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Oxidative Stress Drives Disulfide Bond Formation Between Basic Helix–Loop–Helix Transcription Factors

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ABSTRACT

Basic helix-loop-helix (bHLH) transcription factors including Twist1 and E2a proteins regulate essential processes. These factors bind DNA as homo- or heterodimers and the choice of binding partners determines their functional output. To investigate potential regulators of bHLH dimerization, cells were exposed to the oxidative agent hydrogen peroxide (H_2O_2). Western blot analysis in the presence or absence of reducing agents, revealed that H_2O_2 induces the rapid formation of an intermolecular disulfide bond between Twist1 homodimers and Twist/E2a proteins heterodimers. The disulfide bond is first observed between Twist1 homodimers at 25 mM H_2O_2 and between Twist1 heterodimers at 75 mM H_2O_2. This response is dependent upon cell density as H_2O_2 did not induce disulfide bridge formation between bHLH proteins in cells seeded at high density. In the presence of E proteins, the formation of Twist1/E2a proteins heterodimers is favored over Twist1 homodimers, identifying an oxidative stimulus as an important factor in modulating binding partner specificity. We further demonstrated that a cysteine residue located at the C-terminus of Twist1 and E2a proteins is involved in this response. Disulfide bond formation between Twist1 homodimers significantly reduced its ability to interact with two of its binding partners, Runx2 and HDAC4, indicating that disulfide dimerization in response to H_2O_2 has functional significance. These data support the conclusion that disulfide bond formation in response to an oxidative stimulus contributes to Twist1 homo- and heterodimerization and raises the possibility that the redox status of a cell may represent an important step in bHLH transcriptional regulation. J. Cell. Biochem. 109: 417–424, 2010.

KEY WORDS: bHLH TRANSCRIPTION FACTORS; HYDROGEN PEROXIDE; OXIDATIVE STRESS; DISULFIDE BONDS

Basic-helix-loop-helix (bHLH) family of DNA binding proteins regulates essential processes such as cell cycle control, cell lineage commitment, and cell differentiation. bHLH factors can be classified into several groups based on their partner choice, tissue distribution, DNA binding properties, and structural features [Massari and Murre, 2000]. The dimerization partner of a given bHLH protein can alter its DNA binding preference [Blackwell and Weintraub, 1990] or, as in the case of Id, an HLH protein lacking the basic domain, DNA binding is abolished [Benezra et al., 1990]. It is essential then to identify factors that change the binding preference of bHLH proteins in order to determine which genes are activated when the intracellular concentrations of these proteins are known.

The bHLH transcription factor Twist was first identified in *Drosophila* as a zygotic developmental gene involved in early mesoderm patterning [Thisse et al., 1987]. Two *Twist* genes exist in vertebrates, *Twist1* and *Twist2*. *Twist1* is required for closure of the neural tube during mouse development [Chen and Behringer, 1995]. Mice homozygous for a *Twist2* null allele have elevated expression of proinflammatory cytokines and die perinatally [Sosic et al., 2003]. *Twist1* haploinsufficiency is associated with Saethre–Chotzen syndrome, which is the most common autosomal dominant disorder of craniosynostosis [el Ghouzzi et al., 1997]. Tissue-restricted Twist proteins form heterodimers with the bHLH E proteins that have a wide tissue distribution. The E2a proteins E12 and E47 are alternatively spliced transcripts of the E2a gene with different exons coding for the bHLH domains [Murre et al., 1989; Sun and Baltimore, 1991]. A recent study determined that, depending on the Id level in cells, Twist1 forms functional homodimers which have different activities from Twist1/E2A protein heterodimers [Connerney et al., 2006].

Twist1 transiently inhibit osteoblast differentiation during skeletogenesis through the direct interaction with Runx2, a key transcriptional factor regulating osteogenic gene expression [Bialek et al., 2004]. The anti-osteogenic domain of Twist1 is located at its

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C-terminus and is called the "Twist box." A recently identified binding partner of Twist1 is histone deacetylase 1 (HDAC1), a member of the HDAC family [Hayashi et al., 2007]. HDACs participate in a dynamic process of chromatin remodeling, forming complexes that repress transcription by deacetylating histones and transcription factors [reviewed in Haberland et al., 2009. HDAC4 has a central role in the formation of the skeleton through its ability to delay chondrocyte hypertrophy and thus control the timing and extent of ossification of endochondral bones [Vega et al., 2004]. Very little is known about how bHLH dimerization affects interactions between these transcription factors and their nonbHLH binding partners.

Reactive oxygen species (ROS) include oxygen anions and radicals ($^{\bullet}O_2^-$ and $OH^{\bullet}$) or milder oxidants such as hydrogen peroxide (H_2O_2). H_2O_2 has been used extensively to examine redox regulation of several processes including signal transduction, cell proliferation, and apoptosis. The generation of ROS has been connected to stress responses, apoptosis, and aging resulting from nonspecific damage to vital cellular components such as DNA, lipids, and proteins. However, over the recent years, it has become clear that ROS can also cause specific protein modifications that may lead to a change in the protein's activity or function (reviewed in Barford, 2004]. Protein sulfhydryls can be oxidized to protein disulfides thus, by reacting with ROS, cysteine residues function as detectors of cellular redox status. The vast majority of proteins do not contain disulfide bonds at physiological pH due to the reducing environment of the cytosol. However, proteins which can act as sensors of the intracellular redox state, possess cysteine residues that exist as thiolate anions at neutral pH and thus are more vulnerable to oxidation [Rietsch and Beckwith, 1998]. Recent data demonstrate the existence of cytoplasmic protein disulfide bond formation in nonstressed as well as oxidant-stressed cells [Cumming et al., 2004]. Redox control of transcription factor activity has been demonstrated for various proteins including NF-κB and AP-1, via highly conserved cysteine residues [Sun and Oberley, 1996].

