Cell Growth Inhibition by the Multifunctional Multivalent Zinc-Finger Factor CTCF


Abstract

The 11-zinc finger protein CTCF-binding factor (CTCF) employs different sets of zinc fingers to form distinct complexes with varying CTCF-target sequences (CTSSs) that mediate the repression or activation of gene expression and the creation of hormone-responsive gene silencers and of diverse vertebrate enhancer-blocking elements (chromatin insulators). To determine how these varying effects would integrate in vivo, we engineered a variety of expression systems to study effects of CTCF on cell growth. Here we show that ectopic expression of CTCF in many cell types inhibits cell clonogenicity by causing profound growth retardation without apoptosis. In asynchronous cultures, the cell-cycle profile of CTCF-expressing cells remained unaltered, which suggested that progression through the cycle was slowed at multiple points. Although conditionally induced CTCF caused the S-phase block, CTCF can also arrest cell division. Viable CTCF-expressing cells could be maintained without division for several days. While MYC is the well-characterized CTCF target, the inhibitory effects of CTCF on cell growth could not be ascribed solely to repression of MYC, suggesting that additional CTS-driven genes involved in growth-regulatory circuits, such as p19ARF, are likely to contribute to CTCF-induced growth arrest. These findings indicate that CTCF may regulate cell-cycle progression at multiple steps within the cycle, and add to the growing evidence for the function of CTCF as a tumor suppressor gene.

Introduction

CTCF is the ubiquitously expressed gene upregulated during the S-G2 stage of the cell cycle. It encodes a nuclear factor containing three major, functionally distinct regions with amino acid sequences that were maintained practically identical throughout vertebrate evolution: a DNA-binding domain, composed of the 11 ZFs, and two flanking trans-acting transcriptional repressor/activator regions that account for approximately two-thirds of the entire protein (reviewed in Ref. 1). Recent review of the literature (1) established CTCF as a true “multivalent multifunctional” protein that utilizes different sets of ZFs to form distinct complexes with varying ~50-bp CTSs that mediate distinct functions in regulation of gene expression. These functions include context-dependent promoter repression or activation, creation of modular hormone-responsive gene silencers, and formation of diverse vertebrate-enhancer blocking elements (chromatin insulators or boundaries). Functions of varying CTCF/CTSS complexes may be regulated by posttranslational protein modifications (2); by physical interactions with other multifunctional nuclear proteins, which include, among others, RNA/DNA binding factor YB-1 (3) and the repression-associated mSin3A/HDACs (4); and by attenuation of the interactions with DNA via specific methylation of CpG pairs involved in recognition of specific CTSs by the protein (5). For example, the latter class of conserved targets that require particular sets of CTCF ZFs for formation of the very-high-affinity complexes with CTCF, were characterized (see Ref. 1 for review) within the ICR between growth-regulating gene IGF2 (6) and a candidate tumor suppressor gene, H19 (7). Specific CpG methylation eliminates interaction of CTCF with the ICR, allowing the protein to distinguish normally differentially methylated maternal versus paternal IGF2/H19 alleles in vivo (5). Methylation-regulated formation of CTCF/ICR complexes controls activity and conformation of the chromatin insulator that regulates imprinted IGF2 and H19 expression (see Ref. 1 for review).

In addition to the IGF2/H19 ICR CTSs, critical regulatory regions at the promoters of vertebrate MYC oncogenes have been shown to contain CTSs that mediate negative transcriptional control by CTCF (8, 9) modulated by the COOH-terminal phosphorylation (2). Moreover, a number of novel functional CTSs were identified; in respect to cell proliferation control, some of these are neutral, whereas some others are important, e.g., the CTSs in mouse/human PLK and p19ARF genes and mouse/human PIM1 oncogene among others (see Ref. 1 for review).

