD200 was examined. As shown in Figure 3B, the primer pair used for full-length CD200 did not amplify the template from CD200 RNA lacking exon 2 (truncated form), whereas the primer pairs for truncated CD200 were not

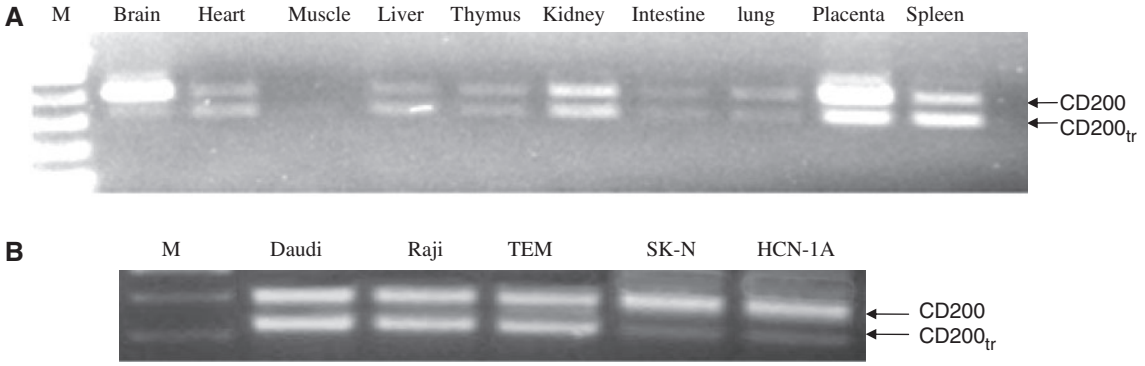

Figure 1. Comparison of transcriptional expression of full-length human CD200 with truncated CD200. Five micrograms of total RNA from different human tissues (**A**) or from the human B cell lines Daudi, Raji and TEM, and human neuronal cell lines SK-N and HCN-1A (**B**) was used for RT-PCR using a sense primer located in exon 1 and an antisense primer located in exon 3. The upper arrow pointed to CD200 containing exon 2 and the lower one indicated the CD200 without exon 2 ($CD200_{tr}$).

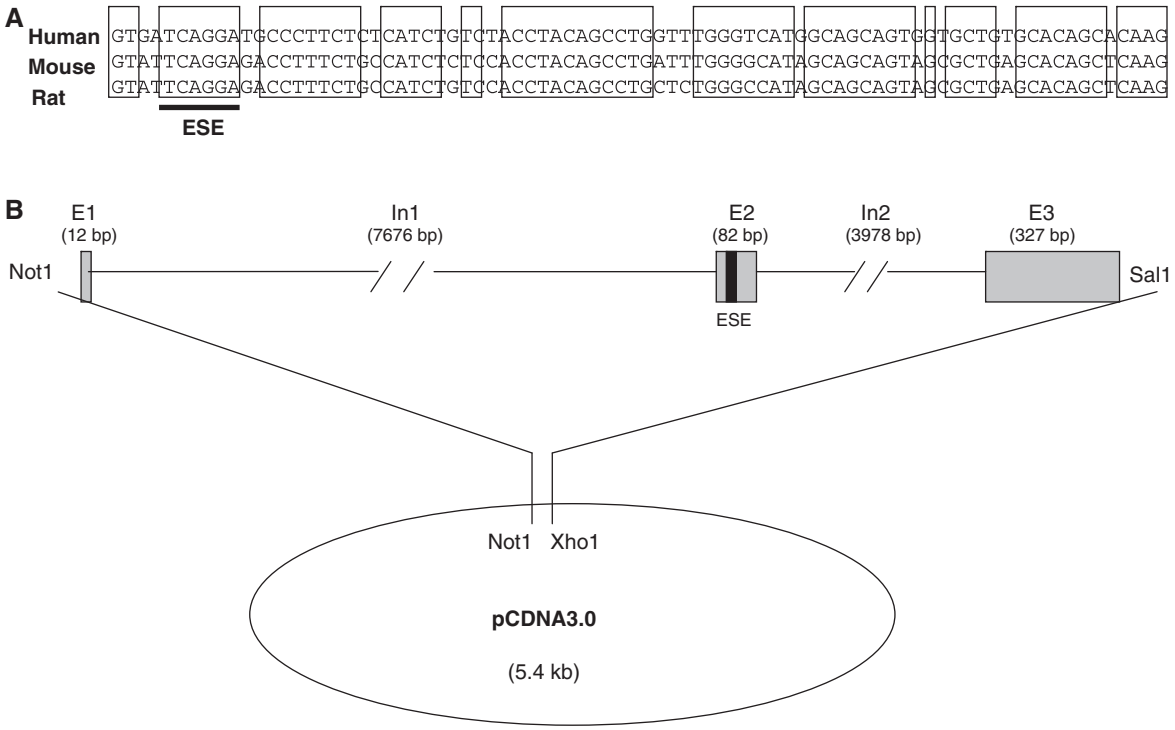

Figure 2. Identification of an ESE in exon 2 of CD200 and schematic drawing of the alternative splicing minigene construct. (**A**) An ESE with the sequence of TCAGGA was located in exon 2 of human, mouse and rat CD200. (**B**) A minigene construct was generated by inserting a ~12-kb fragment including exon 1, intron 1, exon 2, intron 2 and exon 3 of human CD200 into the pcDNA3.0 expression vector. The location of restriction enzyme cleavage sites for ligation of the insert with the vector was indicated in the figure.

