The mouse transcription factor PEBP2 is a heterodimer of two subunits: a DNA binding subunit α and its partner subunit β. The α subunit shares a region of high homology, termed the Runt domain, with the products of the Drosophila melanogaster segmentation gene runt and the human acute myeloid leukemia-related gene AML1. To study the molecular basis for the DNA binding and heterodimerization functions of this factor, we constructed series of deletions of the α and β subunits and examined their activities by electrophoretic mobility shift and affinity column assays. The minimal functional region of the α subunit for DNA binding and dimerization was shown to coincide with the Runt domain. On the other hand, the region of the β subunit required for heterodimerization was localized to the N-terminal 135 amino acids. Furthermore, it was found that the DNA binding activity of the Runt domain is regulated by a reduction/oxidation (redox) mechanism and that its reductively activated state, which is extremely labile, is stabilized by the β subunit. These findings add a new layer to the mechanism and significance of the regulatory interplay between the two subunits of PEBP2.

Transcription factor PEBP2 was originally identified from mice as a factor that binds to the core regions of polyomavirus enhancer, specifically recognizing a consensus sequence, (R/T)\(^3\) as well as the regulatory regions of many cellular genes present in the core motifs of murine leukemia virus enhancer (3) and the regulatory interplay between the two subunits of PEBP2.

These studies led to the discovery that the DNA binding activity of the Runt domain is responsible for both DNA binding and heterodimerization with the β subunit (11, 16). In this study, we extended the deletion analysis with PEBP2αA in more detail to define the exact regions required for the two activities within the Runt domain. Analogous deletion analysis with PEBP2β was also carried out to localize its functional domain for association with the α subunit. We utilized these deletion constructs to further characterize the molecular and functional interactions between the two subunits. These studies led to the discovery that the DNA binding activity of the Runt domain can be sensitively controlled by a reduction/oxidation (redox) mechanism and that the β subunit acts to stabilize the activated state of the Runt domain, apart from and in addition to its known effect to increase the DNA binding affinity.
Functional Domain Analysis of Transcription Factor PEBP2

MATERIALS AND METHODS

Plasmid Construction—To produce α1 and α2, the two alternatively spliced isoforms of the PEBP2α subunit originally isolated (11), as fusions with an N-terminal hexahistidine (His) tag in Escherichia coli, their coding sequences were inserted between the BamHI and HindIII sites of expression vector pQE9 (Qiagen), resulting in pQE-α1 and pQE-α2, respectively. A series of truncations of α were generated from the parental plasmid pQE-α2 by cleaving at appropriate restriction sites within the α coding sequence (Fig. 1). The N- and C-terminal deletions are designated αN and αC followed by numbers indicating the respective termini of the remaining region. Since pQE vector allowed only poor overexpression for α1, α2, αN94C306, and αN113C306, the EcoRI-HindIII fragments encoding these proteins in pQE were recloned into the xgal site of a T7 promoter-driven expression plasmid vector, pET3a (23).

To produce N-terminally His-tagged β subunits, the coding sequences of three alternatively spliced isoforms, β1, β2, and β3, were inserted between the BamHI and SalI sites of plasmid pQE9. Furthermore, to produce N- or C-terminal dihydrofolate reductase (DHFR) fusions of β subunit, which also were His-tagged, the coding sequences of PEBP2β1 and PEBP2β2 subunits were inserted between the BamHI and HindIII sites of plasmid pQE13 (Qiagen). A series of deletions were introduced into β subunit by cleavage at the appropriate restriction sites (Fig. 2). The resulting β deletions were designated in the same way as described for α deletions. Plasmid pET-β2 coding for the tagless β protein was described previously (10).

