Transcriptional repression by the insulator protein CTCF involves histone deacetylases

Marcus Lutz, Les J. Burke, Guillermo Barreto, Frauke Goeman, Heiko Greb, Rüdiger Arnold, Holger Schultheiß, Alexander Brehm, Tony Kouzarides¹, Victor Lobanenkov² and Rainer Renkawitz*

Genetisches Institut der Justus-Liebig-Universität, Heinrich-Buff-Ring 58–62, 35392 Giessen, Germany, 'Wellcome/CRC Institute for Development and Cancer Biology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK and ²National Institute of Allergy and Infectious Diseases, National Institutes of Health, Molecular Pathology Section, Building 7, Room 303, MSC-0760 Bethesda, MD 20892-0760, USA

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ABSTRACT

The highly conserved zinc-finger protein CTCF, is a candidate tumor suppressor protein that binds to highly divergent DNA sequences. CTCF has been connected to multiple functions in chromatin organization and gene regulation including chromatin insulator activity and transcriptional enhancement and silencing. Here we show that CTCF harbors several autonomous repression domains. One of these domains, the zinc-finger cluster, silences transcription in all cell types tested and binds directly to the co-repressor SIN3A. Two distinct regions of SIN3A, the PAH3 domain and the extreme C-terminal region, bind independently to this zinc-finger cluster. Analysis of nuclear extract from HeLa cells revealed that CTCF is also capable of retaining functional histone deacetylase activity. Furthermore, the ability of regions of CTCF to retain deacetylase activity correlates with the ability to bind to SIN3A and to repress gene activity. We suggest that CTCF driven repression is mediated in part by the recruitment of histone deacetylase activity by SIN3A.

INTRODUCTION

The zinc-finger protein CTCF, previously known as NeP1, was one of the first identified factors binding to metazoan silencing elements (1,2). Transcription of the chicken lysozyme gene (3) and the chicken and human myc-genes (4) is repressed by DNA elements, which are bound by CTCF. The identification of CTCF binding to repressive or silencing elements in both types of genes was hampered by the fact that the binding sites are extremely divergent. This divergence is such that the name given CTCF-richness of the binding site at the human c-myc gene is not found at the CTCF binding site at the chicken lysozyme gene (3). The explanation of this apparent paradox is that the 11-zinc-finger DNA binding domain of CTCF allows a selective and specific usage of different combinations of individual zinc-fingers (3–5). These zinc-fingers are of the C₂H₂-type except for the eleventh zinc-finger, which has an unusual amino acid sequence.

The zinc-finger DNA binding domain of about 300 amino acids is 100% identical between mouse, man and chicken. Even the full-length protein of about 700 amino acids is 93% identical between avian and human. This highly conserved protein is ubiquitously expressed in all tissues analyzed so far. Because of this distribution, like a product of a house keeping gene, and because of the high degree of conservation, important and general cellular functions must be mediated by CTCF. In many cases transcriptional repression has been associated with CTCF (2,5,6). Gene activation mediated by CTCF response elements has also been demonstrated (2,7,8). Interestingly, modulation of the effects of CTCF on transcription has been observed in the context of the thyroid hormone receptor (TR). In the case of the chicken lysozyme silencer (S-2.4), synergistic repression of the adjacent CTCF and TR binding sites have been seen in the absence of thyroid hormone, whereas addition of hormone leads to a synergistic activation (2). In the context of a negative thyroid hormone response element (TRE), the thyroid hormone response is reversed such that the presence of hormone synergistically represses an adjacent gene (5).

An even more widespread role of CTCF has been suggested recently. Insulator elements, which act as a barrier to prevent neighboring cis-acting elements from regulating a distal gene, have been found to mediate their function by CTCF (9). All of the vertebrate enhancer-blocking elements examined by this group contain CTCF binding sites. Therefore, given that CTCF has such a fundamental role, it is not surprising that the gene coding for CTCF is a candidate tumor suppressor gene located within a region frequently deleted in several different human malignancies (10).

