The N-terminal Region of the CCAAT Displacement Protein (CDP)/Cux Transcription Factor Functions as an Autoinhibitory Domain that Modulates DNA Binding*

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The CCAAT displacement protein/Cut homeobox (CDP/Cux) transcription factor is expressed as multiple isoforms that may contain up to four DNA-binding domains: Cut repeats 1, 2, and 3 (CR1, CR2, CR3) and the Cut homeodomain (HD). The full-length protein, which contains all four DNA-binding domains, is surprisingly less efficient than the shorter isoforms in DNA binding. Using a panel of recombinant proteins expressed in mammalian or bacterial cells, we have identified a domain at the extreme N terminus of the protein that can inhibit DNA binding. This domain was able to inhibit the activity of full-length CDP/Cux and of proteins containing various combinations of DNA-binding domains: CR1CR2, CR3HD, or CR2CR3HD. Since inhibition of DNA binding was also observed with purified proteins obtained from bacteria, we conclude that autoinhibition does not require post-translational modification or interaction with one or more protein partners. In addition, we show that the relief of autoinhibition can be accomplished via the proteolytic processing of CDP/Cux. Altogether, these results reveal a novel mode of regulation that serves to modulate the DNA binding activity of CDP/Cux.

CDP/Cux/Cut1 (CCAAT displacement protein/cut homeobox) belongs to a family of transcription factors present in all metazoans and involved in the control of proliferation and differentiation (reviewed in Ref. 1). The founding member of this family, the Drosophila melanogaster cut gene, takes its name from the phenotype of a viable mutant that harbored truncated (“cut”) wings (2–5). Other mutations within the cut locus are associated with defects in multiple tissues and often cause a switch in cell type identity (6–10). A “CCAAT displacement activity” was originally described as an activity present in the nuclear extracts of various types of cells that would prevent the binding of a transcriptional activator to a CCAAT motif (11–14). The CDP was eventually purified from HeLa cells and shown to be the human ortholog of cut (15, 16). The mouse ortholog was named cux in accordance with nomenclature rules (17, 18). We use the term CDP/Cux to refer to the mammalian proteins in general, whereas CDP and Cux designate the human and mouse protein, respectively.

The cux-1 knockout mice displayed phenotypes in various organs including curly whiskers, growth retardation, delayed differentiation of lung epithelia, altered hair follicle morphogenesis, male infertility, a deficit in T and B cells, and a surplus of myeloid cells (19–22). In contrast to the small size of the cux-1 knock-out mice, transgenic mice expressing Cux-1 under the control of the cytomegalovirus enhancer/promoter displayed multiorgan hyperplasia and organomegaly (23).

CDP/Cux was found to function in precursor cells of various lineages as a transcriptional repressor that down-modulates genes which later become expressed in terminally differentiated cells (13, 14, 24–26). This function was ascribed to the ability of CDP/Cux to prevent the interaction of various transcriptional activators with their binding sites, probably via its CCAAT displacement activity (27, 28). This type of repression, competition for binding site occupancy, is sometimes referred to as “passive repression,” as opposed to the mechanism of “active repression” whereby a distinct protein domain can down-modulate transcription by other means. The C-terminal domain of CDP/Cux was found to contain two active repression domains that, in a Gal4 reporter assay, were able to repress transcription at a distance from the factor binding site (29). The C-terminal domain was later shown to recruit the HDAC1 deacetylase (30). Thus, CDP/Cux has the potential to repress transcription by two mechanisms, competition for binding site occupancy and active repression (27, 29). In addition, recent findings indicated that CDP/Cux is able to participate in the transcriptional activation of specific promoters (31).

