A differentially methylated imprinting control region within the \textit{Kcnq1} locus harbours a methylation-sensitive chromatin insulator

by

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Abstract

The mechanisms underlying the phenomenon of genomic imprinting remain poorly understood. In one instance, a differentially methylated imprinting control region (ICR) at the $H19$ locus, has been shown to involve a methylation-sensitive chromatin insulator function that apparently partitions the neighbouring $Igf2$ and $H19$ genes in different expression domains in a parent of origin-dependent manner. It is not known, however, if this mechanism is unique to the $Igf2/H19$ locus or if insulator function is a common feature in the regulation of imprinted genes. To address this question, we have studied an ICR in the $Kcnq1$ locus that regulates long-range repression on the paternally derived $p57Kip2$ and $Kcnq1$ alleles in an imprinting domain that includes $Igf2$ and $H19$. We show that this ICR appears to possess a unidirectional chromatin insulator function in somatic cells of both mesodermal and endodermal origins. Moreover, we document that CpG methylation regulates this insulator function suggesting that a methylation-sensitive chromatin insulator is a common theme in the phenomenon of genomic imprinting.
Introduction

Human chromosome 11p15.5 and the distal part of chromosome 7 in mouse harbour a well-characterised cluster of imprinted genes: Ipl, Orctl2, Cdnk1c, Kcnq1, Kcnq1 A-S/LIT1, Mash2, Ins2, Igf2 and H19 (1,2). Disruption of imprinting in 11p15.5 results in the overgrowth and cancer predisposition condition Beckwith-Wiedemann syndrome (BWS) (3). Based on chromosomal break points and methylation changes in BWS patients, a differentially methylated CpG island identified in the intron 10 of the Kcnq1 gene has been proposed to be involved in the regulation of imprinting (4,5). This region is methylated on the active maternal allele, but unmethylated on the inactive paternal allele of Kcnq1 (1) and overlaps with an oppositely oriented and paternally expressed gene known as Kcnq1 A-S or LIT1 (4,5). Targeted deletion of this region in the human paternal chromosome 11 propagated in the chicken DT40 cell line resulted in the activation of the normally silent paternal alleles of KCNQ1 and CDNK1C (6). The suggestions that this region, hereafter termed Kcnq1 imprinting control region (ICR), has a pivotal role in the maintenance of imprinting of neighbouring genes (6,7), has recently been confirmed by targeted deletion experiments in the mouse (Fitzpatrick and Higgins, unpublished observations).

To examine if chromatin insulator properties is a common feature of imprinting control regions, we analysed the activity of the Kcnq1 ICR in enhancer-blocking assays. Our results are consistent with the notion that a CpG
methylation-sensitive chromatin insulator function is not restricted to the H19 ICR, but includes the Kcnq1 ICR as well.

Experimental procedures

Plasmid cloning strategies
The 3.6 kb Kcnq1 ICR segment was inserted into unique Cla I or Not I sites in both orientations in the E-p-neo-scs vector (8) (see Figure 1b). To facilitate cloning into episomal plasmids, we inserted a fragment with multiple cloning sites from the parent pREP4 plasmid (amplified using forward primer AAG CTG ATC TAT CAT GTC TGG ATC CGG CC and reverse primer, AAG CTG ATC CAT TCA CCA CAT TGG TGT GC flanked with either Sal I or Cla I sites at their 5’ ends) into a unique Sal I (with the SV 40 enhancer in the Cla I site) and Cla I (with the SV40 enhancer in the Sal I site) sites of pREPH19 A and C (9). A 3.6 kb Not I-Xho I Kcnq1 ICR fragment was subcloned into the Not I –Xho I sites of multiple cloning fragment at the Sal I and Cla I positions. An opposite orientation of Kcnq1 ICR at the Sal I position was achieved by subcloning a Not I fragment of the Kcnq1 ICR into the Not I site of the multiple cloning site.

The integrated chromatin insulator assay
Equimolar amounts of each experimental and control linearized construct (approx. 1 pmol, 5.0-10.0 µg depending on the construct) was introduced
into $4 \times 10^6$ human T cell leukemia Jurkat cells in triplicate electroporations, as has been described (8). Following recovery on ice in the presence of 20% FCS, the cells were transferred to 10 ml of RPMI 1640, 10% FCS and cultured at $37^\circ$ for 48 hr to allow integration of the plasmid constructs. The cells were then pelleted, resuspended in 1.0 ml RPMI with 10% FCS, and mixed with 30 ml soft agar medium containing 950 ug/ml active G418. The mixture was then poured in 10 cm plates and incubated at $37^\circ$ for 3 weeks to allow growth of drug-resistant colonies.

