Selective Inhibition of Class Switching to IgG and IgE by Recruitment of the HoxC4 and Oct-1 Homeodomain Proteins and Ku70/Ku86 to Newly Identified ATTT cis-Elements

András Saffer‡‡, Edmund C. Kim‡‡, Xiaoping Wu‡, Hong Zan‡, Lucia Testoni‡‡‡, Szilvia Salamon‡, Andrea Cerutti‡, and Paolo Casali‡‡‡

From the Division of Molecular Immunology, Department of Pathology and Laboratory Medicine, Joan and Sanford I. Weill Medical College, Cornell University, New York, New York 10021 and the Center for Immunology, School of Biological Sciences and College of Medicine, University of California, Irvine, California 92697

Immunoglobulin (Ig) class switching is central to the maturation of the antibody response as IgG, IgA, and IgE are endowed with more diverse biological effector functions than IgM. It is induced upon engagement of CD40 on B lymphocytes by CD40L expressed by activated CD4+ T cells and exposure of B cells to T cell-secreted cytokines including interleukin-4 and transforming growth factor-β. It begins with germ line IΔCH transcription and unfolds through class switch DNA recombination (CSR). We show here that the HoxC4 and Oct-1 homeodomain proteins together with the Ku70/Ku86 heterodimer bind as a complex to newly identified switch (S) regulatory ATTT elements (SREs) in the Iγ and Iε promoters and downstream regions to dampen basal germ line IγCγ and IεCe transcriptions and repress CSR to Cγ and Cε. This mechanism is inactive in the Ca1/Ca2 loci because of the lack of SREs in the Iα1/Iα2 promoters. Accordingly, in resting human IgM+IgD+ B cells, HoxC4, Oct-1, and Ku70/Ku86 can be readily identified as bound to the Iγ and Iε promoters but not the Iα1/Iα2 promoters. CD40 signaling dissociates the HoxC4-Oct-1-Ku complex from the Iγ and Iε promoter SREs, thereby relieving the IγCγ transcriptional repression and allowing CSR to unfold. Dissociation of HoxC4-Oct-1-Ku from DNA is hampered by CD153 engagement, a CD40-signaling inhibitor. Thus, these findings outline a HoxC4-Oct-1-Ku-dependent mechanism of selective regulation of class switching to IgG and IgE and further suggest distinct co-evolution and shared CSR activation pathways in the Cγ and Cε as opposed to the Ca1/Ca2 loci.

In class switching, the Ig constant H chain μ (Cμ) region is substituted with Cγ, Cα, or Cε. Class-switched Igs are a critical feature of the high affinity late and memory antibody responses, because IgG, IgA, and IgE carry out those effector functions including binding to receptors for the Fc portion of γ, α, and ε chains on phagocytic and proinflammatory cells and passage through “mucosae” that are required for the eradication of microbial pathogens. In general, Ig class switching occurs in germinal center (GC) B cells upon stimulation by CD40L and cytokines expressed by activated CD4+ T cells. It is initiated by transcription of the intervening (I) switch (S), and C regions of the upstream (donor) and downstream (acceptor) Cγ1 loci (1–3). Splicing of the nascent IγS-Cγ1 RNA yields germ line IγCγ transcripts and probably couples transcription with the cleavage step of CSR, possibly through the action of activation-induced cytidine deaminase (AID) (4). CSR is effected through double-strand DNA breaks of the donor and acceptor S regions, excision of intervening DNA as a switch circle (SC) (2), and non-homologous end-joining (NHEJ) of the DNA ends (1–3) coupled with mismatch repair (5).

Germ line IγCγ transcript is driven by the Iγ promoter lying upstream of each Iγ region (6–8) upon CD40L-, IL-4-, and/or transforming growth factor-β-induced binding of the NF-κB/Rel, Stat6, Smad, activating protein-1, or B cell lineage-specific activator protein transcription factors to Iγ promoter-specific cis-elements (1, 6, 8–13). In CD40-induced (GC) B cells, germ line IγCγ transcription and CSR can be effectively down-regulated by bidirectional CD30:CD153-dependent signaling, which interferes with the recruitment of tumor necrosis factor receptor-associated factor molecules to CD40 and inhibits NF-κB activation (14, 15). However, the regulation of germ line IγCγ transcription and CSR in general and in non-CD40-induced (pre-GC) B cells in particular remains to be defined.

We report here a mechanism of selective inhibition of class switching to IgG and IgE. This mechanism relies on the binding of HoxC4, Oct-1, and Ku70/Ku86 to newly identified SREs...

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that exists in the 1y and 1e but not in the 1x/1o2 promoters. Such a binding dampens basal germ line 1y-Cy and 1e-Ce transcription and represses CSR to Cy and Ce. It is potentiated by CD153 signaling and reversed by CD40 signaling. Thus, by selectively binding to the 1y and 1e promoters, HoxC4, Oct-1, and Ku70/Ku86 differentially regulate class switching to IgG, IgE, and IgA and would minimize S region double-stranded DNA breaks, thereby contributing to the stability of the Ig H chain locus.