A mechanism mediating these effects has not yet been elucidated. Although specific DNA bending has been found to be induced by CTCF (11), it is not very likely that all of the biological effects induced by CTCF will be mediated just by a change in the DNA conformation. Since many of the CTCF functions are repressive, we wondered whether co-repressor proteins might bind to CTCF. Here we find, in contrast to other repressive
zinc-finger proteins, that the zinc-finger domain of CTCF binds directly to the co-repressor SIN3A. In addition, we demonstrate that histone deacetylases from nuclear extracts are bound by the CTCF zinc-finger domain, indicating that CTCF may repress transcription by recruitment of histone deacetylases.

**MATERIALS AND METHODS**

**Plasmids**

Regions of CTCF were generated by PCR using Pfu polymerase (Stratagene) and specific primers using pSG5–CTCF (7) as a template and cloned into pBluescript II (Stratagene). CTCF-FL, CTCF-NT, CTCF-ZF, CTCF-ZF/CT and CTCF-CT encode amino acids 2–728, 2–268, 269–577, 269–738 and 577–738 of chicken CTCF, respectively (see Fig. 1). All junctions were sequenced with a T7 Sequenase kit (Amersham Pharmacia Biotech AB). The GST–CTCF and Gal–CTCF fusion constructs were generated by fusing fragments of CTCF cDNA from the above pBluescript II SK clones, in frame into GST-linker (12) that encodes for glutathione S-transferase (GST) or pAbGal94 (13), which encodes for amino acids 1–94 of the Gal4 DNA binding domain. To create pGST–NCoR, the EcoRV/SalI fragment of pCMX-Gal4 NCoR1–312 (14) was cloned in frame into GST-linker. All junctions were sequenced to ensure that proteins would be expressed in frame. The reporter construct, p17mer6x-tk-CAT, has been described previously (2). The series of 35S-labeled mSin3A proteins, produced by the T7/T3 TNT-coupled transcription/translation system (Promega) in 200 µl of binding buffer [100 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.5% NP-40, 5 µg of ethidium bromide, 100 µg of BSA]. After 0.5 h incubation at room temperature the beads were washed eight times with 1 ml of binding buffer without ethidium bromide and BSA. The bound proteins were eluted with SDS sample buffer, fractionated on SDS–PAGE and after treatment with sodium salicylate, visualized by fluorography.

**GST pull-downs from nuclear extracts**

GST pull-downs from nuclear extracts were carried out as described previously (20,21). GST fusion proteins bound to glutathione–Sepharose beads were incubated with 30 µl of HeLa nuclear extract (Computer Cell Culture Centre, Moens, Belgium) in 250 µl of IPH buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40) at 4°C for 1–2 h. Beads were washed three times with 1 ml of IPH buffer and resuspended in SDS–PAGE loading buffer or 100 µl of IPH buffer (for histone deacetylase assays). Samples were separated by SDS–PAGE and analyzed by western blotting with an antibody specific for Sin3A (K20, Santa Cruz).

**Histone deacetylase assays**

Histone deacetylase assays were carried out essentially as described previously (20,21) in a volume of 100 µl of IPH buffer containing 2.5 × 105 c.p.m. of 3H-labeled acetylated histone H4 peptide.

**Preparation of stably transfected cells**

To create NIH 3T3 6TCP cells, 6 × 150-mm dishes of NIH 3T3 cells were transfected at ~40% confluence using the calcium phosphate mediated procedure using 60 µg of p17mer6x-tk-CAT and 6 µg of pcDNA3.1 (Invitrogen) per dish. Cells were then grown for a further 48 h to allow for cell recovery and for high-level pcDNA3.1 expression before selection with 400 µg/ml G418. Stable transfectants (approximately 150 colonies) were pooled after 14 days selection.