The episome insulator assay

The pREP4-based episomal vectors were transfected into Hep3B cells, as has been described (9). The RNase protection expression analysis was performed as previously described using a 365 bp $H19$ antisense probe and a 150 bp GAP (Glyceraldehyde 3-phosphate dehydrogenase) antisense probe as control (9). 10 µg of RNA (including various amounts of total cell RNA depending on episome copy number and yeast tRNA) was hybridised with the antisense probes (300 000 cpm/reaction for $H19$ and 20 000 cpm/reaction for GAP) overnight at 45°C. All procedures were performed according to the manufacturer’s protocol of the RPAIII kit (Ambion). Quantification of individual protected fragments was done using a Fuji FLA 3000 Phosphorimager. The $H19$ expression was corrected with respect to both internal control (GAP) and episome copy number as determined by Southern blot analysis of Bgl II restricted DNA, hybridised with $H19$ and PDGFB probes (9).
**The methylated cassette approach**

A purified Kcnq1 ICR 3.6 kb fragment was methylated with 2 U/µg Sss I methyltransferase in the presence of 180 µM S-adenosyl methionine for 16 hours at 37°C. The methylation reaction was terminated by heat-inactivation at 65°C for 15 min and the methylation status of the purified fragment was analysed by digestion with Hha I. The mock-methylated fragment was treated in the same way without the addition of Sss I. Following linearisation of pREPH19A with Xho I and Not I, the methylated and mock-methylated ICR fragments were ligated within the vector overnight at 14°C. Each ligation mix was then phenol:chloroform-extracted (1:1) and used directly for transfection.

**Chromatin conformation analyses**

Hygromycin-selected clones were individually harvested and cells from each clone were equally divided for DNA and RNA extraction. DNA was extracted by lysing the cells overnight with 1% SDS supplemented with Proteinase K. DNA was then purified with phenol:chloroform (1:1) extraction. The RNA was extracted with an RNA kit (Clonetech) according to the manufacturers recommendations. For genotyping, DNA from cell clones was restricted with Bst BI/BstZ17I, and for methylation analysis the DNA was digested with Pst I and Hha I and analysed by standard Southern blot hybridisation protocols (9) using probe A, a 3.6 kb Pst I fragment covering the entire Kcnq1 ICR (see Figure1a).

Nuclei from cell clones and mouse fetal liver were isolated and treated with
DNase I as previously described (9). Twenty μg of digested DNA was restricted with restriction enzymes and electrophoresed on a 1.7 % gel and blotted to a Hybond N+ membrane (Amersham) followed by hybridisation with probes; a 810 bp Eag I/Pst I fragment (Probe B), a 860bp NsiI-BsaHI fragment and 1.5 kb Bgl II PDGFB fragment (Probe D) (see Figure1a) according to standard protocols. All probe fragments were radiolabelled using a multi-prime labelling kit (Amersham) and [α-32P] dCTP.

Results

To examine the possibility that the Kcnq1 ICR possesses a chromatin insulator function similar to the H19 ICR (9-13), we exploited two different insulator assays which both monitor position-dependent or independent gene silencing. These different parameters are often used to distinguish between chromatin insulator and silencer functions (14). In the first assay, the reporter consisted of a neo-resistance gene cassette driven by the Vδ1 promoter in combination with its cognate endogenous Eδ4 enhancer (8). The known insulator element active in mammalian cells, scs (8), was included in the vector to prevent interaction of the Vδ1 promoter with the enhancer on adjacent copies in tandem arrays. The 3.6 kb Kcnq1 ICR fragment (Figure 1a) was inserted between the Vδ1 promoter and the Eδ4 enhancer (Figure 1b). The number of neo-resistant colonies that formed following electroporation of p-neo-scs (Figure 1b) provided a baseline for Vδ1-driven
neo-cassette expression in the absence of an enhancer. This is the level that one would expect to see in constructs carrying insulators or silencers that ablate the influence of an enhancer completely. The inclusion of the Eδ.4 enhancer increased the number of colonies more than 10-fold (Figure 1b). This level of neo-reporter activity was set to 100%, to which all other constructs were compared (Figure 1b). As a negative control, construct E-2.7-p-neo-scs was used which contains 2.7 kb of anonymous DNA placed between the enhancer and promoter. No significant reduction in colony number was observed with this construct (Figure 1b). However, when the Kcnq1 ICR was inserted between the enhancer and the promoter, there was a strong reduction of colony numbers. Insertion upstream of the enhancer had no repressive effect, thus documenting a position-dependent function typical of chromatin insulators (Figure 1b). Interestingly, this insulator function depended on the orientation of the Kcnq1 ICR, as has been shown earlier for the H19 ICR insulator (9).