able to amplify the template from CD200 RNA containing exon 2 (full-length form). Each primer pair generated only a single product (Supplementary Figure 1A) and the standard curves generated from each primer pair are parallel with slopes between −3.1 and −3.6 (Supplementary Figure 1B). The exogenous expression of CD200/$CD200_{tr}$ had a similar pattern to the corresponding endogenous one in Daudi cells or SK-N cells (Figure 3D and E).

Exon 2 inclusion was abolished by mutation or deletion of the ESE

To examine further whether the ESE in exon 2 of CD200 determined the fate of the exon (inclusion or exclusion), site-directed mutagenesis was performed to mutate the ESE element in the alternative splicing construct, replacing the ESE (TCCTGA) with a restriction enzyme BsiWI site (CGTACG) (Figure 3C) or to delete the ESE. After characterizing the mutation or deletion construct by sequencing, the splicing construct was transfected to Daudi and SK-N cells. Total RNA was extracted from cells 48 h after transfection and real-time RT-PCR was carried out. As shown in Figures 3C and D, and 4A and B, expression of the full-length transcript (exon 2 inclusion) was reduced in both Daudi and SK-N cells after mutation or deletion of the ESE in exon 2. These data suggest that the ESE in exon 2 of CD200 promotes exon 2 inclusion.

A splicing regulatory protein SF2/ASF directly binds to the ESE and determines the fate of exon 2 of CD200

Since the ESE described above is known to contain a putative binding site for SF2/ASF, we investigated whether SF2/ASF binds to the ESE. An RNA–EMSA was performed. As shown in Figure 5, an RNA–protein complex was detected after the SF2/ASF recombinant protein with ΔRS domain was mixed with a radiolabeled RNA oligonucleotide containing the ESE site. This protein/RNA interaction is specific since SF2/ASF did not bind to a radiolabeled RNA oligonucleotide containing mutated ESE site and the above binding was eliminated by competing 100× unlabelled oligonucleotide containing the same ESE (Figure 5). Moreover, this binding was not competed by the same level of cold oligonucleotide with the ESE site mutated (data not shown).

As previously described, the full-length CD200 was expressed predominantly in brain and neuronal cells. One explanation of this observation is that the expression of SF2/ASF is higher in neuronal cells and brain. To test this hypothesis, we assessed SF2/ASF levels in Daudi and SK-N cells by Western blotting. As shown in Figure 6A, the natural level of SF2/ASF was clearly higher in SK-N cells than in Daudi cells.

To gain further insight into the role of SF2/ASF in controlling alternative splicing of CD200, an siRNA against SF2/ASF was employed to knock down SF2/ASF in Daudi and SK-N cells. A scramble siRNA was used as a negative control. After 48 h, cells were collected