In this report, oxidative stress was found to be an important modulator of bHLH dimerization specificity. We demonstrate that in response to H_2O_2 exposure, Twist1 protein has a strong propensity to form an intermolecular disulfide bond between Twist1/Twist1 homodimers as well as Twist1-E protein heterodimers. Furthermore, there is a significant preference for HDAC4 and Runx2 interaction with Twist1 that is not disulfide linked. In the case of Runx2, these findings indicate that at least for the period that Twist1 responds to H_2O_2, a significant portion of Twist1 does not interact with Runx2 and thus is not able to interfere with Runx2 function. These data suggest that the redox state of a bHLH protein can significantly alter its dimerization status and possibly affect gene regulation.

MATERIALS AND METHODS

CONSTRUCT GENERATION

Xenopus Twist-1 (xTwist-1) was subcloned into pcs4$^+$ using the restriction sites *Eco*RI and *Xho*I. Flag and HA-xTwist-1 were generated by subcloning xTwist-1 into the pcs4$^+$-3FLAG and pcs4$^+$-3HA vectors, respectively. Mouse Twist-1 (mTwist-1; IMAGE clone 4935230) was subcloned into pcs4$^+$, using the restriction sites *Bgl*II and *Xho*I. Flag and HA mouse Twist-1 were generated by subcloning mouse Twist-1 into the pcs4$^+$-3Flag and pcs4$^+$-3HA vectors, respectively. CMV5-HA-human E12 was a kind gift from Andrew Lassar (Harvard Medical School, Boston, MA). Pcs4$^+$ human E47 was generated by PCR-amplification of *Bgl*II and *Eco*RI sites onto human E47 obtained from Cornelius Murre (University of California, San Diego, La Jolla, CA). One-step site-directed PCR-based mutagenesis was performed as previously described [Makarova et al., 2000] to generate the following cysteine (Cys) to alanine (Ala) mutants: Cys124Ala xTwist-1, Cys139Ala xTwist-1, Cys179Ala mTwist-1, Cys573Ala hE47, and Cys614Ala hE47. Human HDAC4 (a generous gift from Eric Olson, University of Texas, Dallas, TX) was subcloned into pcs4-3HA$^+$ using the restriction sites *Bgl*II and *Xba*I. Mouse Runx2 (a generous gift from Bjorn Olson, Harvard Medical School), was subcloned into pcs4-3HA$^+$ using the restriction sites *Bgl*II and *Xba*I.

TISSUE CULTURE AND CELLULAR TRANSFECTIONS

293T human kidney epithelial cells were cultured in Dulbecco's modified Eagle' medium (DMEM) containing 10% fetal bovine serum (Invitrogen), 50 IU/ml penicillin–streptomycin (Mediatech, Herndon, VA). Cells were transfected using 25-kDa linear polyethyleneimine (Polysciences, Warrington, PA) as described previously [Durocher et al., 2002].

HYDROGEN PEROXIDE STIMULATION

After 24 h, transfection cells were washed twice with phosphate-buffered saline (PBS). H_2O_2 (Sigma) was added to serum-free, antibiotic-free medium for the indicated times.

WESTERN BLOT ANALYSIS AND IMMUNOPRECIPITATIONS

Cultured 293T cells were rinsed twice in ice-cold PBS then lysed in modified RIPA buffer [(150 mM NaCl, 50 mM Tris (pH 8), 25 mM β-glycerophosphate, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 2× Complete EDTA-free protease inhibitor mixture (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride), 2 mM EDTA, 1% Nonidet P-40 (NP-40)]. To prevent disulfide bond formation during handling of the extracts, cells were lysed in the presence of 10 mM *N*-ethylmaleimide (NEM; Sigma), a sulfhydryl alkylating reagent. Protein samples were prepared in the presence or absence of the reducing agent dithiothreitol (DTT; Sigma; 100 mM final concentration). Lysates were centrifuged and supernatants were collected.

The following antibodies were used for immunoprecipitations. Anti-HA rat monoclonal antibody (clone 3F10; Roche Applied Science), followed by incubation with protein A matrix preincubated in rat anti-rabbit IgG (Jackson Laboratories, West Grove, PA). Anti-Flag matrix was purchased from Sigma. Both immunoprecipitations were performed for 3 h at 4°C with gentle rocking. For HDAC4 and Runx2 immunoprecipitations, precleared lysates were incubated with HA.11 antibody (Covance) overnight at 4°C with gentle rocking followed by 1 h incubation with protein A/G plus matrix (Santa Cruz Biotechnology, Inc.). At the last wash, beads were divided equally and either boiled for 5 min in DTT-containing loading buffer or incubated for 5 min at room temperature in loading buffer without DTT.