EXPERIMENTAL PROCEDURES

Human Cγ, Cα, and Cε Loci Sequence Analysis—S repeats and ATT motifs were identified within the 1p promoters, S regions, and flanking areas using the Pustell dot-matrix comparison (16) and nucleotide sequence search (MacVector, Inc., Cary, NC). B Cell Preparation and Culture—The human monoclonal 1G11 IgM-IgD B cell line was reported previously (8, 15, 17–21). In the absence of stimulation, these cells maintain their IgM IgD phenotype in culture but switch to all of the downstream isotypes upon exposure to CD40L and IL-4. In addition, they hypermutate the expressed Ig VH DH genes and BCL6 when co-cultured with activated CD4+ T cells upon B cell receptor cross-linking (18–21). 7D7 and 4B6 are subclones of IgM-IgD CL-01 cells that were selected for spontaneous and ongoing activation using IgA, IgG, and IgE. Human peripheral blood CD19+ B cells and tonsil B cell subsets were separated as reported previously (15). 7D7 and 4B6 are subclones of the respective human 5'-S regions (a gift from Dr. E. Max, Food and Drug Administration, Bethesda, MD) (23) and cloned into pGL3 vector (Promega, Madison, WI). Mutations including the 5/-3/, or both 5/-3/ promoter ATT motifs to 5'/3' were introduced by PCR-based mutagenesis. The Ku70mutHIM lacking the homeodomain (HD) interaction motif (Ku70mutHIM) was used to observe the binding of HoxC and Oct-1, 7k7, and Ku86 cDNAs were cloned into the pGEX-6P1 vector (Amersham Biosciences) to generate the respective GST fusion proteins. The pBSKII plasmid (Stratagene Corp., La Jolla, CA) was used to determine the binding of HoxC4, Oct-1, Ku70, and Ku86 to circular DNA containing the ATT motif.

Antibodies—Anti-Ku70 (Ab-5), anti-Ku86 (Ab-2), and anti-Ku70/Ku86 (Ab-3) mAbs were from Lab Vision/NeoMarkers (Fremont, CA). Anti-Oct-1 (YL15) mAb was from Upstate Biotech (Waltham, MA); rabbit anti-Oct-1 Ab (sc-232) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-HoxC4 and anti-HoxC8 mAbs were from CRP Inc. (Berkeley, CA). Goat anti-Oct-2 (C-20) Ab was from Santa Cruz Biotechnology, Inc. Controls were MOPC-21 mouse IgG mAb (Sigma), rabbit, and goat polyclonal IgGs (Santa Cruz Biotechnology, Inc.).

Gene Reporter Assay—CL-01 B cells were transfected with firefly luciferase gene reporter pGL3 vector (10 μg) and Renilla luciferase gene control pRL-CMV vector (10 ng) (Promega). 7D7 and 4B6 B cells were transfected with the same reporter and control vectors together with expression vector(s) (2 μg each). The DNA was precipitated previously (8) and transfected into 60 μm 24 microtiter plate luminometer (Thermo Labystems, Chantilly, VA). mRNA, cDNA, and RT-PCR—mRNA isolation, first strand cDNA synthesis, and RT-PCR were performed as described previously (8). PCR conditions and cycle numbers were as follows: denaturation for 1 min at 94 °C, annealing for 1 min at 68 °C, and extension for 1 min at 72 °C. Before each RT-PCR, cDNAs were denatured for 5 min at 94 °C.

Electrophoretic Mobility Shift Assays (EMSA) and Protein Purification—Cytoplasmic and nuclear protein extraction, probe labeling, EMSA, and supershift reactions were performed as reported previously (8). γ-32P]ATP-labeled and cold 1p SRE double-stranded oligonucleotides encompassed the sequences depicted in Fig. 1B. The sequences of the S3y and Sre SRE oligonucleotides were 5/-CAGCGCGCAGACCA-GATAGGCGG-3/ and 5/-GGGTTGGGTTAGTAAAATGAGT-3/, respectively, encompassing the ATT motif immediately 5/- of the S3y region and the first ATT motif of the Sre region. The sequence of the Sp1 oligonucleotide was 5/-ATTCGACGGCCGCGGCGGAGC-3/.

The SRE binding activity of CL-01 nuclear extracts (75 mg) was first enriched by 20% (NH4)2SO4 precipitation, dialyzed against DNA binding buffer (DBB) (10 mM Tris-HCl, pH 7.6, 200 mM KCl, 1 mM EDTA, 1 mM diithiothreitol, 10% glycerol), and then fractionated on a Centricon concentrator using a 100-kDa molecular mass cut-off membrane (Millipore Corp., Bedford, MA). The >100-kDa fraction was applied to a Superose 6 gel filtration column (Amersham Biosciences), which was eluted at a 100 μl/min rate with DBB containing 20% glycerol. Fractions (500 μl) were collected and tested for SRE binding activity by EMSA. The SRE binding fractions were pooled and loaded onto a DNA column consisting of an agarose matrix bearing streptavidin (Pierce) and loaded with 5'-biotinylated pentamerized double-stranded 5/-SRE oligonucleotides (Qiagen Sciences, Valencia, CA). The column was washed with 200 mM KCl DBB containing 20% glycerol. The DNA-bound proteins were eluted using a 300–800 mM KCl gradient in DBB 20% glycerol and collected in 500-μl fractions, which were monitored for SRE binding activity and protein content. Proteins were visualized in SDS-PAGE using the Rapid Silver Stain Plus kit (Bio-Rad). The fractions eluted at 300 mM KCl contained the strongest SRE binding activity and were pooled, concentrated on Centricon filters, and applied to SDS-PAGE. The resolved protein bands were stained by Comassie Brilliant Blue G-250 (Bio-Rad), excised, and subjected to in-gel proteolysis by trypsin. Peptides mixtures were separated and identified on a C8 reverse-phase column with a linear 0–60% CH3CN gradient in 0.1% trifluoroacetic acid for 1 h. The molecular mass of the peptide mixtures were determined by MALDI-TOF mass spectrometry using a Voyager-DE short tandem repeat (PerSeptive Biosystems, Inc., Framingham, MA). The complete list of accurately measured masses of the trypptic peptides was used to search for protein candidates in the OWL protein sequence data base with the program ProFound (prowl.rockefeller.edu/cgi-bin/ProFound). Internal sequencing of trypptic peptides was performed as described previously (25).

