Homeobox gene DLx3 is regulated by p63 during ectoderm development: relevance in the pathogenesis of ectodermal dysplasias

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Ectodermal dysplasias (EDs) are a group of human pathological conditions characterized by anomalies in organs derived from epithelial-mesenchymal interactions during development. Dlx3 and p63 act as part of the transcriptional regulatory pathways relevant in ectoderm derivatives, and autosomal mutations in either of these genes are associated with human EDs. However, the functional relationship between both proteins is unknown. Here, we demonstrate that Dlx3 is regulated by p63 during ectoderm development. Dlx3 and p63 act as part of the transcriptional regulatory pathways relevant in the pathogenesis of ectodermal dysplasias (EEC), Limb-mammary syndrome (LMS) and split hand-foot malformation (SHFM) dysplasias. Our results unravel aspects of the transcriptional cascade of events that contribute to ectoderm development and pathogenesis associated with p63 mutations.

KEY WORDS: Dlx3, p63, Transcription, Ectodermal dysplasias, Mouse development

INTRODUCTION

During embryonic development and organ formation, a series of signals between epithelial cells and the underlying mesenchymal cells are the basis for the formation of a variety of appendages and/or organs (Pispa and Thesleff, 2003). Anomalies in epithelial-mesenchymal-derived organs are characteristics of human pathological conditions defined as ectodermal dysplasias (EDs) (Priolo and Lagana, 2001).

Mutations in DLX3 and p63, among other genes, have been directly linked with EDs. The Dlx and p63 families of transcriptional effectors are essential for the development of the epidermis and/or embryonic appendages (Panganiban and Rubenstein, 2002; Merlo et al., 2003; Morasso and Radoja, 2005; Koster and Roop, 2004). Dlx3 expression has been detected in the hair follicle, tooth, limb bud, branchial arches, labyrinthine layer of the placenta, osteoblasts and epidermis (Morasso et al., 1995; Morasso et al., 1999; Hassan et al., 2004). Here, we present evidence that Dlx3 is regulated by p63 as part of a transcriptional regulatory pathway relevant to specific EDs.

p63 regulates multiple signaling pathways, such as the bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) pathways (Laurikka et al., 2006; Barbieri and Pitenpol, 2006). Transgenic and knockout (KO) mouse models indicate that p63 has essential roles in the development and maintenance of the stratified epidermis (Yang et al., 1999; Mills et al., 1999; Koster et al., 2004; Koster and Roop, 2004). The p63 gene is transcribed from two distinct promoters, giving rise to proteins that either contain (TAp63) or lack (∆Np63) the amino terminal transactivating domain. The TA and ∆N isoforms both possess the DNA-binding and oligomerization domains, and, by alternative splicing at the 3’ end, produce isoforms with different C-termini, termed alpha (α), beta (β) and gamma (γ) (Yang et al., 1998). The α isoforms contain a sterile α motif (SAM) – a domain with reputed importance in protein-protein interactions (Qiao and Bowie, 2005). p63 isoforms act as transcriptional activators and/or repressors (Ghioni et al., 2002; King et al., 2003; Wu et al., 2005), and bind to two or more tandem repeats of RRRCGTGYYY, but preferentially activate the RRRCGTGYYY sequence (Osada et al., 2005).

Mutations in the p63 gene have been associated with EDs that include ectrodactyly-ectodermal dysplasia-cleft lip/palate (EEC), limb-mammary syndrome (LMS), split hand-foot malformation (SHFM) and ankyloblepharon-ectodermal dysplasia-clefting (AEC) syndrome. There is a correlation between the position of the mutation and the observed abnormal phenotype (van Bokhoven et al., 2001; McGrath et al., 2001; van Bokhoven and Brunner, 2002). Mutations in the DLX3 gene are linked to tricho-dento-osseus (TDO) syndrome, which, like AEC, is characterized by defects in the development of hair, teeth and bone, and by absence of overt limb malformations (Price et al., 1998).