Proteins were electrophoretically separated on polyacrylamide gels and then transferred onto nitrocellulose membranes. The following antibodies were used for Western blotting: anti-HA-peroxidase rat monoclonal peroxidase-conjugated antibody (clone 3F10, Roche Applied Science), anti-Flag-peroxidase mouse monoclonal peroxidase-conjugated antibody (Sigma), and anti-actin mouse monoclonal antibody (Sigma). Where applicable, densitometry data were analyzed using the ImageJ Analysis software (rsbweb.nih.gov/ij/).

RESULTS

INTERMOLECULAR DISULFIDE BOND FORMATION IN TWIST1 HOMODIMERS AND TWIST1-E PROTEINS HETERODIMERS IN RESPONSE TO AN OXIDATIVE STIMULUS

293T cells were transfected with constructs expressing Flag-tagged mouse Twist1 (mTwist1) or Flag-tagged mTwist1 and HA-tagged E12. Protein samples were prepared in the presence or absence of the reducing agent DTT. As Figure 1A illustrates, when mTwist1 was transfected alone and cells were stimulated with at least 25 μM H_2O_2, in the absence of DTT, a species of twice the predicted molecular mass of mTwist1 (approximately 54 kDa) was observed as well as a species migrating at the predicted molecular mass for monomeric mTwist1. The intensity of the signal for the 54 kDa band was dependent on the H_2O_2 concentration. This behavior is diagnostic of an intermolecular disulfide bond. In addition, a 50 kDa band is also observed that is not DTT sensitive and not H_2O_2 dependent. The identity of this band is presently unknown but it most likely represents a nonspecific signal.

When cells were co-transfected with Flag-tagged mTwist1 and HA-tagged E12, DTT-sensitive bands were observed at a concentration of 50 μM H_2O_2 and above. The band corresponding to the mTwist1 homodimer at 54 kDa was first observed at a concentration of 75 μM H_2O_2 and was weak in comparison with the Twist1-E12 heterodimer band observed at approximately 100 kDa. Although the results illustrated were obtained with mouse Twist1 constructs, similar findings were observed when xTwist1 was used instead (data not shown). Similar results were obtained using HA-tagged E47 instead of E12 (data not shown). Data presented in Figure 1A also illustrates that H_2O_2-induced disulfide bonds favors the formation of heterodimers (Twist1-E proteins) over homodimers (Twist1/Twist1); in response to 75–100 mM H_2O_2 most of the dimers formed were Twist1/E12 instead of Twist1/Twist1.

Figure 1B demonstrates that H_2O_2-induced heterodimer formation is strongest at 5 min of stimulation, decreases rapidly, and returns to baseline by 30 min.

To confirm the finding that H_2O_2 causes increased heterodimerization of Twist1 and E12, cells were co-transfected with HA-tagged E12 and mTwist1 or xTwist1. After stimulation with H_2O_2, cells were lysed and a flag immunoprecipitation was performed. Figure 2 illustrates that the increased interaction of Twist1 and E12 is observed in co-immunoprecipitation (Fig. 2, upper panels) as well as in whole cell lysates (Fig. 2, lower panels). The homo- or heterodimers observed in the presence of H_2O_2 were DTT sensitive (Fig. 2, lower panel) as no signal was observed at approximately 100 kDa which corresponds to the molecular weight of Twist-E12