Detection of Reciprocal Recombination SCs—Genomic DNA was extracted from B cells using the QIAmp DNA mini kit (Qiagen). Specific Sγ-Sμ, Sε-Sμ, and Sμ-Sε reciprocal SCs were amplified from genomic DNA (500 ng) using nested PCRs and Sμ, Sε (y1–4), and Sμ (a1/a2) primers (17). Specific primers (17) were used to detect the presence of the reciprocal peptide mixtures were determined by MALDI-TOF mass spectrometry using a Voyager-DE short tandem repeat (PerSeptive Biosystems, Inc., Framingham, MA). The complete list of accurately measured masses of the trypptic peptides was used to search for protein candidates in the OWL protein sequence data base with the program ProFound (prowl.rockefeller.edu/cgi-bin/ProFound). Internal sequencing of trypptic peptides was performed as described previously (25).

Phosphatase Treatment of Nuclear Extracts—Extracts prepared from freshly isolated human IgM+ B cells (9, 10) were incubated with 5 and 20 milligrams of acid phosphatase (Grade I, Roche Applied Sciences) in DBB in a final volume of 30 μl at 25 °C for 10 min. A 10-μl aliquot of the phosphatase-treated sample was then tested in each binding assay.

GST Proteins and Pull-down Assays—GST fusion proteins were expressed, purified using GSHagarose beads according to the manufacturer’s protocol (Amersham Biosciences), and affinity purified using SDS-PAGE and silver staining. 1-35S]Methionine-labeled proteins were translated using the TNT Quick coupled transcription/translation system (Promega) method. For pull-down experiments, 5 μl of in vitro translated protein was mixed with 50 μg of nuclear extract and applied
ATTT SREs are found in the human Iγ and Iε promoters and related Iγ-S regions but not in Iα1/Iα2 or Iα1/Iα2-Sα1/Sα2. A schematic depiction of the human Iγ1-Cγ1, Iγ2-Cγ2, Iγ3-Cγ3, Iγ4-Cγ4, Iε-Cε, and Iα1/Sα1/Iε DNA sequences. Yellow boxes denote promoters encompassing evolutionarily conserved sequence and flanking areas (8, 48–51). Red boxes denote S regions. Residue 1 in the γ sequences is as described by Mills et al. (23) and in the ε and α sequences is according to GenBank™ references below. Major initiation of transcription sites depicted by turned arrows is as follows: γ1, 310; γ2, 466; γ3, 449; γ4, 544; ε, 592; and α1, 748. 5’-SRE (ATTT) and IL-4 RE-Stat6 (TTCNNNNGAA) encompassed the following residue numbers: Iγ1, 360–363 and 362–371; Iγ2, 350–353 and 352–361; Iγ3, 362–365 and 364–373; Iγ4, 360–363 and 362–371; and ε, 502–505 and 438–447, respectively. The gray box within the Iα1/Sα1/Sγ2 region denotes an unidentified area of ~200 bp as deduced from the equivalent mouse genomic DNA. Compilation is based on the GenBank™ sequences with accession numbers as follows: AL122127 (γ1 and γ3), U39934 (γ2), HSG481A (γ4), X56797 and J00222 (ε), L04540 and L19121 (α1), and L04541 (α2). B, SRE and flanking areas within the Iγ1, Iγ2, Iγ3, Iγ4, and Iε promoters. Upper cluster, 5’-Iγ1 promoter SRE; lower cluster, 5’-Iγ2 promoter SRE (residue numbers are indicated). Gaps (-) are used to align ATTT stretches. SRE and IL-4 RE-Stat6 are boxed. Mutations introduced in the different Iγ and Iε promoter-driven luciferase gene reporter pGL3 vectors are indicated by small italic letters. The position of residues identical in both WT and mutSRE sequences are underscored.

ATTT SREs critically regulate Iγ and Iε promoter activity. CL-01 B cells were transfected with WT (wtSRE) or double mutSRE Iγ or Iε promoter-driven luciferase gene reporter pGL3 vectors and then cultured with or without htcCD40L for 24 h in the presence or absence of agonistic anti-CD153 mAb M81. Luciferase activity was measured and expressed as relative light units. Values are means ± 1 S.D. of three experiments.
deoxycholate, 0.5% Sarkosyl, pH 8.0), re-suspended in immunoprecipitation buffer (20 mM Tris-HCl, 200 mM NaCl, 2 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitors), and sonicated to yield 500–1000bp DNA fragments. These were precleared with agarose beads bearing protein A or G (Santa Cruz Biotechnology, Inc.) and then incubated with mAb to HoxC4, Ab to Oct-1, or mAb to Ku70/Ku86 overnight at 4 °C. The immune complexes were isolated using beads bearing protein A or G (Santa Cruz Biotechnology, Inc.) and then incubated with mAb to HoxC4, Ab to Oct-1, or mAb to Ku70/Ku86 overnight at 4 °C. The immune complexes were isolated using beads bearing protein A or G, eluted with “elution buffer” (50 mM Tris-HCl, 0.5% SDS, 200 mM NaCl, 100 μg/ml proteinase K, pH 8.0), and then heated at 65 °C overnight to reverse cross-links. DNA was recovered by phenol extraction and ethanol precipitation and then solubilized in Tris-EDTA buffer. The recovered DNA was specified by cloning and sequencing of the PCR product amplifying the yeast promoter forward (281–300, 5’-TGGGCGGCCAGTTCAATC-3’) and reverse (444–424, 5’-GTCTACGCCCTTCTGTGTG-3’) primers, the Ie promoter forward (441–461, 5’-CCAAGACAGAGAAGGAGG-3’) and reverse (615–598, 5’-ATCAGGTGGGAGAGGTGATC-3’) primers, or the Ia promoter forward (127–193, 5’-ACAGGTTAAGAGACCGTCTCCG-3’) and reverse (353–332, 5’-ATCAGGTGGGAGAGGTGATC-3’) primers.

