Two Isoforms of Protein Disulfide Isomerase Alter the Dimerization Status of E2A Proteins by a Redox Mechanism*

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We have shown previously that E2A helix-loop-helix proteins spontaneously form an intermolecular disulfide cross-link that is required for stable homodimer binding to DNA (Benezra, R. (1994) Cell 79, 1057–1067). These homodimers are important for the development of B lymphocytes but are not present in other cell lineages. We have purified two proteins that are capable of regulating the formation of this disulfide bond and found them to be members of the protein disulfide isomerase (PDI) family. By regulating the formation of the disulfide cross-link, these proteins are capable of regulating the dimerization state of E proteins. PDI-mediated reduction appears to dissociate E protein homodimers and favors heterodimer formation with other basic helix-loop-helix proteins in both a purified protein system and in cellular extracts. These studies suggest that PDI may play an important role in the regulation of E2A transcription factor dimerization and the development of the B lymphocyte lineage.

The basic helix-loop-helix (bHLH) proteins are a family of lineage- and development-regulated transcription factors that appear to regulate cell type-specific transcription and differentiation (for reviews, see Refs. 2 and 3). bHLH proteins are distinguished by a dimerization domain consisting of two amphipathic α helices linked by a loop segment. The basic domain is responsible for DNA binding and is characterized rich in the basic amino acids lysine and arginine (4–6). The bHLH family is divided into several groups. In mammals, the E proteins, consisting of the E2A gene products (the alternate splice products E12, E47, and E2–5), E2–2, and HEB (5, 7–12), are expressed in most tissues during all stages of development and play a critical role in immunoglobulin rearrangement and B cell differentiation as evidenced by the loss of function mutations in mice (33, 37, 40, 41). In addition, overexpression of E47 is sufficient to stimulate sterile transcription from the IgH enhancer in fibroblasts and immunoglobulin rearrangement in a pre-T cell (42, 43).

Restiction of E2A homodimers to the B cell lineage may in part be explained by the formation of a tissue-specific intermolecular disulfide bond that is required for stable homodimer DNA binding (1). Importantly, this disulfide bond is formed in the majority of B cell-specific DNA-binding complexes as detected by electrophoretic mobility shift assays. The bond is formed between cysteine residues located at position 570 in the first helical region of the bHLH domain. E protein homodimer formation in cells other than pre- and mature B cells is inhibited by the Id proteins, lower E2A gene expression, and an activity that apparently reduces the disulfide bond necessary for E2A homodimer stability at physiologic temperatures (1, 33, 44).

We report here the purification and identification of two proteins from HeLa cell extracts which are capable of regulating the E protein dimerization state via a redox mechanism. These proteins are both members of the protein disulfide isomerase (PDI) family and are capable of reducing a disulfide bond that links E protein homodimers. Reduction of this bond prevents E protein homodimerization and fosters E protein heterodimerization in both a purified protein system and with endogenous bHLH complexes from cell extracts. Thus, the PDI family of redox active proteins is capable of regulating E protein dimerization and may play a role in the B cell developmental pathway.

MATERIALS AND METHODS

Preparation of Recombinant Proteins and Electrophoretic Mobility Shift Assays—E47, Flag-E47, PDI I, and PDI II were cloned into pET28 (Novagen), expressed in BL21/DE3 Escherichia coli, and purified by nickel-chelate chromatography as recommended by the manufacturer. Flag-E47 contains the sequence DYKDDDDK consisting of the core Flag epitope tag NH2-terminal to the E47 coding sequence. HMK-E47 was created by removing the NH2-terminal T7 epitope tag from Flag-E47 and replacing it with the sequence PRASSV, containing the phosphorylation sequence for bovine heart muscle kinase (HMK). This protein was then radiolabeled as described in Blanar and Rutter (45).
Protein preparations were dialyzed extensively against a buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA, and 350 mM KCl. Recombinant Id-1 was cloned into pET19b (Novagen), expressed in E. coli, and purified by nickel-chelate chromatography as recommended by the manufacturer. PDI was also prepared from insect cells using the baculovirus system (used in Fig. 3D) and the discontinuous SDS gel. The protein composition of immunodepleted material was analyzed by silver staining (ICN Biomedicals, Inc.).

