TNF Signaling via the Ligand-Receptor Pair Ectodysplasin and Edar Controls the Function of Epithelial Signaling Centers and Is Regulated by Wnt and Activin during Tooth Organogenesis

Johanna Laurikkala,* Marja Mikkola,* Tuija Mustonen,* Thomas Åberg,* Petra Hopin,* Johanna Pispa,* Pekka Nieminen,* Juan Galceran,† Rudolf Grosschedl,† and Irma Thesleff*

*Institute of Biotechnology, Viikki Biocenter, University of Helsinki, 00014 Helsinki, Finland; and †Gene Center and Institute of Biochemistry, University of Munich, Feodor Lynen Strasse 25, 81377 Munich, Germany

Ectodermal dysplasia syndromes affect the development of several organs, including hair, teeth, and glands. The recent cloning of two genes responsible for these syndromes has led to the identification of a novel TNF family ligand, ectodysplasin, and TNF receptor, edar. This has indicated a developmental regulatory role for TNFs for the first time. Our in situ hybridization analysis of the expression of ectodysplasin (encoded by the Tabby gene) and edar (encoded by the downless gene) during mouse tooth morphogenesis showed that they are expressed in complementary patterns exclusively in ectodermal tissue layer. Edar was expressed reiteratively in signaling centers regulating key steps in morphogenesis. The analysis of the effects of eight signaling molecules in the TGFβ, FGF, Hh, Wnt, and EGF families in tooth explant cultures revealed that the expression of edar was induced by activin B, whereas Wnt6 induced ectodysplasin expression. Moreover, ectodysplasin expression was downregulated in branchial arch epithelium and in tooth germs of Lef1 mutant mice, suggesting that signaling by ectodysplasin is regulated by LEF-1-mediated Wnt signals. The analysis of the signaling centers in tooth germs of Tabby mice (ectodysplasin null mutants) indicated that in the absence of ectodysplasin the signaling centers were small. However, no downstream targets of ectodysplasin signaling were identified among several genes expressed in the signaling centers. We conclude that ectodysplasin functions as a planar signal between ectodermal compartments and regulates the function, but not the induction, of epithelial signaling centers. This TNF signaling is tightly associated with epithelial-mesenchymal interactions and with other signaling pathways regulating organogenesis.

Key Words: Tabby; downless; epithelial-mesenchymal interactions; enamel knot; tooth development; Lef1.

INTRODUCTION

Tumor necrosis factors (TNF) are important regulators of embryonic organ development as shown recently by cloning of two genes responsible for ectodermal dysplasia syndromes. The anhidrotic (or hypohidrotic) ectodermal dysplasias are characterized by congenital defects in several organs, including hair, teeth, and some exocrine glands, in particular sweat glands. Positional cloning of the gene defective in the most common X-linked form of ectodermal dysplasia (Kere et al., 1996) and subsequent cloning of its mouse counterpart, the spontaneously mutated gene in Tabby (Ta) mice, led to the identification of a novel TNF ligand named ectodysplasin (Ferguson et al., 1997; Srivastava et al., 1997; Mikkola et al., 1999). Interestingly, the recent cloning of the autosomal gene behind the spontaneous mouse mutant downless (dl), which has a phenotype identical to Tabby, indicated that the gene encodes a novel TNF receptor, named edar (Headon and Overbeek, 1999). The same gene was also shown to be mutated in patients.
with an autosomal form of ectodermal dysplasia (Monreal et al., 1999). Recent biochemical studies have demonstrated that ectodysplasin binds to edar (but not to other known TNF receptors), and that no other ligands bind to edar, thus confirming that ectodysplasin and edar form a ligand–receptor pair (Jürg Tschopp—personal communication).

TNFs constitute a family of about 20 cytokines that bind to an increasing number of specific cell surface receptors (Gruss and Dower, 1995; Baker and Reddy, 1998). TNFs are produced as type II integral cell surface proteins and they exert their effects in paracrine fashion by binding and inducing the trimerization of TNFRs. TNF signaling has been associated almost exclusively with inflammation, host defense, and cancer, but recently novel roles have been revealed, including regulation of osteoclast differentiation and bone resorption (Filvaroff and Derynck, 1998). Ectodysplasin and edar constitute the first TNF and TNFR that regulate embryonic morphogenesis. New members of the TNF and TNFR superfamilies are being rapidly cloned and, interestingly, some of these are expressed in embryonic tissues, suggesting that TNFs may have widespread developmental regulatory roles (Eby et al., 2000; Kojima et al., 2000). However, at present nothing is known about the functions of TNFs during organogenesis or their possible associations with other signaling pathways implicated in morphogenetic regulation.

All organs affected in anhidrotic ectodermal dysplasia syndromes are epithelial appendages which typically develop through a series of epithelial–mesenchymal interactions and it was assumed already decades ago that a failure in these inductive tissue interactions caused the phenotypes (Gruneberg, 1971). The data that have accumulated during the past 15 years have indicated that the molecular mechanisms regulating the morphogenesis of all organs are shared to a great extent (Thesleff et al., 1995). In particular, several conserved families of signal molecules, regulating numerous developmental processes in all animals, also regulate the morphogenesis of ectodermal organs, including teeth, hairs, and glands, and most signals have been specifically implicated in mediation of epithelial–mesenchymal interactions (Chuong, 1998; Jernvall and Thesleff, 2000). The most studied signals fall into four conserved families, FGF, Wnt, Hh, and TGFβ. It is characteristic for the development of an organ that signals in different families are used reiteratively during the advancing morphogenesis. The signals form complex networks and regulatory loops, but the ways in which different signaling pathways interact seem to vary between different developmental systems.