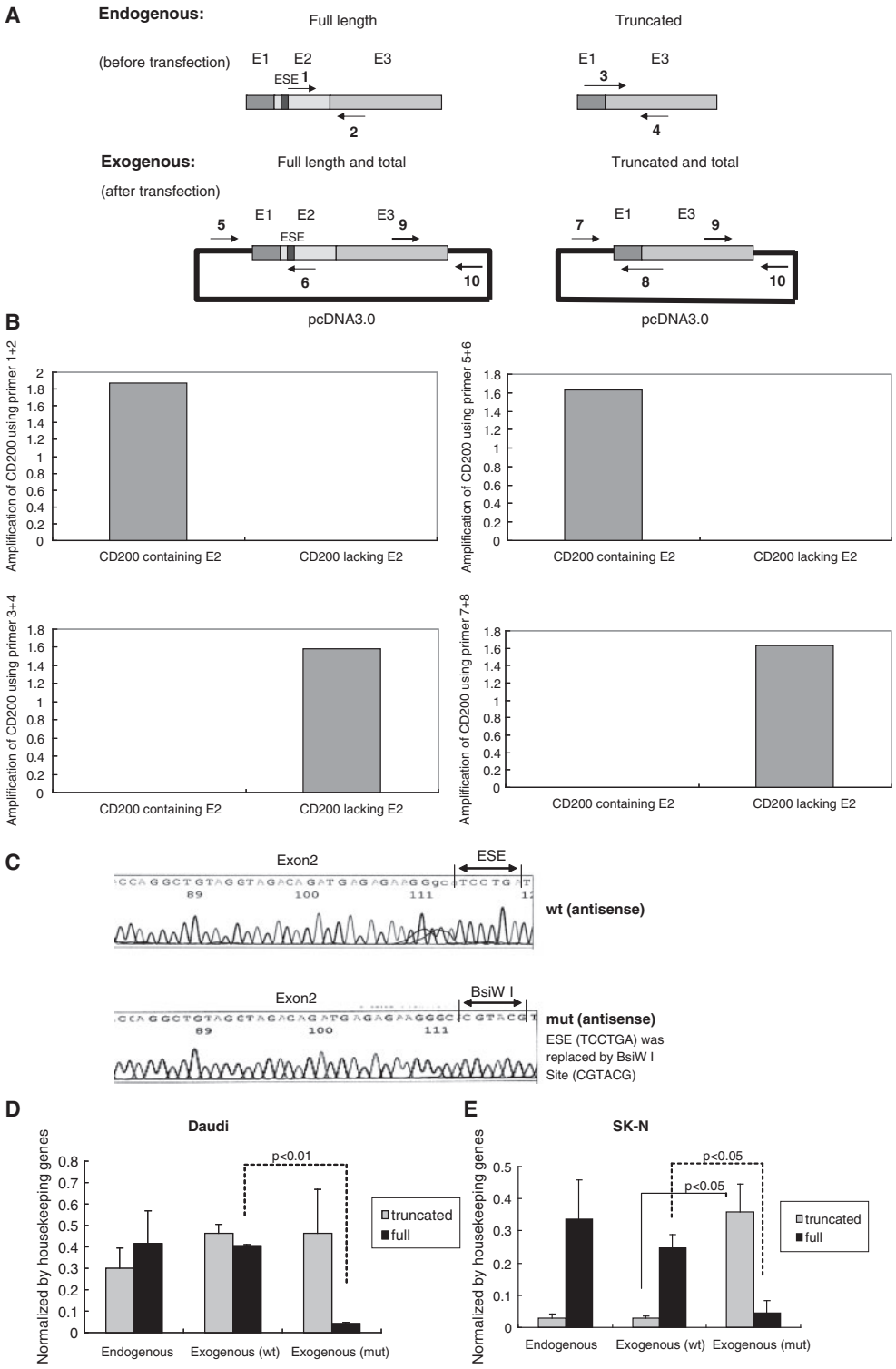

Figure 3. The pattern of expression of exogenous full-length CD200 or truncated CD200 in different cells parallels that of the endogenous molecules and mutation of the ESE in exon 2 abolishes exon 2 inclusion. (**A**) The location of the primers used for real-time RT-PCR. Primers 1 and 2 were used for endogenous expression of full-length CD200; primers 3 and 4 were used for endogenous expression of truncated CD200; primers 5 and 6 were used for exogenous expression of full-length CD200; primers 7 and 8 were used for exogenous expression of truncated CD200; primers 9 and 10 were used for the constitutive expression of V region of CD200. (**B**) The specificity of the primers used for full-length or truncated CD200. CD200 RNA containing exon 2 or lacking exon 2 from *in vitro* transcription was reverse transcribed and used for real-time PCR using the primer pairs labeled in the figure. (**C**) Mutation of the ESE in exon 2 was confirmed by DNA sequencing. (**D**) Endogenous and exogenous expression of the full-length and truncated CD200 in Daudi cells, and exogenous expression of two isoforms after mutation of the ESE. (**E**) Endogenous and exogenous expression of the full-length and truncated CD200 in SK-N cells, and exogenous expression of two isoforms after mutation of the ESE. The data represent the mean ± SE (three independent experiments, triplicate determinations). Broken lines reflect exogenous expression of the full-length CD200 decreased after mutation of the ESE relative to that of wild type ($P < 0.01$ in Daudi; $P < 0.05$ in SK-N). Continuous lines reflect exogenous expression of the truncated CD200 increased after mutation of the ESE relative to that of wild type in SK-N cells ($P < 0.05$).

