Conserved Features and Evolutionary Shifts of the EDA Signaling Pathway Involved in Vertebrate Skin Appendage Development

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It is widely accepted that evolutionary changes in conserved developmental signaling pathways play an important role in morphological evolution. However, few in silico studies were interested in tracking such changes in a signaling pathway. The Ectodysplasin (EDA) pathway provides an opportunity to fill this gap because it is involved in vertebrate skin appendage development such as scales, teeth, hair, and feathers that take an obvious part in the adaptation of species to their environment. We benefited from the large amount of genomic data now available to explore the evolution of the upstream genes of the EDA pathway. In mammals, these genes are *eda* (encoding 2 ligands, EDA-A1 and EDA-A2), *edar* (EDA-A1 receptor), *edaradd* (EDA receptor [EDAR] adapter), *xedar* (EDA-A2 receptor), and *troy* (a XEDAR-related receptor). We show that the evolution of EDA pathway genes combines both strongly conserved features and evolutionary shifts. These shifts are found at different signaling levels (from the ligand to intracellular signaling) and at different taxonomic levels (class, suborder, and genera). Although conserved features likely participate to the similarities found in the early development of vertebrate skin appendages, these shifts might account for innovations and specializations. Moreover, our study demonstrates that we can now benefit from the large number of sequenced vertebrate genomes to explore the evolution of specific signaling pathways and thereby to open new perspectives for developmental biology and evolutionary developmental biology.

Introduction

Morphological evolution of complex organisms is thought to arise through the evolution of the developmental mechanisms controlling morphogenesis. Among these mechanisms, "embryonic induction" refers to the ability of cells to induce their neighbors to change their behavior or their fate. Cells do so by secreting signals (ligands) that, upon reception (by receptors), are transduced into the cell through complex cascades leading, for example, to changes in gene expression. Research in the past 2 decades have shown that despite their wide morphological diversity, bilaterians only use a few types of developmental signaling pathways, such as Hedgehog, Wnt, TGFβ, Notch, FGF, and nuclear hormone pathways (for review, see Pires-daSilva and Sommer 2003). Nevertheless, many evolutionary changes in these signaling pathways can, at least in principle, shape morphological evolution, like, for example, 1) structural mutations in the signaling pathway components: ligand, receptor, transcription factor, and so on (e.g., the melanocortin receptor, reviewed in Hoekstra and Coyne 2007); 2) the functional diversification of the signaling pathway components through gene duplications (e.g., the FGF superfamily, Itoh and Ornitz 2004; Popovici et al. 2005 or the nuclear receptor superfamily, Bertrand et al. 2004); 3) the co-option of existing signaling pathways and networks into new structures (as exemplified by the Hedgehog pathway in butterfly wing eyespot development, Keys et al. 1999); 4) changes in the signaling network (e.g., vulva development in *Caenorhabditis elegans*, Felix 2005); and 5) changes in *cis*-regulation (and thus spatiotemporal expression) of signaling pathway components or target genes (e.g., BMP4 in finches beaks, Abzhanov et al. 2004 or Wnt signaling in the mouse and chicken face, Brugmann et al. 2007; for recent reviews on *cis* regulation in morphological evolution, Wray 2007; Prud'homme et al. 2007).

Despite this large conceptual framework, only few studies have been able to pinpoint specific genetic changes in a signaling pathway gene as a key event for morphological evolution. Even in silico studies of signaling pathway genes were, to our knowledge, limited to comparative studies at very high taxonomic scales (e.g., bilaterians; Pires-daSilva and Sommer 2003) or to reconstructions of gene family histories. But thanks to the large number of vertebrate genome and EST (Expressed Sequence Tag) sequencing programs, we have now the opportunity to trace signaling pathway gene evolution while scanning for those clade specificities that may be associated with morphological evolution.

For this purpose, we have chosen the Ectodysplasin (EDA) pathway, which presents an obvious interest for morphological evolution. This pathway is involved in early organogenesis of vertebrate skin appendages, such as teleost fish scale or tooth (Kondo et al. 2001; Harris MP, Rohner N, Konstantinidis P, Schwarz H, Nüsslein-Volhard C, unpublished data), bird feather (Houghton et al. 2005; Drew et al. 2007), or mammal tooth, hair, and glands (Courtney et al. 2005; Mikkola 2007). As specialized interfaces with the environment, skin appendages are known hot spots of morphological evolution, both at the macroevolutionary and microevolutionary levels, and the study of their origin and evolution has been a major topic in evolutionary biology (see e.g., Reif 1982; Peterkova et al. 2006). They share many similarities in the early steps of their development, which use a common set of genes, among which the genes of the EDA pathway (Sharpe 2001; Mikkola 2007). It is unclear whether this common genetic network is the