Production and Purification of PEBP2 α and β Subunits—E. coli strain M15 with pQE plasmids and BL21 with pET plasmids were grown in LB broth and induced with 0.5 mM isopropyl β-D-thiogalacto-side. Harvested cells were lysed by suspending them in buffer A (0.1 M sodium phosphate, 10 mM Tris, 6 mM guanidine-HCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol, and 0.1% Nonidet P-40, pH 8.0). The lysate was centrifuged at 15,000 rpm at 4 °C for 30 min, and the resulting supernatant was sonicated to break down DNA. The supernatant was applied onto a nickel nitriotropic resin (Ni-NTA column) (Qiagen) and washed with buffer A containing 0.8 M imidazole. His-tagged proteins were eluted with 12–250 mM stepwise imidazole gradients in buffer A containing 20% glycerol. The purified proteins were renatured by dialysis against 100 volumes of buffer D (0.1 M sodium phosphate, 10 mM Tris, 10 mM DTT, and 30% glycerol, pH 8.0) at 4 °C overnight. The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of the purified α and β derivatives after the step of the Ni-NTA column is shown in Fig. 3. A and B. Since α1 recovered from the Ni-NTA column still contained substantial contaminants or its proteolytic degradation products, its full-length polypeptide was eluted from the SDS-polyacrylamide gel and renatured by dialysis as described previously (2). The tagless β protein was expressed from pET-β2 in E. coli and purified under non-denaturing conditions as described previously (10).

Electrophoretic Mobility Shift Assay (EMSA)—A DNA probe containing the wild-type PEBP2 binding site was prepared by annealing an oligonucleotide pair, 5'-CATGGTAACTGACCGCAGAGGGC-3' and 5'-CATGGCCTCTGGCGGTCAGTTAC-3' (the PEBP2-binding site is underlined), and labeled with [α-32P]dATP in a standard reaction using the Klenow fragment. Unless specified otherwise, the DNA binding reaction (final volume, 10 μl) was routinely carried out at room temperature for 15 min in EMSA buffer (20 mM HEPES-KOH, 4% Ficoll, 2 mM EDTA, 1 mM DTT, 100 mM KCl, 1 μg of poly(dI-dC), 6% glycerol, 0.2 mg/ml bovine serum albumin, 0.04% bromphenol blue and 10 fmol of labeled probe). The reaction mixture was loaded onto a 10% nondenaturing polyacrylamide gel (acrylamide:bisacrylamide, 39:1) in 0.25 × TBE (22.5 mM Tris borate and 0.1 mM EDTA, pH 8.0) and electrophoresed at room temperature.

Affinity Column Assay of α Subunit Association—To assay the heterodimerization activity of a protein derivatives independent of their DNA binding activity, an affinity column assay was performed. About 1 μg of the His-tagged α subunit and the tag-less β subunit (about 0.5 μg) were incubated in 100 μl of affinity column assay buffer (0.1 M sodium phosphate, 10 mM Tris, 1 mM β-mercaptoethanol, and 20% glycerol, pH 8.0) at 4 °C for 30 min. The reaction mixture was loaded onto a Ni-NTA column. This column was successively washed with affinity column assay buffer containing 40 mM imidazole and 250 mM imidazole. Proteins eluted in each fraction were analyzed by SDS-PAGE followed by silver staining.

RESULTS

Functional Domain Analysis of PEBP2α Subunit—To localize the minimal regions of PEBP2α subunit required for its DNA binding and heterodimerization activities, we produced a series of its deletions as His-tagged forms in E. coli (Figs. 1 and 3A). The resultant α derivatives were purified on a Ni-NTA column under denaturing conditions, renatured by dialysis, and subjected to EMSA in the presence and absence of the β2 subunit (Fig. 4). In this assay, the heterodimerization activity

1 The abbreviations used are: Ni-NTA, nickel nitriotropic resin; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; DHFR, dihydrofolate reductase; RD, Runt domain; ADF, adult T cell leukemia-derived factor.
was readily detected by supershifts of protein-DNA complexes. In the pilot study with α1 and α2, the two alternatively spliced isoforms of PEBP2αA, we noticed that the 513-amino acid α1 protein tended to undergo extensive proteolytic degradation in its C-terminal region either within bacterial cells or during the isolation procedure. Moreover, its full-length polypeptide, as purified by gel electrophoresis, gave a barely detectable band shift in EMSA (however, see below for its assay under improved conditions). In contrast, the 306-amino acid α2 protein was more resistant to proteolysis and showed a strong DNA binding activity at a relatively low dose of 5–10 ng/reaction. Hence, α2 was used as the starting material for constructing the deletion from either end and also as the reference in comparing the DNA binding capacities of various α protein constructs.