Partial-overlapping mRNA expression and phenotypes of specific human malformations caused by molecular lesions in either p63 or DLX3 suggest that these genes are components of common signaling pathways during embryonic development. Here, we show that p63 is able to bind and transactivate Dlx3 both in vitro and in vivo. Mutant p63 proteins derived from AEC patients exhibit an impaired ability to transactivate Dlx3, indicating that the misregulation of the DLX3 gene is involved in the pathogenesis of human syndromes associated with AEC.
DEVELOPMENT

MATERIALS AND METHODS

DNA constructs

The −117 to +60 DNA fragment of the Dlx3 promoter (Park and Morasso, 1999) was inserted into the pGL3-Basic and pCAT-Basic vectors (Promega). Mutations in the p63-binding sites of the Dlx3 promoter region containing the two overlapping p63-binding sites (p63 site1 and p63 site2; underlined) and CCAAT box (italics). The mutated Dlx3 sequences are shown in gray. (C-D) Dlx3-117/+60, Dlx3p63M1 and Dlx3p63M2 constructs were co-transfected with vectors expressing the TAp63 (C) and ΔNp63 (D) isoforms, with activity shown relative to the basic activity of Dlx3-117/+60. Transient transfection experiments were performed using mouse keratinocytes (C-D) and Saos-2 cells (E). Basal activity of the reporter was set to 1. Each histogram bar represents the mean of three independent transfection duplicates. Standard deviations are indicated. Dlx3 p63M1, mutated at p63-binding-site 1; Dlx3p63M2, mutated at p63-binding-site 2.

newborn foreskin and cultured in K-SFM (Invitrogen). The human osteosarcoma Saos-2 cell line was maintained in RPMI 1640 and 10% FCS. The immortalized human keratinocyte HaCaT and H1299 non-small-lung-carcinoma cell lines were grown in DMEM and 10% FBS.

Transient transfection

Transient transfections of keratinocytes were performed with FuGENE6 (Roche) in a 1:3 ratio. PRL-SV40 vector was used as an internal control. Luciferase activity was measured 24–36 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega). Transient transfections of Saos-2 and H1299 cells were performed according to Calabrò et al. (Calabrò et al., 2002). CAT reaction was performed 48 hours after transfection using 90 μg of cell extract. β-Gal was used to normalize for transfection efficiency.

EMSA analysis

Nuclear extracts and EMSA analysis were carried out according to Park and Morasso (Park and Morasso, 1999) using the Dlx3 p63site1+2 and non-specific competitor AP-2 binding site.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed with chromatin from mouse keratinocytes and embryonic tissues, the following oligonucleotides: for mouse, Dlx3(F) 5′-GAGAAAGCGCCGAGGCGT-GTTTTGCC-3′ and Dlx3(R) 5′-CCGGCTGTCGGTCAGTCG CTGCGT-3′; for human, Dlx3(F) 5′-AGAGGGGAGGAGAGGAGAAGGA-3′ and Dlx3(R) 5′-GAGGAGGGAGGAGAGGAGAAGGA-3′; and for JAG2(F) 5′-CAAGTGGTGAACAAGGGAGACT-3′ and JAG2(R) 5′-ACTGTGTCCTTCTGGAACACT-3′. Data are presented as fold differences relative to input and values are obtained by IgG with the formula

\[ \text{Fold-induction} = \frac{(Ct_{\text{Input}} - Ct_{\text{IgG}})}{(Ct_{\text{Ab}} - Ct_{\text{Input}})} \]

where Ct is threshold cycles, IgG is normal rabbit IgG, Ab is specific antibody and Input is input genomic DNA. ACHR amplification was performed as a control for H1299 transfected with Tap63α using ACHR(F) 5′-TGCTCTCCGAGTAACTAGTG-3′ and ACHR(R) 5′-GCCTCATTCGTTGGGAACT-3′.

Real-time PCR

For analysis of the expression of p63 in mouse keratinocytes and embryonic tissues, the following oligonucleotides were used: TAP63(F) 5′-AGAAGGCACTCGAGCGA-3′ and TAP63(R) 5′-TGGCGATACATCCC-3′. For JAG2(F) 5′-ATGTTTGATCTGCTGAAGCTGAGTTG-3′ and JAG2(R) 5′-ACTGTGTCCTTCTGGAACACT-3′. These oligonucleotides are designed in regions of the mRNA common to all isoforms (α, β and γ), and do not distinguish between these variants.

For Dlx3 expression in mouse keratinocytes, the following oligonucleotides were used: Dlx3(F) 5′-ATTACACGCCTCCCTCACATCAGT-3′ and Dlx3(R) 5′-GCCTTATAAGATCCCGCTTAG-3′. In embryonic
tissues: Dlx3(F) 5'-CGTCCAGACACCGCAGCTAC-3' and Dlx3(R) 5'-CGTTAGGATGATGTT-3'. For normalization: GAPDH(F) 5'-TTCAGTCATGAAATATCG-3' and GAPDH(R) 5'-TGTAGGGAGGATGTTC-3'.