Disrupting the spectrum of functional CTCF/CTSS complexes either (a) by selective ZF point-mutations observed in some tumors with frequent LOH at CTCF locus mapped on chromosome 16q22 (10) or (b) by abnormal CpG-methylation of CTSs that constitute insulator sites upstream of IGF2, observed in tumors with loss of imprinting, is associated with cancer development (reviewed in Refs. 1 and 11). These findings provisionally defined CTCF as a tumor suppressor gene. However, despite the fact that CTCF emerged as an important player in networks linking expression domains with epigenetics and cell-growth regulatory processes, there was no investigation of the possible effects, if any, of CTCF on cell growth. We expected that a cumulative effect of CTCF on CTS-driven growth-regulating genes
would be suppression of cell growth. Here we provide, for the first time, direct evidence in support of this suggestion. In different experimental systems, ectopic expression of CTCF does not lead to an acute cell death but results in a severe cell-growth inhibition involving a nearly-complete blockade of DNA replication and cell division. Together, these events lead to a dramatic inhibition of cell clonogenic capacity. Our results, obtained with transiently and stably transfected GFP-CTCF fusion proteins and with the level-controlled inducible CTCF, ruled out any possibility of a trivial toxicity effect. We also show that one of the major growth-controlling CTCF-target genes, MYC, could not alone fully account for these effects of CTCF.

Materials and Methods

Expression Vectors. For stable selectable expression of CTCF, we constructed a bi-cistronic vector, pCIIN, which permits coupled coexpression of CTCF and of the selectable neoR marker (Fig. 1A). The nonselectable pCI/CTCF vector based on the pCI construct (Promega) was described earlier (9). “Green CTCF” (in-frame fusion of CTCF and EGFP) was prepared by in-frame ligation of the EcoRI-Agel fragment from the CTCF clone p7.1 into the EcoRI and Xmal sites of the pEGFP-N1 (Clontech). As a control, the fusion of EGFP with the three NLSs from the SV40 large T-antigen was also prepared (Fig. 2A). The MYC-expression vector was described earlier (12). The pLNCG vector contains the EGFP-coding sequence from the pEGFP-1 plasmid (Clontech) cloned into the retroviral vector pLNCX under the internal CMV promoter.

Conditional CTCF-Expression System. The pLK-neo dex-responsive vector was of a variant MMTV LTR promoter and a neoR gene in a SV40 transcription unit (13) was used to construct pLK-neo/chtCTCF containing full-length 3.8-kb chicken CTCF cDNA (Ref. 14; Fig. 3). To establish a conditional system, CTCF-expressing or the empty pLK-neo control vectors were transfected into NIH 3T3 cells. G418-resistant clones were isolated and analyzed for dex-controlled CTCF expression (Fig. 3).

Cell Lines, Transfections, and Colony Assays. NIH 3T3, 293, and HeLa cells were grown in DMEM supplemented with 10% calf serum; PC3, K562, and Raji cell lines were grown in RPMI supplemented with 10% FCS. These cells were transfected by electroporation with a Bio-Rad apparatus at 270 V and 960 μF. The “no-Myc” TGR15 and control cells (15) (kindly provided by J. Sedivy, Brown University, Providence, RI) were transfected by the CaPO4-mediated procedure. Adherent cells were plated in growth medium supplemented with 400 μg/ml of G418. After 15–21 days, antibiotic-resistant colonies were stained with Coomassie Blue and counted. For K562 and Raji cells, 20,000 cells were seeded in Iscove’s medium containing 15% calf serum, 0.3% agar (Difco), and 400 μg/ml G418. Thirty 35-mm plates were prepared for each transfection. Control plates without G418 were seeded with 2,000 cells.

Analyses of CTCF Effects on Cell Division. To study cell division of CTCF-overexpressing cells, we utilized a technique based on the sequential halving at each division step of an irreversible cellular fluorescent stain, CFSE (Molecular Probes, Eugene, OR; Ref. 16). Human 293 cells were cotransfected with either “empty” vector pCI (15 μg) or pCI/CTCF (15 μg), and the pLAPSN vector (1.5 μg) expressing AP. After incubation for 48 h to allow protein expression from transfected plasmids, cells were harvested and stained with biotinylated mouse antibody against human AP (clone SB6; Dako). AP staining was revealed by incubation with streptavidin-phycocerythrin. AP-positive, PI-excluding viable cells were obtained with >95% purity using a FACS Vantage II (Becton Dickinson, Palo Alto, CA). One set of these two populations of transfected cells, AP(+)CTCF(−) and AP(+)CTCF(+), was immediately stained with 10 μl CFSE (16). Within 3 h of CFSE staining (day 0), live (PI-negative) cells were analyzed for CFSE distribution, and then, on the following 3 days, were analyzed using the FACS Calibur flow cytometer (Becton Dickinson) to assess cell division. The protocol for these experiments, outlined in Fig. 4A, also included monitoring of cell proliferation in the second set of the sorted cells cultured for 7 days without selective pressure.