In the N-terminal deletion series, αN94C306 was as active as α2 in both DNA binding and heterodimerization with the β subunit. The next deletion, αN113C306, was still weakly active in DNA binding but not in heterodimerization. Although this deletion gave two distinct bands, the lower one is supposed to represent a proteolytic degradation product, which tended to accumulate gradually during its storage. Further deletion to position 140 resulted in a complete loss of DNA binding activity, giving no discernible shifted band even when its dose was increased to 100 ng/reaction. In the C-terminal deletion series, αC226 was strongly active in DNA binding and heterodimerization, but the next further deletion, αC216, no longer showed any DNA binding activity. These results indicate that the minimal region for DNA binding resides between amino acids 113 and 226, although an additional segment up to amino acid 94 is further required for full activity. The region from amino acid 94 to 226 was similarly shown to be sufficient for the heterodimerization activity. However, its exact C-terminal boundary could not be determined by EMSA, because C-terminal deletions more extensive than αC226 did not bind to DNA. For such non-DNA binding deletions, we measured their heterodimerization activity by their ability to cause co-adsorption of tagless β subunit to a Ni-NTA column as described under "Materials and Methods." This direct assay indicated that the heterodimerization activity was still retained in αC216 but not in deletions with further C-terminal truncations (Fig. 5).

Taking all of these results together, we conclude that the functional regions for DNA binding (amino acids 113–226) and heterodimerization (amino acids 94–216) extensively overlap each other and encompass together almost the entire expanse of the Runt domain (amino acids 93–220) as originally defined from sequence homologies alone (16) (see "Discussion" for further detailed considerations). During this deletion analysis, we also noticed a general trend that α derivatives retaining the C-terminal sequence up to position 306 showed greater -fold stimulation in their DNA binding by the β subunit than those lacking this region (Fig. 4, compare the lanes for αA1, αA2, and αN94C306 against those for αC226 and αN94C226). This suggested the intriguing possibility that the C-terminal region might act to modulate the regulatory interaction between the

![Fig. 2. Structure and activities of PEBP2β deletion derivatives. The horizontal bars diagrammatically depict β derivatives constructed. The region shared by all three β isoforms is indicated by open boxes, and C-terminal divergent areas are variously decorated as follows. Darkly shaded, common to β1 and β2; lightly shaded, unique to β2; hatched, common to β1 and β3. The symbols in the left-hand column indicate heterodimerization and DNA binding stimulation activities as estimated from the results shown in Fig. 6: 1, active; 2, weakly active; 3, inactive; NEG, inhibitory. The open and filled arrows indicate the minimum regions required for heterodimerization (amino acids 1–135) and simulating activity (amino acids 1–117), respectively.](image)

![Fig. 3. SDS-PAGE patterns of α and β derivatives. His-tagged derivatives of PEBP2α (A) and PEBP2β (B) were purified with the NTA column, subjected to SDS-PAGE, and visualized by silver staining.](image)
Functional Domain Analysis of Transcription Factor PEBP2

Fig. 4. DNA binding and complex formation assays of deleted variants of PEBP2α subunit by EMSA. The EMSA reaction was performed with the following deletions in specified amounts: αA1, 50 ng; αA2, 10 ng; αN94C226, 5 ng; αN113C306, 50 ng; αN140C306, 100 ng; αC216, 5 ng; αC216, 100 ng; αC202, 100 ng; αN94C226, 2 ng; αN113C226, 60 ng; DHFR, 100 ng; + and − indicate the presence and absence of the β2 subunit (100 ng).