Classic tissue recombination experiments showed that tooth morphogenesis is initiated by signals from early oral epithelium (E9–E11 in mouse) to the underlying neural crest-derived mesenchyme which subsequently signals back to the epithelium and regulates its morphogenesis (Mina and Kollar, 1987; Lumsden, 1988). Functions have been indicated for epithelial FGFs, BMPs, Wnts, and Shh in regulating tooth initiation and many transcription factors in the mesenchyme have been pointed out as their targets (Jernvall and Thesleff, 2000). As a result reciprocal signals in the mesenchyme are induced, including BMP, FGF, and activinβA, a member of the TGFβ superfamily. These subsequently regulate epithelial budding, folding, and growth, and the advancing tooth morphogenesis then continues to be regulated by reciprocal interactions between the two tissues (Jernvall and Thesleff, 2000). Targeted deletion of many genes in these signaling networks results in arrested tooth development in mutant mice. These genes include several transcription factors as well as activinβA. Mutations in some of the same genes have also been identified as causes for missing teeth in man (Stockton et al., 2000).

A characteristic feature of progressive tooth morphogenesis is the appearance of epithelial signaling centers at several key morphogenetic steps. These centers are restricted epithelial compartments which express several signaling molecules belonging to the four families. The first centers appear at the onset of epithelial budding (Keränen et al., 1998). The next centers, the enamel knots, appear at the tips of the tooth buds and are fully developed after transition to the cap stage. The life histories of the enamel knots have been analyzed in detail, and they have been shown to be induced by mesenchymal BMP4, to express at least 10 different signal molecules during a 24-h period, and subsequently to be largely removed by apoptosis (Jernvall et al., 1998). The last set of signaling centers, the secondary enamel knots, appear in the epithelium of molar teeth at the sites of cusp formation and their locations thus determine the patterns of tooth crowns (Jernvall and Thesleff, 2000). Our recent analysis of the tooth phenotype in Tabby mice indicated that they have a cusp patterning defect and we showed that this resulted from defective formation of the signaling centers at the enamel knots (Pispa et al., 1999).

The aim of the present study was to examine how TNF signaling mediated by ectodysplasin and edar regulates epithelial morphogenesis and how it is associated with other signaling pathways implicated in morphogenetic control. Our results on the expression patterns of Tabby (Ta; encoding the TNF ligand ectodysplasin) and downless (dl; encoding the TNF receptor edar) during tooth morphogenesis indicate that they mediate signaling within the epithelial cell layer between epithelial compartments. The striking punctuated expression of dl in the dental epithelium indicates that TNF signaling regulates the function of the epithelial signaling centers and explains our previous observations on the tooth phenotype of Tabby mice. The analysis of the signaling centers in tooth germs of Tabby mice indicated that in the absence of ectodysplasin the signaling centers were small. However, no downstream targets of ectodysplasin signaling were identified among several genes expressed in the signaling centers. In vitro assays in which the effects of several signaling molecules were analyzed on Ta and dl expression in dental epithelium indicated that Ta is regulated by Wnt signaling, whereas dl expression is stimulated by activinβA. Tabby expression
was downregulated in Lef1 mutant embryos, indicating that the Wnt signals regulating Tabby expression are transduced by LEF-1. As activinA is expressed in mesenchyme, the results indicate that TNF signaling is controlled by epithelial–mesenchymal interactions and by a cooperation of the Wnt and activin signaling pathways. Wnt regulating the expression of the ligand and activin regulating that of the receptor.

MATERIALS AND METHODS

Animals

Wild-type embryonic mice were from the NMRI strain. Mice were mated overnight, and the appearance of the vaginal plug was taken as day 0 of embryogenesis. The Tabby allele used, B6CBA-C-A+/A-Ta, was obtained from The Jackson Laboratory, Bar Harbor, Maine (Stock JR 0314), and was kept by breeding Ta0 females to Ta0 males. All embryos from the cross were either Ta0 or TaTa females or Ta0/Y males and displayed the Tabby phenotype (Pispa et al., 1999). The generation and analysis of the Lef1 null mutant mice have been described previously (van Genderen et al., 1994; Kratochwil et al., 1996).

Organ Cultures

The area of the lower molar teeth from E12 mouse embryos (NMRI × NMRI) was dissected in Dulbecco's phosphate-buffered saline (PBS; pH 7.4) under a stereomicroscope. Explants were cultured in a Trowell-type organ culture on Nuclepore filters 24 h at 37°C in medium containing Dulbecco's minimum essential medium (Sigma) supplemented with 10% fetal calf serum (PAA Laboratories GmbH) and 1% penicillin–streptomycin (Gibco). Medium (Sigma) was mated overnight, and the appearance of the vaginal plug was taken as day 0 of embryogenesis. The Tabby allele used, B6CBA-C-A+/A-Ta, was obtained from The Jackson Laboratory, Bar Harbor, Maine (Stock JR 0314), and was kept by breeding Ta0 females to Ta0 males. All embryos from the cross were either Ta0 or TaTa females or Ta0/Y males and displayed the Tabby phenotype (Pispa et al., 1999). The generation and analysis of the Lef1 null mutant mice have been described previously (van Genderen et al., 1994; Kratochwil et al., 1996).