Reduction Assay and Protease Studies—For reduction assays samples and fractions were added to 20 ng of E47, Flag-E47, or HMK-E47 substrate and incubated at 37 °C for the indicated times. SDS loading buffer was then added, and samples were immediately incubated at 95 °C for 5 min. Monomeric and dimeric E47 were resolved by discontinuous 7.5% SDS-PAGE. In experiments using HMK-E47 substrate, gels were fixed in 10% acetic acid, dried, visualized by autoradiography, and quantified by PhosphorImager analysis. In experiments using E47 and Flag-E47 substrates, proteins were transferred into nitrocellulose filters, and E47 was detected by Western blot using anti-T7 or anti-Flag monoclonal antibodies, respectively.

For protease studies, 2 µg of HeLa cell extract was incubated with the indicated amounts of trypsin for 15 min at 37 °C. Reactions were stopped by the addition of protease inhibitors (1 ng of leupeptin, 1 ng of antipain, 10 ng of benzamide hydrochloride, 10 ng of soybean trypsin inhibitor). Treated extracts were then used in the reduction assay described above.

Protein Microsequencing—Proteins were resolved by SDS-PAGE and transferred into nitrocellulose membranes. Porcine S-stained bands were excised, digested in situ with trypsin, and resulting peptides were fractionated by reverse phase high performance liquid chromatography using a 0.8-mm Vydac C18 column (52). Selected peptides were analyzed by a combination of matrix-assisted laser desorption time-of-flight mass spectrometry (Reflex III; Bruker-Franzen, Bremen, Germany) and chemical microsequencing (477A; Applied Biosystems, Foster City, CA). 12 tryptic peptides derived from the PDI immunoprecipitation were analyzed by chemical sequencing/matrix-assisted laser absorption ionization mass spectrometry. The sequences (K)VHSFPTLK(F), (K)KAEGSIEIR(L), (R)TETFCHR(F), (R)TVDIYNGER(T), (R)EADDIVNLKK(K), (K)QLYLDKDGVVFV, (K)KPEENVNTRL, (K)KLCVYKHENIVIAK(K), (K)KFDEGRNNPEGEVTKE, (R)ILEFGLKKECPAVR(L), (K)IKPHLMSQELPEDWDKPKVPK(V), and (K)QFLQFAQAEIDIPGFITSNSDFVSFKY( ) are identical to sequences found in PDI (54).

Nine tryptic peptides derived from the 57-kDa heparin-agarose protein were analyzed by MALDI mass spectrometry. Their predicted sequences (R)TADGIVSHILKK(Q), (K)QMVMQESFR(D), (K)LKRDPIVLKMK(M), (R)ELSDFISSLGRL(E), (K)KVQVDANT-NTCNK(Y), (R)FLQVDFTKDN-LKRY(Y), (R)EATNPVQIEEKKK(K), (K)YLKSEPIPE-SNDGPV(K), and (R)FAQHNIVSLNVEYDNGEGLIRFPLHLTNK(F) are identical to sequences found in phospholipase Ca (55).

RESULTS

We have demonstrated previously that homodimers of the bHLH protein E12 spontaneously form disulfide cross-links that span the two bHLH domains (1). Fig. 1A demonstrates that E47 (the major splice product of the E2A gene (38)) is isolated from bacteria as a 50% mixture of species with monomeric and dimeric apparent molecular weights. The dimer is not observed in the presence of the strong reducing agent β-mercaptoethanol, suggesting that there is a linkage mediated by a disulfide bond similar to that of E12. Electrophoretic mobility shift analysis demonstrates that under nonreducing conditions E47 is fully functional in binding DNA as a homodimer (Fig. 1B). Inhibition of binding by heterodimerization with the dominant negative HLH protein Id-1 is dependent upon inclusion of the reducing agent DTT in the binding reaction (lane 5). It appears likely that, similar to E12, reduction of the disulfide bond in E47 homodimers is necessary for the formation of heterodimeric interactions.

