Redox Regulation of the DNA Binding Activity in Transcription Factor PEBP2

THE ROLES OF TWO CONSERVED CYSTEINE RESIDUES

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Transcription factor PEBP2/CFB consists of a DNA binding subunit, α, and a regulatory subunit, β. The α subunit has an evolutionarily conserved 128-amino acid region termed “Runt domain” that is responsible for both DNA binding and heterodimerization with the β subunit. The Runt domain in all mammalian submembers of the α subunit contains two conserved cysteine residues, and its DNA binding activity undergoes redox regulation. To investigate the mechanism of this redox regulation, we performed site-directed mutagenesis of the two conserved cysteines in the Runt domain of the mouse PEBP2αA homolog. Substitution of Cys-115 to serine resulted in a partially impaired DNA binding, which remained highly sensitive to a thiol-oxidizing reagent, diamide. Conversely, the corresponding substitution of Cys-124 caused an increased DNA binding concomitant with an increased resistance to diamide. In contrast, substitution of either cysteine to aspartate was destructive to DNA binding to marked extents. These results have revealed that both Cys-115 and Cys-124 are responsible for the redox regulation in their own ways with low and high oxidizabilities, respectively. We have also found that two cellular thiol-reactive proteins, thioredoxin and Ref-1, work effectively and synergistically for activation of the Runt domain. Interestingly, the β subunit further enhanced the activation by these proteins and reciprocally prevented the oxidative inactivation by diamide. These findings collectively suggest the possibility that the Runt domain’s function in vivo could be dynamically regulated by the redox mechanism with Trx, Ref-1, and the β subunit as key modulators.

Polymy virus enhancer-binding protein 2 (PEBP2)1 (1), also called core binding factor (2), is a heterodimeric transcription factor composed of two different subunits, α and β. The α subunit binds directly to a specific DNA sequence, RACCRCA, while the β subunit does not by itself contact with DNA but facilitates the DNA binding activity of the α subunit through allosteric interactions (1, 2). The α subunit shares a 128-amino acid region of high homology with the Drosophila segmentation gene runt (3) and the human AML1 gene (4). The conserved region, termed “Runt domain,” is responsible for both DNA binding and heterodimerization with the β subunit (5, 6). After the identification of the first member of the α subunit, PEBP2αA (1), two additional members of the Runt family have subsequently been isolated in mice and humans: PEBP2αB (mouse homolog of AML1) (7), and PEBP2αC (8) or AML2 (9).

In mammals, PEBP2 has been implicated in the transcriptional regulation of lymphoid cell-specific genes such as T-cell receptors, CD3, and myeloperoxidase, neutrophil elastase, granulocyte/macrophage colony-stimulating factor, macrophage colony-stimulating factor receptors, interleukin-3, and granzyme B (for review, see Ref. 10). Chromosomal translocations involving the human AML1 gene, such as t(5;21), t(3;21), and t(12;21), lead to various types of leukemia including acute myeloid leukemia, the blast crisis of chronic myeloid leukemia, and B-lineage acute lymphoblastic leukemia, respectively (for review, see Refs. 10 and 11). Moreover, an inversion in human chromosome 16 that gives rise to a fusion product, PEBP2β-SMMHC (smooth muscle myosin heavy chain), was also found to cause a M4Eo subtype acute myeloid leukemia (12). Recent gene disruption studies in mice of AML1 (13) as well as PEBP2/CFB studies (14) have confirmed that these genes are essential for definitive hematopoiesis.