Cell Cycle Analyses and Proliferation Assays. For cell cycle analysis of transiently transfected cells, human 293 cells were cotransfected with both the pLNCG, which expresses neo and GFP (1.5 μg) to mark transfected cells, and either pCI/CTCF or empty pCI vector (15 μg). After 48 h, cells were analyzed on the FACS Vantage II for GFP expression and cell cycle as described previously (17). To observe effects of dex-inducible CTCF expression on cell proliferation, 0.5 × 10⁶ control cells, or cells carrying either the empty vector pLK-neo or the pLK-neo/chtCTCF vector, were plated into 6-well plates with...
RESULTS AND DISCUSSION

In preliminary experiments, we found it impossible to produce stable packaging cells for retroviral constructs designed to express full-length CTCF. In attempts to identify a cell line that could tolerate CTCF expression, we generated series of cell lines stably transfected with full-length CTCF-containing vectors. Consistently, there was a marked reduction in colony formation after selection (Fig. 1B, left panel). Using the pCIN-based bi-cistronic expression vectors, cell-growth suppression by the ZF domain was significantly less than the growth inhibition noted with the full-length CTCF, even though the levels of expression were comparable (not shown). In contrast, the vector harboring only the COOH-terminal domain (CTCF-C) exhibited no significant suppressive activity (Fig. 1B, middle panel). In these experiments, initial expression levels of all three His-tagged CTCF proteins, estimated by immunoblotting with anti-tag antibodies and 28-fold at day 10. Cell cycle analyses of GFP-expressing cells expressing CTCF was reduced 45- to 70-fold at day 7 after transfection, consistent with the marked reduction in colony formation after selection. Colonies that grew under G418 selection might derive from cells with a low level of the CTCF-IRES-neo mRNA, which produced amounts of CTCF insufficient for growth inhibition but sufficient levels of neomycin phosphotransferase to maintain drug resistance. Quite unexpectedly, we found that colonies surviving selection did not express detectable levels of ectopic CTCF (not shown). The COOH-terminal phosphorylation of CTCF, which modulates transcriptional repression mediated through binding to CTS in MYC, appears to play an important role in blocking cell clonogenicity (2). The results of these studies, thus, suggested that CTCF might suppress cell growth, induce cell death, or both.

Because expression of CTCF and Neo was coupled in the bi-cistronic vectors, it was possible that a link between levels of neo and CTCF expression could influence outcome of the experiments described above. To avoid this potential bias, we compared the effects of CTCF on cell clonogenicity in the presence and absence of selection. A GFP-expression plasmid was cotransfected either with the CTCF-expressing vector, pCI/CTCF, or with the empty control pCI vector in 293 cells. Cotransfection efficiency was equal for both vectors: 46–47%. After 48 h, equal numbers of cells, sorted for GFP expression, were seeded in media with or without G418. Because the GFP plasmid was cotransfected at a 1:1 ratio relative to the pCI plasmids, nearly all GFP-positive cells should bear the second vector, either control or CTCF-expressing. Whether selected or not, colony formation by cells expressing CTCF was reduced 45- to 70-fold at day 7 after transfection and 28-fold at day 10. Cell cycle analyses of GFP-expressing cells, either CTCF-transfected or control, revealed no differences, which indicated that CTCF-induced growth inhibition did not result in an accumulation of cells at any particular checkpoint in the cell cycle.