Active activity present in cellular extracts can reduce the intermolecular disulfide bond in E47 homodimers. To monitor this activity we designed an assay that directly detects the presence of the disulfide bond in a Western blot analysis. In the following assays, bacterially expressed, epitope-tagged E47 protein was purified and diazyed into nonreducing buffers. Aliquots of this protein were mixed with cellular extracts and incubated at
37 °C. Monomeric and dimeric E47 proteins were then resolved by nonreducing SD±-PAGE and detected with antibodies against the epitope tag. As demonstrated in Fig. 2A, an activity present in C2C12 myoblast extracts is able to convert oxidized, homodimeric E47 to the monomeric state in a time-dependent manner. To investigate the distribution of this activity, we tested extracts from several different cell types. This activity appears to be present in extracts from many different cell types including lines derived from myoblast (C2C12), epithelial (HeLa), and erythroid (MEL) lineages but is conspicuously reduced in a B lymphocyte cell line (Namalwa) (Fig. 2B, left). Quantitative analyses of B cell extracts (assay described below) demonstrate that lymphocytes contain less than 50% of the activity present in HeLa cells (Fig. 2B, right). We also note that neither thioredoxin, NADH, NADPH, FADH2, nor CoA is capable of converting the dimer to a monomer (data not shown). This indicates that the factor responsible for the activity has widespread tissue distribution, is reduced significantly in B lymphocytes, and is not any of a number of small, physiologic reducing agents.

To demonstrate that this reduction activity is a protein, protease studies were performed. Fig. 2C demonstrates that cellular extracts predigested with the serine protease trypsin lose significant activity (compare lane 3 with lanes 4–6) compared with extracts treated with protease inhibitors alone. This indicates that the reduction activity depends on at least one protein component. Extracts containing this activity can also be inactivated by incubation at 85 °C (data not shown).

We sought to purify this activity by standard biochemical fractionation procedures. First, to obtain quantitative estimates of specific activity, we added a bovine HMK site to our substrate E47 and radiolabeled the protein using [γ-32P]ATP. Use of this radiolabeled protein allows rapid, quantitative analysis of E47 conversion by PhosphorImager analysis. HeLa cytoplasmic lysates were used as a source of activity because these cells could be grown to high density and are particularly rich in the activity. As a first step, 2.5 g of crude cytoplasmic HeLa lysate was dialyzed against buffer A, applied to a 120-ml Q-Sepharose column, and eluted with a linear gradient of KCl (Fig. 3A). The reduction activity is plotted as the amount of monomeric E47 generated after incubation of dimeric E47 with each fraction at 37 °C for 5 min. The reduction activity clearly separates into two active peaks, one eluting at approximately 200 mM KCl and one at 350 mM KCl. We designated these two peaks Qp1 and Qp2, respectively. In these reduction assays, we define 1 unit of activity as that which produces a 25% increase in monomeric E47 in a 5-min reaction at 37 °C. The input cytoplasmic lysate contained 1.81*10^6 units with a specific activity of 725 units/mg of protein, Qp1 contained 3.49*10^6 units with a specific activity of 2.8*10^4 units/mg, Qp2 contained 1.33*10^6 units with a specific activity of 1.1*10^4 units/mg, and flow-through and washes contained no significant amounts of activity. This net increase in activity is probably the result of removal of proteins present in the crude lysate which inhibit or interfere with the reduction activity.

The enzyme PDI has recently been demonstrated to be important for the redox regulation of several mammalian and chloroplast transcription and translation factors and can catalyze the net reduction of proteins (56–58). Because our reduction activity behaves similarly to PDI on several purification resins, we sought to determine whether PDI was responsible for this activity. For this purpose, we performed Western blots on fractions eluted from the Q-Sepharose column using an anti-PDI monoclonal antibody. This antibody reacts with a 57-kDa band that coelutes with Qp2 (Fig. 3A, lower panel). Pooled active Qp2 fractions were then immunodepleted using either anti-PDI antibody or an unrelated monoclonal antibody (anti-T7, Novagen), and supernatants were assayed for activity. Fractions depleted with control antibody retain significant activity, whereas those depleted using anti-PDI lose all activity (Fig. 3B). Immunoprecipitated material from the immunodepletion analysis was then resolved by SDS-PAGE and visualized by silver staining. The immunoprecipitated material contained a single major protein of approximately 57 kDa with no significant contaminating species (Fig. 3C, lanes 2 and 3). The band marked with a line in lane 3 corresponds to full-length immunoglobulin that can be dissociated into heavy and light chains by inclusion of β-mercaptoethanol in the SDS loading buffer (lane 2, heavy chain marked with line). Microsequence analysis of several tryptic peptides derived from this 57-kDa protein identified it as a PDI family member (54), which we will call PDI I. To prove that PDI I is capable of performing this reduction reaction, recombinant PDI I was produced using the baculovirus system and demonstrated reduction activity with a specific activity of 2.81*10^4 units/mg of protein (Fig. 3D).

