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The D-domain of Sp3 modulates its protein levels and activation of the p21$^{CIP1/WAF1}$ promoter

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Abstract

A variety of signals result in the transcriptional induction of the p21$^{CIP1/WAF1}$ promoter and both Sp1 and the related Sp3 proteins have been implicated in this induction. We have characterized the role of the C-terminal D-domains of both Sp1 and Sp3 proteins in the activation of this promoter in response to butyrate treatment of Hep G2 cells. We have defined a negative regulatory domain present in the C-terminus of Sp3. This domain decreases Sp3 protein levels, and this property can be transferred to Sp1. Changes in Sp3 protein levels may bring about growth arrest through the induction of inhibitors of the cell cycle such as p21$^{CIP1/WAF1}$.

Keywords: Sp1; Sp3; p21$^{CIP1/WAF1}$; Transcription; Cyclin-dependent kinase inhibitor; Zinc finger; Protein; Regulation; Differentiation; Growth arrest

The p21$^{CIP1/WAF1}$ gene is a key negative regulator of the cell cycle. It is a member of the dual specificity class of cyclin-dependent kinase inhibitors and interacts with cyclin D containing complexes. The activation of the p53 gene in response to DNA damage results in an increase in p21$^{CIP1/WAF1}$ transcription through two p53 response elements located approximately 2 kbp upstream of the start site [1]. This in turn brings about growth arrest through the ability of p21$^{CIP1/WAF1}$ protein to inhibit cyclin/cyclin-dependent kinase activity. The p21$^{CIP1/WAF1}$ gene is also induced during terminal differentiation and in response to a wide variety of treatments and signals. These include growth factors such as TGF-β [2], NGF-1 [3], PDGF [4] as well as chemical inducers such as okadaic acid [5], trichostatin A [6], and butyrate [7]. In all of these cases, a series of Sp1 recognition elements have been identified as being essential for p21$^{CIP1/WAF1}$ induction.

One Sp1 site in particular, located at approximately nt-93, has been shown to be critical for the transcriptional responsiveness of the p21$^{CIP1/WAF1}$ promoter to many of the known inducers. The spacing between this element and more proximal parts of the promoter has been shown to be important for the appropriate regulation of the promoter suggesting this element does not act alone [2]. Activation of the p21$^{CIP1/WAF1}$ promoter appears to involve Sp1 [3,8–12] and the closely related protein Sp3 [6,13–15].

Sp1 and Sp3 are ubiquitously expressed zinc finger proteins which share a high level of sequence identity through their zinc finger domains. Both are required for viability of the mouse, though Sp1 null mice are embryonic lethal [16], while the Sp3 null dies at birth [17] and appears to have defects in hematopoiesis [18]. They bind to identical recognition elements and depending on the promoter context both can function either as positive or negative transcriptional activators [19,20]. There have been four domains mapped in Sp1 which are required for transcriptional activation. The A and B domains are the classic glutamine rich domains which interact with

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$TAFII_{110}$ [21,22]. There is also a C domain which is rich in charged amino acids and which is amino terminal to the zinc finger domain. The D-domain is comprised of the C-terminal region, adjacent to the zinc finger domain [23]. This domain has been implicated in the formation of higher order structures with other Sp1 molecules [24,25]. The combined zinc and D-domain regions are known to interact with a variety of proteins such as YY1 [26], E2F1 [27], GATA-1 [28], HDAC1 [29] and corepressors such as SMRT, NcoR, and Bcor [30]. Previously, we have demonstrated that phosphorylation of Sp1 decreases its DNA binding activity and that this phosphorylation increases during terminal differentiation in vivo [31]. This phosphorylation may be mediated by casein kinase II and involves sites in both the zinc finger domain and in the D-domain [32]. Sp3 also contains a C-terminal domain of a similar size to that of Sp1, however no function has been associated with this region, and it has only limited homology to the Sp1 D-domain. Given the role of these domains in mediating protein–protein interactions, and their modification during terminal differentiation, it suggests that they might play a role in the induction of the $p21^{CIP1/WAF1}$ promoter.

Butyrate treatment of Hep G2 cells induces $p21^{CIP1/WAF1}$ expression and results in the growth arrest of these cells [7]. Cotransfection of expression vectors for wildtype Sp1 or Sp3 produced a limited increase in the reporter construct only with Sp3. In order to examine the role of the D-domains of both proteins in mediating transactivation, chimeric proteins were created in which the D-domains of Sp1 and Sp3 were exchanged. This had no effect on the activity of the Sp1 chimera but the transactivation activity of the Sp3 protein with the Sp1 D-domain was dramatically increased. Deletion analysis of the Sp3 chimeric protein suggested that it was the loss of inhibitory Sp3 D-domain sequences, rather than the gain of Sp1 sequences which was important for activation. Analysis of protein levels revealed that removal of this region increased protein levels significantly. This modulatory element could be localized to a region of some 40 amino acids. Transfer of this element to Sp1 resulted in dramatically decreased protein levels, indicating it is a portable functional domain. Sp3 protein levels increase during Caco-2 cell differentiation and are associated with activation of $p21^{CIP1/WAF1}$ [15] and Sp3 protein levels also increase during adipocyte differentiation [33] suggesting that modulation of its levels may be an important regulatory mechanism.