Our previous functional characterization of PEBP2 (6) has revealed that the DNA binding activity of the Runt domain is subject to regulation by a reduction/oxidation-dependent mechanism (redox regulation). Since the finding of human thioredoxin/adult T-cell leukemia-derived factor (15), the importance of the thiol-mediated redox regulation has been well recognized in various biological responses (16, 17). Particularly notable are accumulating examples of redox-responsive transcription factors, such as the AP-1 family (18, 19), the Rel family (20–22), Myb (23), Ets-1 (24), and p53 (25). In these transcription factors, the reduced state of cysteine in the DNA-binding domain is essential for their DNA binding. Coincidentally, the Runt domain in all three mammalian submembers of the α subunit contain two cysteine residues that are perfectly conserved among them (8). Our recent random mutagenesis study with the Runt domain of PEBP2αA (26) has suggested that one target for its redox regulation should be Cys-124, because substitution of this residue to serine resulted in an enhanced DNA binding concomitant with decreased redox-dependence. However, the possibility remains that the other cysteine residue, Cys-115, could also be redox-responsive. To further define the

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1 The abbreviations used are: PEBP, polyoma virus enhancer-binding protein; Trx, thioredoxin; TrxR, thioredoxin reductase; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol.
potential roles of the two conserved cysteine residues in the redox regulation of PEBP2, we performed their site-directed mutagenesis and examined the DNA binding ability of resulting mutants under various redox conditions in vitro.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis of the Runt Domain**—Plasmid pQE-RD (6) encoding the Runt domain of PEBP2αA with an N-terminal hexahistidine tag (for its structure, see Fig. 1A) was used as a vector for construction and over-expression of Runt domain mutants. Each or both of Cys-115 and Cys-124 were mutagenized to serine or aspartate by polymerase chain reactions using Vent DNA polymerase (New England Biolabs) as described (27). The substitutions of the targeted residues were confirmed by sequencing.

**Production and Purification of Proteins**—His-tagged derivatives of the Runt domain and the β subunit were expressed in Escherichia coli and purified on a nickel-nitrilotriacetic acid resin (QIAGEN) as described (6). Trx was purchased from Aijinomoto. TrxR was purified as described (28). Ref-1 was purified as described (29).

**Electrophoretic Mobility Shift Assay (EMSA)**—A DNA probe containing the wild type PEBP2 binding site was prepared as described (6). The DNA binding reaction was routinely carried out for 10 min at 25 °C in 10 mM Tris-Cl pH 8.0, 0.25 mM EDTA, 4% glycerol, 0.2 mg/ml bovine serum albumin, 0.04% bromphenol blue, 10 fmol of 32P-labeled probe, 5 ng of purified His-tagged Runt domain, and the β subunit (50 ng) where indicated. In some experiments, the Runt domain was pretreated with redox reagents such as diamide, DTT, Trx, and Ref-1. The reaction mixture was loaded on a 10% nondenaturing polyacrylamide gel (acrylamide:bisacrylamide, 39:1) in 0.25× Tris-borate-EDTA buffer and electrophoresed at room temperature. Gels were dried and visualized by a phosphorimagor (Fuji film BAS 2000) or autoradiography with x-ray films.

**RESULTS**

The two conserved cysteine residues in the Runt domain of PEBP2αA to redox regulation were substituted to serine or aspartate, either separately or simultaneously (Fig. 1A). In the following, the resultant mutants are denoted by abbreviations in the form XY, in which the first and second letters represent amino acids in one-letter codes that replace Cys-115 and Cys-124, respectively. All the serine mutants showed readily detectable DNA binding, although those with Cys-115 substituted to serine (SC and SS) were severalfold less active than the wild type (CC) in the absence of the β subunit (Fig. 1B, left panel; see also Fig. 1C for quantitative comparison). In confirmation of our previous observation (26), mutant CS displayed stronger than normal DNA binding. On the other hand, the aspartate mutants invariably showed drastically impaired DNA binding, which was virtually undetectable in DC and DD, and barely recognizable in CD even after a prolonged exposure for autoradiography (Fig. 1B, right panel). When the β subunit was present, the wild type and all the mutants with any recognizable DNA binding activity gave supershifted DNA bands, whose intensitites, except for CS, were prominently increased over those observed without the β subunit. This indicates that substitution of either cysteine residue to serine or aspartate is tolerable for the heterodimerization between the Runt domain and the β subunit as well as their allosteric regulatory interaction.