For the analysis of p63 and Dlx3 mRNA levels in embryonic tissues, E10.5, E11.5 and E12.5 anterior or posterior limb buds from wild-type embryos were dissected, pooled in TRIZOL (Roche) and extracted, as indicated by the manufacturer. Real-time PCR was performed with a LightCycler (Roche) using FastStart DNA MasterPLUS SYBR-Green I (Roche). Standard curves were performed using wild-type cDNA with four calibration points: TQ: 1:2; 1:4 and 1:8. All samples were done in duplicates and the analysis was repeated twice. GAPDH was used for normalization, calculated using LightCycler Software 3.5.3. The results are expressed with the value relative to E10.5 (set at 1) for each mRNA.

**RESULTS AND DISCUSSION**

The existence of malformations caused by either p63 or DLX3 gene mutations that translate to partially overlapping phenotypes suggests that these genes are transcriptional effectors in common signaling cascades regulating epidermal development. The severity of the phenotype in p63-null mice suggests that it is a crucial upstream regulator of these signaling pathways (Mills et al., 1999; Yang et al., 1999). Detailed analysis of the Dlx3 promoter region revealed a sequence with two p63-like overlapping binding sites immediately upstream of the CCAAT box, located from –89 to –80 bp (site 1) and from –84 to –75 bp (site 2) of the transcriptional start site (Park and Morasso, 1999). Because the expression patterns of p63 and Dlx3 overlap throughout embryonic development (Morasso and Radoja, 2005), we proceeded to test the ability of different p63 isoforms to transactivate the Dlx3 promoter. The Dlx3 promoter contains the two overlapping sites, which contains the two overlapping sites, was transiently transfected into primary mouse keratinocytes in either the absence or presence of expression vectors encoding TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β or ΔNp63γ. The TA isoforms activated the Dlx3 promoter at a magnitude of twelve-, three- and seven-
development and TAp63 and p63 indicate limb buds. M, mesoderm; E, ectoderm. Scale bars: 10 μM in C. wt, wild type.

In order to test each overlapping site (p63-binding-site 1 and 2), we compared the activities of Dlx3–117/+60, Dlx3–117/+60 p63M1 (mutated p63 site 1) and Dlx3–117/+60 p63M2 (mutated p63 site 2) constructs (Fig. 1B) by cotransfection performed in the presence of the TA and ΔNp63 promoters (Fig. 1C,D). Our results indicate that TAp63α induction of Dlx3 is mediated through either of the two p63 sites, whereas TAp63γ required an intact regulatory region. These results are not cell-type specific, because a similar profile was obtained using Saos-2 cells (Fig. 1E).

Since Dlx3 is induced in keratinocytes cultured in 0.12 mM Ca²⁺ (Park and Morasso, 1999), we compared the endogenous expression of the p63 isoforms with Dlx3 after 12- and 24-hours of 0.12 mM Ca²⁺ treatment (Fig. 2A-C). The real-time PCR results showed a correlation between the upregulation of Dlx3 and TAp63 and the downregulation of ΔNp63 mRNAs associated with Ca²⁺-induced differentiation, and are consistent with the recent report by King et al. (King et al., 2006). The specificity of the PCR products was corroborated by sequencing (data not shown). TAp63 proteins are found in normal adult tissues (Nylander et al., 2002) and during mouse embryonic development (Koster et al., 2004) (also our own data). Findings of Koster et al. (Koster et al., 2004) support a role for TAp63 as a molecular switch for the initiation of epithelial stratification. Our findings support a working model in which, once transactivated by TAp63α, Dlx3 will in turn regulate the expression of terminal differentiation markers (Morasso et al., 1996).

In order to demonstrate direct binding to the p63 region in the Dlx3 promoter, we performed EMSA with a fragment comprising −89 to −75 bp (Dlx3 p63 sites 1 and 2) using nuclear extracts from primary keratinocytes (NE). A shift was detected (Fig. 2D, lane NE), and the complexes were competed with a specific competitor (Fig. 2D, lane SC), but not with a nonspecific DNA competitor (Fig. 2D, lane NC). We next evaluated whether p63 bound this region of the Dlx3 promoter in vivo. In mouse keratinocytes, ChIP experiments were performed with a p63-specific antibody and analyzed by regular PCR (Fig. 2E) and real-time PCR (Fig. 2F). The data shows that p63 specifically binds to the Dlx3 promoter in vivo (eightfold higher than with IgG control). Moreover, ChIP experiments on TAp63α-transfected H1299 cells, which are devoid of p63, demonstrated the direct binding of TAp63α to the Dlx3 promoter (Fig. 2G).