One of the most fascinating features of CDP/Cux proteins is that they contain up to four evolutionarily conserved DNA-binding domains: three Cut repeats (CR1, CR2, and CR3) and a Cut homeodomain (HD) (15, 32–34). Despite early claims made by us and others on the basis of results obtained with glutathione S-transferase (GST) fusion proteins (32–36), individual Cut repeats cannot bind to DNA on their own but need to cooperate with a second Cut repeat or with the Cut homeodomain (28). In vitro DNA binding studies revealed that
CR1CR2 makes a rapid but transient interaction with DNA, whereas CR2CR3HD and CR3HD bind more slowly, but stably, to DNA (28). These combinations of domains also exhibited different DNA binding specificities. Although fusion proteins that included the Cut homeodomain displayed preference for the ATCRAT motif (where r = A or G), the CR1CR2 domains bound better to sequences including two CRAT motifs positioned in either orientation and at various distances from one another (28, 32–35). Thus, CDP/Cux contains several DNA-binding domains that, in various combinations, can carry distinct DNA binding activities.

In cells, three major CDP/Cux isoforms have been described so far. The full-length protein, which we refer to as CDP/Cux p200, contains all four DNA-binding domains but displays DNA binding activities similar to those of CR1CR2 (37). DNA binding by CR3HD appears to be inhibited in the context of the p200, contains all four DNA-binding domains but displays so far. The full-length protein, which we refer to as CDP/Cux tinct DNA binding activities.

The protein c, which comprises CR2, CR3, and HD (37, 38), in addition to the two alternate, tissue-specific mRNA species were found to code for a CDP/Cux p75 isoform that contains only two DNA-binding domains: CR3 and HD (39, 40). Predictably, CDP/Cux p110 and p75 exhibited DNA binding properties similar to those of CR2CR3HD and CR3HD (37, 40). The three major CDP/Cux isoforms, therefore, carry different DNA binding activities and, potentially, different transcriptional functions. Consistent with their ability to make a stable interaction with DNA, the p75 and p110 isoforms were shown to be able to participate in the transcriptional activation of specific promoters (31). How CDP/Cux may function as a repressor on some promoters and as an activator on other promoters remains to be fully investigated. Interestingly, the DNA binding and transcriptional activities of CDP/Cux are regulated by a number of post-translational modifications including phosphorylation by protein kinase C, casein kinase II, and cyclin A/Cdk1, dephosphorylation by Cdc25A, acetylation by p300/CBP-associated factor, and proteolytic processing by caspase L (37, 38, 41–45). Interaction with various protein partners may also affect CDP/Cux activities (30, 46–49).

The present study was triggered from an observation made with our first CDP/Cux cDNA clones. We noticed that those cDNA clones that were incomplete at their 5’ end generated N-terminally truncated proteins that appeared to bind more efficiently to DNA than the full-length protein. We therefore decided to carry a systematic analysis of recombinant CDP/Cux proteins that were truncated at strategic positions. One question we sought to answer was why the full-length p200 protein was able to bind to DNA only transiently despite the presence of the Cut repeat 3 and the Cut homeodomain, which, on their own, are capable of making a very stable interaction with DNA. Our results indicated that the simultaneous presence of CR1 and CR2 within a protein somehow prevents stable DNA binding. In addition, our results revealed that a region at the extreme N terminus of CDP/Cux p200 is able, on its own, to inhibit DNA binding by the full-length protein or any combination of CDP/Cux DNA-binding domains.

**MATERIALS AND METHODS**

**Cell Culture and Transfection—**NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and transfected using ExGen5000 reagent (MBI Fermentas) according to the manufacturer’s instructions. Ha578T breast cancer epithelial cells were grown in Dulbecco’s modified Eagle’s medium plus 5% fetal bovine serum (50) and were transfected using GeneJuice (Novagen) according to the manufacturer’s instructions.

**CDP/Cux Isoforms—**Plasmid sequences and maps will be provided upon request. The following recombinant CDP/Cux proteins include the indicated amino acids and were expressed as histidine-tagged protein using the pTriEx-2 vector (Novagen): (a) 1–150; (b) 324–1505; (c) 547–1238; (d) 612–1238; (g) 1–1505; (h) 659–1505; (i) 1505–1818; 1505–659. Other proteins were expressed using the pm139 mammalian expressing vector (51): (e) 1–15053256–659; (f) 659–1505; (h) 1029–1505; (i), 1–1029. The PET-15b vector (Novagen) was used to express (j) 1027; 1–101/CR3HD(1125–1308); 1–183/CR3HD; 1–281/CR3HD; 1–324/CR3HD; 1–101/CR1CR2 (252–1027); 1–324/CR1CR2; 94–324/CR1CR2.