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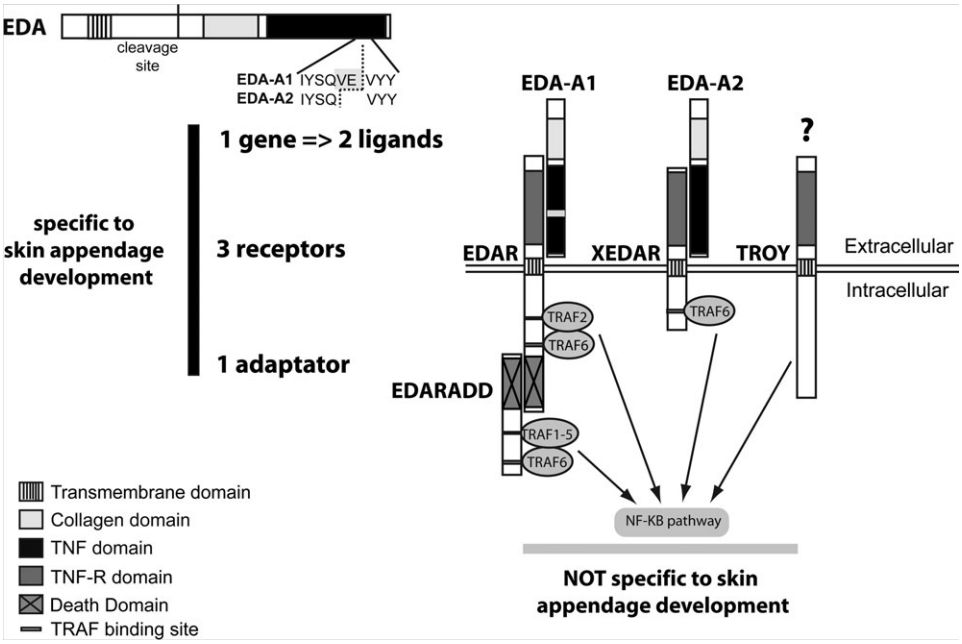

FIG. 1.—The EDA pathway, senso lato, as known in mammals. Only the upstream players of the EDA pathway are specific to skin appendage development. Note that the *eda* gene gives rise to 2 isoforms, which differ only in 2 amino acids of the TNF domain. Proteases act at the cleavage site to free the active ligand, which contains a collagenous domain and the TNF domain.

result of direct lineage relationships between different types of skin appendages or the result of co-option events or both. Lineage relationships have been demonstrated for some cases (e.g., from the keratinized scales of dinosaur to bird feathers Wu et al. 2004) but are still debated for others (from placoid scales of early cartilaginous fishes to teeth Stock 2001; Donoghue 2002). Whatever the case, we can expect that the evolution of the EDA pathway has been linked to the evolution of skin appendages. We already have an example for this since the *eda* gene is responsible for adaptive variation of the body armor plate in freshwater populations of a teleost fish, the threespine stickleback (Colosimo et al. 2005; Knecht et al. 2007). Moreover, 2 other genes of the EDA pathway are strong candidates in recent adaptation of human populations in Asia (Sabeti et al. 2007; Williamson et al. 2007). Albeit in those cases, we do not know the selected trait, the Asian-specific allele of 1 of these 2 genes (*edar*) has been clearly associated with increased hair thickness (Fujimoto et al. 2007).