To further explore the relationship of Dlx3 and p63 in vivo, we analyzed their colocalization by immunofluorescence on E10.5 embryonic forelimb sections with anti-p63 and anti-distal-less antibodies. The latter reagent recognizes Dlx3 in the limb ectoderm. p63 and Dlx3 immunoactivity were found to colocalize in the same nuclei (Fig. 3A). Comparison of the expression of Dlx3, TAp63 and ΔNp63 in the limbs at embryonic stages E10.5, E11.5 and E12.5 was performed by real-time PCR. Between E11.5 and E12.5, the relative abundance of both TAp63 and Dlx3 mRNA increased from three- to eight-fold relative to their expression at E10.5 in the anterior (AL) and posterior limbs (PL) (Fig. 3B), whereas expression of ΔNp63 was only moderately increased. These results for p63 in the limb ectoderm of embryos at E10.5-E12.5 are in agreement with reported data (Koster et al., 2004) and show for the first time that a good correlation is observed between the expressions of Dlx3 and TAp63.

To provide further evidence that p63 is an upstream regulator of Dlx3, we studied the effect of p63 ablation on Dlx3 mRNA and protein expression in the Brdm2/p63 KO mice (Mills et al., 1999) (Fig. 3C-D). As assessed by immunohistochemistry, the abundance of Dlx3 protein was significantly reduced in p63-KO limb ectoderm (Fig. 3C). Analysis by whole-mount in situ hybridization with a Dlx3 antisense probe demonstrated that the absence of p63 led to a downregulation of Dlx3 (Fig. 3D).

We next studied the clinical relevance of p63-mediated regulation of Dlx3 expression. We examined the transcriptional activity of the Dlx3 promoter in the presence of p63 mutants causative of human AEC, EEC, SHFM or LMS (Fig. 4A) in the H1299 cell line. The p63 AEC mutants – L518V, L518F and Q540L – are all point

Fig. 3. p63 and Dlx3 colocalize in the embryonic ectoderm, and Dlx3 expression is downregulated in p63-KO embryos. (A) Histochemistry with anti-distal-less (pan-anti-Dlx; red) and p63 (green) antibodies on the dorsal forelimb ectoderm of E10.5 wild-type embryos (merge of Dlx and p63 expression is yellow). DAPI staining is also shown (blue). Arrows indicate the dlx-p63 double-positive nucleus. (B) Relative mRNA abundance, determined by real-time PCR, for Dlx3, TAp63 and ΔNp63 in the anterior limb (AL) and posterior limb (PL) of E10.5, E11.5 and E12.5 wild-type embryos. The relative abundance is expressed as fold-induction relative to the value at E10.5 (set at 1). (C) Histochemistry with anti-p63 and anti-Dlx on E10.5 wild-type and p63-KO limb-bud ectoderm. (D) Whole-mount in situ hybridization on wild-type and p63-KO E10.5 embryos with a Dlx3 antisense probe. Arrows indicate limb buds. M, mesoderm; E, ectoderm. Scale bars: 10 μM in A and 40 μM in C. wt, wild type.
substitutions within the SAM domain, present only in TAp63α and ΔNp63α. The p63αFS mutant contains a mutation found in EEC that generates a frameshift at amino acid 525, which leads to a premature stop. The E639X mutation, in exon 14, was isolated in a SHFM patient, whereas the 2-bp deletion, in exon 14, was found in a LMS patient and affects all p63 isoforms. Co-transfection experiments were performed with expression vectors encoding TAp63 and ΔNp63 mutants. As shown in Fig. 4B and 4C, the AEC mutants failed to yield a significant level of reporter-gene expression despite having intact amino-terminal and DNA-binding domains. These results point to a crucial role of the C-terminus of p63α on the regulation of Dlx3 transcription. The LMS-derived mutants G76W and ΔAA, as well as the FSEE C mutant, SHFM-derived E639X TA and ΔN proteins; showed a similar mode of regulation of Dlx3-promoter activity compared with the corresponding wild-type proteins (Fig. 4B,C). A similar profile, albeit with a lower amount of induction, was obtained upon transfection in HaCaT cells (data not shown). The level of expression of the mutant proteins was corroborated by immunoblot analysis with anti-p63 antibody (Fig. 4D).