**Preparation of Nuclear Extracts—**Nuclear extracts were prepared as described (Moon et al. (37)).

**Expression and Purification of Protein Produced in Bacteria—**Bacterially expressed His-tagged fusion isoforms were expressed and purified as described (Moon et al. (37)).

**Electrophoretic Mobility Shift Assays (EMSA)—**EMSA were performed as described (Moon et al. (37)) except that we used 60 ng of poly(dI-dC) and 3 μg of bovine serum albumin as nonspecific competitors. Depending on the level of the isoform expressed, 0.5–5 μg of nuclear extract was used to obtain the same quantity of specific protein in each assay. In the case of purified proteins, 40 ng of protein was used (see Fig. 3) unless otherwise indicated (see Fig. 5). Samples were loaded on 3.5% Tris-glycine or 0.5% Tris-borate–EDTA (see Fig. 5) polyacrylamide gels.

**Oligonucleotides—**EMSA analysis was done with the oligonucleotides indicated in the figure legends. The sequences are as follows: CTGATATCGA (universal CDP/Cux consensus binding site), 5’-CTGA-

**Western Blot Analysis—**Visualization and control for protein normalization were done by Western blot analysis as described (Moon et al. (37)) using the antibodies specified in each figure.

**Luciferase Assay—**Luciferase assays were performed as described previously (37). Because the internal control plasmid cdpl is itself often repressed by CDP/Cux, as a control for transfection efficiency, the pT7-8-galactosidase protein (Sigma) was included in the transfection mix, as described previously (52). The luciferase activity was then normalized based on β-galactosidase activity.

**RESULTS**

**The Presence of Cut Repeat 1 (CR1) within CDP/Cux Determines Its DNA Binding Specificity—**Previous studies have established that the DNA binding properties of CDP/Cux isoforms are greatly affected by the presence or absence of the Cut repeat 1 (28, 37). This is illustrated in Fig. 1 with recombinant proteins c and d. Plasmid vectors expressing various CDP/Cux recombinant proteins were introduced into NIH3T3 cells. Two days later, nuclear extracts were prepared and analyzed in EMSAs. A diagram showing the maps of the recombinant proteins is shown in Fig. 1C. Western blot analysis confirmed that all recombinant proteins were expressed (Fig. 1B). Importantly, the amounts of nuclear extracts used in the Western and EMSA analyses were too small to permit the detection of endogenous CDP/Cux species in either assay (Fig. 1B, lane 1, and 1A, lanes 1 and 10). Two DNA probes were used in EMSA. One probe, which contained a direct repeat of the CRAT motif (r = A or G), was previously shown to represent an optimal binding site for the composite DNA-binding domains, CR1 and CR2 (CR1CR2) (28). The second probe included the ATCGAT sequence, which is recognized by composite domains containing the Cut homeodomain and either one or two Cut repeats (CR3HD and CR2CR3HD). The protein d, which includes CR2, CR3, and the HD, but not CR1, was able to form a complex with the ATCGAT probe but not with the CAAT/CGAT probe (Fig. 1A, compare lanes 8 and 17). Note that the addition of the CDP/Cux 861 antibody was able to supershift the retarded complex formed with the ATCGAT probe (Fig. 1A, lane 18). In contrast, the protein c, which comprises CR1, CR2, CR3, and the HD, was able to bind to the CAAT/CGAT probe but not to the ATCGAT probe (Fig. 1A, compare lanes 6 and 15). Again, the addition of a specific antibody was able to supershift the
Autoinhibition of CDP/Cux DNA Binding

An autoinhibitory domain is present at the N terminus of CDP/Cux. A, NIH3T3 cells were transfected with vectors expressing recombinant CDP/Cux proteins with N- and C-terminal deletions. Nuclear extracts were prepared and analyzed in EMSA with double-stranded oligonucleotides containing binding sites for CR1CR2 (top panel) or CR3HD (bottom panel) (28). Where indicated (+), the DNA and proteins were incubated with an antibody (Ab) that recognizes the recombinant protein, anti-hemagglutinin in the case of proteins a and b or 861 in the case of proteins c and d. B, nuclear extracts from untransfected and transfected NIH3T3 cells were separated by electrophoresis on 6% polyacrylamide gel and analyzed by Western blot with the CDP/Cux 861 antibody. C, a diagram is shown indicating the boundaries of each recombinant protein, the positions of evolutionarily conserved domains, and the region recognized by the 861 antibodies. CC, coiled-coil; HA, hemagglutinin tag.