To confirm the chromatin insulator activity of the Kcnq1 ICR, and to allow the examination of the effect of methylation on this activity (see below), we tested this locus in another enhancer blocking assay, using the SV40 enhancer and a mouse H19 reporter gene. This assay is based on the stable propagation of episome-based plasmids in cultured cells and analysis of reporter gene expression by RNase protection analysis (RPA) (9). Following transfection of plasmids into Hep3B cells, hygromycin-selection and expansion for 30 days or more, RNA extracted from these cells was subjected to RPA and adjusted
for episome copy numbers as has been described (9). We have previously showed that the SV40 enhancer activates the H19 reporter gene more than 200-fold (9). When the Kcnq1 ICR was positioned between the reporter gene and the enhancer, the steady-state levels of mouse H19 reporter transcripts were reduced more than 40-fold (Figure 1c). Conversely, when the Kcnq1 ICR fragment was inserted in the opposite orientation or outside the reporter gene-enhancer context, there was little or no effect on reporter gene expression levels (Figure 1c). We conclude that two independent assays document that the Kcnq1 ICR possesses a polar/unidirectional chromatin insulator function in two different types of somatic cells.

Next, we were interested in determining whether or not the unidirectional insulating property of Kcnq1 ICR depended on the presence of enhancer elements in the 3.6 kb fragment. To this end, we deleted SV40 enhancer from episomal insulator constructs and examined reporter gene activity in the Kcnq1 ICR-containing pREP constructs by scoring for numbers of hygromycin-resistant colonies of transfected Hep3B cells. Figure 1c shows that the deletion of the SV40 enhancer from the episomal Kcnq1 insulator constructs (termed: - enh) results in no detectable hygromycin promoter activity. This result was obtained with the Kcnq1 ICR inserted in either orientation ruling out that the unidirectional insulating property depends on an endogenous enhancer.

We have earlier shown that the insulator activity of the H19 ICR is CpG
methylation-sensitive (13). By analogy, CpG methylation might control the insulating properties of the *Kcnq1* ICR which is methylated only on the maternal allele. To test the effect of DNA methylation on the insulating function of the *Kcnq1* ICR we generated *in vitro*-methylated and mock-methylated 3.6 kb *Kcnq1* ICR fragments. The procedure for obtaining mock-methylated and methylated fragments between the reporter gene and the SV40 enhancer in the episomal vector, has been described and discussed earlier (13). In brief, *in vitro*-methylated and mock-methylated fragments were ligated into unmethylated episomal vector and transfected into Hep-3B cells. Following hygromycin selection for 3 to 4 weeks, 20 clones were expanded from each of the mock-methylated and methylated transfections and genotyped/epigenotyped as described earlier (9). Based on extensive Southern blot hybridisation analyses, we selected three clones harbouring methylated *Kcnq1* ICR and one clone containing the mock-methylated *Kcnq1* ICR fragments which were unrearranged and maintained episomally (data not shown). To document absence of *de novo* methylation or demethylation in the expanded clones, the extracted DNA was digested with the methylation sensitive enzyme, *Hha I*. Figure 2a shows that while the insert of the mock-methylated clone remained unmethylated, whereas the *in vitro* preimposed methylation patterns were generally maintained in the other expanded clones with insignificant exception in clones 1 and 2 which displayed a minor proportion of unmethylated ICR sequences.

The effects of CpG methylation were monitored by first examining the
chromatin conformation at the Kcnq1 ICR. Figure 2b shows that three DNase I hypersensitive sites map within the Kcnq1 ICR in both mouse fetal and adult liver and that these sites were recapitulated in the mock-methylated clone, but not in the methylated clone 1 (Figures 2c-e), documenting epigenetic control of DNA-protein interactions in this region. In order to make sure that DNase I equally degraded mock and methylated clones, the blots were reprobed with a 1.5 kb Bgl II fragment human PDGFB gene. As can be seen in Figure 2d, the PDGFB gene is degraded equally between methylated and mock-methylated samples. The differentially sensitivity of methylated Kcnq1 ICR to DNase I could be due to methylation-induced chromatin compaction. We next analysed the effect of DNA methylation on the unidirectional insulator activity of the Kcnq1 ICR. Importantly, expression analysis of the H19 reporter gene revealed full insulator function in the mock-methylated clone whereas the insulator function was significantly reduced in the clones with a methylated insert (Figure 3). The various degrees of insulating properties of the methylated clones 1 to 3 appear to reflect the stringency of methylation status of the Kcnq1 ICR insert (Figure 3). Figure 4 summarises these observations leading us to propose that the Kcnq1 ICR is a methylation-sensitive chromatin insulator that operates in a polar/unidirectional fashion.

Discussion

The observations reported here support an emerging consensus that epigenetically controlled chromatin insulator functions are common
denominators with respect to the manifestation of imprinted states. The methylation-sensitivity of the chromatin insulator function in the Kcnq1 ICR explains the effect of loss of methylation at the KCNQ1 ICR on the maternally inherited chromosome. This is the most frequent abnormality observed in children with BWS, accounting for roughly 50% of non-UPD cases (15).