RESULTS

ATTT SREs Critically Modulate Iγ and Ie but Not Iα Promoter Activity—We have suggested the existence of a repression of germ line Iγ3-Cγ3 transcription in resting human IgM(IgD+ B cells (8) and tentatively ascribed it to Iγ3 promoter tetramer ATTT SREs. In the Iy1, Iy2, Iγ3, and Iy4 promoters, such SREs exist in two identical copies, one straddling the 5′ boundary of the IL-4-responsive element Stat6-binding site (IL-4 RE-Stat6) and the other straddling the 3′ boundary of the IL-4 RE-Stat6. Two SREs were also identified in the Ie promoter, both of them 3′ of the IL-4 RE-Stat6 (Fig. 2). Additional ATTT motifs exist in the downstream Iγ3-S regions and flanking areas: 3, 3, 2, 3, and 13 in the γ1, γ2, γ3, γ4, and ε loci, respectively. No SRE was found in the Iα/Ion2 promoters or Iα-S regions.

Transfection of human IgM(IgD+ CL-01 B cells, our model of inducible CSR and somatic hypermutation (8, 17, 19), with Iγ or Ie promoter-driven luciferase gene reporter pGL3 vectors containing WT or mutated (ATTT to gTT) mutSRE(s) was performed to analyze the role of these SREs in Iγ promoter activity (Fig. 3). Mutation of both the 5′- and 3′-SREs (double mutSRE) in the Iγ and Ie promoters resulted in a 10- (Iγ) to 15-fold (Ie) increase of basal reporter gene transcription and a 4- (Iγ) to 6-fold (Ie) increase of human trimeric (ht) CD40L-induced transcription (Fig. 3). This possibly reflects the suboptimal induction of transfected CL-01 cells by soluble CD40L as underscored by our previous findings that similar culture conditions induce switching in approximately one-third of CL-01 cells (17). The double mutSRE also reverted the ability of CD153 signaling to reduce the basal (no reduction in double mutSRE versus a 25–40% reduction in WT SRE) and htCD40L-induced Iγ and Ie promoter-driven reporter gene transcription (no reduction in double mutSRE versus a 79–84% reduction in WT SRE). Mutation of the 5′-SRE alone resulted in enhancement of basal and htCD40L-induced Iγ or Ie promoter-driven
transcription that was 28–56% lower than that of the double mutSRE (data of supplemental Fig. 2A versus those of Fig. 2). Finally, the absence of the ATTT SRE in Iα1/Iα2 was associated with a significantly higher basal activity of this promoter as compared with the I1, Iγ2, Iγ3, Iγ4, and Iε promoters (supplemental Fig. 2B). Thus, the ATTT SREs critically mediated basal and CD153-induced inhibition of Iγ and Iε promoter-driven transcription.

Iγ and Iε SREs Recruit HoxC4, Oct-1, and Ku70/Ku86—EMSA were performed using radiolabeled oligonucleotides encompassing the Iγ3 or Iε 5′-SRE, the SRE immediately 5′ of the Sy3 region (Sy3 SRE) or the most 5′-SRE in the Se region (Se SRE), and nuclear extracts from freshly isolated human peripheral blood IgM+IgD+ B cells to characterize the trans-factors specifically binding to the identified Iγ and Iε SREs. Two specific and closely migrating SRE-protein complexes (complexes A and B) were identified by all of the four probes (Fig. 3A). The formation of such complexes was inhibited by cold WT but not mutSRE (ATTT to ggTT) oligonucleotides containing the Iγ1, Iγ2, Iγ3, Iγ4, Iε, Sy3, or Se 5′- or 3′-SRE (data not shown). To analyze the composition of complexes A and B, nuclear extracts from CL-01 IgM+IgD+ B cells were subjected to sequential (NH₄)₂SO₄ precipitation, gel filtration, and SRE DNA affinity chromatography (DAC), which eventually yielded proteins of 100, 89, 72, and 34-kDa apparent molecular masses (Fig. 3, B-C). These proteins were identified as Oct-1, Ku86, Ku70, and HoxC4 by in gel trypsin digestion, peptide mass fingerprinting, internal sequencing, and specific mAbs in eluates from a DAC column bearing SRE Iγ3 oligonucleotides (Fig. 3D, left panel). Additional proteins of 190, 144, and 120-kDa apparent molecular masses were also detected by silver staining, but they accounted for bands of minor intensity and were not sequenced (Fig. 3C). The binding of HoxC4, Oct-1, and Ku70/Ku86 to DNA was mediated specifically by the SRE and was not attributed to “stickiness” of these proteins for free DNA ends as shown by the following: (i) the failure of HoxC4, Oct-1, and Ku70/Ku86 to bind to a mutSRE DAC column (Fig. 3D, left panel); (ii) the efficient pull-down of HoxC4, Oct-1, and Ku70/Ku86 by beads bearing circular pBSKII plasmid DNA containing 29 copies of the ATTT motif; and (iii) the inhibition of this pull-down by 100-fold molar excess of WT but not mutSRE oligonucleotides (Fig. 3D, right panel). Thus, whether in the Iγ1 promoter or S region context, the ATTT SREs recruit the HoxC4 and Oct-1 HD proteins together with the Ku70/Ku86 heterodimer in a sequence-specific and DNA end-independent fashion.