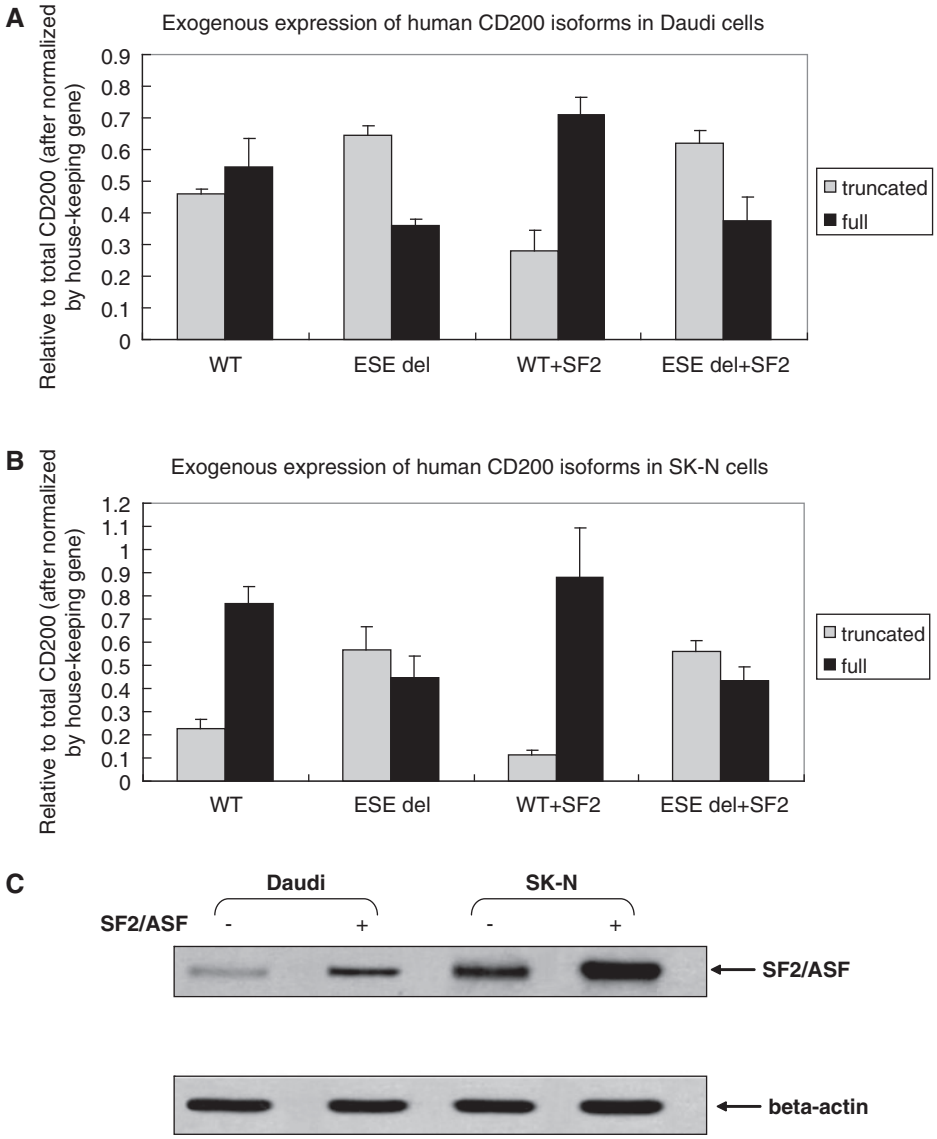

Figure 4. ESE deletion or overexpression of SF2/ASF affects exogenous expression patterns of CD200 isoforms. Ten micrograms of the wild-type minigene construct, the minigene construct with the ESE site deleted, the minigene construct plus SF2/ASF expression vector, or the minigene construct with the ESE site deleted plus SF2/ASF expression vector was transfected into Daudi or SK-N cells by electroporation. After 48 h, cells were collected and total RNA was isolated for real-time RT-PCR.The expression levels of the full-length and truncated CD200 as well as total CD200 in Daudi (**A**) and SK-N (**B**) were normalized to the housekeeping genes GAPDH and HPRT. The data shown are expression levels of full-length or truncated CD200 relative to total CD200. The data represent the mean ± SE (three independent experiments, triplicate determinations). (**C**) Overexpression of SF2/ASF. Daudi and SK-N cells were transfected with 10 μg of the minigene construct with the ESE site deleted plus SF2/ASF expression vector. Western blotting was performed using 20 μg of nuclear extracts from the cells and an antibody against SF2/ASF.

and total RNAs extracted for real-time RT-PCR, along with nuclear proteins for western blot. As shown in Figure 6A, SF2/ASF expression was eliminated after treatment with 2.5 μg of siRNA. β-Actin was used as a loading control. Real-time RT-PCR was then performed using RNA samples treated with siRNA. As shown in Figure 6B and C, the endogenous expression of full-length CD200 (exon 2 inclusion) was reduced in both Daudi and SK-N cells, compared with Mock (no siRNA) or scramble siRNA-treated cells. The same pattern was observed for the exogenous expression of CD200 in Daudi (Figure 6D or SK-N cells (Figure 6E) following silencing SF2/ASF. Consistent with the observation resulting from the ESE mutation or deletion, the expression pattern of full-length versus truncated CD200 was reversed in SK-N cells after knockdown of SF2/ASF.

To investigate further the function of SF2/ASF in exon 2 inclusion or exclusion, we performed over-expression analysis by transfection of SF2/ASF expression vector to Daudi or SK-N cells and examined the fate of exon 2. As shown in Figure 4A–C, overexpression of SF2/ASF induced exon 2 inclusion but this function was abolished in the absence of the ESE in exon 2, indicating that SF2/ASF regulates CD200 isoforms only via the ESE.

These results support the hypothesis that the splicing regulatory protein SF2/ASF, acting through binding to the ESE in exon 2 of CD200, plays an important role in controlling alternative splicing of CD200, and regulates