Fig. 5. Complex formation assays by protein-protein affinity column. The β2 subunit, not containing any histidine tag, was incubated with the following His-tagged proteins bound to Ni-NTA resin. Lane 1, αN80C226 as a positive control; lane 2, DHFR as a negative control; lane 3, αC216; lane 4, αC216; lane 5, αC202; lane 6, αC160. Bound proteins were eluted using a stepwise imidazole gradient as described under "Materials and Methods." Eluates were resolved by SDS-PAGE and visualized by silver staining. The electrophoretograms are shown only for the main fraction eluted with 250 mM imidazole.

Runt domain and the β subunit. This issue will be readdressed below in reference to the redox regulation of the α subunit function.

Functional Domain Analysis of PEBP2β Subunit—We have previously reported that two isoforms of the β subunit, β1 and β2, and a β1-derived C-terminal deletion (β1C141) heterodimerize with the α subunit, whereas the third isoform β3 cannot (10).

To extend that analysis, we constructed N- and C-terminal deletions of the β subunit (Figs. 2 and 3B) and tested their ability to interact with the minimal Runt domain fragment (αN94C226, referred to hereafter as the RD fragment) by EMSA (Fig. 6). In the C-terminal deletion series, which were readily produced and purified as His-tagged forms, those truncated up to amino acid 135 (β1C135) were functional, but the next further deletion (β1C117) became inactive. On the other hand, we were unable to produce any N-terminal deletions as His-tagged fusions, because the growth of bacterial hosts was severely inhibited upon their expression from plasmids encoding them. After various trials, we finally succeeded in overproducing them as N- or C-terminal fusions with DHFR. However, the N-terminal DHFR fusion with β1 (and also β2; data not shown) apparently inhibited the DNA binding by the RD fragment without showing any supershift. This implies that DHFR-β1 can interact with the Runt domain but that the resultant complex is inactive in DNA binding. In contrast, the corresponding C-terminal DHFR fusion with β1 produced a highly smeared supershifted band, suggesting that it formed a complex with the RD fragment being somewhat unstable, although active in DNA binding. On the other hand, N-terminal β deletions (β1N41, β1N55, β1N72, and β1N86) fused to DHFR on either end had no visible effect at all on the DNA binding signal due to the RD fragment (data not shown). Taken together, these results indicate that the N-terminal 135-amino acid region and its unperturbed steric or conformational state at the N-terminal end are important for its effective interaction with the Runt domain.

Surprisingly, β3 and β1C117 subunits caused notable stimulation in the DNA-binding activity of the RD fragment, although these subunits did not produce any supershifted band. This effect seems specific to β derivatives, since no such phenomenon was observed with other unrelated proteins such as DHFR (data not shown) and bovine serum albumin (always included in the EMSA buffer). We infer that these β subunits can interact with the RD fragment in solution to form heterodimers that bind more efficiently to DNA than does the RD fragment alone but that they may fall apart from the DNA-RD complex upon entering polyacrylamide gels. Such an event could happen if the association between those β derivatives and the RD fragment is substantially weaker than that between the RD fragment and DNA. Thus, the N-terminal 117 amino acids appear to contain the region minimally essential for the stimulating effect.

Redox Regulation of the Runt Domain Function—While performing EMSA as described above, we found that the DNA binding activity of a subunit tended to decrease gradually during its storage but that such an aged sample could be effectively reactivated by incubation with a high concentration of sulfhydryl protecting agent, DTT (Fig. 7A, lane 2). Coincidentally, the three Runt family proteins identified in mouse and human share two conserved cysteine residues in their Runt domain sequence, and one of them (Cys124 in αA) is thought to be highly sensitive to oxidation, since it is surrounded by positively charged residues (24). These observations suggested to us that PEBP2 might be subject to the so-called redox regulation as had been known for Jun/Fos (25) and NF-κB (26).