The EDA pathway belongs to an ancient type of signaling pathway, the Tumor Necrosis Factor (TNF) signaling pathway, which is common to bilaterians and involves ligands of the TNF superfamily binding to receptors of the Tumor Necrosis Factor Receptor (TNFR) superfamily. Like for many other signaling pathways, both the ligands and receptors of these superfamilies were extensively duplicated during early vertebrate evolution (Collette et al. 2003). In human and mouse, the term "EDA pathway" is used to describe 2 TNF pathways of which respective ligands, EDA-A1 and EDA-A2, are produced by alternative splicing from the same gene, *eda* (fig. 1). First, the EDA-A1 pathway involves the TNF-like ligand EDA-A1, the TNFR-like receptor EDAR (Ectodysplasin Receptor), and a death domain adapter, EDAR adapter with death domain (EDAR-ADD), that is recruited by EDAR death domain after ligand binding (Thesleff and Mikkola 2002; Courtney et al. 2005) (fig. 1). Second, the EDA-A2/XEDAR pathway involves the EDA-A2 ligand, which differs from EDA-A1 by only 2 missing amino acids (fig. 1), and the XEDAR receptor, whose TNFR domain is related to that of EDAR (Yan et al. 2000) (fig. 1). From an evolutionary point of view, a third pathway, the TROY pathway, could also be included in an EDA pathway, senso lato, because the TNFR domain of TROY is closely related to that of XEDAR. TROY (also known as TAJ-1, TRADE, or TNFRSF19 [Eby et al. 2000; Kojima et al. 2000]), however, does not bind either EDA-A2 or EDA-A1 and is still an orphan receptor to date (Bossen et al. 2006). In all, the EDA pathway, senso lato, involves signaling through the 3 related TNFR receptors: EDAR, XEDAR, and TROY (fig. 1). Despite their radically different cytoplasmic tail, signaling through all 3 receptors converges on Tumor Necrosis Factor Receptor-Associated Factor (TRAF) proteins binding to receptors and EDAR-ADD) and NF-κB signaling (Nuclear Factor κB) (Courtney et al. 2005) (fig. 1), which are commonly involved in TNF pathways and, as a consequence, are also involved in other processes like osteogenesis or immunity (Zonana et al. 2000; Ohazama et al. 2004). That these 3 pathways should be taken as a whole is confirmed by their broad involvement in skin appendage development. In mammals, the EDA-A1/EDAR/EDARADD pathway is necessary for skin appendage development and its impairment results in hypohidrotic ectodermal dysplasia (HED), characterized both in human and mouse by defects in tooth, hair, and glands (Mikkola and Thesleff 2003). EDAR signaling is also necessary for feather development in chicken (Drew et al. 2007) and for tooth and scale development in teleost fishes (Kondo et al. 2001; Harris MP, Rohner N, Konstantinidis P, Schwarz H,

Nüsslein-Volhard C, unpublished data). The *xedar* and *troy* knockout mice show no obvious phenotype (Newton et al. 2004; Shao et al. 2005). However, both genes are expressed during skin appendage development in both mouse and chicken (Pispa et al. 2003; Drew et al. 2007) and, in chicken, their loss results in feather development defects (Drew et al. 2007). Thus, at least in amniotes, all 3 receptors share an intimate and related role in skin appendage development. We thus consider that the EDA pathway, senso lato, includes the signaling related to skin appendage development through all 3 receptors.

In summary, the EDA pathway is a promising model for studying how the evolution of a signaling pathway can be involved in morphological evolution. In addition, while being relatively simple, it is also quite representative of other signaling pathways. Indeed, it provides a typical case of duplicated receptors that evolved different ligand binding and intracellular signaling specificities, which, like very often, were studied mainly in mammals. An important consequence is that, except in human and mouse, there are no biochemical data about the precise ligand–receptor relationships of the 3 receptors to the EDA ligands. For all these reasons, we chose the EDA pathway as a model and made use of the large number of vertebrate genomes to explore the evolution of this specific signaling pathway during vertebrate evolution. This allows us to provide the first comparative genomic overview of the evolution of a signaling pathway at the scale of vertebrates.

Materials and Methods

Gene Prediction

The origin of sequences used in this study is described in supplementary table S1 (Supplementary Material online). Reference sequences stored in "Refseq" from GenBank were used in priority when available as well as the Ensembl gene predictions. Importantly, all these predictions were checked manually to detect annotation errors especially around splicing sites, which are very frequent. Similarity searches were performed using TBlastN against assembled genomes (http://ensembl.org; http://esharkgenome.imcb.a-star.edu.sg/index.html), EST data (from http://ncbi.org), and TRACE data (deposited in the trace archive of GenBank) followed by manual compilation of data to predict further genes or exons missing from Ensembl predictions (small exons). Criteria for accepting an exon were high sequence similarity, adequacy of the splicing sites, and assignment of all exons of a gene to the same genomic region (assembled genomes only). Each time our prediction or even an Ensembl prediction did not fit with this criteria, the corresponding sequence was replaced by question marks. EST data were checked against genomic data when possible. EST and Trace data being susceptible to sequencing errors, we compared 2 or more sequences with each other when feasible. When the sequences diverged, we added a question mark except when one of the sequences exhibited a conserved amino acid, in which case we included this conservative version. For small exons (like EDARADD exons 1A and 1B), we also used conservation of noncoding regions immediately around to screen databases by BlastN.

cDNA Cloning

We cloned *Macropus eugenii edaradd* isoform-A, *Mesocricetus auratus edaradd* isoform-B, *Mus (Nannomys) minutoides edaradd* isoform-B, *Cavia porcellus edaradd* isoform-A, and *M. eugenii eda* cDNAs from adult kidney, total RNA of respective species with primers included in the supplementary table S1 (Supplementary Material online). *Macropus* RNA was kindly provided by Dr Kevin Nicholas from Melbourne University. The corresponding sequences were deposited in GenBank (accession numbers: EU410404, EU410405, EU410406, EU410407, and EU410403, respectively).