HoxC4, Oct-1, and Ku70/Ku86 Form a DNA-binding Complex in B Cell Nuclei—The nature of the SRE-binding proteins was further verified by EMAS utilizing nuclear extracts from freshly isolated IgM+IgD+ B cells, radiolabeled Iγ3 and Iε 5′-SRE as well as Sy3 and Se SRE probes, and specific anti-HoxC4, anti-Oct-1, anti-Ku70, and Ku70/Ku86 mAbs. Anti-Ku70, anti-Ku86, and anti-Ku70/Ku86 mAbs but not control HoxC8- or Oct-2-specific Abs-supershifted complex A, whereas anti-HoxC4 and anti-Oct-1 mAbs inhibited the formation of complex B (Fig. 4A). Comparable results were obtained in EMAS utilizing Iγ3 and Iε 3′-SRE probes as well as Iγ2, Iγ3, or Iγ4 5′- or 3′-SRE probes (data not shown). Pull-down experiments using GST-HoxC4, GST-Oct-1, and GST-Ku70 fusion proteins, GSH-agarse beads, and in vitro translated 35S-labeled HoxC4 and Oct-1 proteins premixed with freshly isolated IgM+IgD+ B cell nuclear extracts showed that HoxC4, Oct-1, and Ku effectively interact with one another in B cell nuclei. Both 35S-labeled HoxC4 and 35S-labeled Oct-1 bound to GST-HoxC4, GST-Oct-1, and GST-Ku70, indicating a significant self-association among HoxC4 and Oct-1 proteins as well...
as direct physical interaction between HoxC4 and Oct-1, HoxC4 and Ku70, and Oct-1 and Ku70 (neither 35S-labeled HoxC4 nor 35S-labeled Oct-1 bound to GST alone) (Fig. 4B). Consistent with the critical role of the C-terminal HIM (24) in Ku70 binding, GST-Ku70wtHIM, a GST fusion protein encoding Ku70 lacking its HIM, reacted with neither 35S-labeled HoxC4 nor 35S-labeled Oct-1. Also, a mAb that specifically recognizes the Ku70/Ku86 heterodimer interface (26) co-precipitated HoxC4 and Oct-1 from CL-01 nuclear extracts, and an anti-Oct-1 mAb co-precipitated HoxC4, Ku70, and Ku86 (Fig. 4C), indicating that Ku interacts with these HD proteins in B cell nuclei. Finally, ChIP assays in which the Iγ and Iε promoters were sequenced in the DNA that had been precipitated from freshly isolated IgM* IgD+ B cells by anti-Oct-1 Ab, anti-HoxC4 mAb, or anti-Ku mAb demonstrated direct binding of HoxC4-Oct-1-Ku to the Iγ and Iε but not Iα promoters (Fig. 4D). Thus, HoxC4, Oct-1, and Ku70/Ku86 can exist as discrete components of a HD-dependent nuclear complex and specifically bind to the Iγ and Iε promoters and S region DNA in vitro and in vivo.

CD40 and IL-4 Signaling Dissociates HoxC4, Oct-1, and Ku70/Ku86 from SRE in a Dephosphorylation-dependent Manner.—If binding of HoxC4-Oct-1-Ku to the Iγ promoter SREs is responsible for the basal repression of germ line IgM* IgD+ transcription, then hCD40L-induced germ line IgM* IgD+ transcription and subsequent CSR should entail the dissociation of the HoxC4-Oct-1-Ku complex from SREs, and this dissociation should be prevented by physiological CD40-signaling inhibitors such as CD153 (15) EMSAs using Iγ and Iε promoter 5′-SRE as well as 5′-Syγ and Se SRE probes showed that freshly isolated IgM* IgD+ B cells cultured for 2 days with either hCD40L alone or hCD40L and IL-4 but not an agonistic anti-CD153 mAb or IL-4 alone decreased the level of SRE-bound HoxC4-Oct-1-Ku complexes A and B by >95% (Fig. 5A). Comparable results were obtained utilizing Iγ3 and Iε3 3′-SRE probes as well as Iγ1 and Iε1 3′- and 5′-SRE probes (data not shown). The hCD40L-induced dissociation of HoxC4-Oct-1-Ku from SREs was efficiently inhibited by cycloheximide, a protein synthesis inhibitor, or CD153 cross-linking, which has been shown to dampen germ line IgM* IgD+ transcription and repress CSR (15, 27). It was concomitant with increase of nuclear HoxC4, Oct-1, and Ku70/Ku86 proteins (Fig. 5B), suggesting a posttranslational modification in the hCD40L-induced dissociation of HoxC4-Oct-1-Ku from SRE. Incubation of nuclear extracts from freshly isolated IgM* IgD+ B cells with increasing amounts of acid phosphatase prior to the addition of the SRE probes and separation on native gel resulted in decreased SRE-binding by HoxC4-Oct-1-Ku (Fig. 5C). This was prevented by sodium phosphate, an inhibitor of acid phosphatase. Accordingly, pretreatment of B cells with okadaic acid, a Ser/Thr phosphatase inhibitor, efficiently abrogated the CD40-induced dissociation of HoxC4-Oct-1-Ku from the SRE (Fig. 5D).