To test this possibility, we systematically examined the effects of sulfhydryl reducing and oxidizing agents on the function of the Runt domain in EMSA. When the RD fragment was treated with a mild oxidant, diamide, its DNA binding activity...
was completely inhibited at 0.25 mM or more (Fig. 7A, lane 3). However, this lost activity was completely recovered by subsequent incubation with a molar excess of DTT (Fig. 7B). The restored activity increased proportionately with increments in the concentration of added DTT up to 300 mM as far as tested. Such an extreme dependence on DTT is characteristic of redox-responsive DNA binding proteins (24–26). Interestingly, the oxidative inactivation of the RD fragment was effectively prevented by the addition of the β subunit before but not after the treatment with diamide (Fig. 7A, compare lanes 7 and 8 with lane 5 as a control). However, the β subunit did not interfere with the reductive reactivation of the preoxidized RD fragment when it was added before DTT (Fig. 7A, compare lanes 8 and 10). Thus, the β subunit may selectively act to mask the putative redox-sensitive cysteine residue(s) of the Runt domain from the action of diamide but not DTT, either by steric hindrance or through conformational influence. This implies that the β subunit could promote the reduction of the RD fragment either in its reaction kinetics or in its overall equilibrium. In accord with this view, the β-dependent -fold increase in the DNA activity was apparently greater at lower concentrations of DTT (Fig. 7B, compare lanes 2–5 versus lanes 6–9). To further investigate this issue, we compared the time course of the reductive reactivation of the RD fragment in the presence and absence of the β subunit. The result showed that the reactivation could actually take place with similar rapidity with or without the β subunit, rising to half of the maximal level already at the earliest time of sampling (0.5 min) and reaching a peak at 10 min after the addition of DTT (Fig. 8, A and B, left parts). Rather surprisingly, the resumed activity of the RD fragment by itself began to rapidly diminish thereafter to less than one-fourth the peak level at 30 min and almost null at 60 min. In a dramatic contrast, little inactivation occurred in the presence of the β subunit. Since the inactivation of the RD fragment proceeded despite the presence of a vast molar excess of DTT, it could not be due to a simple reoxidation, but it might involve some other irreversible processes such as thermal protein denaturation or oxyradical reaction. In any case, these observations have disclosed that the activated state of the RD fragment is extremely labile and that it can be greatly stabilized by the association with the β subunit.

We also performed similar analyses with the intact αA1 protein (Fig. 8A, right panel). It showed essentially the same patterns of activation by DTT and protection by the β subunit as did the RD fragment, except that its activated state was even more unstable than that of the RD fragment. The activity of αA1 alone was completely lost within 30 min, and it diminished to less than one-third the peak level at 60 min even in the presence of the β subunit (Fig. 8B, right part). In consequence, its DNA binding activity turned out to appear almost abso-
lutely dependent on the \( \beta \) subunit when the incubation was kept longer than 10 min (see the dashed lines in Fig. 8B). Retrospectively, this circumstance may explain why the DNA binding activity of \( \alpha A1 \) appeared to be so weak and stringently dependent on the \( \beta \) subunit in the earlier EMSA analysis (Fig. 4), in which the DTT concentration was insufficient (1 mM) and the incubation time was longer than optimal (15 min).

Quantitative Reevaluation of the Functional Roles of the \( \beta \) Subunit in DNA Binding by PEBP2—Ogawa et al. (10) have previously reported that the intrinsic function of the \( \beta \) subunit is to decrease the dissociation constant \( (K_d) \) of the \( \alpha \) subunit for DNA without changing its maximal binding capacity \( (P_{\text{max}}) \) (10). As described in the preceding section, however, the \( \beta \) subunits could also augment the apparent \( P_{\text{max}} \) level through its protective action in a manner dependent on both the redox state and the structural context of the \( \alpha \) subunit. To evaluate these dual functional contributions of the \( \beta \) subunit more quantitatively, we studied the DNA binding kinetics of both the RD fragment and \( \alpha A1 \) by EMSA in the presence or absence of the \( \beta \) subunit at low and high concentrations of DTT (Fig. 9). In this analysis, extreme care was taken to perform the EMSA manipulations at a constant and optimal timing, so that the DNA-protein complex could be applied to gel electrophoresis while it stayed at a maximally activated state. In the assay of the RD fragment (Fig. 9A) with 10 mM DTT, the \( \beta \) subunit caused not only a severalfold decrease in the \( K_d \) (from 1.4 to 0.2 nM) but also a substantial increase in the \( P_{\text{max}} \) (about 2-fold).