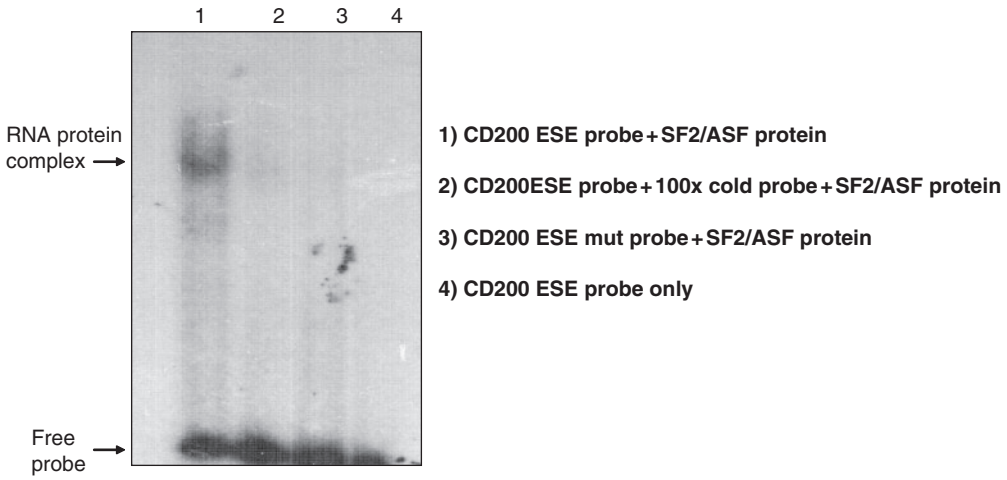

Figure 5. Direct binding of SF2/ASF to the ESE in exon 2 of CD200. A recombinant SF2/ASF (ΔRS domain) was incubated with a radiolabeled RNA oligonucleotide probe containing the ESE in exon 2 of CD200 (lane 1), with a radiolabeled RNA oligonucleotide plus 100× cold probe containing the same ESE (lane 2), or with a mutated ESE (lane 3). Lane 4 represents a radiolabeled RNA oligonucleotide only. The sequences of the RNA oligonucleotides are shown in 'Materials and methods' section. Free RNA probes and RNA–protein complexes were resolved by 5% nondenaturing polyacrylamide gel. The figure shown is representative of three independent experiments with similar results.