In Situ Hybridization

Radioactive in situ hybridization procedures for tissue sections were carried out as described by Wilkinson and Green (1990). The tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and serially sectioned. Probes were labeled with 35S-UTP (Amerham); exposure time was 14 days. Whole-mount in situ hybridization was done as described earlier (Raatikainen-Ahokas et al., 2000). The digoxigenin-labeled probes were detected with alkaline phosphatase-coupled anti-digoxigenin antibodies (Boehringer Mannheim) using NBT (Boehringer Mannheim) and BCIP (Boehringer Mannheim) as the color substrates.

RESULTS

Expression of Tabby and downless mRNA Is Restricted to Epithelium and Shows Complementary Patterns during Advancing Tooth Morphogenesis

We compared the expression patterns of Tabby (the TNF ligand ectodysplasin) and downless (the TNF receptor edar) with in situ hybridization in the mandibular arches and in developing teeth from E10 to newborn mice. Ta transcripts were observed already at E10 throughout the simple epithelium covering the mandibular arch (Fig. 1A). This stage precedes morphological tooth formation and the presumptive dental epithelium cannot yet be discerned from the oral epithelium. During the initiation of mandibular molar development (E11) a thickening becomes apparent in the presumptive dental epithelium, and at this time Ta hybridization signal was reduced in the epithelial thickening and continued in the oral epithelium (Fig. 1C). At E12 the dental epithelium starts to bud to the underlying mesenchyme and at this stage Ta expression was absent from the budding epithelium, whereas expression remained in the oral epithelium (Fig. 1E). At the fully developed bud stage (E13), the dental epithelium consists of an outer layer of basal cells surrounding inner loose epithelial tissue. Ta expression was intense in the basal cells of the bud except at the tip of the bud from where it was strikingly absent (Fig. 1G). These Ta-negative cells correspond to the forming enamel knot. At cap stage (E14) the epithelial bud has undergone folding morphogenesis and the basal epithelial cells of the bud have contributed to the inner enamel epithelium facing the forming dental papilla mesenchyme and to the outer enamel epithelium facing the dental follicle cells, which separate the tooth germ from the surrounding jaw mesenchyme. As we reported previously, at this stage Ta expression is intense in the outer enamel epithelium, whereas the inner enamel epithelium is negative (Fig. 1I). (Pispa et al., 1999).
The expression in the outer enamel epithelium persisted throughout the bell stage of development and was still evident in the molars of newborn mice (Figs. 1K and 1M).

The expression pattern of dl was strikingly different from that of Ta during tooth morphogenesis. dl transcripts seemed to be absent from the branchial arch epithelium before morphological tooth formation (E10), but during the initiation of tooth development (E11) intense dl expression appeared in the thickened presumptive dental epithelium (Figs. 1B and 1D). At E12, when the budding of dental epithelium has started, dl mRNAs became restricted to the budding cells, whereas the dental epithelial cells contacting oral epithelium were devoid of transcripts (Fig. 1F). Interestingly, the dl-positive cells correspond closely to the cells of the early epithelial signaling center expressing several signal molecules (Keränen et al., 1998). At the bud stage (E13), dl expression remained intense and was restricted to the tip of the tooth bud and hence corresponded to the Ta-negative cells of the forming enamel knot (Fig. 1H). Intense expression remained in the fully developed enamel knot throughout the cap stage (E14), whereas all other dental cells were completely negative (Fig. 1J). The enamel knot disappears largely through apoptosis during E15 (Jernvall et al., 1998) and some dl transcripts were still seen in the area (not shown). Some expression was seen in the inner enamel epithelium in the intercuspal area at E17 (Fig. 1L) but no dl expression was detected in the tooth germs of newborn mice (Fig. 1N). Notably, no dl expression appeared in the secondary enamel knots which form in the inner enamel epithelium in a sequential manner during E15–E17 and express several signal molecules (Keränen et al., 1998; Jernvall and Thesleff, 2000). We conclude that during tooth morphogenesis Ta and dl are expressed in a largely complementary fashion and restricted to the epithelium.

Regulation of Ta and dl Expression by Signaling Molecules

The expression patterns of Ta and dl in the dental epithelium indicated that they are developmentally regulated and suggested that they may be associated with the epithelial-mesenchymal interactions and signaling net-

FIG. 1. Expression of Tabby (Ta, encoding ectodysplasin) and downless (dl, encoding edar) during mouse tooth development. (A, B) Prior to morphological tooth formation in E10 embryos, Ta transcripts are seen throughout the simple oral epithelium, whereas dl expression is not detected. (C–F) During the initiation (E11) and early bud stages (E12) Ta expression continues in the oral epithelium but is downregulated in the thickened and budding dental epithelium, which starts to express dl intensely. (G, H) At the bud stage (E13), Ta expression is intense in the outer epithelium of the tooth germ except for the forming signaling center at the tip of the tooth bud (arrow). This Ta-negative cell population expresses dl intensely (arrow). (I, J) During cap stage (E14) Ta expression continues in the outer enamel epithelium and oral epithelium, whereas dl expression is restricted to the signaling center at the enamel knot (arrows). (K–N) At bell stage (E17–NB) Ta expression continues in the outer enamel epithelium (arrows in K, M), whereas dl remains first weakly expressed in the intercuspal area of the inner enamel epithelium (arrow in L) and is thereafter downregulated (N). ek, enamel knot; oe, oral epithelium. Bar, (A–L) 95.4 μm, (M, N) 239 μm.
works implicated in tooth morphogenesis. The coexpression of \( dl \) with many signal molecules in the enamel knot also suggested the possibility that \( dl \) expression may be