We then used three serine mutants together with the wild type to evaluate the susceptibilities of the two cysteine residues to a thiol oxidizing reagent, diamide (Fig. 2A). Fig. 2B shows changes in the relative DNA binding activity of these constructs after their treatment with increasing concentrations of diamide relative to the respective mock-treated controls. In the absence of the β subunit (open circles), SC remained nearly as sensitive as the wild type to diamide, showing more than 75% inhibition at 1 mM. In contrast, CS was only weakly affected (25% inhibition) at 1 mM. However, the activity of this mutant was progressively and extensively decreased with further increments of diamide up to 100 mM. Thus we conclude that Cys-115 is also responsive to redox regulation, although being much less sensitive than Cys-124. When the β subunit was present, all variants having one or both cysteine residues showed decreased sensitivities to diamide. CC and SC remained 90% active at 1 mM, although they became drastically inactivated at higher concentrations; CS was only moderately inhibited even at 100 mM. Thus, the β subunit appeared to render both Cys-115 and Cys-124 tolerable to proportionally increased dosages of diamide by one order of magnitude or more. No such β subunit-dependent protection was observed with the cysteine-less mutant, SS, as expected.

In Fig. 2B are also indicated changes in the overall -fold stimulation of DNA binding by the β subunit with the diamide concentration for each Runt domain construct (crosses on dotted lines). Note first that SS showed moderate stimulation (severalfold), which is almost invariant with the diamide concentration and hence supposed to represent the redox-indepen dent regulatory action of the β subunit in augmenting the intrinsic affinity of the Runt domain for DNA as previously defined by Kagoshima et al. (6). By contrast, CC and SC exhibited much greater stimulation than SS, attaining a peak of 30-fold or more at 1 mM diamide. This enormous stimulation over that observed with SS is taken to reflect the above-noted anti-oxidation effect of the β subunit on Cys-124 as target. Although the -fold stimulation of CS was comparatively very...
low, it still showed a gradual rise with increasing concentrations of diamide, eventually surpassing that of SS at 100 mM. Evidently, therefore, Cys-115 is also subject to the protective action of the β subunit.

We further addressed the question of what cellular component(s) could serve for reductive activation of the Runt domain. Three known factors were tested as candidates: glutathione (GSH), Trx/ADF, and Ref-1. Trx/ADF is known to activate transcription factor NF-κB (GSH), Trx/ADF, and Ref-1. Trx/ADF is known to activate the Runt domain in the absence of the subunit(s) could serve for reductive activation of the Runt domain. The DNA binding activities of each construct measured above the level of those with no added diamide. Crosses on dotted lines show fold stimulation by the β subunit.

**DISCUSSION**

The present mutational study has demonstrated that both of the two cysteine residues within the Runt domain are responsible for redox regulation in their own ways, which are unique in comparison with known other redox-responsive transcription factors. Cys-124 apparently resembles the redox-responsive cysteine identified in Jun and Fos (18) in its high oxidizability due to its substitution to serine. Conversely, the lesser redox sensitivity of Cys-115 is likely attributable to the absence of any basic amino acid in its vicinity (Fig. 1A). Nevertheless, the reduced DNA binding activity caused by its substitution to serine as well as aspartate suggests that the free sulfhydryl group per se might be functionally essential. Similar mutational effects have previously been reported for NF-κB (21), Myb (23), and Ets-1 (24). Indeed, the x-ray structural analysis of NF-κB has demonstrated direct contacts of the free sulfhydryl group of cysteine with its target DNA (31). A priori, the two cysteines in the Runt domain might form a reversible disulfide bridge, as reported for p53 (25). However, their differential redox susceptibilities as observed rather favor the view that the two cysteines undergo redox reactions independently from each other. Such a two-pronged mechanism would enable more flexible regulation of DNA binding in response to diverse cellular redox signals than otherwise.