By contrast, the \( P_{\text{max}} \) was increased at 100 mM DTT to nearly the same level either in the presence or absence of the \( \beta \) subunit. Under this assay condition, the \( \beta \) subunit solely exhibited the \( K_d \) effect.

In the case of the full-length \( \alpha A1 \) protein (Fig. 9B), the \( \beta \) subunit similarly caused a decrease in the \( K_d \) value at either concentration of DTT tested. However, the absolute magnitude of its \( K_d \) in the presence of \( \beta \) (around 0.4 nM) was noticeably higher than that obtained with the RD fragment (0.2 nM). In addition, the effect of \( \beta \) to augment the \( P_{\text{max}} \) was still observed at the higher DTT concentration (about 3-fold). By extrapolations from the result shown in Fig. 8B, a 3-fold inactivation of the \( \alpha A1 \) protein could readily occur in the absence of the \( \beta \) subunit during the 10 min of incubation as adopted.

**DISCUSSION**

The results of this study lend support to and further refine our previous proposal that the Runt domain is a structural core responsible for both DNA binding and heteromeric protein-protein interaction (summarized in Fig. 1). The present deletion analysis localized the DNA binding and heterodimerization domain within amino acids 113–226 and 94–216, respectively. If other available evidence is also taken into account, the respective functional regions can be further confined within the conserved region originally defined from sequence homology between the Runt protein and AML1 (27). The DNA binding activity has been demonstrated in various Runt family proteins and their chimeric derivatives that have variable sequences distal to the C-terminal end of their Runt domain (amino acid 220 in \( \alpha A1 \)). The heterodimerization activity was similarly detected in a deletion product of AML1 containing...
amino acids 59–190, which corresponds to amino acids 102–233 in α (16). The localization of the dual functions in the same Runt domain for its whole expanse may explain why its sequence has been conserved so highly from insects to mammals. Intriguing from both the mechanistic and experimental points of view is the finding that the regions for DNA binding and heterodimerization are slightly staggered from each other by several amino acids toward the C-terminal and N-terminal ends, respectively. Although the DNA binding activity is detectable in αN113C306 or αN113C226, its magnitude per protein basis is much less than that of the full Runt domain fragment (cf. legend to Fig. 4). Evidently, therefore, the adjacent N-terminal segment, at least up to amino acid 93, is functionally important not only for heterodimerization with the β subunit but also for efficient DNA binding. This N-terminal proximal region may be directly involved in both DNA binding and its regulatory modulation by the β subunit. In support of this view, we have recently observed that a mutation in amino acid 97 (Ile → Thr) can cause a marked change in the DNA binding activity (28). Suggestive further in this connection is that this region is spatially close to and hence could be functionally interrelated with the two cysteine residues (Cys115 and Cys124) that are implicated in the redox regulation of the Runt domain function as discussed below. By contrast, the 4-amino acid segment on the C-terminal side (217–220, REPR) is dispensable for heterodimerization, but it is essential for DNA binding. Thus, deletions or site-directed mutagenesis of these segments would be useful in constructing mutants of the α subunit differentially altered in DNA binding or heterodimerization, which should provide valuable probes for studying the regulatory interplay between the α and β subunits of PEBP2 in vivo.