retarded complex (Fig. 1A, lane 7). Thus, proteins c and d exhibited opposite DNA binding specificities. These results demonstrate that the DNA binding specificity of CDP/Cux isoforms is determined by the presence or absence of CR1.

The Presence of the N-terminal Region within the Full-length CDP/Cux Protein Inhibits Its DNA Binding Activity—The protein b, which also included all four DNA-binding domains, exhibited a DNA binding specificity similar to that of protein c; it bound to the CAAT/GAT probe but not to the ATCGAT probe (Fig. 1A, compare lanes 4 and 13). Interestingly, the protein a, which started at amino acid 1 and also included all four DNA binding domains, did not appreciably bind to either probe (Fig. 1A, lanes 2, 3, 11, and 12). These results suggested that the N-terminal region of CDP/Cux somehow interferes with DNA binding.

The N-terminal Region Inhibits DNA Binding by CR1CR2, CR3HD, and CR2CR3HD—To further investigate the effect of the N-terminal region, we generated shorter recombinant proteins that contained various combinations of DNA-binding domains fused or not to the N-terminal region of CDP/Cux: CR1CR2 (i and j), CR3HD (g and h), and CR2CR3HD (l and m) (diagram in Fig. 2C). Proteins were expressed in NIH3T3 cells and were tested in Western blot and EMSA analysis, as described in Fig. 1. Western blot analysis confirmed that the recombinant proteins were expressed at comparable levels (Fig. 2A). To be able to compare the results obtained with various proteins in EMSA, we used a probe that contains a direct repeat of the CGAT motif in addition to the ATCGAT sequence. This probe was previously shown to contain excellent binding sites for all combinations of Cut repeats and the Cut homeodomain (28, 37). In each case, the inclusion of various lengths of N-terminal sequences greatly reduced the ability of the proteins to bind to DNA (Fig. 2B, compare i and j, lanes 2 and 3; g and h, lanes 5 and 6; and l and m, lanes 7 and 9). These results demonstrate that the N-terminal region of CDP/Cux is able to inhibit DNA binding by various combinations of CDP/Cux DNA binding domains.

The N-terminal Region of CDP/Cux Inhibits DNA Binding without the Intervention of a Partner Protein—We considered two mechanisms to account for the inhibitory effect of the N-terminal region. This region may act as a binding site for a protein that inhibits CDP/Cux activity. Alternatively, the reduction in DNA binding may involve a direct effect of the N-terminal region. To distinguish between these two hypotheses and to map the inhibitory domain more precisely, we engineered vectors for the expression of proteins in bacteria and their purification by affinity chromatography on nickel beads. We prepared a panel of proteins that included the CR3HD or the CR1CR2 region fused to various segments of the N-terminal region (Fig. 3C). All proteins were obtained in comparable amounts (Fig. 3A, lanes 6–10, and 3B, lanes 5–8). Proteins that included the N-terminal region from amino acids 1 to 324, 1 to 281, 1 to 183, or 1 to 101 were impaired in their ability to form a complex with the DNA probe (Fig. 3A, lanes 1–4, and 3B, lanes 1–3). In contrast, the protein 94–324 was able to bind to DNA efficiently (Fig. 3A, lane 5; Fig. 3B, lane 4). In conclusion, the region from amino acids 1 to 101 is able to inhibit DNA binding by CR1CR2 or CR3HD. Furthermore, these results exclude the possibility that the mechanism of inhibition proceeds via an interaction with another mammalian protein that functions as an inhibitor of CDP/Cux activity. Instead, these results support the notion that the inhibition is a direct effect of the N-terminal region. Thus, our findings define an autoinhibitory domain at the N terminus of CDP/Cux.