Western blot analysis revealed that Qp1 does not contain PDI I (Fig. 3A, lower panel). It seemed likely, therefore, that a second protein is responsible for the activity in Qp1, and we attempted to purify the activity. The pooled Qp1 fractions were dialyzed against buffer B, loaded onto a 20-ml SP-Sepharose column and eluted using a KCl gradient. Activity was recovered in a single major peak that eluted at approximately 150 mM KCl (Fig. 4A). Active fractions were pooled and found to contain approximately 26% of the starting activity, yielding an 83-fold increase in specific activity.

SD±-PAGE analysis of protein in the SP active peak demonstrated a major protein species of approximately 57 kDa (Fig. 4C, lanes 4 and 9). To purify the activity away from remaining contaminants further, the active SP peak was dialyzed against buffer C, loaded onto a 4-ml heparin column, and eluted with a linear gradient of KCl. Activity was again recovered in a single major peak that eluted at approximately 200 mM KCl (Fig. 4B).
FIG. 2. Characterization of the reducing activity. Panel A, 2 μg of C2C12 myoblast whole cell lysate was incubated with 150 ng of purified Flag-E47 for the indicated times at 37 °C. The reaction mixture was then separated by 7.5% discontinuous SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-Flag M2 monoclonal antibody (Sigma). Panel B, left, 150 ng of Flag-E47 was exposed to buffer alone (lane 1), 10 mM βME (lane 2), or 2 μg of extract from the indicated cell lines (lanes 3–6) for 30 min at 37 °C before separation by SDS-PAGE, as described in panel A. Monomeric and dimeric E47 are indicated. Right, reduction time course of HMK-E47 using 4 μg of extract from Namalwa (∗) or HeLa (■) cells. HMK-E47 was incubated with extracts at 37 °C for indicated times. Monomeric E47 was resolved by SDS-PAGE and quantitated by PhosphorImager analysis. The y axis is marked in arbitrary PhosphorImager units. Panel C, HeLa cell extract (2 μg) was incubated with 0, 0.5, 0.25, or 0.125 μg of trypsin (lanes 3–6) for 15 min at 37 °C. Protease reactions were stopped by the addition of protease inhibitors. Protease-treated extracts were then incubated with 100 ng of Flag-E47 for 20 min at 37 °C. Reduced and oxidized Flag-E47 was then resolved by discontinuous SDS-PAGE and visualized by Western blot using anti-Flag antibody. Lane 1, Flag-E47 input; lane 2, Flag-E47 incubated with 10 mM β-mercaptoethanol.
The active fractions were pooled and represented approximately a 4.6% yield of initial activity with a specific activity of \(2.8 \times 10^4\) units/mg of protein, yielding a 31.5-fold purification over the initial lysates. The low yield and fold purification may be the result of removal of cofactors during the process of purification. A similar problem arose during Ref-1 purification and was corrected by the addition of thioredoxin (59). However, neither thioredoxin, NADH, NADPH, nor FADH2 rescued reduction activity in our preparations (data not shown). Active fractions again contained a single major 57-kDa protein (Fig. 4A, lanes 5 and 10).

Mass spectroscopy of several tryptic peptides derived from the 57-kDa protein led to its identification as phospholipase C\(\alpha\), a second member of the PDI family (55). Because this protein has PDI activity but lacks phospholipase activity (60, 61) (it was inaccurately named on initial identification (55)) it will be referred to as PDI II in this report. To prove that PDI II is capable of performing this reaction, recombinant PDI II was produced in bacteria and demonstrated reducing activity with a specific activity of \(1 \times 10^6\) units/mg of protein (Fig. 4D).