To prove that in vivo activation of germ line IgM* IgD+ tran-
IgD-sorted human tonsil B lymphocytes into four fractions representing sequential stages of differentiation as follows: mature VHDJH-C\(_{\text{H}}\)-scrambled B cells/centrocytes, and is extinct in memory B cells, whereas pre-GC B cells, appears in early centroblasts, peaks in centroblasts/centrocytes, which harbored germ line IgD\(-C\gamma\) and IgD\(-C\varepsilon\) transcripts as well as—consistent, because no inhibition could be measured when overexpression of both HoxC4 and Oct-1 or Ku70/Ku86. After culture, B cells with high GFP expression were sorted germ line IgD\(-C\gamma\) and IgD\(-C\varepsilon\) expression and CSR to C\(\gamma\) and C\(\varepsilon\) but not germ line IgD\(-C\alpha\) transcription and CSR to C\(\alpha\). A 7D7 B cells were transiently co-transfected with pIRE2 expression constructs encoding the indicated proteins as well as with I\(\gamma\)3, I\(\varepsilon\), or I\(\alpha1/\alpha2\) promoter-driven luciferase (luc) gene reporter pGL3 vectors. Luciferase activity was measured after 24 h, normalized, and expressed as the percentage of the activity measured in cells transfected with empty pIRE2 vector. Comparable results were obtained in experiments involving I\(\gamma\)3, I\(\varepsilon\), or I\(\alpha1/\alpha2\) promoter-driven reporter vectors and 4B6 B cells (data not shown). B 7D7 and 4B6 B cells were transfected with empty pIRE2 vectors or with pIRE2 expression vectors encoding the indicated proteins. Transfected cells were cultured in complete medium for 4 days. Sorted GFP\({}^{\text{high}}\) cells were analyzed for expression of germ line I\(\gamma3\)-Ch3, I\(\varepsilon\)-Ch4, and I\(\alpha1/\alpha2\)-Caslo2 promoters; mature V\(\gamma\)3D\(\gamma\)3-C\(\gamma\), V\(\gamma\)3D\(\gamma\)3-C\(\varepsilon\), and V\(\gamma\)3D\(\gamma\)3-Caslo2 promoters; and AID and 3-actin transcripts (left panels) as well as Sx-Sx, Sx-Sv, Sx-Sv, and Sx-Sv SCs (right panels). Sx-Sv, Sx-Sv SCs represent SCs from all of the four \(\gamma\) isotypes; Sx-Sx SCs include SCs from both \(\alpha1\) and \(\alpha2\) isotypes.

**Fig. 7** Overexpression of HoxC4, Oct-1, and Ku70/Ku86 dampens germ line IgD\(-C\gamma\) and IgD\(-C\varepsilon\) transcription and represses CSR to C\(\gamma\) and C\(\varepsilon\). A. 7D7 B cells were transiently co-transfected with pIRE2 expression constructs encoding the indicated proteins as well as with I\(\gamma\)3, I\(\varepsilon\), or I\(\alpha1/\alpha2\) promoter-driven luciferase (luc) gene reporter pGL3 vectors. Luciferase activity was measured after 24 h, normalized, and expressed as the percentage of the activity measured in cells transfected with empty pIRE2 vector. Comparable results were obtained in experiments involving I\(\gamma\)3, I\(\varepsilon\), or I\(\alpha1/\alpha2\) promoter-driven reporter vectors and 4B6 B cells (data not shown). B 7D7 and 4B6 B cells were transfected with empty pIRE2 vectors or with pIRE2 expression vectors encoding the indicated proteins. Transfected cells were cultured in complete medium for 4 days. Sorted GFP\({}^{\text{high}}\) cells were analyzed for expression of germ line I\(\gamma3\)-Ch3, I\(\varepsilon\)-Ch4, and I\(\alpha1/\alpha2\)-Caslo2 promoters; mature V\(\gamma\)3D\(\gamma\)3-C\(\gamma\), V\(\gamma\)3D\(\gamma\)3-C\(\varepsilon\), and V\(\gamma\)3D\(\gamma\)3-Caslo2 promoters; and AID and 3-actin transcripts (left panels) as well as Sx-Sx, Sx-Sv, Sx-Sv, and Sx-Sv SCs (right panels). Sx-Sv, Sx-Sv SCs represent SCs from all of the four \(\gamma\) isotypes; Sx-Sx SCs include SCs from both \(\alpha1\) and \(\alpha2\) isotypes.

**Overexpression of HoxC4, Oct-1, and Ku70/Ku86 Represses CSR to C\(\gamma\) and C\(\varepsilon\) but Not C\(\alpha\).** To prove that HoxC4, Oct-1, and Ku70/Ku86 critically repress germ line I\(\gamma3\)-Ch3 transcription as well as CSR in the C\(\gamma\) and C\(\varepsilon\) loci, we co-transfected 7D7 and 4B6 IgM\+ IgD\+ B cells, both CL-01 cell subclones selected for spontaneous switching to IgG, IgA, and IgE, with a pIRE2 expression vector containing nil or cDNA encoding HoxC4, Oct-1, Ku70, Ku70mutHIM, and/or Ku86 together with the I\(\gamma3\), I\(\varepsilon\), or I\(\alpha1/\alpha2\) promoter-driven luciferase gene reporter pGL3 vector. Overexpression of HoxC4 or Oct-1 alone reduced only moderately the activity of co-transfected I\(\gamma3\) or I\(\varepsilon\) promoters, whereas overexpression of both HoxC4 and Oct-1 or Ku70/ Ku86 reduced the I\(\gamma3\) or I\(\varepsilon\) promoter activity by up to 85% but had no effect on basal I\(\alpha1/\alpha2\) promoter activity (Fig. 7A). This was specific, because no inhibition could be measured when double mutSRE I\(\gamma3\) and I\(\varepsilon\) promoters were used (supplemental Fig. 7A). The C-terminal HIM was critically required, because overexpression of Ku70mutHIM and Ku86 or Ku70mutHIM alone failed to inhibit I\(\gamma3\) and I\(\varepsilon\) promoter-driven gene reporter transcription and ablated the HoxC4- or Oct-1-mediated inhibition of I\(\gamma3\) and I\(\varepsilon\) promoter activity.