Antibodies against the N-terminal Region Can Stimulate DNA Binding—DNA binding by the p53 protein cannot easily be detected in EMSA. It is believed that the C-terminal domain of the protein folds back on the specific DNA-binding domain and prevents it from interacting with DNA (53). In practice, the addition of p53 C-terminal antibodies increases its specific DNA binding activity (54, 55). By analogy with p53, we considered the possibility that antibodies that recognize the N-terminal region of CDP/Cux might alleviate the inhibitory effect on DNA binding and improve the detection of a protein/DNA complex in EMSA. We have recently raised antibodies against a GST protein containing amino acids 23–50 of CDP/Cux, Ab-23. The effect of these antibodies on the DNA binding activity of the full-length CDP/Cux protein was tested using nuclear extracts from NIH3T3 cells transfected or not with a vector expressing a recombinant full-length CDP/Cux protein. Prior to adding the double-stranded oligonucleotides to the reactions, the nuclear extracts were preincubated for 10 min in the presence of the Abs. Antibodies raised against the GST protein alone were used as control. Although the GST antibodies had no effect, DNA binding by the endogenous and the recombinant CDP/Cux protein was moderately increased when the extracts were preincubated with Ab-23 (Fig. 4, compare lane 3 with lanes 2 and 4 for the recombinant protein and compare lane 8 with lanes 9 and 10 for the endogenous protein). The recombinant CDP/Cux protein used in this experiment included a Myc epitope tag at its N terminus. We therefore
tested whether DNA binding would be increased when the reaction was performed in the presence of anti-Myc antibodies. Indeed, DNA binding was increased in the presence of Myc antibodies (Fig. 4, compare lane 5 with lanes 6 and 7). In contrast, anti-Myc antibodies had no effect on the DNA binding activity of the endogenous CDP/Cux protein, which does not contain a Myc epitope tag (data not shown). Moreover, anti-hemagglutinin antibodies, which recognize the hemagglutinin epitope tag at the C terminus of the recombinant CDP/Cux protein, did not increase DNA binding (data not shown; see also Fig. 3 in Ref. 37). Globally, these results indicate that the interaction between an antibody and the N-terminal region of CDP/Cux can stimulate DNA binding.

**The N-terminal Region of CDP/Cux Inhibits Its Transcriptional Activity**—We have previously shown that proteolytic processing of CDP/Cux at the G1/S transition generates a 110-
Autoinhibition of CDP/Cux DNA Binding

Our results showed that the N-terminal region of CDP/Cux reduces its DNA binding efficiency when assayed in nuclear extracts or with purified proteins (Figs. 1, 2, 3, and 6C). The fact that this down-regulatory effect was observed with purified, bacterially expressed proteins allows us to exclude a mechanism of inhibition that requires the intervention of other proteins that would bind to CDP/Cux in mammalian cells (Figs. 3 and 4). Instead, it appears that the N-terminal region has an intrinsic ability to inhibit DNA binding by several CDP/Cux DNA-binding domains and thus functions as an autoinhibitory domain.

The phenomenon of autoinhibition has been documented for a number of transcription factors as well as for other types of proteins (reviewed in Ref. 56). In the case of transcription factors, autoinhibition may take different forms and involve the retention of the factor outside of the nucleus, the inhibition of DNA binding, or an interaction with a protein that attenuates the transcriptional activity of the factor. An example of the latter is provided by the interaction between the lung Kruppel-like factor (LKLF) and the WWP1 ubiquitin-protein isopeptide ligase (E3) ubiquitin ligase (57). Sequestration of transcription factors in the cytoplasm or through membrane anchoring has been reported for NFκB p105, the Cubitus interruptus factor, sterol regulatory element-binding proteins (SREBPs), ATF6, and the Notch receptor (58–68). Release of inhibition in all these cases involves proteolytic processing, whether via “regulated intramembrane proteolysis” (RIP) or “ubiquitin/proteasome-dependent processing” (RUP) (69, 70). In the case of the Smad2/Smad4 heterodimer, the N-terminal inhibitory domain of Smad2 interacts with its C-terminal domain, thereby preventing the association of Smad2 with Smad4 (71). Inactivation of the Smad2/Smad4 tumor suppressors in some cancers was found to involve mutations that increase autoinhibition. DNA binding by several members of the ets gene family of transcription factors is negatively regulated by an intramolecular mechanism involving two regions flanking the DNA-binding domain (72–74). Autoinhibition of Els factors can be reinforced by phosphorylation, or alternatively, relieved by interaction with protein partners (75–80).