beads except Wnt6, which was introduced by cell aggregates. Gene expression was analyzed by whole-mount in situ hybridization after 24 h. (A) Ta expression was induced by Wnt6-expressing cells. (B–H) None of the other tested signal proteins (activin\( \beta \)A, BMP2, BMP4, FGF4, FGF9, EGF, and Shh) stimulated Ta expression. (I–N) Control cultures. (I, J) BMP2 and BMP4 induced Msx2 expression. (K) Shh induced Ptc expression. (L) Wnt6-expressing cells induced Lef1 expression. (M, N) Control beads soaked in BSA and control NIH3T3 cells failed to induce \( dl \) and Ta expression.

**FIG. 3.** Induction of downless expression by activin\( \beta \)A in dental epithelium. (A) Wnt6-expressing cells did not induce \( dl \) expression. (B) \( dl \) expression was induced by beads releasing activin\( \beta \)A. (C–H) None of the other signals tested (BMP2, BMP4, FGF4, FGF9, EGF, and Shh) had stimulatory effects on \( dl \) expression.

**FIG. 2.** Induction of Tabby expression by Wnt6 in dental epithelium. Signal proteins were applied on E12 tooth explants with

---

Copyright © 2000 by Academic Press. All rights of reproduction in any form reserved.
controlled by some of the enamel knot signals or, alternatively, by signals in the adjacent mesenchyme. We studied therefore the effects of several signaling molecules on the expression of Ta and dl in the dental epithelium. The signal proteins were applied locally on explants of E12 tooth germs. After culture, the expression of Ta and dl was analyzed by in situ hybridization in whole mounts. Because the sensitivity of whole-mount in situ hybridization is lower than that of radioactive in situ hybridization, endogenous Ta and dl expressions were not clearly detectable by whole-mount in situ hybridization. Also, the negative results were confirmed in most cases by radioactive probes applied on tissue sections.

We analyzed the effects of several signals in the four most studied signal families: activinA, BMP2, and BMP4 in the TGFβ family; FGF4 and FGF10 in the FGF family; Shh in the hedgehog family; and Wnt6. In addition we analyzed the effects of EGF, which has been implicated in ectodermal dysplasias, as EGF partly rescues the sweat gland phenotype in Tabby mice (Blecher et al., 1993). Except for Wnt6, which was introduced by cell aggregates expressing the protein, the signaling molecules were applied on the tissues by beads soaked in high concentrations of the recombinant proteins. The activities of these proteins were confirmed by their earlier well-documented inductive effects in dental mesenchyme as follows: Mx1 expression by FGF4 (not shown) (Kettunen and Thesleff, 1998), Mx2 expression by BMP2 and BMP4 (Figs. 2I and 2J) (Vainio et al., 1993), Ptc expression by Shh (Fig. 2K), and Lef1 expression by Wnt6 (Fig. 2L) (Kettunen et al., 2000). No induction of Ta nor dl transcripts was observed around BSA-soaked beads used in control explants. Analysis of tissue sections by radioactive in situ hybridization revealed the normal expression patterns of Ta and dl, which were only weakly detectable by whole-mount in situ hybridization. The numbers of explants and the effects of the signal proteins are summarized in Table 1.

**Wnt6-Expressing Cells Induce Ta Expression in the Dental Epithelium**

Interestingly, the 3T3 cells expressing Wnt6 induced a clear zone of Ta transcripts in the dental epithelium (Fig. 2A). The analysis of Vibratome sections of the explants confirmed that the induced expression indeed was in the epithelial tissue (Figs. 4A and 4B). The induction was seen in all 14 explants analyzed. The control 3T3 cell aggregates had no observable effects (Fig. 2N). On the other hand, none of the beads releasing other signal molecules stimulated Ta expression. These included activinβA, BMP2, BMP4, FGF4, FGF10, EGF, and Shh (Figs. 2B–2H, Table 1). The lack of effects of BMP2, BMP4, FGF4, FGF10, EGF, and Shh on Ta expression was confirmed also by radioactive in situ hybridization of sections cut through the explants (not shown). The results thus indicate a very specific effect of Wnt signaling on the regulation of Ta expression.