Reduction of the Cys-570 disulfide is critical for inhibition of E protein homodimerization and for fostering heterodimeric interactions with other HLH proteins (1). We used electrophoretic mobility shift assays to investigate whether PDI is capable of regulating the dimerization state of E proteins. For these assays we used the alternate splice product of the E2A gene, E12, because E12 forms heterodimers more efficiently than E47 in our assays. We included purified Hsp-90 in the DNA binding reactions to enhance the affinity of MBP-E12 for DNA (48). Full-length E12 binds DNA as a homodimer but is resistant to heterodimerization with the HLH protein MyoD in the absence of reducing agents such as DTT (Fig. 5A; compare lanes 4 and 5). Incubation of E12 with recombinant PDI I or PDI II strongly fosters heterodimer formation while completely abrogating the homodimer-dependent shift in the presence of MyoD (lanes 7 and 9). It is important to point out here that redox inhibition of E protein homodimerization using this purified protein system seems to be dependent on the presence of dimerization partners such as MyoD (Fig. 5A) or Id-1 (see below). Homodimer-dependent shifts observed under reducing conditions in the absence of heterodimeric partners (Fig. 5A, lanes 3, 6, and 8) could be the result of either DNA binding by the reduced form of the homodimer in this purified protein system or spontaneous reoxidation of the disulfide during elec-
trophoresis (as has been observed previously (1)). In this assay, free probe has been electrophoresed off the bottom of the gel to achieve greater resolution of MyoD-E and E-E complexes. Shorter gel runs have demonstrated that there is an excess of free probe in each lane (data not shown).

The dominant negative HLH protein Id disrupts E protein homodimers in solution and prevents DNA binding (20, 21, 33). To see if PDI is also capable of fostering this heterodimeric association, recombinant Id-1 protein was used in electrophoretic mobility shift assays. A 15-fold molar excess of Id-1 is incapable of preventing E protein homodimerization in the absence of DTT (Fig. 5B, compare lanes 4 and 5). Similar to the results with MyoD, recombinant PDI I and PDI II were extremely efficient in fostering Id heterodimerization when included in the binding reaction (lanes 6–9).

Because E protein homodimerization appears to be stabilized by disulfide cross-linkage in vitro (this paper and Ref. 1), we sought to determine if the dimerization state of the E proteins in different cellular extracts could be regulated by changes in redox. For this purpose we used three cell lines, 3T3 (a fibroblast line), RH18 (a myoblast line), and Namalwa (a pre-B lymphocyte line). An electrophoretic mobility shift assay (Fig. 6A) confirms previous observations that BCF-1, a high molecular weight E box-binding species consisting of E protein homodimers (35, 38, 39), is present only in B cell nuclear extracts (34, 36, 37). This endogenous E protein homodimeric species can be dissociated by reduction with DTT (Fig. 6A, lane 8, and Ref. 1). Similarly, incubation of B cell nuclear extracts with
FIG. 4. Purification and identification PDI II as an E2A-reducing enzyme. Reduction activity analysis of fraction eluted from each column is shown: panel A, SP-Sepharose; panel B, heparin. The area between the gray lines in each profile represents the pooled peak fraction containing reduction activity. Panel C, 3.5 µg of pooled active fractions from each column was resolved on a 15% (lanes 1–5) and 10% (lanes 6–10) SDS-polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue dye. Lanes 1 and 6, molecular weight markers; lanes 2 and 7, input cytoplasmic lysate; lanes 3 and 8, pooled Qp1 fractions; lanes 4 and 9, pooled SP-Sepharose active fractions; lanes 5 and 10, pooled heparin active fractions. Panel D, HMK-E47 was incubated with 200 ng of bacterially produced PDI II (□) or buffer alone (○) at 37 °C for indicated times. Monomeric E47 generated was resolved by SDS-PAGE and quantitated by PhosphorImager analysis.
PDI Alters the Dimerization Status of E2A Proteins

Fig. 5. PDI can alter the dimerization potential of E proteins. Panel A, purified MBP-E12 (150 ng) mixed with MBP-hsp90 (150 ng) was incubated with or without MyoD (150 ng) as indicated. Reactions also contained buffer alone (lanes 2 and 4), 10 mM DTT (lanes 3 and 5), 100 ng of bacterially produced PDI I (lanes 6 and 7), or 100 ng of PDI II (lanes 8 and 9). Reactions were heated to 37 °C for 5 min, then transferred to room temperature for 5 min and assayed using electrophoretic mobility shift assays. Lane 1, probe alone; lane 10, MyoD alone. The free probe is below the region of the gel displayed. Panel B, identical to panel A except 500 ng of Id-1 was used instead of MyoD.