There are currently no structural data on CDP/Cux from which we could derive a mechanistic model of the autoinhibition. In the simplest model, the inhibitory domain may sterically mask the DNA-binding domain. Inhibition of DNA binding was observed with recombinant proteins containing CR1CR2, CR3HD, CR2CR3HD, or CR1CR2CR3HD as DNA-binding domains. Since a Cut repeat was present in all cases, this down-regulatory effect was observed with purified, bacterially expressed proteins allows us to exclude a mechanism of inhibition that requires the intervention of other proteins that would bind to CDP/Cux in mammalian cells (Figs. 3 and 4).

DISCUSSION

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In summary, our results showed that the N-terminal region of CDP/Cux reduces its DNA binding efficiency when assayed in nuclear extracts or with purified proteins (Figs. 1, 2, 3, and 6C). The fact that this down-regulatory effect was observed with purified, bacterially expressed proteins allows us to exclude a mechanism of inhibition that requires the intervention of other proteins that would bind to CDP/Cux in mammalian cells (Figs. 3 and 4). Instead, it appears that the N-terminal region has an intrinsic ability to inhibit DNA binding by several CDP/Cux DNA-binding domains and thus functions as an autoinhibitory domain.

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main (data not shown). However, these assays were designed for the detection of intermolecular interactions. We cannot exclude the possibility that the N-terminal domain might exhibit a weak affinity for CDP/Cux DNA-binding domains and that an intramolecular interaction could take place when these domains are present within the same protein. An alternative mechanism of action would be that the autoinhibitory domain forces the protein to adopt a conformation that prevents an efficient interaction with DNA. Both of these hypotheses are consistent with the findings that antibodies which recognize the N-terminal region of CDP/Cux were able, albeit weakly, to stimulate DNA binding (Fig. 4).

The fact that autoinhibition was observed with bacterially expressed proteins suggests that autoinhibition does not require post-translational modifications. Clearly, an unmodified N-terminal domain can inhibit DNA binding. On the other hand, we suspect that in mammalian cells, post-translational modifications or interaction with other proteins can prevent the inhibitory effect of the N-terminal domain since DNA binding by the full-length CDP/Cux protein, in particular the CCAAT displacement activity, has previously been documented by us and others in numerous reports (13, 26, 27, 37, 47, 81–85). Altogether, these findings imply that the autoinhibition is not absolute but can be modulated. In agreement with the notion that autoinhibition can be relieved in cells, we noticed in transfection experiments that autoinhibition was less...
intense for a period of time after the medium had been replenished with serum (data not shown). We considered the possibility that the increase in CDP activity following re-entry into the cell cycle may be associated with a reduction in autoinhibition. However, serum-starvation and restimulation experiments following transfection of proteins that included or not the N-terminal region did not lend support to this hypothesis. We are currently investigating various post-translational mechanisms by which autoinhibition could be modulated in cells. Such a mechanism of regulation is likely to play an important role in the control of CDP/Cux activity in certain physiological situations. For example, in myeloid cells that are induced to differentiate, it is not yet known how CDP/Cux DNA binding is inhibited despite the fact that the protein continues to be expressed (13, 86, 87).

One obvious mechanism to relieve autoinhibition is the removal of the N-terminal region by proteolytic processing (37). We had originally considered that the major changes in the DNA binding properties of the p110 processed isoform resulted mainly from the removal of Cut repeat 1. Indeed, the presence of Cut repeat 1 appears to dictate the DNA binding specificity and kinetics. Proteins that contain the CR1 domain rapidly but mainly from the removal of Cut repeat 1. Indeed, the presence of CR3HD domains, exhibit preference for CGAT or CAAT repeats and kinetics. Proteins that contain the CR1 domain rapidly but

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