### Table 1

<table>
<thead>
<tr>
<th>Signaling molecule</th>
<th>Tabby</th>
<th>downless</th>
<th>Lef1</th>
<th>Mx1</th>
<th>Mx2</th>
<th>Ptc</th>
</tr>
</thead>
<tbody>
<tr>
<td>ActivinβA</td>
<td>0/5</td>
<td>14/15</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>BMP2</td>
<td>0/5</td>
<td>0/6</td>
<td>—</td>
<td>—</td>
<td>6/6</td>
<td>—</td>
</tr>
<tr>
<td>BMP4</td>
<td>0/6</td>
<td>0/7</td>
<td>—</td>
<td>6/6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>EGF</td>
<td>0/6</td>
<td>0/5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>FGF4</td>
<td>0/6</td>
<td>0/6</td>
<td>3/3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>FGF10</td>
<td>0/6</td>
<td>0/6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Shh</td>
<td>0/6</td>
<td>0/8</td>
<td>—</td>
<td>—</td>
<td>6/6</td>
<td>—</td>
</tr>
<tr>
<td>Wnt6</td>
<td>14/14</td>
<td>10/10</td>
<td>8/8</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>BSA control</td>
<td>0/5</td>
<td>0/8</td>
<td>0/3</td>
<td>0/4</td>
<td>0/3</td>
<td>—</td>
</tr>
<tr>
<td>3T3 cell control</td>
<td>0/8</td>
<td>0/8</td>
<td>0/6</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

**Note.** Numbers indicate the amounts of positive/total explants. Expression was analyzed by in situ hybridization after 24 h culture with beads releasing the signals or cells expressing the protein (Wnt6).

### ActivinβA Induces dl Expression in the Dental Epithelium

The expression of dl was monitored in a series of experiments similar to that for Ta, and interestingly, of the tested signals only activinβA stimulated the expression of dl in E12 dental epithelium (Figs. 3B, 4C, and 4D). BMP2-, BMP4-, FGF4-, FGF10-, EGF-, and Shh-releasing beads and 3T3 cell aggregates expressing Wnt6 consistently failed to stimulate dl (Figs. 3A and 3C–3H). BSA control beads (Fig. 2M) and 3T3 control cells (not shown) had no effects on the expression of dl. The lack of effects of BMP2, BMP4, FGF4, FGF10, EGF, and Shh on dl expression was confirmed also by radioactive in situ hybridization of sections cut through the explants (not shown). Hence, although Bmp2, Bmp4, Fgf4, and Shh are coexpressed with dl in the epithelial signaling centers they did not regulate its expression. Instead, dl expression in the dental epithelium appears to be regulated by mesenchymal–epithelial interactions, because activinβA expression is confined to dental mesenchyme. activinβA expression in dental mesenchyme is necessary for normal tooth development (Ferguson et al., 1998), and our results indicate that at least one of its functions is to stimulate dl expression in the dental epithelium and thereby to make the signaling centers responsive to TNF signaling.

### Ta but Not dl Expression Requires LEF-1

Wnt signaling is mediated by association of β-catenin with members of the LEF1/TCF family of transcription factors (Eastman and Grosschedl, 1999). Lef1 knockout mice have defects in the same organs as the Tabby and downless mutants, although the phenotype is more severe.
in the Lef1 mutants (van Genderen et al., 1994; Pispa et al., 1999). Our finding that Wnt6 regulates Ta, but not dl, expression is in line with the possibility that LEF-1 could act upstream of Ta. Moreover, a conserved binding site for LEF-1 was identified in the promoter of the human and mouse EDA and Ta genes (Kere et al., 1996; Srivastava et al., 1997). Thus, Lef1 may act in the same pathway as Tabby. Ta and Lef1 show coexpression in the oral epithelium during the initiation stages of tooth development as well as in the dental epithelium during bud and cap stages (Oosterwegel et al., 1993; Keränen et al., 1998). In addition, Lef1 is intensely expressed in the early signaling centers and enamel knots (Keränen et al., 1998) and hence is coexpressed with dl. We therefore analyzed the expression of both Ta and dl in tooth germs of Lef1 mutant embryos.

Tooth development is arrested at the bud stage in Lef1-deficient embryos, and we performed in situ hybridization analysis of sections through the heads (E10–E12) and through arrested bud stage tooth germs (E13 and E14) of Lef1 mutants. Ta expression was not detectable in the ectoderm covering the mandibular and maxillary processes of E10–E12 Lef1 mutants, whereas the wild-type embryos showed continuous expression on the surfaces of branchial arches (Figs. 5A and 5B). In E13 Lef1 mutant embryos Ta expression was significantly downregulated in the dental epithelium as well as in the oral ectoderm flanking the tooth buds (Figs. 5C and 5D). No Ta expression was detected on the lingual aspect of the mutant tooth buds but variable weak expression was often seen on the buccal side (Fig. 5D). At E14 the Lef1 mutant teeth had been arrested during transition to cap stage and Ta expression was evident on the buccal but not the lingual aspect of the tooth germs (Figs. 5E and 5F). These results indicate that LEF-1 is required for Ta expression in the dental epithelium. The transcripts detected in the bud stage dental epithelium suggest that in addition to LEF-1 other factors influence Ta expression.

In contrast, dl expression was unaltered in the E11 dental epithelium (not shown), as well as in E13 tooth buds of the Lef1 mutants. The intense and restricted dl signal at the tip of the mutant tooth buds was identical to the wild types (Figs. 5G and 5H). Hence, although Lef1 transcripts are intensely expressed with dl in the signaling centers of the tooth, our results indicate that Lef1 is not required for dl expression. Taken together, our data suggest that Wnt signals that regulate Ta expression at early stages of tooth organogenesis are transduced by LEF-1.