Fig. 6. PDI can disrupt endogenous E protein homodimers. Panel A, 5 μg of extracts from RH18 myotubes (lanes 3–6) or Namalwa pre-B lymphocytes (lanes 7–10) was mixed with buffer alone (lanes 3 and 7), 100 mM DTT (lanes 4 and 8), 500 ng of bacterially produced PDI I (lanes 5 and 9), or 500 ng of PDI II (lanes 6 and 10). Reactions were incubated at 37 °C for 5 min and then analyzed in a 37 °C DNA binding assay using a radiolabeled E box-containing probe. Lane 1, probe alone; lane 2, NIH-3T3 fibroblast extract. Panel B, identical to panel A except DNA binding assays were performed using an octamer binding site-containing oligonucleotide. The free probe is below the region of the gel displayed.

of regulating the formation of this bond. These proteins, both members of the PDI family, can reduce the disulfide bond in preformed E protein homodimeric complexes. Upon reduction, E proteins are rendered competent to form heterodimeric interactions with other bHLH proteins, and in cellular extracts this reduction is sufficient to prevent these proteins from forming homodimers. These results suggest that PDIs are capable of regulating E protein dimerization by a redox mechanism.

PDIs are a family of redox active enzymes expressed in most cell types and have been characterized as abundant ER resident enzymes. PDI family members are characterized by an NH2-terminal ER localization sequence and a COOH-terminal KDEL sequence that allows ER retention. Redox properties are conferred by two thio-redoxin-like domains that contain the core sequence Cys-Gly-His-Cys (CGHC). The cysteine residues in this motif are highly redox-reactive and participate in both the disulfide rearrangement and reduction activities of PDI (54, 62–64). Although classically thought of as ER proteins, PDIs also play a role outside the ER. PDI family members have been found in the nuclei of maturing spermatids where they may play a role in the redox-dependent condensation of spermatid chromatin (65–67). PDI family members have also been found in chloroplasts where they appear to be responsible for the redox control of chloroplast polyadenylate-binding protein (56). Redox alterations in chloroplast polyadenylate-binding protein alter its ability to bind the 5′-untranslated region and regulate the translation of psbA mRNA, which encodes the photosynthetic reaction center protein D1 from the green alga Chlamydomonas reinhardtii (68–71). PDIs have also been found to be important in the redox regulation of factor binding to enhancers of interferon α-induced genes (57) and may play a role in the redox control of NF-κB and AP-1 transcription factor (58). These data suggest that PDI may play an important role in the redox regulation of many non-ER proteins.

DISCUSSION

Previous studies have demonstrated that homodimers of the bHLH proteins known as E proteins are stabilized by an intermolecular disulfide bond (1). In the present study, we have purified and characterized two cellular factors that are capable

recombinant PDI I or PDI II can also lead to the complete abrogation of this signal (lanes 9 and 10). In contrast, the myogenic specific DNA-binding species, the result of a Myo-D-E protein heterodimeric complex (14), is completely resistant to both DTT and PDI (lanes 4, 5, and 6), demonstrating that the reduced form of E proteins is fully functional but can only participate in heterodimeric interactions. It is noteworthy to point out here again that E protein homodimeric DNA-binding species present in complex cellular lysates such as nuclear extracts (Fig. 6) or reticulocyte lysates (1) can be abrogated by incubation with reducing agents such as PDI or DTT, whereas purified E protein homodimer disruption requires partners such as Id or MyoD in addition to redox changes (Fig. 5, A and B) (see also “Discussion”).

In contrast to E protein complexes, the octamer binding proteins are not altered by changes in redox state. As shown in Fig. 6B, Oct-1 from RH18 and Namalwa cells (and Oct-2 from Namalwa cells) binds DNA even in the presence of DTT or when preincubated with PDI I or PDI II. This indicates that neither DTT nor PDI leads to a global disruption of DNA binding and is presumably specific for redox-responsive species such as BCF-1. Shorter gel runs of the experiments in Fig. 6, A and B, have demonstrated that there is an excess of free probe in each lane (data not shown).

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Members of the E protein family are expressed in most tissues during many stages of development but fail to form homodimers in cells outside the B lymphoid lineage. E protein homodimeric complexes are required for B cell differentiation and are termed BCF-1 (35, 37–39). In non-B cells, it has been hypothesized that homodimerization is prevented by members of the dominant negative HLH family, the Id proteins. However, even in the absence of such dimerization inhibitors, for example in cells grown under low serum conditions, E proteins do not homodimerize in non-B cells. Logically, then, there must be inhibitory factors, presumably other than Id proteins, which are expressed in non-B lymphoid lineages but are absent, sequestered, or inhibited in maturing B cells. We have demonstrated that PDIs are present in non-B cell lysates and are sufficient to dissociate preformed E protein homodimers present in B lymphocyte extracts. We have also demonstrated that B cells contain significantly reduced amounts of the PDI dimerization activity compared with non-B cells. Because preliminary results indicate that there is not a significant difference in the absolute levels of PDI in B cells and non-B cells, we are lead to speculate that PDI activity is regulated by inhibitors or sequestration in B lymphocytes.