**TSA experiments**

For TSA experiments, NIH 3T3 6TCP cells (2 × 105 cells per well) were grown on 6-well plates. Cells were transfected with 2.3 µg of expression plasmids using TRANFAST reagent (Promega) according to the manufacturer’s manual. TSA (BIOMOL) was added 32 h after transfection at a concentration of 10 ng/ml. Cells were harvested 12 h of TSA treatment and CAT assays were carried out as described above.
RESULTS

CTCF harbors several autonomous repression domains

Previously, the highly conserved and ubiquitous zinc-finger protein CTCF was shown to repress transcriptional activity of genes. This transcriptional repression is seen when CTCF is directly targeted to promoter sequences (3,4). Recently it has been demonstrated that CTCF plays a central role in the function of vertebrate insulators (9). Here we wanted to test whether CTCF harbors autonomous silencing domains. Therefore we fused the coding region of full-length CTCF to the sequence coding for the DNA binding domain of Gal 4 (Fig. 1). Co-transfection of the Gal–CTCF expression plasmid with the p17mer6xs-tk-CAT reporter repressed the reporter activity, when tested in several vertebrate cell lines (Fig. 2). Repression is expressed relative to the effect mediated by the empty expression vector Gal. In order to identify a repression domain within CTCF, the coding sequence was dissected into different parts containing the N-terminus, the C-terminus and the region coding for the 11 zinc-fingers from the center of the protein. Fusions to the Gal DNA binding domain were tested for repression and showed the strongest repression effect for the zinc-finger region attached to the C-terminus. Dissection of this region into the zinc-finger domain by itself and the remaining C-terminal part showed repression for both of these regions, such that apparently the zinc-finger plus C-terminal region consists of at least two repressive domains. In contrast, the N-terminal domain showed no repression in some cell lines (COS1 and CV1 cells are shown in Fig. 2), whereas in the chicken erythroblast line, HD3, a strong repression was mediated by the N-terminal domain as well. This apparent tissue-specific effect was not further analyzed since we wanted to focus on the general transcriptional mechanisms conferred by the ubiquitous CTCF protein.

Thus, CTCF harbors strong transcriptional silencing domains, one of which is located in the zinc-finger region.

CTCF binds to the co-repressor SIN3A

Since CTCF contains autonomous repression domains, we wondered whether at least one of the repression functions of CTCF might be mediated by one of the known co-repressors. Therefore we replaced the Gal DNA binding domain from the Gal–CTCF fusion constructs (Fig. 1) with GST. We then tested *Escherichia coli* expressed GST–CTCF fusions for the ability to bind several published co-repressors (data not shown). Among these, *in vitro* translated SIN3A was bound to the GST–CTCF fusions. The binding was specific, since SIN3A did not bind to GST alone (Fig. 3A, lane 2) nor did any GST–CTCF fusion bind to *in vitro* translated luciferase (data not shown). As a positive control we used the *E.coli* expressed GST–N-CoR fusion, which previously has been shown to bind SIN3A (14,22,23). Analysis of the individual CTCF domains fused to GST showed binding of SIN3A to full-length CTCF, to the zinc-finger plus C-terminal region and to the zinc-finger domain itself. The C-terminal domain without the zinc-finger region showed almost no binding. The N-terminal domain was also negative for SIN3A binding. The binding of SIN3A to the zinc-finger domain was much stronger as compared to full-length CTCF. Such a finding, that a subdomain interacts or functions much stronger as compared to the full-length construct, has been seen in many other cases as well. This may indicate a potential shielding of the binding domain by other regions of the protein. Furthermore, we wanted to know whether *in vivo* produced SIN3A could bind to CTCF as well. Therefore we used the same GST–CTCF fusion proteins linked to Sepharose beads and carried out a co-precipitation with nuclear extract from HeLa cells. The bound proteins were analyzed by western blotting with an antibody against SIN3A. The western blot (Fig. 3B) shows a clear SIN3A signal precipitated by full-length CTCF, the zinc-finger plus C-terminal region...
and the zinc-finger domain by itself. No signal was detected after binding to either the N- or C-terminal region alone. There are some differences in the relative binding efficiencies, when binding of in vitro translated material is compared with in vivo produced HeLa SIN3A, nevertheless the common finding of both types of analyses shows that the zinc-finger domain by itself is sufficient to bind SIN3A.