To directly monitor CTCF in cells, we made constructs expressing green CTCF, an in-frame fusion of CTCF and GFP (Fig. 2). In transiently-transfected 293 cells, ~30% of cells manifested green nuclear fluorescence with control, nuclear-localized NLS-GFP, and CTCF-GFP proteins (Fig. 2, B and D). No dead cells with green fluorescence were observed for 3 days, which indicated that expression of ectopic CTCF-GFP was not immediately toxic. After selection...
for stable expression, 90% of colonies transfected with NLS-GFP expressed GFP (Fig. 2C). In striking contrast, colony formation by cells transfected with green CTCF was 10% that of control (Fig. 2C), and none of the colonies expressed CTCF-GFP (Fig. 2E). Thus, expression of CTCF, while not acutely toxic for cells, is incompatible with cell growth. This concept was reinforced by studies of BrdUrd incorporation in transiently transfected cells that revealed essentially no uptake in cells expressing green CTCF (not shown).

To study CTCF-induced cell growth inhibition in more detail, we developed a system for conditional expression of chicken CTCF in mouse cells (Fig. 3). Using affinity-purified polyclonal rabbit antibodies, Ab2, which distinguish avian and mammalian CTCFs because of the minor difference in the NH2-terminal sequences (1), we could specifically monitor production in mammalian cells of CTCF not tagged with any foreign peptide. NIH 3T3 cells were stably transfected with the pLK-neo/chCTCF (dex)-responsive vector (Fig. 3A) or empty pLK-neo as a control. Randomly picked colonies were expanded and cultured for several weeks in no-dex media to establish cell lines. Two single-cell-derived cell lines 232 and 242, which on dex-stimulation showed similar CTCF levels detected with Ab2 in nuclei of all cells, were selected for detailed analyses. dex-dependent expression of exogenous CTCF in these cell lines was verified by both immunofluorescence and Western immunoblotting (Fig. 3, B and C).

Control cells plus-or-minus empty vector plus-or-minus dex grew rapidly during the first 4 days of culture and reached a plateau at high density (Fig. 3D). Similar results were obtained with cells containing the CTCF vector. In the absence of dex, when no “leaking” of exogenous CTCF could be detected by Western blot assay (Fig. 3B), 232 cells continued to grow logarithmically for 5 days until reaching confluence (Fig. 3D). In sharp contrast, cells with the CTCF vector, induced with dex, exhibited only doubling (approximately) in cell number in 6 days. Again, overexpression of CTCF was not associated with the appearance of increased numbers of apoptotic cells. Using the same system, BrdUrd incorporation studies showed that ~95% of control cell populations in log growth phase exhibited uptake (Fig. 3E), whereas cells expressing CTCF were almost completely negative. In addition, cell cycle studies showed no differences between CTCF-positive and CTCF-negative cultures (data not shown), a finding fully consistent with the results from transient expression systems. Because both 232 and 242 cell lines gave practically identical results, clonal variations in response to conditional CTCF expression could be ruled out.

The lack of notable changes in the cell cycle profiles in cells arrested by CTCF, expressed by different methods described here, could be explained if, in addition to the S-phase block, CTCF also inhibits progression through other major phases of the cell cycle. This tentative conclusion was evaluated using cells marked with CFSE to monitor cell-traversing division stage (Fig. 4A). Equal partitioning of CFSE between daughter cells leads to a stepwise diminution in fluorescence intensity with the completion of each cell cycle (16). In this experiment, 293 cells were transfected with a 10-fold excess of CTCF-expressing or empty vector to a vector expressing AP, AP-expressing CTCF(−) and CTCF(+) cells were separated by flow cytometry; one-half were followed for CFSE levels and one-half for proliferation. The results of these studies showed that the effect of CTCF is progressively more pronounced for each successive day of the analysis with an approximately 10-fold difference in the percentage of live undivided cells by day 3 after staining with CFSE. A rather remarkable finding was that, after 3 days in culture, when nearly 98% of control cells had undergone at least one round of division, ~20% of CTCF-expressing cells had not divided (Fig. 4B). Thus, ectopic expression of CTCF resulted in a marked inhibition of cell division, keeping significant percentage of cells senescent, but not dying, for several days. This reduced rate of cell division was also reflected in a nearly 7-fold difference in cell recovery after 6 days in culture (Fig. 4C).