**Search for Downstream Targets of Ectodysplasin-Edar Signaling in the Enamel Knot**

Our earlier analysis of the phenotype of the first molars in Tabby mice indicated that the development was affected already at bud stage and at cap stage the teeth were hypoplastic. The enamel knot formed but it was small in size and morphologically abnormal. We also showed that transcripts of several enamel knot signals, including Fgf4, Bmp4, Shh, and Wnt10a, were present in the enamel knots of Tabby teeth although in smaller than normal domains (Pispa et al., 1999). Our present finding that dl expression is actually restricted to the enamel knot provided further support to the assumption that the function of the enamel knot is directly affected in Tabby mice, and this prompted us to continue the search for possible targets of TNF signaling among the genes expressed in the enamel knots.

Radioactive in situ hybridization was performed on sections of E14 cap stage teeth from wild-type and Tabby mutant embryos with probes for Lef1, Msx2, and Bmp2. They are all expressed in the enamel knot in wild-type mice (Keränen et al., 1998), and we detected their transcripts also in the epithelium of Tabby molars although in smaller domains (Fig. 6). As shown earlier, the development of Ta teeth was delayed and the formation of enamel knots was aberrant. Hence, like the signal molecules analyzed previously (Pispa et al., 1999), the Lef1, Msx2, and Bmp2 genes do not appear to be targets of ectodysplasin signaling in the enamel knot. We also found that the dl gene was expressed in the enamel knots of Tabby teeth (Fig. 6), indicating that the expression of the TNF receptor edar does not depend on its own signaling.

**DISCUSSION**

**Ectodysplasin and Edar Mediate Signaling between Epithelial Compartments and Regulate the Function of Signaling Centers**

The expression of both Ta and dl was restricted to ectodermal tissue during tooth morphogenesis, indicating that TNF signaling here takes place within epithelial tissue and not across the epithelial and mesenchymal tissue layers like most other signals so far analyzed in developing teeth (Jernvall and Thesleff, 2000). Ta was expressed in the branchial arch ectoderm already prior to the onset of morphological tooth development, whereas dl transcripts were not detected. Interestingly, the initiation of tooth development was accompanied by downregulation of Ta and upregulation of dl expression in the thickened presumptive dental epithelium. Subsequently, dl expression became restricted to the tips of the forming tooth buds, whereas Ta expression was excluded from these sites and continued in the budding epithelium outside the dl-expressing tip. A similar pattern continued during advancing morphogenesis when the dental epithelium folded and formed a cap. dl expression was very intense and restricted to the enamel knots at the sites of folding, and Ta was expressed in the outer enamel epithelium. Hence, ectodysplasin–edar signaling appears to take place between epithelial compartments in a reiterative manner during advancing morphogenesis.

It is of particular interest that the epithelial compartments in which dl, i.e., the TNF receptor, is expressed...
consist of cells which actively produce signal molecules. During tooth development, the early epithelial signaling centers at the tips of the forming buds in E12 embryos as well as the enamel knot cells in E13–E14 bud to cap stage tooth germs have previously been characterized by restricted expression of more than 10 signaling molecules in the BMP, FGF, Wnt, and Shh families. The comparison of in situ hybridization analyses of our present and previous studies indicate exact colocalization of \( dl \) expression with these signals (Vahtokari et al., 1996; Jernvall et al., 1998; Kettunen et al., 1998; Keränen et al., 1998). Because the TNF ligand ectodysplasin is produced by epithelial cells outside the signaling centers, this indicates that interactions between epithelial compartments regulate the function of the signaling centers. \( dl \) is so far the only signal receptor restricted to the enamel knot. Interestingly, none of the FGF receptors is expressed in the enamel knots (Kettunen et al., 1998).

It is intriguing that Ta and dl seem to have analogous expression patterns during hair development. In both mouse and human embryos, ectodysplasin is expressed widely in early skin ectoderm and it is excluded from the epithelial thickenings during hair initiation (Montonen et al., 1998; Mikkola et al., 1999). \( dl \), on the other hand, is expressed in restricted spots at the sites of hair follicles and apparently it colocalizes with the expression of many signal molecules (Headon and Overbeek, 1999; our unpublished observations). Hence, it can be suggested that ectodysplasin and edar are involved in the regulation of signaling tissues also during hair organogenesis. We speculate that ectodysplasin and edar may regulate interactions between epithelial compartments in the different organs affected in the ectodermal dysplasias during the initiation of development, as the cells which will form the bud respond to ectodysplasin signal from the surrounding ectoderm (Fig. 7).

Ectodysplasin like all TNFs is produced as a transmembrane protein and exerts its effects by binding to specific cell surface receptors. Some, but not all, TNFs are shed from the cell surface and can thus act also on cells which are not in intimate contact (Gruss and Dower, 1995; Baker and Reddy, 1998). It is not known at present whether

FIG. 4. Induction of Ta and dl expression in the epithelium of the tooth explants. Vibratome sections through the whole mounts as in Figs. 2A and 3B. (A, B) Epithelial expression of Ta has been induced by Wnt6-expressing cells. (C, D) Epithelial expression of dl has been induced by activin\( \alpha \A

FIG. 5. Ta but not dl expression requires LEF-1. (A) Ta expression in the oral and presumptive dental epithelium in mandible and maxilla of an E11 wild-type embryo. (B) No Ta expression is detected in the Lef1 mutant. (C) In a bud stage tooth germ of an E13 wild-type mouse embryo Ta transcripts are seen in the outer epithelium of the bud. (D) In the tooth germ of a Lef1 null mutant Ta expression is absent in the lingual epithelium but faint expression is seen in the buccal side. (E) Intense Ta expression in the outer enamel epithelium of a wild-type E14 cap stage tooth. (F) In the arrested E14 Lef1 mutant tooth germ expression is absent from the lingual side but transcripts are seen on the buccal aspect. (G, H) dl expression is unaffected in the forming enamel knot of a Lef-1 \(-/-\) embryo compared to the wild type. Bar, 95.4 \( \mu \)m.
ectodysplasin is cleaved, but the expression pattern at the cap stage, when Ta- and dl-expressing cells are separated by several cells, indicates that if ectodysplasin has effects at this stage it must be shed. On the other hand, during the initiation and bud stage of tooth development the patterns of Ta and dl are complementary, and signaling could take place at the expression boundaries even if ectodysplasin remains anchored to the cell membrane.