Materials and methods

Plasmid constructs

Cloning HA-tagged FL Sp1 and FL Sp3 in pSCT-Gal. An HA tag was positioned at the 5′ end of Sp1 using a PCR based approach with the ULT Sp1-GGA GAT CTA GAC CAT GGC ATA CCC ATA CGA CGT GCC TGA CTACGC CTC CAC AGG TGA GC and ULT Sp1 comp-CAG CTG TTG CCC ATC AAC GG primers. This was also done for Sp3 using the RealULT Sp3-GGG GAT CCA CCA TGG CAT ACC CAT ACG ACG TGC CTG ACT ACG CCT CCG CGG CCG CCG CAG C and RealULT Sp3 comp-ATT GTC TGA GAA CTG CCC G primers. Both of these fragments were then combined with the 3′ parts of both genes cloned into the pSCT-gal vector, which is a CMV based mammalian expression vector.

Cloning HA-tagged FL Sp1/3D and Sp3/1D vectors. Sp3 sequences corresponding to the D-domain were substituted for the D-domain of Sp1 using primers corresponding to the fusion junction Sp1/3D plus-CA GAA TAA GAA GGG AAT TCA CTC TAG CAG TAC and Sp1/3D negative-GTA CTG CTA GAG TGA ATT CCC TTC TTA TTC TGG as well as a 5′ primer from Sp1 and a 3′ primer from Sp3. Similarly, the Sp1 D-domain was substituted for the Sp3 D-domain using the Sp3/1D plus-CCA GAA TAA AAA AGG TGG CCC AGG TGT AGC and Sp3/1D negative-GCT ACA CCT GGG CCA CCT TTT TTA TTC TGG primers in conjunction with the a 5′ primer from Sp3 and a 3′ primer from Sp1.

Cloning deletion mutants. Deletions of the Sp1/3D, Sp3/1D, and Sp3 vectors were created using PCR with primers specific to each end point and with appropriately located common primers. Additional information is available upon request. The sequence of all clones was confirmed by sequencing.

p21 93S pGL2 and pRL CMV. p21 93S pGL2, the p21 promoter reporter construct, as well as the series of mutant promoters was a gift from Xiao-Fan Wang and has previously been described [2]. pRL CMV was used as an internal control in transfection experiments and is as described by Promega.

Transient transfections

HepG2 cells were obtained from the American Type Culture Collection and were grown in minimal essential medium containing 10% fetal bovine serum and 1% penicillin and streptomycin (Gibco/Sigma). Cells were plated onto 24-well plates 24 h prior to transfection at 200,000 cells/mL in 500 μL. Transfection was carried out using polyethanolamine-25 (PEI-25), with 2 μg of DNA mixed with the reagent at a ratio of 24:1 N/P in a total of 100 μL. The DNA was added as 500 ng sample DNA, 1 μg p21 93S pGL2 reporter, 50 ng pRL CMV internal control, and 450 ng of either pSCT-Gal or the appropriate vector to make up the weight to 2 μg. Both the PEI reagent and the DNA were made in NaCl to a final concentration of 150 mM NaCl. The PEI reagent was added to the NaCl and allowed to sit for 15 min before addition to the DNA. The solution was then allowed to sit for another 10 min before being added to the cells. Cells were incubated at 37 °C for 2 h, washed with phosphate-buffered saline (PBS) and fresh medium was added. After 24 h, the medium on the cells to be induced was changed to medium containing 2 mM *N*-butyrate. Fresh medium was also added to the uninduced wells at that time. Cells were harvested with 75 μL 1× passive lysis buffer as described by Promega 24–48 h later. Dual luciferase assays were performed on 20 μL lysate using 50 μL of each luciferase reagent according to conditions described by Promega.

Western blot analysis

HepG2 cells were maintained as above. Cells were plated onto 100 mm plates 24 h prior to transfection at a 200,000 cells/mL in 14 mL. Transfection was carried out using PEI-25 as above using a total of 10 μg sample DNA and 46.7 μg salmon sperm DNA to give a total DNA weight of 56.7 μg DNA per plate. Cells were treated with butyrate 24 h post-transfection. After another 24–48 h, cells were harvested and nuclear extracts prepared. Cells were washed with PBS, scrapped and transferred to 15 mL Falcon tubes and spun at 1000*g* for