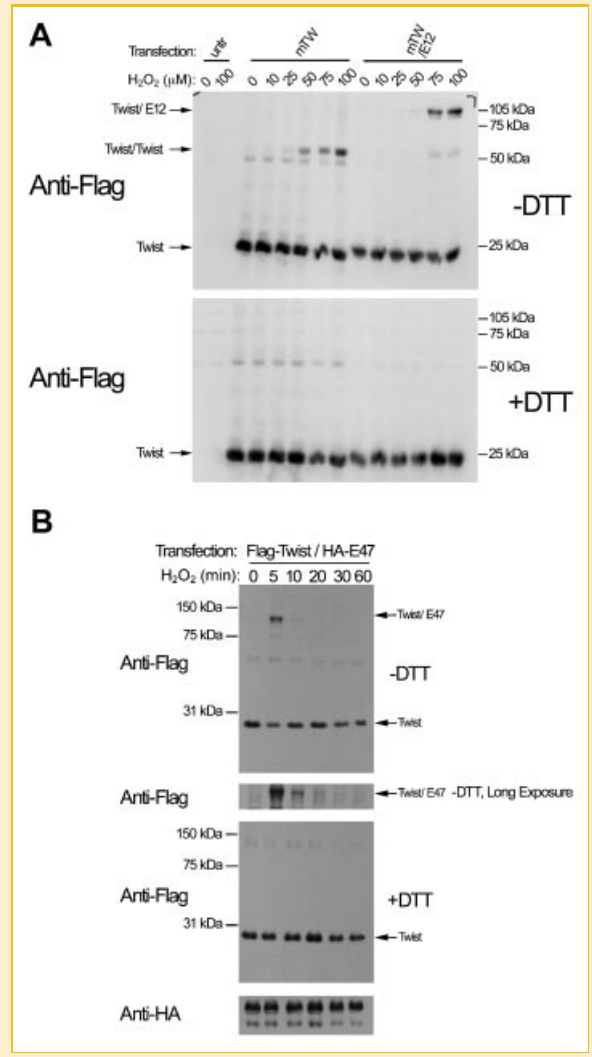

Fig. 1. A: Intermolecular disulfide bond formation in Twist1 homodimers and Twist1-E proteins heterodimers in response to an oxidative stimulus. 293T cells transfected with Flag-tagged Twist1 and HA-tagged E12 were stimulated for 5 min with H_2O_2 in serum-free media at concentrations ranging from 0 to 100 mM. Upper panel (Flag immunoblot) shows Twist1 homodimer and Twist/E12 heterodimer formation in response to H_2O_2 treatment. These dimers are visible only in the absence of DTT in the gel-loading buffer. Lower panel (flag immunoblot) illustrates the absence of dimers in the presence of DTT. B: Time course of Twist1 homo and heterodimer formation in cells stimulated with H_2O_2. 293T cells transfected with Flag-tagged Twist1 and HA-tagged E47 were stimulated for various times with 500 mM H_2O_2. Upper panel (flag immunoblot) demonstrates the formation of Twist1 homodimers and Twist/E12 heterodimers in response to H_2O_2 treatment. These dimers are visible up to 20 min after stimulation and only in the absence of DTT in the gel-loading buffer. Lower panel (Flag immunoblot) illustrates the absence of dimers in the presence of DTT.

heterodimers. We conclude from these experiments that H_2O_2 causes an intermolecular disulfide bond to form between Twist1 homodimers and Twist1-E12 heterodimers.

IDENTIFICATION OF CYSTEINE RESIDUE RESPONSIBLE FOR DISULFIDE BOND FORMATION

We hypothesized that the cysteine residue located at the C-terminus after the bHLH domain and conserved among all species examined

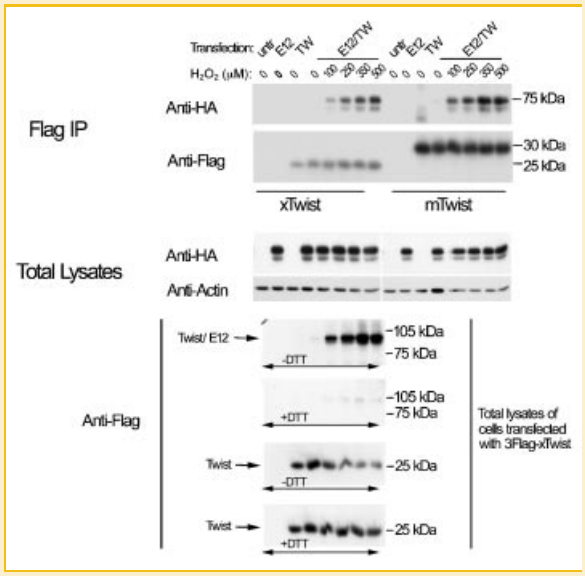

Fig. 2. H_2O_2 stimulates association of E12 with mouse or *Xenopus* Twist1: Co-immunoprecipitation of Twist1 and E12. 293T cells were co-transfected with HA-tagged E12 and mTwist1 or xTwist1. After 5 min stimulation with H_2O_2 at various concentrations, cells were lysed and a Flag immunoprecipitation was performed. Upper panels are of the flag immunoprecipitations blotted with anti-HA antibody or with an anti-Flag antibody to detect Twist-E12 heterodimers and Twist monomers, respectively. Middle panels indicate that an equal amount of HA-tagged E12 protein was expressed in each condition. Actin was used as a loading control. Total lysates of cells used for the experiment in the upper and middle panels of this figure, were blotted with an anti-Flag antibody illustrating the presence of DTT-sensitive Twist-E12 heterodimers (data shown for the cells transfected with xTwist only).

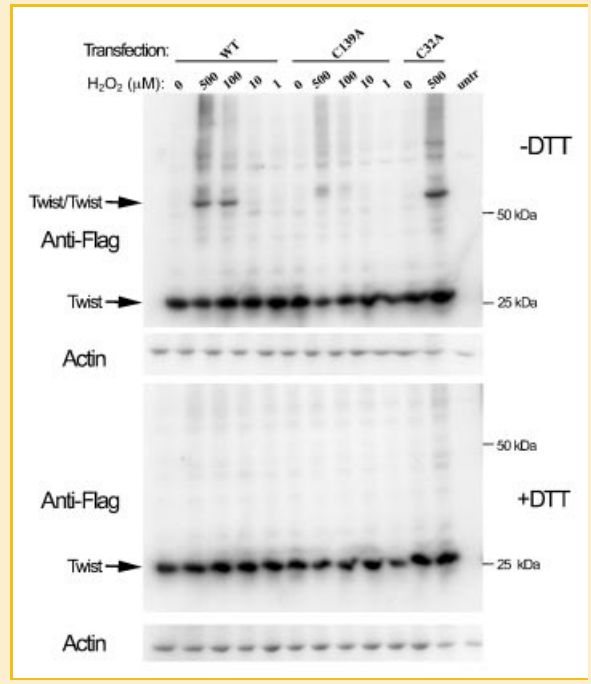

Fig. 3. Identification of the cysteine residue in Twist1 responsible for formation of an intracellular disulfide bridge. Flag-tagged *Xenopus* Twist1 and two cysteine mutants (Cys139Ala and Cys32Ala xTwist1) were transfected into 293T cells. Cells were then stimulated with various concentrations of H_2O_2 for 5 min. Upper panel illustrates the formation of DTT-sensitive homodimers of wild-type xTwist1 and Cys32Ala xTwist1 mutant. The Cys129Ala xTwist mutant fails to form homodimers in response to H_2O_2 (flag immunoblots; C, cys: cysteine; A, ala: alanine).