The inhibition of I\(\gamma\)3 and I\(\varepsilon\) promoter-driven transcription by HoxC4, Oct-1, and Ku70/Ku86 reflects the ability of these trans-factors to effectively dampen endogenous germ line I\(\gamma3\)-Ch3 transcription and repress CSR to C\(\gamma\) and C\(\varepsilon\). 7D7 and 4B6 B cells were transfected with a bicistronic pIRE2 expression vector encoding GFP and HoxC4, Oct-1, Ku70, Ku70mutHIM, and/or Ku86. After culture, B cells with high GFP expression were sorted and used as a source of mRNA and genomic DNA for the analysis of germ line I\(\gamma3\)-Ch3, mature V\(\gamma\)3D\(\gamma\)3-Ch3 (detected as FR3-Ch3 sequences) and AID transcripts as well as Sx-Ss, Sx-Sv SCs. Overexpression of HoxC4 and/or Oct-1 or Ku70/Ku86 repressed endogenous I\(\gamma3\)-
Cy3 and Ie-Ce transcripts, direct Sμ → Sγ, Sμ → Se, and sequential Sγ → Se CSR as indicated by the low levels of Sγ-Sμ, Se-Sμ, and Se-Sy SCs, and mature V\textsubscript{H}DJ\textsubscript{H}-Cy3 and V\textsubscript{H}DJ\textsubscript{H}-Ce transcripts. It did not affect germ line I\textsubscript{α}1/1/I\textsubscript{α}2-Cα1/Cα2, AID, and β-actin transcripts or CSR to CaI/Ca2 as indicated by the normal level of Se-Sμ SCs and mature V\textsubscript{H}DJ\textsubscript{H}-Cy3 and V\textsubscript{H}DJ\textsubscript{H}-Ce transcripts, although in some experiments, a more profound repression was observed (data not shown). Overexpression of HoxC4 or Oct-1 alone partially lowered the levels of germ line I\textsubscript{α}3-Cy3 and Ie-Ce transcripts as well as mature V\textsubscript{H}DJ\textsubscript{H}-Cy3 and V\textsubscript{H}DJ\textsubscript{H}-Ce transcripts, although in some experiments, a more profound repression was observed (data not shown). Consistent with the failure to repress basal I\textsubscript{α}3 and Ie promoter-driven reporter gene transcription, overexpression of Ku70\textsubscript{mut}HM/Ku86 failed to affect the level of endogenous I\textsubscript{α}3-Cy3 and Ie-Ce transcripts. It also resulted in higher levels of mature V\textsubscript{H}DJ\textsubscript{H}-Cy3 and V\textsubscript{H}DJ\textsubscript{H}-Ce transcripts as well as Sγ-Sμ and Se-Sμ and Se-Sy SCs without affecting the level of Sα-Sμ SCs and mature V\textsubscript{H}DJ\textsubscript{H}-CaI/Ca2 transcripts. An analysis of germ line and mature transcripts in the Cy1 (supplemental Fig. 7B), Cγ2, or Cγ4 loci yielded comparable results (data not shown). Thus, HoxC4, Oct-1, and Ku70/Ku86 effectively repress germ line Iγ-Cγ and Ie-Ce transcription and CSR to Cy and Ce but not germ line Iα-Cα transcription and CSR to CaI/Ca2 in a fashion that is dependent on the HIM of Ku70.

**DISCUSSION**

We have defined here a novel mechanism that inhibits class switching to IgG and IgE but not IgA. By identifying the key elements of this inhibitory mechanism, we provide the first evidence for an important role of a Hox protein and a novel function for Oct-1 and Ku70/Ku86 in B cell differentiation. We show that HoxC4 and Oct-1 together with the Ku70/Ku86 heterodimer form a HD-dependent complex, which is recruited to ATTT motifs in the human Iγ and Ie promoters to dampen germ line Iγ-Cγ and Ie-Ce transcription and repress direct and sequential CSR to Cy and Ce. The HoxC4-Oct-1-Ku-dependent inhibitory mechanism is operational in the presence of AID that plays a critical role in CSR (28) and would provide a threshold of transcriptional and recombinational inertia that must be overcome for effective initiation and unfolding of CSR, thereby contributing to the homeostasis of the H chain locus.

Hox proteins are phylogenetically conserved HD-containing trans-factors that serve principally as transcriptional repressors. They modulate transcription by binding to the HD-specific ATTt/A core-motif (29, 30) and critically regulate not only embryonic pattern formation, axis specification, and organogenesis (31) but also adult cellular processes including selective hematopoietic lineage differentiation and stem cell renewal (32). Oct-1 is a ubiquitous member of the POU (Pit, Oct, Une) family of transcription factors that regulates both general and cell type-specific genes (33) including V and C genes in the Ig locus (34). Ku70/Ku86, the ATP-dependent DNA helicase II subunits of the DNA-dependent protein kinase, serves as DNA end-binding and alignment factors in NHEJ DNA repair. NHEJ is critical not only in Ig VDJ gene recombination and CSR but also in overall genome maintenance (3, 35).

The HoxC4-Oct-1-Ku complex may function as a common effector in the modulation of IgG and IgE class switching at different stages of the B cell natural history. In pre-GC and perhaps memory B cells, the complex would maintain the basal
repression of CSR in the Cy and Ce loci. The partial overlap of the 5′-SRE with the IL-4 RE-Stat6 (1γ) or the proximity of these cis-elements (1e) would entail a complex regulation of the 1γ and 1e promoters, allowing for competition and/or interplay among the respective trans-factors. In GC B cells, CD40 engagement and exposure to IL-4 induce the binding of NF-κB to the CD40 RE and binding of Stat6 to the IL-4 RE. This would result in Bcl6 displacement (36) and dissociation of HoxC4-Oct-1-Ku from the SREs, which would in turn lift the inhibition off of the Ig H chain locus and activate germ line 1γ-Cy and 1e-Ce transcription and CSR to Cy and Ce (Fig. 8). These processes are counteracted by a CSR inhibitory signal from B cell CD153 upon engagement by CD200 on suppressor T cells. Here, we demonstrate that CD153 signaling, which inhibits the CD40-induced activation of NF-κB (14, 15), effectively prevents the CD40-dependent dissociation of HoxC4-Oct-1-Ku from SREs. Thus, in addition to repressing basal germ line 1H-Cy transcription and CSR in non-CD40-induced (pre-GC) B cells, HoxC4-Oct-1-Ku mediates the CD153-dependent inhibition of germ line 1H-Cy and Sce transcription and CSR in CD40-induced (GC) B cells.