**TNF Signaling Mediated by Ectodysplasin and Edar Is Intimately Linked with Epithelial-Mesenchymal Interactions and Regulated by Wnt and Activin Pathways**

Although TNF signaling mediated by ectodysplasin and edar operates exclusively within epithelial tissue in tooth morphogenesis, our analysis of the effects of signal molecules on their expression in explant cultures indicates intimate links with the epithelial-mesenchymal interactions governing tooth morphogenesis. We cultured tooth explants with cells expressing Wnt6 and with beads soaked in EGF, FGF4, FGF10, BMP2, BMP4, Shh, and activinβA. Of these signaling molecules, only Wnt6 induced Ta expression and activinβA induced dl expression. This indicates that TNF signaling is controlled by two different signals, an (presumably) epithelial signal (Wnt) and a mesenchymal signal (activinβA) regulating the expression of the ligand and receptor, respectively (Fig. 7). This is the first demonstration of integration of the three signaling pathways, Wnt, activin, and TNF.

ActivinβA and BMP4 have been identified as the earliest mesenchymal signals regulating tooth development. They are induced by signals from the presumptive dental epithelium before E11.5; Bmp4 is autoinduced by BMP4 (Vainio et al., 1993) and activinβA is induced by FGF8 (Ferguson et al., 1998). The early expression of activinβA is necessary for the morphogenesis of all teeth except upper molars from bud stage onward (Matzuk et al., 1995; Ferguson et al., 1998). However, downstream targets of activin and the pathway which it affects were not identified by analyzing tooth buds of activinβA null mutant embryos. Our bead experiments indicate that activin regulates the TNF pathway in epithelium by upregulating dl expression. dl is conceivably not the only target of activin signaling because the phenotype of the activin null mutants is more severe than that of Tabby and downless mutants (Gruneberg, 1965; Ferguson et al., 1998).

Our finding that mesenchymal activinβA induces dl expression in E12 dental epithelium suggests that an im-
portant function of activin is to regulate the function of the early signaling center at the tip of the budding epithelium (Fig. 7). In this way TNF signaling would be intimately linked with the epithelial–mesenchymal interactions regulating tooth morphogenesis. ActivinβA continues to be expressed in the dental mesenchyme during cap stage and therefore it may regulate dl expression also in the enamel knot. Although tissue recombination studies have shown that activin is not required for tooth development any more during cap stage (Ferguson et al., 1998) it is possible that loss of activin at this stage causes only slight impairment in tooth development which was not detected. This would be in line with the rather mild and variable tooth phenotype in Tabby and downless mutants.

BMP4 did not induce dl expression although it was considered a good candidate as it is the only signal so far implicated in the induction of epithelial signaling centers in teeth. We have shown earlier that BMP4 from dental mesenchyme regulates the expression of the enamel knot markers p21 and Msx2 in epithelium (Jenvall et al., 1998). Furthermore, loss of mesenchymal Bmp4 expression has been associated with bud stage arrest of tooth development in mouse knockouts of Msx1 and Pax9 in which the enamel knot does not develop (Chen et al., 1996; Peters et al., 1998). Hence, we speculate that the BMP pathway induces the formation of the signaling centers, and it may be parallel rather than integrated with the activin pathway. By inducing dl expression in the signaling tissue activin controls responsiveness to TNF signaling which in turn regulates the function of the signaling centers (Fig. 7). As activinβA is also expressed in dermal mesenchyme and necessary for the development of vibrissae (Matzuk et al., 1995) it is possible that dl expression (and thereby TNF signaling) is regulated by activin also during hair organogenesis and perhaps in other organs affected in ectodermal dysplasia syndromes.

**Ta Expression Is Regulated by Wnt Signals and Requires Lef1**

Of the different signaling molecules tested on dental explants, only Wnt6 stimulated Ta expression. Wnt6 is expressed throughout the oral and dental epithelia during active tooth morphogenesis and it is thus coexpressed with Ta and could be an autocrine regulator of Ta. However, also several other Wnts are expressed in the epithelium and one (Wnt5) in the mesenchyme during early tooth development (Sarkar and Sharpe, 1999) and it is therefore possible that Wnt6 mimicked the effect of another Wnt protein. It is also possible that different Wnts regulate Ta expression at various developmental stages. One candidate regulator of the early Ta expression is Wnt7b which is expressed in oral epithelium but is excluded from the dental epithelium and hence shows coexpression with Ta during the time of tooth initiation, but not later. Wnt7b was recently implicated in the formation of the boundary between oral and dental ectoderm together with Shh, which is expressed exclusively in dental epithelium (Sarkar et al., 2000). Like ectodysplasin and edar, Wnt7b and Shh were suggested to mediate planar signaling within the epithelium and to regulate the formation of the tooth bud from the dental lamina.