The parallel deletion analysis with the β subunit has revealed that the first 135 amino acids are required for its stable association with the α subunit as monitored by supershifts of EMSA bands (summarized in Fig. 2). Recently, Drosophila has been shown to possess two kinds of β homologs, designated Brother (Bro) and Big brother (Bgb) (29). These two proteins share high homologies with the N-terminal 137 amino acid of the mouse β subunit except for a diverged internal segment that corresponds to positions 69–86. In a fair agreement with our results, a deletion of Bro containing the first 132 amino acids (137 in mouse β) was fully active in heterodimerization with its cognate partner Runt, but shorter derivatives lacking the first seven amino acids or ending at position 127 (132 in mouse β) were not. Furthermore, it was also reported that the heterodimerization activity was undetectable or significantly weakened in mammalian β subunit derivatives that retained only the first 133 amino acids: the mouse β3 (Ref. 10 and this work) and an internal deletion product (CDC32/M) of the human CBFβ-SMMC chimera (22). From these observations, the two conserved amino acids at positions 134 and 135 (Glu and Asp) might be important for an efficient interaction between the α and β subunits.

On the other hand, the capacity of β to enhance DNA binding was mapped within the first 117-amino acid region. This implies the occurrence of weak but functionally significant interactions between the N-terminal proximal region of β and the Runt domain. Moreover, the fusion of DHFR to the intact β1 subunit caused inhibition, rather than stimulation, of DNA binding, suggesting that it could interact with the α subunit, although in an unproductive way. The bulky DHFR moiety may either act as a direct steric block against the access of the α subunit to DNA or induce an unfavorable conformation change in the αβ complex. If this is the case, the DHFR-β fusion might be useful as a dominant negative mutant in studying the functional role of the β subunit in vivo.

The redox regulation mechanism has previously been known for a number of transcription factors such as Jun/Fos (25, 30), Myb (31, 32), NF-κB (26), and Ets-1 (33). Their DNA binding activity is greatly stimulated by sulfhydryl protecting agents such as DTT and mercaptoethanol or by cellular redox cofactors, Refl/APEX for Jun/Fos (34, 35) and ADF/thioredoxin for NF-κB (36). These factors contain cysteine residues that are surrounded by basic amino acids and hence made hypersensitive to oxidation (24). Under insufficient reducing conditions or upon exposure to a mild oxidant, typically diamide, the thiol moiety of such cysteines is readily converted into a more acidic state that is inhibitory to DNA binding, although the exact chemical nature of this inactivated state remains to be determined. The present study has revealed that the DNA binding activity of PEBP2α requires the presence of a high concentration of DTT and is conversely inactivated by diamide in a manner closely mimicking the above noted precedent. In addition, the Runt domain of PEBP2α contains two conserved cysteines, one of which is surrounded by basic amino acids (Arg-Cys124-Asn-Lys) and located close to the N-terminal boundary of the minimal DNA binding region. Thus, it is very likely that PEBP2α is also subject to the redox regulation. Notable here is that the Drosophila Runt protein is also redox-sensitive,2 although it has serine at the position corresponding to the Cys124 residue. Instead, the Runt protein contains three extra cysteines, two of which are flanked by basic amino acids and, hence, could be redox-sensitive.

Further investigations of the redox sensitivity of the Runt

\[ \text{Bound DNA} = P_{max} \times \frac{[\text{free DNA}]}{K_d + [\text{free DNA}]} \]