Relation of MYC regulation to growth suppression by CTCF is an important issue because MYC is the well-established target for CTCF,
and, in many cell types, down-regulation of MYC is associated with cessation of division and terminal differentiation or apoptosis. Although CTCF has multiple targets, it was nonetheless possible that control of MYC alone was critical to regulating cell growth. To evaluate this possibility, we first analyzed cells cotransfected with the expression vectors for both CTCF and MYC (Fig. 1B, right panel). These studies showed that MYC only partially alleviated the block to cell growth that was imposed by CTCF. In a second set of experiments, Rat1-derived TGR cells, deficient in expression of all MYC family members, were stably transfected with pCIIN-CTCF (Fig. 1B, left panel); the frequency of clonal recovery did not differ significantly from that of control cells that were suppressed by CTCF as for the other lines examined. Together, these two experiments suggest that, although regulation of MYC may contribute to growth suppression by CTCF, another gene or genes may be of greater importance.

Several lines of evidence, summarized in Ref. 1 suggest that CTCF has a general role in neoplasia: (a) many of CTCF-regulated genes are frequently deregulated in human tumors; (b) the human CTCF locus localizes within one of the smallest regions of overlap for LOH frequently observed at chromosome 16q22.1 in different cancers; (c) cancer-associated missense somatic mutations in CTCF ZFs selectively impair binding to CTSs in growth-controlling genes versus growth-inert genes; and (d) an aberrant region-specific CpG methylation eliminates CTCF-DNA interactions at certain CTSs in tumors.

All of these data together and all of those above suggest that CTCF expression markedly inhibits cell growth of a number of different immortalized mammalian cell lines, including those representing fibroblasts and epithelial and hematopoietic cell lines. Perhaps the most remarkable aspect of these studies was the demonstration that none of the colonies that were formed were of a variety of cell types initially expressing CTCF and Neo tested positive for CTCF protein. This was not an artifact of stable transfection and Neo selection, because identical conclusions could be drawn from the studies of cells transiently expressing EGFP-CTCF fusion protein. The ability of CTCF-expressing 293 cells to remain viable and to slowly divide (or not to divide at all) over several days to a week is, thus, unusual and additional studies of this exception could provide important clues to the identity of genes that are critical for growth regulation by CTCF. The fact that 293 cells lack functional p53 and Rb is certainly worth noting.

Because CTCF expression markedly inhibits cell growth of a number of different immortalized mammalian cell lines, including those representing fibroblasts and epithelial and hematopoietic cell lines, control of CTCF expression is a potential novel pathway of cell proliferation control conserved in mammals.
regulation of CTCF itself that may include (as reviewed in Ref. 1) regulation of CTCF promoter activity during cell cycle, posttranslational modifications of the protein including phosphorylation, interactions with multifunctional protein partners, and other mechanisms yet to be discovered. Furthermore, it is conceivable that effects of CTCF on cell cycle progression may depend not only on transcriptional regulation of a variety of target genes by CTCF, but also on DNA-independent direct interactions of CTCF with certain components and regulators of the cyclin-CDK complexes, and with some of the proteins directly involved in replication machinery and in cytokinesis. Our preliminary studies of numerous CTCF-interacting functional partners, identified by an affinity chromatography of total cell extracts on matrix-immobilized pure recombinant CTCF (3), by GST-CTCF-mediated protein “pull-down” approach with nuclear extracts, and by the yeast two-hybrid method, have provided evidence that CTCF may indeed be a subject of such interactions. Thus, CTCF functions may go beyond being just a transcription factor. However, it is clear that better understanding of cell growth regulation by CTCF will require an in-depth evaluation of a complete repertoire of CTCF target genes and CTCF-interacting partners.

Acknowledgments

We thank Drs. I. R. Ghattas and J. E. Majors (Washington University School of Medicine, St. Louis, MO) for providing the pLZIN plasmid containing the IRES-Neo cassette, Dr. N. Fasel (University of Lausanne, Epalinges, Switzerland) for the pLKneo plasmid, and Drs. J. Breen and R. Ohlsson for critically reading the manuscript.

References

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