In extension of our previous observation (6), the results of this study also highlighted the potential significance of the β subunit as a modulator of redox sensitivity of the Runt domain, in addition to its role in allosterically enhancing the intrinsic DNA binding affinity of the Runt domain. The β subunit acted protect both cysteines from oxidation by diamide. Puzzlingly, however, the β subunit did not hinder the access of the Runt domain by much larger molecules, Trx and Ref-1. How then was the β subunit able to block the action of diamide?
that the intrinsic oxidizability of these cysteine residues could be modulated by β subunit-induced alterations in the local environment around them in terms of their spatial alignment with neighboring charged amino acids or their involvement in intra- or inter-molecular hydrogen bonding, rather than a simple steric hindrance.

The finding that Trx and Ref-1 can efficiently activate the Runt domain in a manner synergistic with each other and enforceable by the β subunit raises the question of where and how these protein factors interact with each other within the cell. While the α subunit is a nuclear protein, the β subunit by itself is localized in the cytoplasm and transported to the nucleus through association with the α subunit (32). Available evidence (6, 32) has suggested that the heteromeric interaction of the β subunit with the intact α subunit is somewhat restricted both in vitro and in vivo by a putative intramolecular interference between the Runt domain and its flanking regions and that a specific mechanism would exist to promote this heterodimerization. Likewise, Ref-1 is a nuclear protein (19), and that a specific mechanism would exist to promote this restricted both in vitro and in vivo by a putative intramolecular interference between the Runt domain and its flanking regions and that a specific mechanism would exist to promote this heterodimerization. Likewise, Ref-1 is a nuclear protein (19), and that a specific mechanism would exist to promote this restricted both in vitro and in vivo by a putative intramolecular interference between the Runt domain and its flanking regions and that a specific mechanism would exist to promote this heterodimerization. Likewise, Ref-1 is a nuclear protein (19), and that a specific mechanism would exist to promote this restricted both in vitro and in vivo by a putative intramolecular interference between the Runt domain and its flanking regions and that a specific mechanism would exist to promote this restricted both in vitro and in vivo by a putative intramolecular interference between the Runt domain and its flanking regions and that a specific mechanism would exist to promote this restricted both in vitro and in vivo by a putative intramolecular interference between the Runt domain and its flanking regions and that a specific mechanism would exist to promote this restricted both in vitro and in vivo by a putative intramolecular interference between the Runt domain and its flanking regions and that a specific mechanism would exist to promote this restricted both in vitro and in vivo by a putative intramolecular interference between the Runt domain and its flanking regions and that a specific mechanism would exist to promote this.

While this work was in progress, in vitro evidence underscoring the functional importance of cysteine residues in the Runt domain protein was provided by the report (35) that the transactivation and cell-transforming potentials of AML1/PEBP2ab were markedly weakened by substitution of serine for Cys-72 (corresponding to Cys-115 in PEBP2ab). To further delineate the dynamics and significance of redox regulation of PEBP2 to their full extents, it should be informative to extend such functional studies using a wider variety of mutants as constructed here in combination with various conditions affecting the cellular redox state.

Worth noting finally is the pattern of evolutionary conservation of cysteine in the Runt domain proteins. This protein family has been identified in a wide range of animal species from Caenorhabditis elegans (deduced from the C. elegans genomic data base) to mammals, and shown thus far to contain at least three subclasses in mammals (8–10) and two in Drosophila (Runt (3) and Lozenge (36)). As indicated in Fig. 4, all the members identified in species classified to deuterostomia including sea urchin (37), chicken (38), and mammals (8–10) share the two conserved cysteine residues at positions 115 and 124. On the other hand, those found in Drosophila and C. elegans, which belong to protoptostomia, have one or both of these cysteines altered to serine or tyrosine. Discernible also is an opposite change from cysteine to serine at position 157 in an apparent association with the advent of vertebrate. It thus seems that there were a number of possible ways in utilizing cysteine to control DNA binding by the Runt domain and nature had shifted its choices among them at major steps of evolutionary divergence in the animal kingdom. Further comparative studies of the Runt domain proteins are awaited to explore the potential evolutionary implications of its redox regulation in full scope.

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