(Cys139 in xTwist, Cys179 in mTwist1, Cys175 in human Twist1, and Cys614 in human E47), is involved in the intermolecular disulfide bond in Twist1 homodimers and Twist1-E proteins heterodimers. Figure 3 demonstrates that wild-type xTwist1 forms a disulfide bridge between Twist1 homodimers in the presence of 100 and 500 μM H_2O_2. When cysteine 139 was mutated to alanine in xTwist1, this response is abolished while mutating the other cysteine in xTwist1 at position 32 to alanine, had no effect on disulfide-linked homodimers. Furthermore, the cysteine at position 139 in xTwist1 is also essential for H_2O_2-induced disulfide bond formation between Twist1 and E12 heterodimers as Figure 4 demonstrates.

E proteins have two cysteine residues after the bHLH domain corresponding to cysteine 573 and cysteine 614 in human E47. In order to identify the residue responsible for disulfide bond formation during heterodimerization with Twist1, we mutated both residues to alanines. Figure 5 illustrates that while Cys573Ala E47 mutant behaved in a similar manner to wild-type E47 in response to H_2O_2, Cys614Ala E47 mutant lost the ability to form intermolecular disulfide bridges with Twist1 suggesting that cysteine residue at position 614 in human E47 is responsible for the formation of a disulfide bond with Twist1.

POSSIBLE STABILIZATION OF TWIST1-E PROTEINS HETERODIMERS THROUGH DISULFIDE BOND FORMATION

The increased signal corresponding to Twist1-E proteins heterodimers seen in the presence of H_2O_2 could be due to either increased interaction of the two proteins or increased stabilization of the heterodimer through intermolecular disulfide bond formation. Flag-tagged xTwist and Cys139Ala xTwist were transfected in the presence or absence of HA-tagged E12. Figure 6 illustrates that while H_2O_2 causes an increased co-immunoprecipitation of Twist1 and E12, this response is abolished when cysteine 139 was mutated to alanine. These results were confirmed with the mTwist1 construct where the corresponding cysteine (cysteine 179) was mutated to alanine (Fig. 7). These data illustrate that H_2O_2 causes increased stabilization of the heterodimer through disulfide bond formation as H_2O_2 does not cause an increase in co-immunoprecipitation of E proteins and the Cys139Ala xTwist1 mutant which is not able to form disulfide bonds with E proteins.

CELL DENSITY CONTROLS HOMO- AND HETERODIMERIZATION OF bHLH PROTEINS IN RESPONSE TO H_2O_2

Since increasing cell density is associated with increasing ROS levels in transformed cells such as HeLa [Limoli et al., 2004], we hypothesized that cell culture conditions would affect Twist1 homo- and heterodimer formation. As Figure 7 illustrates, cell density did not affect homo- or heterodimer formation in unstimulated cells; however, the H_2O_2-induced dimerization was abolished in cells grown at high density. Cells were plated at the following densities (per well of a 6-well plate) 24 h prior to transfection: 1×10^5 (20%

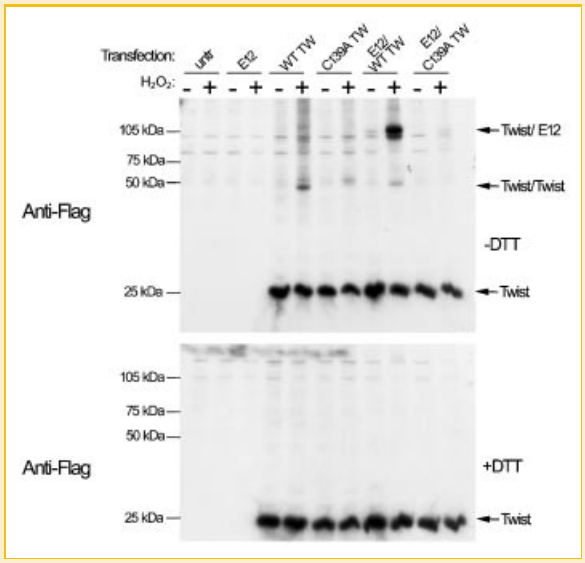

Fig. 4. Cysteine 139 of xTwist1 is essential for H_2O_2-induced heterodimerization with E12. 293T cells transfected with Flag-tagged wild-type xTwist1 or Cys139Ala xTwist1 in the presence or absence of HA-tagged E12 were stimulated for 5 min with 500 mM H_2O_2. Samples were run in the absence (upper panel) or presence (lower panel) of DTT (Flag immunoblots; C: cysteine, A: alanine).

density at stimulation), 5×10^5 (50%), 8×10^5 (80%), and 1×10^6 (100%). Twenty-four hours after transfection, cells were washed with PBS and the medium was changed to serum-free DMEM containing H_2O_2 or vehicle thus excluding the possibility that a secreted factor was responsible for the observed changes.