Our findings suggest a role for a CD40 signaling-induced Ser/Thr phosphatase in the dissociation of the HoxC4-Oct-1-Ku complex from SREs. In vitro Ser/Thr-specific dephosphorylation of HoxC4-Oct-1-Ku abrogated their SRE binding, and pretreatment of freshly isolated B cells with okadaic acid prevented CD40-induced dissociation of HoxC4-Oct-1-Ku from SREs (Fig. 5D). A similar dephosphorylation-dependent regulation of Hox protein activity has been reported previously (37) in Drosophila melanogaster. The overexpression of HoxC4-Oct-1 and/or Ku70/Ku86 (Fig. 7) would probably overcome the dephosphorylation activity of endogenous phosphatase(s), thereby allowing for the expression of the CSR inhibitory activity by the repressor complex. Upon CD40-induced dissociation from SREs, the amount of HoxC4, Oct-1, and Ku proteins increased in cell nuclei (Fig. 5B), probably as a result of increased transcription and protein synthesis (HoxC4 and Oct-1) or cytoplasmic-to-nuclear translocation (Ku70/Ku86) (38), suggesting that these proteins play a role in later CSR-related events such as NHEJ (39) or regulate transcription of CSR-related genes by binding other HD recognition sequences.

For efficient inhibition of 1γ and 1e promoter activity, HoxC4 and Oct-1 rely on the recruitment of the Ku70/Ku86 heterodimer. Overexpression of Ku70mutHIM reverted HoxC4-Oct-1-dependent IκB β–H1 promoter inhibition and enhanced basal promoter activity (Fig. 7, A and B), possibly through displacement of endogenous Ku70 and formation of Ku70mutHIM/Ku86 heterodimers, which would effectively reduce the availability of the functional endogenous Ku70/Ku86 heterodimer but not associate with HD proteins. Importantly, forced expression of HoxC4 and Oct-1 as well as Ku70/Ku86 effectively repressed both germ line 1γ-Cy and 1e-Ce transcription and CSR to Cy and Ce. Again, this repression was dependent on the ability of Ku70 to interact with HD proteins, because overexpression of the Ku70mutHIM/Ku86 heterodimer failed to repress germ line 1H-Cy transcription and enhanced CSR to IgG and IgE as detected by the increased level of mature VH-DJH-Cγ and VH-DJH-Cε transcripts and reciprocal SCs. The mechanism of Ku-dependent transcriptional inhibition was not addressed in this study. It may include DNA-dependent protein kinase-dependent phosphorylation of 1γ promoter- and/or S region-bound trans-factors (40), inhibition of histone acetyltransferases (41), or recruitment of histone deacetylases including Sir2-related proteins (42).

Inhibition of IgG and IgE class switching by HoxC4-Oct-1-Ku is consistent with the Cy- and Ce-shared CSR activation pathway, which includes CD40 and IL-4R signaling, and possibly reflects the co-evolution of the Cy and Ce loci arising from the duplication of a single ancestral locus (43, 44). The lack of ATTT motifs within the 1α/1ο promoters accounts for the failure of HoxC4-Oct-1-Ku to inhibit IgA class switching and emphasizes the difference in regulation (transforming growth factor-β versus IL-4) and ancestral origin (early versus late) of the CD1/C2 versus the Cyγ and Ce loci. Also, it probably underlies the T cell CD40L independence and distinct anatomic compartmentalization of IgA-secreting cells, which are mainly CD5+ (B-1 lymphocytes). B-1 lymphocytes accumulate preferentially in the splanchic district and near external membranes and play a critical role in the first line of defense against microbial pathogens (45). Similar to B-1 cells, IgA appears early in phylogeny, emerging prior to IgG and IgE as the first of the “mature” isotypes in birds. The lack of regulation of IgA class switching by HoxC4-Oct-1-Ku may be compensated by other mechanisms. In the mouse, germ line Iα-ε transcription and Sµ– Sζ CSR are repressed by B cell lineage-specific activator protein, which binds to a specific cis-element within the Iα promoter (46) or by the late SV40 factor, which binds to appropriately spaced CTGG repeats within Sµ and Sζ regions, thereby recruiting histone deacetylases and the co-repressor Sin3A (47).

The inhibitory activity unveiled here is probably part of a broader regulation of the Ig H chain locus by HoxC4-Oct-1-Ku. Our preliminary experiments suggest that these trans-factors also regulate the human H chain 3′-hs1,2 enhancer element, which probably plays a role in Ig class switching.2 Because of the wider recurrence of ATTT motifs in the human genome, HoxC4-Oct-1-Ku or other HD protein-Ku complexes could be involved in general transcriptional inhibition and anti-recombinogenic functions as part of the overall genomic caretaker activity as suggested by the extreme genomic instability of Ku70−/− and Ku86−/− mice (35). Dysregulation of the HoxC4-Oct-1-Ku-mediated inhibitory function could cause aberrant CSR and chromosomal translocation and contribute to B cell lymphomagenesis.

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Selective Inhibition of Class Switching to IgG and IgE by Recruitment of the HoxC4 and Oct-1 Homeodomain Proteins and Ku70/Ku86 to Newly Identified ATTT cis -Elements

András Schaffer, Edmund C. Kim, Xiaoping Wu, Hong Zan, Lucia Testoni, Szilvia Salamon, Andrea Cerutti and Paolo Casali

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