Importantly, the chromatin insulator function must be physically positioned between enhancers and target promoters to function. Given the position of the chromatin insulator function at the Kcnq1 ICR, any blocked enhancer function with respect to the Kcnq1 promoter must be located 5' of the ICR. Conversely, the unidirectional feature of the Kcnq1 insulator implies that enhancers 3' of the ICR are not insulated from promoters upstream of the Kcnq1 ICR. Although the polarity of the insulator clearly does not depend on an endogenous enhancer element, as shown here, it might impinge on the promoter for the antisense transcript, which maps within the Kcnq1 ICR (Kanduri et al, unpublished observation). However, the observations that the Kcnq1 ICR is also vital for the paternal-specific repression of the upstream Ipl, Orctl2 and Cdnk1c genes (6) (Fitzpatrick and Higgins, unpublished observation) and that several lineage-specific enhancers have been identified in the intergenic region between Cdnk1c and Kcnq1 (16) are not compatible with a unidirectional chromatin insulator, suggesting that additional cis elements are involved in long-range repression emanating from the Kcnq1 ICR. Several of these cis elements map to CTCF binding sites (Fitzpatrick et al, unpublished observation), underscoring a commonality in the link between
insulators, CTCf and imprinting control regions.

In the mouse, imprinting of Kcnq1 is relaxed during late gestation becoming completely biallelic in adults (17). However, the Kcnq1 ICR possesses similar chromatin conformation in both fetal and adult liver tissues suggesting that the Kcnq1 ICR has no role by itself in the developmental de-repression of the paternal Kcnq1 allele. The developmentally regulated activation of the paternal Kcnq1 allele could hence be explained by a switch in usage from upstream to downstream (with respect to the Kcnq1 ICR) enhancers in adult cells (Figure 4)

Footnotes

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Abbreviations

BWS. Beckwith-Wiedemann syndrome; FCS, fetal calf serum; GAP, glyceraldehyde 3-phosphate dehydrogenase; IGF2, insulin-like growth factor 2; RPA, RNase protection analysis.

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Figure legends
**Figure 1.** The *Kcnq1* ICR is a cell type-specific insulator/silencer. (a) Schematic map over the *Cdk1c*, *Kcnq1* and *Mash2* cluster of imprinted genes. The 3.6 kb *Kcnq1* ICR fragment used in the silencer studies maps at the differentially methylated region that regulates the imprinting status of the *Cdk1c* and *Kcnq1*. (b) The 3.6 kb *Kcnq1* ICR fragment was inserted into the E-p-neo-scs vector in various positions with respect to the Vd1 promoter and the Ed.4 enhancer, as indicated in the panel. Following transfection into Jurkat cells, neo-resistant colonies were counted and the numbers represented as bars for each individual construct, as has been described (8). (c) The insulating/silencing properties of the *Kcnq1* ICR fragment was examined in an episomal assay, as has been described (9). The same set of constructs, indicated in the panel, were transfected into Hep3B cells. The levels of expression of the mouse *H19* reporter gene (cerise bars) were normalised to both total RNA input as well as episome copy numbers, as has been described (9). The numbers of hygromycin-resistant clones (blue bars) were used to assess the bidirectional silencing by the *Kcnq1* ICR. The mean deviation of minimally three different experiments is indicated for each vector construct, unless the differences were too small to allow visualization. The SV40 enhancer-driven expression of the modified pREPH19A construct was, for convenience, assigned a value of 100 and all other samples were related to this value.

**Figure 2.** Propagation of methylation marks and their effects on DNA-protein interactions in transfected cells. (a) shows that the *in vitro* imposed
methylation status of the Kcnq1 ICR fragments in episomal vectors were maintained in isolated Hep3B cell clones. (b) The Kcnq1 ICR harbours DNase I hypersensitive sites in mouse fetal and adult liver which are methylation-sensitive (c). (d) Reprobing of blot of Figure 3c with BgIII fragment of the human PDGFB gene (9). (e) Fine-mapping of the DNase I hypersensitive sites in the Kcnq1 ICR. (f) Schematic representation of the DNase I hypersensitive sites in the Kcnq1 ICR.

**Figure 3.** The Kcnq1 ICR insulator function is methylation-sensitive. H19 reporter gene activity in Hep3B cell clones, with methylated or mock-methylated inserts as indicated in the Figure, was assessed by RNase protection analysis. See legend of Figure 1c for additional details.

**Figure 4.** Model explaining how the methylation-sensitive chromatin insulator function reported here might explain manifestation of the tissue-specific imprinting status of Kcnq1 depending on the position of hypothetical enhancers.
Figure 1, Kanduri et al

Figure 2, Kanduri et al
Figure 3, Kanduri et al

Figure 4, Kanduri et al
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