Previously identified domains of SIN3A that interact with other proteins include the so-called PAH domains (24), four of which are found in SIN3A (Fig. 4A). In order to identify a region of the SIN3A protein binding to the CTCF zinc-finger domain we generated different SIN3A truncations for in vitro translation. The GST–CTCF zinc-finger domain fusion showed binding to full-length SIN3A, but not to SIN3A regions encompassing the PAH1 domain or PAH1+2 (Fig. 4B). Longer SIN3A constructs, containing PAH domains 1–3 or 1–4 are efficiently bound. Furthermore, in vitro translated PAH2–3 was also efficiently bound by the GST–CTCF zinc-finger fusion protein. These data indicate that PAH domain 3 of SIN3A is involved in binding of the CTCF zinc-finger domain. Similar results were achieved with the full-length CTCF molecule fused to GST (data not shown). Since interaction of CTCF could be with more than one region of SIN3A, we tested the very C-terminal part of SIN3A for possible CTCF binding. The in vitro translated C-terminal region of SIN3A (amino acids 1001–1219) showed efficient binding to the zinc-finger domain of CTCF as well as to full-length CTCF (Fig. 3B).

Thus, the CTCF zinc-finger domain is bound by two different regions of SIN3A, one region containing the PAH3 domain and another region encompassing the C-terminal 200 amino acids.

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**Figure 3.** Binding of CTCF to SIN3A. (A) In vitro translated SIN3A binds to GST–CTCF fusions. The indicated GST fusions were expressed in E.coli, affinity purified and incubated with a mixture of in vitro translated and [35S]methionine-labeled SIN3A. Lane 1 contains 10% of the input used for the precipitations. The positive control, GST–N-CoR, bound SIN3A (lane 8), whereas GST alone (lane 2) resulted in no binding. (B) Endogenous SIN3A protein from HeLa nuclear extract binds to GST–CTCF. The indicated GST–CTCF fusions were used to precipitate proteins from HeLa nuclear extracts. The precipitates were analyzed by a western-blot using an antibody directed against SIN3A.

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**Figure 4.** The zinc-finger domain of CTCF interacts with two regions of SIN3A. (A) Schematic representation of mSin3A indicating the characteristic PAH domains (53). The deletion constructs used are indicated below. (B) Binding of mSin3A deletions to CTCF in vitro. The indicated [35S]labeled full-length SIN3A (SIN3A-FL) and SIN3A deletions were incubated with the GST domain (G) or with the fusion of GST with the CTCF zinc-finger domain (G-ZF). The input (I) is 10% of the in vitro translated product used in the assay. The in vitro translated C-terminal region of SIN3A (C-TERM) was incubated with GST or the GST fusion with full-length CTCF (GST-FL) or with GST fused to the CTCF zinc-finger domain (GST–ZF).
CTCF recruits histone deacetylation activity

Several authors have demonstrated that SIN3A can bind to histone deacetylases (14,22,23,25–28). Therefore we wondered whether CTCF can bind to nuclear complexes containing histone deacetylase activity. Again, E.coli expressed GST–CTCF was used to bind factors provided by nuclear extracts from HeLa cells. The bound material was assayed for enzymatic activity resulting in histone deacetylation (20). HDAC activity is readily detectable in the GST–CTCF bound fraction (Fig. 5A), in contrast to the negative controls, empty beads and GST alone. The CTCF bound HDAC activity reached about half of that bound by a GST retinoblastoma protein fusion, which served as a positive control (20). To determine which areas of CTCF were responsible for this recruitment of HDAC activity, we used fusions of the individual N-terminal, C-terminal and zinc-finger domains of CTCF with GST in the HDAC binding assay (Fig. 5B). Clearly, full-length CTCF as well as the zinc-finger domain containing constructs bind an activity from HeLa nuclear extracts, which mediates deacetylation of histones. Neither the N-terminal domain nor the C-terminal region of CTCF alone show HDAC activity above background. Since in vitro translated HDAC-1 or baculovirus expressed HDAC-1 does not bind to GST–CTCF (data not shown), we conclude that HDAC binding to CTCF is indirect. The likely mediator of HDAC binding is SIN3A, which binds directly to CTCF (see above) and which has been shown to bind directly to HDACs (14,22,23,25–28).