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domain have led us to the additional findings of potential mechanistic and biological importance that its activated state is extremely unstable and that the β subunit has a remarkable protective effect on this activated state. This effect, as reflected by an apparent increase in the maximal DNA binding capacity ($P_{\text{max}}$) effect), is distinct from and manifests in superposition to the known function of β to increase the intrinsic DNA binding affinity ($K_d$) effect) (10). While the $K_d$ effect is relatively constant and modest (2–3-fold and 5–7-fold with αA1 and the RD fragment, respectively), the $P_{\text{max}}$ effect is extremely variable with assay conditions. When the DT concentration was sufficiently high and the reaction time in EMSA was kept short (10 min), this effect was minimized to near unity with the RD fragment and 2–3-fold with αA1. If one or both of these conditions were inadequate, the DNA binding activity of the Runt domain by itself could rapidly diminish to an undetectable level with a concomitant upshot in the $P_{\text{max}}$ effect (see dashed lines in Fig. 8). Thus, the incidence of β-dependent stimulation by more than severalfold should be taken as a potential symptom of ill-suppressed inactivation of the α subunit without the β subunit. Such a situation might be relevant to the Droso phila Runt protein, whose DNA binding activity was reported to be almost absolutely dependent on its cognate β subunit, Bro or Bg (29), or the mouse PEBP2β (16). Although it was speculated that the Runt protein by itself, unlike its mammalian homologs, might favor a conformation that could bind to DNA poorly (29), it remains to be tested whether this protein alone could show appreciable DNA binding activity under improved conditions.

Apart from the preceding stability issue, a slight but noticeable difference was also observed between the RD fragment and the intact αA1 in their absolute $K_d$ values attainable in the presence of the β subunit (0.2 and 0.4 nM for the RD fragment and αA1, respectively). The higher $K_d$ value of αA1, together with its weaker β-mediated protection, suggests the possibility that the C-terminal region present in the intact αA1 might exert negative steric or conformational influence on the interaction between the Runt domain and the β subunit. Similar views have also been presented from functional studies with PEBP2β/AML1 in vivo and in vitro. The DNA binding activity of the mouse αβ subunit is considerably strengthened when its 60-amino acid sequence immediately C-terminal to the Runt domain is deleted either by in vitro DNA recombination or by natural alternative splicing (37); the human 250-amino acid AML1a protein has a stronger DNA binding affinity than do the longer isoform AML1b (equivalent to mouse αB1) and the AML1-Evi1 chimeric protein (38); αB1 derivatives deleted of regions outside the Runt domain are more potent than the intact one in their ability to promote the translocation of the β subunit from the cytoplasm to the nucleus (39). Such intramolecular functional modulations of the Runt domain proteins and their chimeric derivatives could have important implications in hematopoietic differentiation and leukemogenesis, as frequently suggested (16, 37–40). Thus, it would be interesting to make detailed comparative analysis of these proteins with respect to their DNA binding and heterodimerization functions under improved assay conditions established here.

A number of crucial questions remain regarding 1) the identity of the physiological reducing agent, 2) which one of the two conserved cysteine residues is the actual target of redox regulation, and 3) whether the redox regulation of PEBP2 occurs in vivo and, if so, what its exact regulatory significance is. As for question 1, we have found that DTT can be effectively substituted by thioredoxin/ADF or Ref1/APEX. To address question 2, we are carrying out site-directed mutagenesis of the two cysteine residues. Preliminary results suggest that the both cysteines are important. Regarding the most crucial question, question 3, Fos/Jun and NF-κB would again serve as suggestive precedents (41). In the Fos oncoprotein, a point mutation of the redox-sensitive cysteine residue to serine causes increased in its DNA binding activity in vitro as well as its transformation potential in vivo (42). As a similar, naturally occurring mutant, the v-Jun oncoprotein has a serine residue at the position corresponding to the redox-sensitive cysteine in the c-Jun proto-oncoprotein (43). NF-κB has also been implicated in the redox-dependent regulation of various cellular and viral genes specifically expressed in lymphoid cells such as cytokines (interleukin-2, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-α, and interleukin-6) and their receptors (interleukin-2 receptor α chain and major histocompatibility complex class I genes (reviewed in Ref. 44), and human immunodeficiency virus long terminal repeat (45, 46). Since the PEBP2/AML1 protein family is also essential for a similar repertoire of lymphoid-specific genes and differentiation of hematopoietic cell lineages as outlined in the Introduction, it is tempting to speculate that its redox regulation might have important roles, similar to the case of NF-κB. Work is under way to experimentally address this possibility in vivo by exploiting the above noted mutants of PEBP2α with altered redox responses.

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