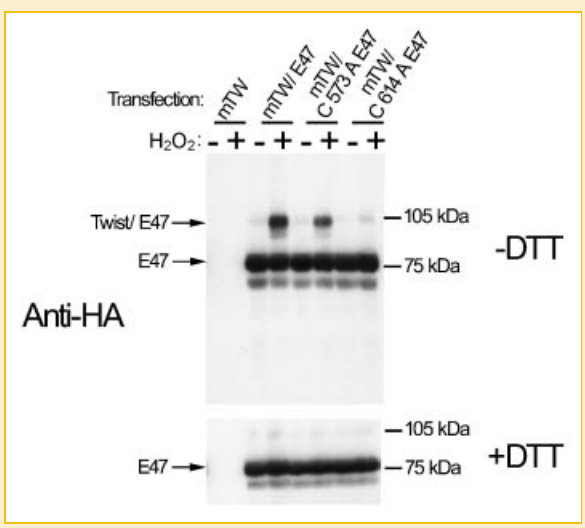

Fig. 5. Identification of the cysteine residue of E47 responsible for forming an intermolecular disulfide bridge with Twist1. 293T cells transfected with Flag-tagged mouse Twist1 in the presence or absence of HA-tagged wild-type E47, Cys573Ala E47, or Cys614Ala E47, were stimulated for 5 min with 500 mM H_2O_2. DTT-sensitive heterodimers are observed between Twist1 and wild type or the Cys573Ala E47 mutant (HA immunoblot; C, cys: cysteine; A, ala: alanine).

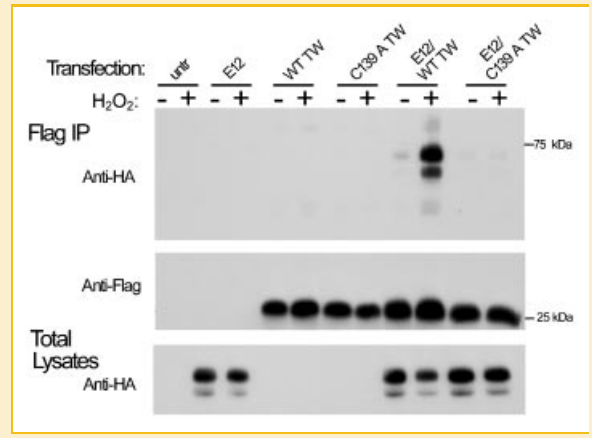

Fig. 6. Increased stabilization of the Twist1-E12 heterodimer through disulfide bond formation. 293T cells overexpressing Flag-tagged wild-type xTwist1 or Cys139Ala xTwist1 in the presence or absence of overexpressed HA-tagged E12, were stimulated for 5 min with 500 mM H_2O_2. Immunoprecipitation against Flag (the epitope of xTwist1) was performed followed by immunoblotting against HA (tag of E12) and Flag. The anti-HA immunoblot of total lysates (lower panel) demonstrates equal levels of E12 expression in total lysates.

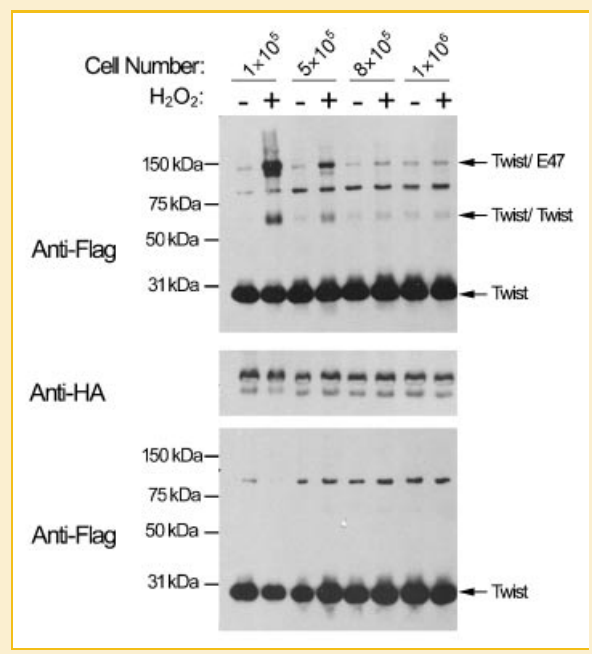

Fig. 7. Cell density affects DTT-sensitive Twist1-E47 heterodimer formation in the presence of H_2O_2. 293T cells plated at various densities (1×10^5, 5×10^5, 8×10^5, and 1×10^6) in a single well of a 6-well dish were transfected with Flag-tagged xTwist1 and HA-tagged E47. Twenty-four hours posttransfection, cells were stimulated for 5 min with 500 mM H_2O_2 in serum-free media. Upper panel illustrates heterodimer formation in the absence of DTT; the lower panel illustrates that the heterodimer formation is DTT sensitive. The middle panel is an anti-HA immunoblot illustrating equal levels of E47 protein at all densities tested. This figure demonstrates that H_2O_2-induced heterodimerization is significantly reduced in cultures where cells were seeded at a density of 5×10^5 cells per well and abolished at a density of 1×10^6 (Flag immunoblot).