In order to test the functional relevance of HDAC activity in the context of CTCF mediated repression we tested the effect of an HDAC inhibitor. Although effects of histone acetylation and deacetylation can be studied in transient transfection protocols, we decided to generate cell pools which have reporter gene sequences stably integrated into the genome. For this purpose we used NIH 3T3 cells that contain a stably integrated reporter gene p17mer6xs-tk-CAT. A pool of these cell clones shows a reporter gene driven CAT activity, which is high enough to analyze transcriptional repression. Transient transfection of an expression plasmid coding for a Gal DNA binding domain fused to the zinc-finger plus C-terminal region of CTCF results in measurable repression (Fig. 6). As expected, this repression is much lower than that achieved with transient co-transfection of the reporter plasmid (compare Fig. 2), since the expression plasmid is only taken up by ~10 % of the cells (measured by transfection of an EGFP expression plasmid; data not shown). Addition of the HDAC inhibitor, TSA, clearly interferes with the CTCF induced repression (Fig. 6), indicating that recruitment of HDAC activity is contributing to the repression mediated by the zinc-finger plus C-terminal region.

Thus, CTCF, which is able to function as an insulator protein (9), harbors an autonomous transcriptional silencing domain which is able to recruit histone deacetylase activity.

DISCUSSION

The multivalent zinc-finger protein CTCF has been shown to be involved in transcriptional enhancement as well as in silencing (3) (2,6–8). In addition, CTCF has been shown to be required for insulator function (9). Until now a mechanism or a co-factor has not been determined for either of these functions. Here we show that CTCF comprises autonomous silencing domains which mediate transcriptional repression when tethered to a promoter sequence. At least one of these domains, the zinc-finger region of CTCF, binds SIN3A and recruits histone deacetylation activity. For the SIN3A co-repressor we identified two different domains that interact independently with the CTCF zinc-finger cluster. One of these regions
contains the PAH3 domain, which has previously been shown to be able to interact with the co-repressor SMRT and with TFIIB (27). The other SIN3A region that interacts with CTCF (the C-terminal 200 amino acids) has not yet been demonstrated to independently bind to other factors. Whether these two domains bind to the zinc-finger cluster simultaneously or whether they constitute alternative binding targets remains to be clarified.

Several other transcriptional factors involved in repression contain zinc-fingers, and for some of these factors co-repressors have been identified that bind to specific domains required for repression of transcriptional activity. These repression domains have been located both outside as well as within the zinc-finger domains. Based on the identity and the function of the co-repressors, a mechanism of transcriptional repression by these factors could be proposed. The POZ domain, found outside of the zinc-finger region of the lymphoma oncogene BCL6/LAZ3 and in many other zinc-finger containing transcriptional repressors, has been shown to bind directly the co-repressors N-CoR and SMRT (29–31). Since these co-repressors have been demonstrated to recruit HDAC proteins, a mechanism of transcriptional repression by zinc-finger domains has been located both outside as well as within the domain containing repressors.

The KRAB domain of many Krüppel-like zinc-finger proteins interacts with the co-repressor KAP-1/TIF1β (32–35). Binding of KAP-1 to members of the HP1 protein family suggests a possible role of these co-repressors in heterochromatin formation. The co-repressors NAB1/2 have been shown to interact with the repression domain of zinc-finger transcription factors encoded by the immediate-early genes (36,37). A functional role for these co-repressors has not yet been established, but the finding that NAB molecules are able to multimerize suggests a repressive chromatin structure may also be involved.