The hampered ability of the AEC mutants, as well as the partial overlapping phenotypes of specific malformations caused by to p63 (i.e. AEC) or Dlx3 (i.e. TDO) gene mutations, suggest that Dlx3 misregulation is involved in aspects of the pathogenesis of AEC. AEC is characterized by ectodermal dysplasia, ankyloblepharon and cleft lip with cleft palate, and by the lack of limb involvement. The absence of limb defects in AEC may reflect the possibility that a putative role of Dlx3 in the limb is compensated for by other Dlx proteins (Panganiban and Rubenstein, 2002; Morasso and Radoja, 2005).

To determine the modulation of endogenous Dlx3 by p63, we used Saos-2 cells to express wild-type and mutant p63 (Fig. 4E). Interestingly, TAp63α increased Dlx3 expression (Fig. 4E, lane 2), and this effect was shared, although to different extents, with the EEC, SHFM and LMS mutants (Fig. 4E; lanes 6, 7 and 8). TAp63β did not alter Dlx3 levels (Fig. 4E, lane 3). Remarkably, an AEC mutant (Fig. 4E, lane 5) and the TAp63γ isoform abolished Dlx3 expression. Transfections of ΔNp63 isoforms, both wild-type and mutant, had no significant effect on, Dlx3 transcription (Fig. 4E, bottom panel). TAp63α and TAp63γ are potent transactivators (Barbieri and Pietenpol, 2006) and, in our in vitro studies, both isoforms were able to transactivate Dlx3 at similar levels. However,
whereas TAp63α induction was mediated through either of the two p63 sites in the Dlx3 promoter. TAp63γ required an intact regulatory region. Surprisingly, a slightly different outcome was obtained for the endogenous Dlx3 regulation, where upregulation of Dlx3 was detected with TAp63α and a complete downregulation was found when expressing TAp63γ in Soas-2 cells. To determine if these results could be attributed to cell context, the experiments were also performed in HaCaT cells. In these cells, overexpression of TAp63α showed upregulation of endogenous Dlx3, whereas TAp63γ once again caused a complete downregulation of Dlx3 expression (Fig. 4F). These differences might be attributed to the specific p63RE-CCAAT-box chromatin architecture. An important feature of the Dlx3 promoter is that the overlapping p63-binding sites are in close proximity to CCAAT box that binds NF-Y in keratinocytes (Park and Morasso, 1999). NF-Y is a general promoter organizer that pres- sets chromatin structure locally. A recent report shows that p63α regulates the transcription of the hsp70 gene through interactions with NF-Y (Wu et al., 2005). Although we have not determined the significance of NF-Y and p63 interactions on Dlx3 transcriptional regulation, it might be proposed that there is a dual role for the overlapping p63-binding sites, and that Dlx3 will be transcriptionally active or repressed depending on the specific p63 isoform bound to the promoter, on which of the p63 sites is occupied and on interactions with NF-Y CCAAT binding factor.

Dlx3 and p63 both function as part of a complex series of cascades that ultimately lead to the formation of ectoderm-derived organs. Unraveling the function of each protein at specific times of embryonic development will prove to be complex because of the differential expression of the p63 isoforms in distinct tissues (Nylander et al., 2002) and the cross-regulation with other developmentally relevant signaling pathways [i.e. FGF, BMP, and Notch (Laurikka et al., 2006; Nguyen et al., 2006)]. The characterization of p63 target genes promises to improve our knowledge of the signaling cascades that directly involved in normal ectodermal development. In summary, our study provides a functional relationship between p63 and Dlx3, with Dlx3 demonstrated to be a direct target of p63. The findings also provide evidence that the misregulation of Dlx3 is involved in the pathogenesis of p63 molecular lesions in AEC.

We thank S. J. Stimpson, Y. Rivera, S. Bertuzzi, T. Lozito, A. Pollice, K. King and G. Caretti for helpful comments; H. van Bokhoven, E. Candi and G. Melino for their gifts of plasmids; M. Simon for NHEK; G. Boekhoff-Falk for the anti-distal-less antibody; and D. Roop for providing the Bmdr2 p63-KO mouse line. The research was supported by a career award from Fondazione Telethon (TCP99003) and Fondazione San Paolo, Italy.

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