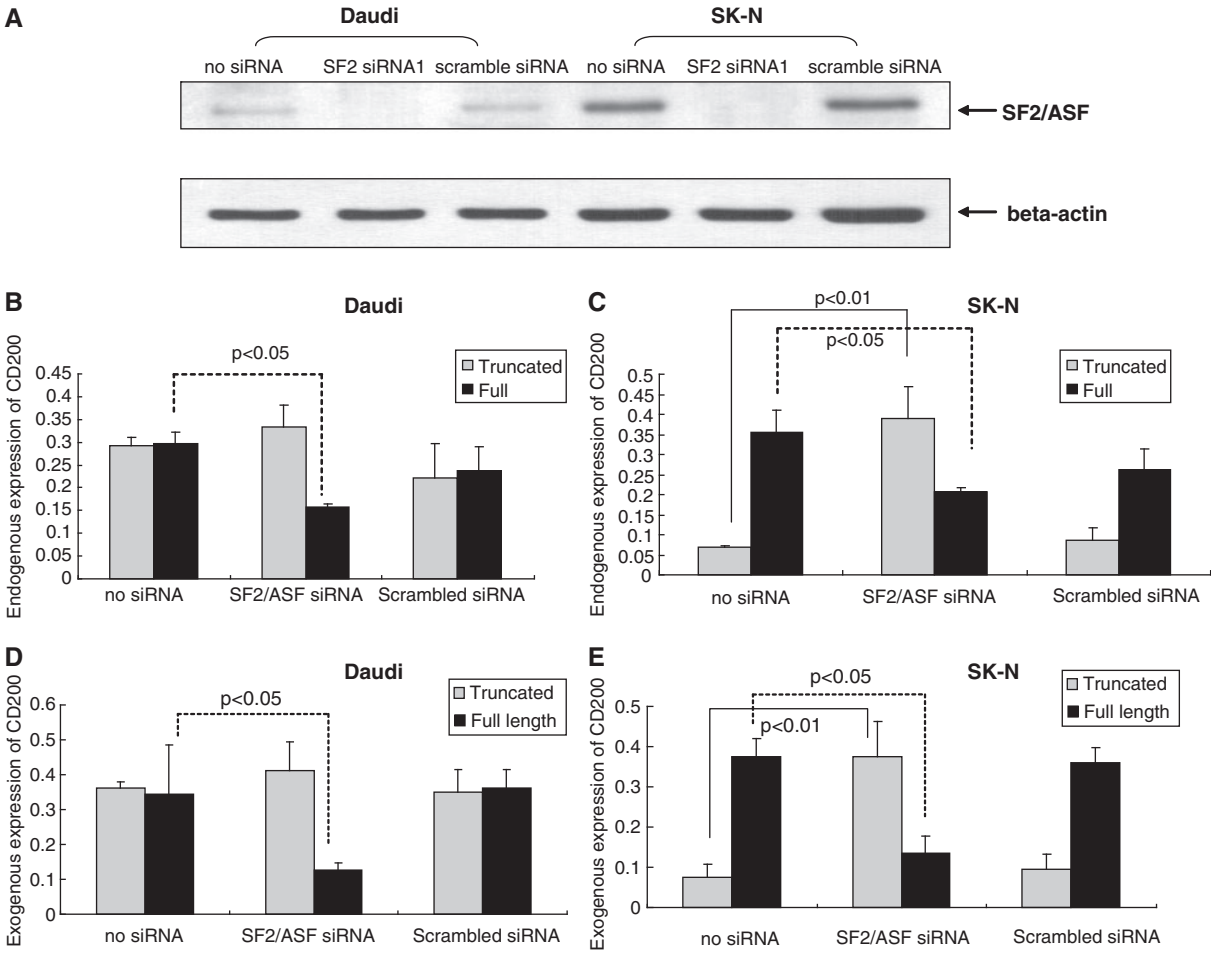

Figure 6. SF2/ASF determines exon 2 inclusion or exclusion in endogenously or exogenously expressed CD200. siRNA against SF2/ASF was used to 'knock-down' its expression. (**A**) Western blotting was employed in Daudi and SK-N cells after treated with 2.5 μg siRNA. (1,4) no siRNA; (2,5) 2.5 μg siRNA; (3,6) scramble siRNA. β-Actin was used as a loading control. Absolute quantitative real-time RT-PCR was performed in Daudi cells (**B** and **D**) and SK-N cells (**C** and **E**) before and after treated with 2.5 μg of SF2/ASF siRNA. Scramble siRNA was used as negative control. Broken lines reflect that endogenous or exogenous expression of full-length CD200 decreased after knockdown of SF2/ASF relative to the control group (no siRNA treatment) ($P < 0.05$). Continuous lines reflect endogenous or exogenous expression of truncated CD200, which are increased after knockdown of SF2/ASF relative to the control group (no siRNA treatment) ($P < 0.01$).