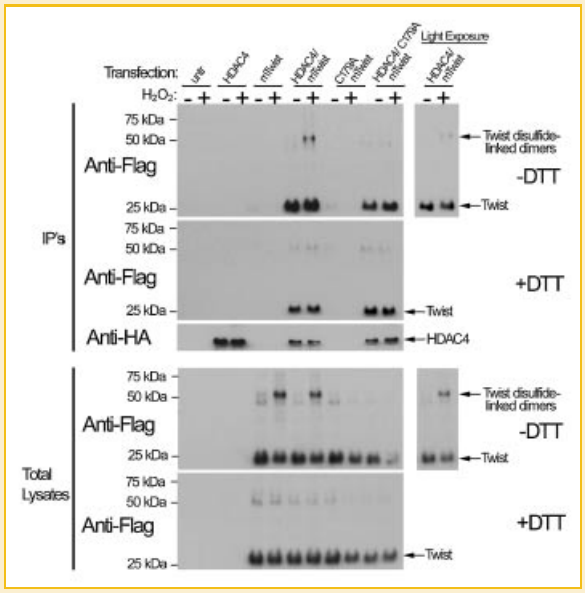

Fig. 8. HDAC4 and Runx2 interact preferentially with Twist1 that is not disulfide linked. 293T cells overexpressing Flag-tagged wild-type mTwist1 or Cys179Ala mTwist1 in the presence or absence of overexpressed HA-tagged HDAC4 were stimulated for 5 min with 500 mM H_2O_2. Immunoprecipitation against HA (the epitope of HDAC4) was performed followed by elution in loading buffer in the presence or absence of DTT. The upper three panels represent Flag and HA immunoblots of the immunoprecipitates. The anti-Flag immunoblot of total lysates (lower two panels) demonstrates Twist disulfide-linked dimers in the absence of DTT and equal levels of Twist1 expression in total lysates. In the whole cell lysates approximately 40% of overexpressed Twist1 forms disulfide-linked bridges, while only 10% of disulfide-linked Twist1 co-immunoprecipitates with HDAC4.

HDAC4 AND RUNX2 INTERACT PREFERENTIALLY WITH TWIST1 THAT IS NOT DISULFIDE LINKED

Lastly, we investigated if the disulfide mediated dimerization of Twist1 alters the interaction between Twist1 and the non-bHLH transcription factor Runx2. As Figure 8 illustrates, while in whole cell lysates approximately 40% of overexpressed Twist1 forms disulfide-linked bridges, only 10% of disulfide-linked Twist1 co-immunoprecipitates with HDAC4. In the case of Runx2, no disulfide-linked Twist1 could be identified as interacting with Runx2. These results indicate that there is a significant preference for HDAC and Runx2 interaction with Twist that is not disulfide linked.

DISCUSSION

The bHLH transcription factor Twist1 forms functional homodimers as well as heterodimers with E proteins and other bHLH/HLH transcription factors; the dimerization partner of Twist then determines the functional output of Twist1 expression [Connerney et al., 2006]. The purpose of this study was to determine if H_2O_2 causes increased dimerization of Twist1 with its binding partners the bHLH proteins E12 and E47.

Phagocytic cells produce a burst of ROS in response to infection. Recent evidence suggests that nonphagocytic cells produce ROS as well but in a regulated manner and at lower levels than in phagocytic cells. It is hypothesized that controlled ROS generation contributes to signaling in response to a variety of physiological stimuli [Lambeth, 2004]. H_2O_2 exhibits properties that are ideal for control of signaling: it is a small, neutral molecule that is rapidly produced and removed in response to physiological stimuli and can cross membranes freely [Finkel, 2003]. Numerous studies have now implicated regulated ROS production in controlling tyrosine phosphorylation-dependent signal transduction [Tonks, 2005]. ROS can elicit various responses ranging from proliferation and growth to differentiation arrest and apoptosis by activating numerous major signaling pathways including phosphoinositide-3-kinase (PI-3K), NF-κB, and mitogen-activated protein kinases (MAPKs) [Martindale and Holbrook, 2002]. Which pathway is affected and what the ultimate physiological outcome is/are dependent upon the magnitude and duration of ROS generation as well as the cell type involved?

Exposure to ROS leads to reversible oxidation of thiol groups of key cysteine residues in numerous proteins including transcriptional regulators, kinases, phosphatases, structural proteins, and metabolic enzymes [Veal et al., 2007]. This modification is associated in many cases with altered activity. For example, the bacterial transcriptional activator OxyR is directly oxidized in response to H_2O_2. Although both the oxidized and reduced form of OxyR are able to bind DNA, only the oxidized form of OxyR can activate the transcription of anti-oxidant genes [Storz et al., 1990]. In eukaryotes, the yeast transcription factor Yap1 plays a similar role as the bacterial OxyR. Upon H_2O_2 treatment Yap1 is activated by oxidation and deactivated by enzymatic reduction [Delaunay et al., 2000] thus providing an H_2O_2-sensing mechanism in eukaryotes that exploits the oxidation of cysteines in order to respond rapidly to ROS. Another example of a transcription factor regulated by ROS is AP-1 which is formed by homo- and heterodimers of the Jun and Fos transcription factor family. The DNA binding domain of AP-1 contains a conserved cysteine residue which can be reversibly oxidized [Abate et al., 1990]. The modification of the residue by reducing agents stimulates AP-1 binding activity in vitro suggesting that modification of cysteine residues of transcription factors alters the function of these proteins. These studies and others demonstrate that ROS-dependent redox cycling of cysteinyl thiols is critical for regulating protein–protein and protein–DNA interactions that in turn, regulate transcription. Further studies will have to be performed to examine the functional implication of disulfide-linked E2A-Twist1 heterodimers in response to oxidative stress. Our results presented in this report that Twist1-E2A heterodimers are favored over Twist1 homodimers in response to an oxidative stimulus (Fig. 1A) suggest the potential for redox control of bHLH-regulated transcription.