Although zinc-finger domains by themselves have been shown in many cases to mediate protein–protein interactions (reviewed in 38), for the transcriptional repressors only a few zinc-finger domains have been demonstrated to bind co-repressors. A single zinc-finger motif within the transcription factor REST is required for CoREST interaction, a co-repressor required for regulation of neural-specific genes (39). The mechanism of repression by CoREST is however unknown. The zinc-finger protein ETO binds directly the co-repressors N-CoR/SMRT but in contrast to BCL6, co-repressor binding to ETO is mediated by two unusual zinc-finger motifs present at the C-terminus (40,41).

Co-repressor binding by the zinc-finger protein GATA again is different from the previous examples. In this case, the interacting partners (FOG1/2) contain zinc-fingers that are involved in binding to the zinc-finger domain of GATA (42–44). This repression mechanism may be mediated by a HDAC, since FOG interacts with CtBP2 (42,43), which is very similar to CtBP1 for which HDAC binding has been demonstrated (45).

Here we show that the zinc-finger domain of CTCF binds directly to SIN3A, which is different from all the other zinc-finger repressors. This direct SIN3A binding is very likely responsible for complexing the HDAC activity. Such a connection of SIN3A and HDAC proteins has been shown for HDAC1 and HDAC2 (reviewed in 46). In addition an interaction of SIN3A with HDAC7 has been published recently (47). Since a direct binding of HDAC-1 to CTCF could not be demonstrated (data not shown) we suggest that CTCF recruits the SIN3–HDAC complex.

The zinc-finger domain of CTCF is 100% identical between chicken and man. This domain shows sequence-specific binding to a large number of different response elements. Specificity of binding is achieved by the usage of different groups of individual zinc-fingers (3–5). Potentially one could envisage that binding of zinc-fingers to SIN3A may be modulated by different DNA response elements, nevertheless, simultaneous binding of zinc-finger to DNA and proteins seems to be possible (reviewed in 38).

A specific property of CTCF is that some CTCF binding sites are found in the vicinity of TR binding sites. This is the case for the chicken lysozyme silencer element (S-2.4) which consists of two binding sites, one for TR and one for CTCF. Interestingly, both factors synergize in repression in the absence of ligand as well as in activation in the presence of thyroid hormone (2,3). A similar functional synergy of these two proteins has also been found for a negative TRE downstream of an unknown gene (5,48). For this element, synergistic effects of the thyroid hormone are reversed such that thyroid hormone mediated transcriptional repression depends on the CTCF binding site.

Recently it was demonstrated that CTCF is able to interact directly with TR in vitro (49). The TR itself has been shown to bind a complex of N-CoR/SMRT and SIN3A as well as HDACs (44,22). Thus, synergy in repression may be attributed to a higher efficiency of HDAC recruitment in the presence of CTCF plus TR. Currently we are testing whether HDAC binding to TR complexes is influenced by CTCF and whether the CTCF recruitment of HDAC is modulated by the hormone-bound or unbound TR.

For most of the zinc-finger repressors and their co-repressors a potential effect on modulating chromatin structures has been shown. Recently, CTCF has been demonstrated to be required for the enhancer blocking activity of vertebrate insulators (9). Molecular mechanisms mediating this enhancer insulation or blocking activity have been discussed controversially (reviewed in 50). A change in chromatin acetylation has been connected to the presence of insulators. A sharp transition is found at the locus of the insulator element demonstrating histone hyper-acetylation of the active chromatin domain and a lower level of acetylation outside of the domain, which is marked by insulator elements (31). Furthermore, maintenance of transgene activity and histone hyper-acetylation have been found to depend on the presence of flanking insulator elements leading to the hypothesis that insulators prevent histone deacetylation of transgenes (52). Our finding that the insulator protein CTCF itself is able to bind histone deacetylases argues for the necessity of directional components that prevent deacetylation of the gene containing unit flanked by insulator elements.

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