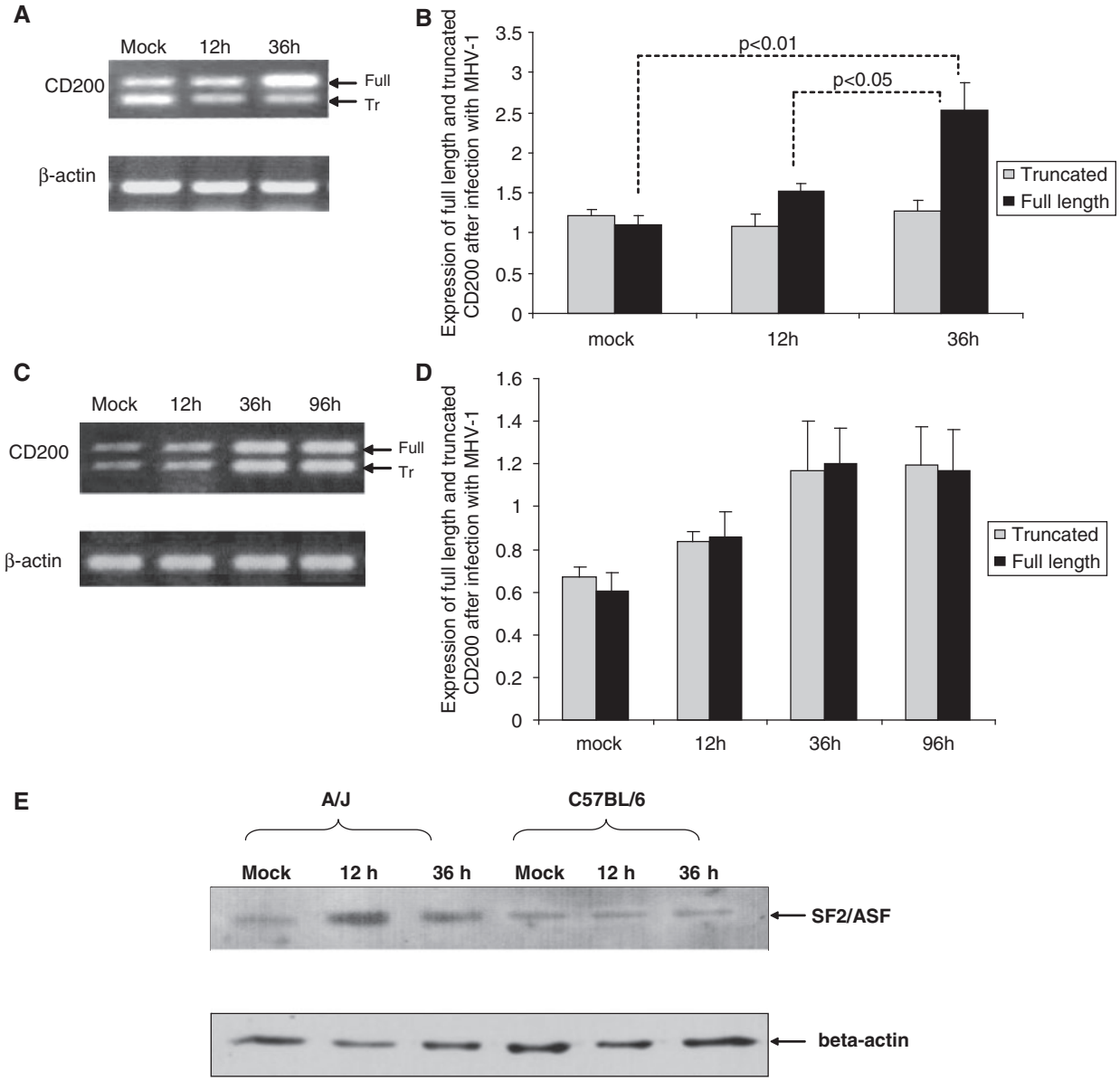

Figure 7. The ratio of full-length CD200 to truncated CD200 is altered *in vivo* in A/J mice infected with MHV-1 but not in infected C57BL/6J mice. Lung tissues from A/J or C57BL/6J mice infected with MHV-1 for 12, 36 or 96 h (C57BL/6J mice only) were collected for total RNA extraction. Regular or quantitative real-time RT-PCR was performed. (**A**) Two transcripts (full-length and truncated CD200) in A/J mice were identified by regular RT-PCR. (1) Lung of A/J mice without infection (Mock); (2) lung of A/J mice infected with MHV-1 for 12 h; (3) lung of A/J mice infected with MHV-1 for 36 h. (**B**) Absolute quantitative real-time RT-PCR for detection of full-length and truncated CD200 in lungs of A/J mice without infection or infected with MHV-1 for 12 or 36 h. (**C**) Two transcripts (full-length and truncated CD200) in C57BL/6 mice were identified by regular RT-PCR. (**D**) Absolute quantitative real-time RT-PCR for detection of full-length and truncated CD200 in lungs of C57BL/6J mice without infection or infected with MHV-1 for 12, 36 or 96 h. The data represent the mean ± S.E (three independent experiments, triplicate determinations). Broken lines reflect expression of full-length CD200 increased after MHV-1 infection for 36 h relative to the group without infection ($P < 0.01$), or the group infected with MHV-1 for 12 h ($P < 0.05$). (**E**) Lung tissues from A/J or C57BL/6J mice were collected and proteins extracted for western blotting using anti-SF2/ASF antibody (1:200). (1,4) No MHV-1 infection; (2,5) 12 h postinfection; (3,6) 36 h postinfection.

the relative ratio of expression of full length to truncated CD200.

The alternative splicing pattern is altered *in vivo* in A/J mice infected with MHV-1

Previous studies have shown that several viruses express a viral protein which mimics human CD200 and down-regulates host immunity to the virus following interaction with a human CD200 receptor on host cells (39–41). Whether viral infection itself affects the expression of CD200 in host is an issue which remains to be explored.

Intranasal infection of A/J mice with the coronavirus murine hepatitis virus strain 1 (MHV-1) has been described to induce pulmonary pathology with features reminiscent of severe acute respiratory syndrome (SARS) (33). To examine the correlation between the

viral (MHV-1) infection and expression of CD200 in host we collected lung tissues from MHV-1 susceptible A/J mice and MHV-1-resistant C57BL/6J mice after infection. RT-PCR was performed using a sense primer located in exon 1 and an antisense primer present in exon 3. Interestingly, we observed a reversal of the normal CD200 splicing pattern in lung tissues of A/J mice postinfection (Figure 7A). Real-time RT-PCR provided a more accurate result of this phenomenon. We documented that the full-length CD200 was increased after viral infection and was 2-fold higher at 36 h postinfection compared with that before infection (Figure 7B). All the susceptible A/J mice were dead at 96 h postinfection. In contrast, the relative ratio of full-length to truncated CD200 did not change in infected C57BL/6J mice (Figure 7C and D). Thus, the pattern of alternative splicing of CD200 was correlated with susceptibility of these strains to viral infection.

Since the above studies have shown that SF2/ASF regulates alternative splicing of CD200, we wondered whether expression of SF2/ASF increased in A/J mice post infection. We performed western blotting using anti-SF2/ASF antibody. As shown in Figure 7E, no obvious difference of SF2/ASF level was seen between A/J and C57BL/6J mice before viral infection. Increased expression of SF2/ASF was detected in lungs of A/J mice 12 h postinfection, whereas no increase of SF2/ASF in C57BL/6J mice even 36 h postinfection, suggesting that the role of virus on host CD200 expression is mediated by SF2/ASF.

DISCUSSION

The studies reported here show that the relative expression of two isoforms (CD200 and $CD200_{tr}$) is tissue and cell specific and the alternative slicing patterns are different between the pattern in the lymphoid tissues and that of neuronal tissues. The relative expression of the two isoforms of CD200 is of interest, given our recent evidence that the truncated form ($CD200_{tr}$) can antagonize the functional suppression induced by full-length CD200 (27). Although Borriello *et al.* (26) reported no change in the alternative splicing pattern of murine CD200 in lymphoid tissue after stimulation by Con A or LPS *in vivo*, in our *in vivo* studies of mouse lung tissues before/after infection of MHV-1 virus we observed that, unlike in the natural condition, following viral infection the expression of total CD200 increased in lung of both MHV-1 susceptible A/J mice and MHV-1-resistant C57BL/6 mice. However, the splicing pattern of CD200 is reversed only in A/J mice, with the full-length transcript, capable of inducing immunosuppresion, becoming the predominant one. In contrast, for C57BL/6J, an MHV-1-resistant mouse strain, no change in the splicing pattern of CD200 was seen in the lung. This result importantly demonstrates that only the splicing pattern, but not the total transcription level, of CD200 determines the murine immune response to MHV-1 and is consistent with the hypothesis that the shift in the balance of expression of $CD200/CD200_{tr}$ to decrease expression of the truncated product allowing CD200 to function in its immunosuppressive role, possibly contributing to the increased susceptibility to MHV-1 in the A/J mice. Further studies showed an increased expression of SF2/ASF in A/J mice postinfection and the increase in SF2/ASF occurred prior to increased full-length CD200, strongly suggesting that the regulation of alternative splicing of CD200 is mediated by SF2/ASF. It remains to be determined what viral proteins of MHV-1 have this effect and how the proteins regulate expression of SF2/ASF. Our studies suggest that viruses escape elimination by the host's immune system not only through producing viral proteins which mimic CD200 but also by inducing host CD200 expression and reducing expression of the antagonist $CD200_{tr}$. Posttranscriptional regulation, including mRNA stability, plays an important role for gene expression (42). Whether the increase of full-length CD200 in A/J mice is also due to differential mRNA stability cannot be ruled out.