E proteins contain two cysteine residues that are conserved in most organisms. One cysteine residue is located in amphipathic helix 1 (Cys 570 in human E12, Cys 573 in human E47), and the other is 14 amino acid residues C-terminal to amphipathic helix 2 (Cys 611 in human E12, Cys 614 in human E47). Mutagenesis experiments presented in this study demonstrate that the second cysteine (Cys 611 and Cys 614 of human E12 and E47, respectively; Fig. 5) is critical for oxidative-stress-induced disulfide bond formation with Twist1 since changing this residue to an alanine

abolished the ability of H_2O_2-induced disulfide bond formation in the E2A/Twist1 heterodimers. Benezra [1994] demonstrated that a disulfide bond forms spontaneously between subunits of E2A homodimers; the intermolecular disulfide bond in the E2A homodimers was shown to be between the two Cys 570 residues of human E12 and this bond was required for high affinity DNA binding at physiologic temperatures. In this study we have demonstrated that E2A protein are targets of oxidative stress leading to the formation of disulfide-linked E2A-Twist1 heterodimers although the E2A residue identified as essential for this response is Cys 611 and Cys 614 of human E12 and E47, respectively.

A significant aspect of Twist1 regulation of numerous biological processes is its ability to interact with nonbHLH binding partners. We reasoned that forming a disulfide bond between Twist1 homo- and heterodimers constrains at least the C-terminus "Twist box" differently from what it would otherwise. Two binding partners were tested: Runx2 which interacts with the "Twist box" and HDAC4 (interaction domain not currently known). Our data indicate that there is a significant preference for HDAC4 and Runx2 interaction with Twist that is not disulfide linked. In the case of Runx2, these findings indicate that at least for the period that Twist1 responds to H_2O_2, a significant proportion of Twist1 does not interact with Runx2 and thus is not able to interfere with Runx2 function. This supports the findings of Douglas Spicer's laboratory [Connerney et al., 2006] suggesting that inhibition of osteoblast differentiation is mediated by Twist1 heterodimerization through the HLH domain, in addition to the Twist box/Runx2 interaction. We believe that this data establish a functional significance to disulfide dimerization of Twist in response to H_2O_2.

Although cell-density effects on H_2O_2-mediated toxicity and biological effects have been addressed by others, we are not aware of any published reports implicating cell density as a regulator of disulfide bond formation between bHLH homo- or heterodimers. Detailed experiments aimed at the role of cell density in regulating disulfide bond formation were beyond the scope of our manuscript. Previous reports [Spitz et al., 1987] indicate that in tissue culture experiments, cytotoxicity of H_2O_2 is cell density dependent and that this effect is in part due to the ability of cells in high-density cultures to deplete H_2O_2 more rapidly. We have tested different time points of H_2O_2 stimulation including 1 min and have not observed disulfide bond formation in cells at cultures at high density—we believe that the lack of response in these cells is due to other unidentified regulatory factors rather than the depletion of H_2O_2 from the medium.

Another relevant published result is that pretreatment with H_2O_2 induces resistance to subsequent H_2O_2 cytotoxicity. Although these points are highly relevant to our research, they will be addressed in the future. More specifically, is the rapid formation of disulfide bonds between bHLH dimers in response to H_2O_2 essential for development of resistance to cytotoxicity? As the ability of Twist to protect cells from apoptosis including its ability to protect cancer cells from death induced by chemotherapeutic agents, is well documented, it would be very relevant to determine what Twist-mediated effects on cell survival are dependent on the ability of this transcription factor to form intermolecular disulfide bonds. Disulfide bond formation in the context of ionizing radiation or in response to cancer chemotherapy both of which processes have been shown to generate H_2O_2, could prove to be an important regulatory step of the anti-apoptotic transcription factor Twist1.

In this study, cells were exposed to concentrations of H_2O_2 (10–500 μM) that did not affect cellular viability (data not shown). We observed a similar phenomenon at high levels of H_2O_2 (1 mM) which can be generated by phagocytic cells (data not shown) [Hampton et al., 1998]. There is some discrepancy in the literature concerning the concentrations of H_2O_2 that represent an accurate physiological stimulus especially in situations of acute oxidative stress. In this study we utilized concentrations that span those previously reported in human tissues and in vitro [Halliwell et al., 2000].

In summary, these data suggest that H_2O_2 treatment leads to oxidation of the cysteine located C-terminally to the bHLH domain of Twist1 which then rapidly forms a disulfide bond with the identical cysteine of Twist1 in a homodimeric complex or a corresponding cysteine in a heterodimeric complex with E proteins. Determination of the molecular basis of H_2O_2 signaling though bHLH transcription factors including Twist would allow the design of targeted therapeutics for a wide range of disease pathologies, including neurodegeneration, hypertension and vascular damage, tumor progression, and systemic inflammation.

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