Wnt and β-catenin have been implicated among the earliest regulatory genes in hair and feather formation (Kat et al., 1998; Widelitz and Chuong, 1998; Noramly et al., 1999). They are expressed widely in the ectoderm and precede the initiation of hair and feather buds. Also Ta is expressed very early in mouse and human skin ectoderm (Montonen et al., 1998; Mikkola et al., 1999) and it is possible that Ta is one of the genes regulated by Wnts during hair development in a manner analogous to what we suggest for tooth organogenesis.

The downregulation of Ta transcripts in the dental epithelium of Lef1 mutant embryos indicates that Ta expression is downstream of Lef1. Lef1 is a TCF transcription factor which mediates Wnt signaling by associating with β-catenin (Behrens et al., 1996). Its role in ectodermal organ development has been revealed in transgenic mice overexpressing or lacking Lef1 (van Gendelen et al., 1994; Zhou et al., 1995). In Lef1 knockout mice, teeth, hair, and mammary glands are arrested early in development and the phenotype thus resembles that of Tabby mice although it is much more severe. Tooth development is arrested at the bud stage in Lef1 mutant embryos, and Lef1 was shown to be required in dental epithelium before bud–cap transition (Kraoctchi et al., 1996). Interestingly, the promoter of the human ectodermal dysplasia and mouse Tabby genes contains a conserved Lef1 binding site (Kere et al., 1996; Srivastava et al., 1997), and a mutation in this region has been reported in one ectodermal dysplasia patient (Kobiljak et al., 1998). Hence, Lef1 may directly regulate the Ta gene. Taken together, these results support a central role for Wnt/β-catenin/Lef1 signaling in the regulation of the expression of ectodysplasin.

**Possible Functions of Ectodysplasin/Edar Signaling during Organogenesis**

Our earlier analysis of the tooth phenotype in Tabby mutants indicated that their third molars were missing in 17% of cases and that all first molars were hypoplastic with a patterning defect of the cusps (Pispa et al., 1999).
The first molar tooth bud when examined at E13 was shorter in anteroposterior dimension as well as slightly narrower in its buccolingual extension. This indicates that development had been disturbed prior to bud stage, and based on the present results of Ta and dl expression it can be assumed that the function of the early signaling center which is visible in the budding epithelia between E12 and 12.5 had been disturbed. This signaling center is, however, formed in Tabby teeth as indicated by the localized expression of Bmp2 (Pispa et al., 1999). The advancing morphogenesis from bud to cap stage was progressively delayed in Tabby molars and the enamel knots were greatly reduced in size (Pispa et al., 1999). However, the enamel knots did form as indicated by the expression of the signal molecules Wnt10a, Shh, Fgf4, and Bmp4. In this study we showed that also Bmp2 and transcription factors Lef1 and Msx2 previously implicated in enamel knot function, as well as dl, were all expressed in Tabby teeth. Hence, it is apparent that the signaling centers had formed in the absence of ectodysplasin, but their reduced size indicates deficient signaling functions.

We conclude that ectodysplasin–edar signaling is not involved in the induction of the signaling centers but rather in regulation of their function. The downstream targets of this TNF signaling remain still to be identified, and therefore the effects at the cellular and molecular levels can only be speculated at present. It is conceivable that like other TNF receptors edar is trimerized upon binding of ectodysplasin and that this elicits intracellular signaling cascades resulting in the activation of NFκB (our unpublished results) and/or JNK pathways. TNF signaling may result in either apoptosis or stimulation of cell survival or proliferation depending on the nature of the TNF receptor and cellular context (Baker and Reddy, 1998). The phenotype of ectodermal dysplasia syndromes, i.e., defective organ development, together with the fact that the mutations in both human and mouse cause loss of gene function (Kere et al., 1996; Srivastava et al., 1997) suggests that ectodysplasin and edar promote cell survival rather than apoptosis. It is noteworthy that the enamel knots are composed of nonproliferative cells, and increased apoptosis occurs in the tissue at the time of termination of the signaling functions (Jernvall et al., 1998). It can be speculated that TNF signaling in these centers supports their survival and protects from too early apoptosis. Interestingly, apoptosis has been associated with other embryonic signaling centers including ZPA and AER in the limb bud, and similar signaling pathways involving BMP4 and FGFs have been implicated in the maintenance of AER and enamel knot (Jernvall et al., 1998; Pizette and Niswander, 1999). It will be interesting to see whether TNFs are involved in the regulation of the function of other signaling centers in the embryo.

ACKNOWLEDGMENTS

We thank Ms. Oxana Arsenieva, Kaija Kettunen, Merja Mäki- nen, and Riikka Santalahi for excellent technical assistance. This study was financially supported by the Academy of Finland, Sigrid Juselius Foundation, and Viikki Graduate School in Biosciences.

Note added in proof. While this paper was in press, contradictory results on the expression of Ta in Lef1 mutants were reported by A. S. Tucker et al., Development 127, 4691–4700, 2000.

REFERENCES


tion between epithelial and mesenchymal tissues during early tooth development. Cell 75, 45-58.


Received for publication August 21, 2000
Accepted September 26, 2000
Published online December 9, 2000