In this report, we searched ESEs in the human and murine exon 2 sequence using two ESE-detecting algorithms RESCUE-ESE and ESE finder (35,43). Only one ESE, which is a putative binding site for SF2/ASF, was detected by both RESCUE-ESE and ESEfinder 2.0. No ESE was identified in the whole exon 2 when using higher stringent ESEfinder 3.0. Thus, we focused on this ESE for the rest of the experiments.

Since an ESE can promote exon inclusion, mutation or deletion of the ESE would lead to less full-length but more truncated CD200. Our results showed that after mutating the ESE in exon 2, expression of full-length CD200 was reduced in both Daudi and SK-N cells. This expression pattern is the reverse of that seen for endogenous CD200 expression in SK-N cells, in which the predominant expression is of full-length CD200. To exclude the possibility that the mutation created a new exonic splicing silencer (ESS) which led to decreased full-length, and increased truncated CD200, we deleted the ESE and examined the changes in $CD200:CD200_{tr}$. Our result showed that deletion of the ESE promoted exon 2 exclusion, the same result as we obtained from mutation analysis, indicating that mutation of the ESE does not create an ESS.

Identification of a putative ESE for SF2/ASF binding does not provide direct evidence that SF2/ASF recognizes and binds to the ESE. To examine whether the identified ESE in exon 2 is bound by SF2/ASF, we performed RNA–EMSA using RNA radiolabeled oligonucleotides bearing the ESE in exon 2 and a recombinant SF2/ASF with ΔRS domain to reduce nonspecific binding. The result showed a binding of SF2/ASF to the ESE and the binding is specific because either mutated ESE or 100× cold oligonucleotides abolished the binding.

Knockdown of SF2/ASF decreased expression of full-length CD200 in both Daudi and SK-N cells. Consistent with data seen following mutation or deletion of the ESE, the expression pattern of CD200 was again reversed in SK-N cells. The western blot performed confirmed the efficiency of knockdown of SF2/ASF. In contrast, overexpression of SF2/ASF increased expression of full-length CD200 but only in the presence of the ESE

in exon 2, highlighting the critical role of the ESE in the mechanism of alternative splicing of CD200. Ubiquitously expressed splicing factors, among them is SF2/ASF, are thought to control tissue specific alternative splicing through their different expression levels in different tissues (44). Our result showed that the natural level of SF2/ASF was higher in the neuronal cell line SK-N than in B cell line Daudi. This may help explain why endogenous full-length CD200 (exon 2 inclusion) is expressed at much higher level than that of truncated CD200 (exon 2 exclusion) in SK-N.

A recent report has described a higher expression level of SF2/ASF in many tumors, including lung, thyroid, kidney, colon, small intestine and melanoma, relative to their respective normal controls. One mechanism to explain this observation is that SF2/ASF abolished the tumor suppressor activity of BIN1, a tumor suppressor gene, by inclusion of exon 12A which interferes with MYC binding (45). In contrast to its roles in transplantation, autoimmune diseases and inflammation, CD200 enhances the growth of malignant tumors and it has been suggested that a novel approach to anticancer therapy might include blockade of CD200 (15,16,46–49). Since $CD200_{tr}$ is an antagonist to CD200 (27), our data are consistent with the hypothesis that increased $CD200_{tr}$ expression and decreased expression of full-length CD200 by blockade of SF2/ASF may also be of potential benefit for cancer treatment.

In conclusion, we have identified an alternative splicing pattern for expressed human CD200 in different cells and tissues, and compared this with the pattern observed *in vivo* following viral infection. Our data suggest that regulation of expression of alternative splicing transcripts may be important in controlling susceptibility to viral infection. An ESE in exon 2 of CD200 is a binding site for a splicing regulatory protein, SF2/ASF, which we have shown to control the alternative splicing pattern of CD200. A drug-mediated manipulation of alternative splicing has recently been reported which includes modulation of SF2/ASF (25). It would be of interest to know if this drug treatment alters the expression ratio of CD200 to $CD200_{tr}$ and thereby produces change in immune function.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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