As noted earlier, DNA binding by E protein homodimers can be disrupted by reduction but only in the presence of accessory factors such as dimerization partners or a factor found in cellular lysates. It is possible that Id proteins play this accessory role in immature B cells. However, the mature B cell line used in this study is known not to express Id or any other known HLH protein, yet their E protein homodimers can be disrupted by exogenous PDI. Therefore, it seems likely that there is an unknown cellular factor responsible for preventing homodimerization of reduced E proteins. We hypothesize that this factor is present in both B cells and non-B cells and acts in concert with redox to disrupt the reduced form of the homodimer.

One alternate proposed mechanism of cell type-specific regulation of E protein homodimerization involves casein kinase II. Studies have elucidated two casein kinase II sites in E47 which prevent DNA binding by E47 homodimers. This phosphorylation event appears to occur in non-B cells only and may serve as one means of inhibiting the E protein homodimer-DNA complex. Importantly, phosphorylation does not disrupt E protein homodimers but merely prevents DNA binding once these dimers are formed (72). Phosphorylation-dependent regulation may act in concert with redox regulation to prevent DNA binding by any homodimers that escape redox control.

Redox regulation of transcription factor function has emerged as a potentially important and widespread mechanism of gene regulation. The growing list of redox-regulated factors currently includes such well known proteins as AP-1, NF-κB, and p53 (73–80). Each of these proteins has a decreased affinity for DNA binding under nonreducing conditions. DNA binding capacity can be rescued by strong reducing agents such as DTT or a nuclear bipotential reducing factor/DNA repair enzyme called Ref-1 (59). Redox susceptibility has been mapped to specific cysteine residues in the DNA binding domains of each of these proteins that undergo reversible redox alterations depending on environmental redox potentials. In contrast to the behavior of AP-1 and NF-κB, the transcription factor E2A is fully capable of binding DNA under oxidizing conditions (this report and Ref. 1). For E proteins, redox alterations seem to alter the dimerization potential of the molecule, encouraging the formation of homodimers under oxidizing conditions and heterodimers under strongly reducing conditions. This method of regulation is also utilized by the transcription factors OxyR and NF-Y. The bacterial transcription factor OxyR binds DNA as a homotetramer and acts as a transcriptional repressor (81, 82). Upon oxidative stress, each OxyR molecule in the tetramer forms an intramolecular disulfide bond resulting in a significant alteration in the conformational state of the complex as determined by DNase I footprint analysis (83). Oxidation also results in conversion of the molecule to a transcriptional activator that promotes transcription of several genes responsible for defense against oxidative stress (84, 85).

NF-Y is a eukaryotic transcription factor that binds the CCAAT-containing promoter and enhancer elements in many RNA polymerase II-transcribed genes. It is a heterotrimeric complex of three proteins, NF-YA, NF-YB, and NF-YC. Under oxidizing conditions, NF-YB fails to bind NF-YA and NF-YC and forms intramolecular disulfide cross-linked homodimers. Incubation of NF-YB with DTT reduces these disulfide bonds and fosters association with NF-YA and NF-YB. Although the function of these NF-YB homodimers is unknown, it is possible that they may function as a distinct DNA-binding species that is subject to redox regulation similar to that of E2A (85). In summary, the present studies demonstrate that members of the PDI family can regulate E protein homodimerization by a redox mechanism. This dimerization event is critical for the formation of the B lymphoid lineage, so PDIs may play an important role in B lymphopoiesis. Interestingly, E2A gene products, in addition to being required for B cell development, are also required for postnatal survival and prevention of T cell lymphomagenesis (86, 87), and it has been speculated that this may be because of their roles as heterodimeric partners for other bHLH proteins. Mutation of the critical Cys-570 residue of the endogenous E2A gene in mice may provide a method of examining the role of heterodimers specifically as this mutation prevents E protein homodimers from forming while leaving heterodimeric complexes intact.

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