Sequence Alignments

Sequences were aligned using Muscle (http://www.drive5.com/muscle/) (Edgar 2004) followed by manual refinements. In the provided alignments (supplementary figs. S1–S5, Supplementary Material online), we used a color code to underline amino acids shared by different groups of species (for details, see legend of supplementary figs. S1–S5, Supplementary Material online).

Phylogenetic Reconstructions

Phylogenetic trees were calculated on specific protein domains. Alignments used can be found in fasta format in supplementary table S2 (Supplementary Material online). Maximum likelihood (ML) reconstructions were conducted with PHYML (Guindon and Gascuel 2003) using the JTT model of amino acid substitution, with among-site rate heterogeneity model by a gamma distribution with 4 categories and an estimated proportion of invariable sites. Indeed, JTT model with a gamma distribution had been found to best fit to the data, as tested with ProtTest 1.4 (Abascal et al. 2005). For the "free topology tree," the tree was calculated using NNI (Nearest Neighbor Interchange) moves on a BioNJ starting tree and 500 bootstrap replicates were performed. For the "imposed topology," the tree topology was constrained according to the accepted phylogeny of organisms with only branch lengths being estimated by the software. The imposed species phylogeny used was established on Murphy et al. (2001) for mammals and Lavoue et al. (2005) for fish and can be found on supplementary figures S1–S5 (Supplementary Material online).

3D Modeling of EDA-A1 and EDA-A2

3D models of EDA-A1 and EDA-A2 of the various species were modeled on the human EDA-A1 (1RJ7) and EDA-A2 (1RJ8) homotrimers using the Swiss-PDB Viewer software (v3.9) and the Web-based Swiss-Model server. Electrostatic potential was computed using the Swiss-PDB Viewer software.

Identification of Shifts in Site-Specific Selective Constraints during Mammal Evolution

In order to identify amino acid undergoing shifts in their selective constraints during mammal evolutionary history, we performed phylogenetic analyses using ML

methods and codon-based substitution models. Nucleic sequences were first aligned by reference to protein alignments. Because gap positions are discarded before ML computations, different sets of sequences (supplementary table S3, Supplementary Material online) were analyzed favoring either a maximum number of positions (but a reduced species number) or a maximum number of species (but a reduced position number, i.e., a subpart of the protein). We used the software PAML v3.14 (Yang 1997) that allows site-specific computation of nonsynonymous/synonymous substitution rate ratios (ω or *K*a/*K*s) for a predefined branch versus other branches of a phylogenetic tree. We carried out all computations with an unrooted consensus topology of mammals (topology after Murphy et al. [2001] and Huchon et al. [2002] for rodents). In order to test site-specific changes in selective constraints for predefined branches, we used the likelihood ratio tests based on branch-site models developed by Zhang and colleagues (Yang 1997; Zhang et al. 2005). These tests consist of applying 3 models to the data and comparing their respective likelihood value: (I) the nearly neutral model (M1a) only considers constrained and neutral residues with no possibility of shift between the 2 categories along the branches of the phylogenetic tree; (II) the modified branch-site model A, which allows some sites to undergo a nonsynonymous/synonymous substitution rate ratio (ω_2) greater than 1 for a predefined branch of the tree, considering thus possible positive selection events along a specific lineage; (III) a branch model built in the same way as the modified branch-site model A but with $\omega_2 = 1$ fixed, considering thus site-specific relaxation along the same lineage (Zhang et al. 2005). Because these models are nested, likelihood ratio tests based on twice the log-likelihood difference as a statistic can be constructed to test the existence of positive selection or relaxation events on given branches (Yang 1997). Test 1 of Zhang et al. (2005)—that is, branch selection model (II) as alternative versus nearly neutral model (I) as null model using a conservative χ^2 with 2 degrees of freedom (df)—was employed here to test the relaxation in a given branch. The relaxation was also tested with a supplementary likelihood ratio test called "test 1bis," considering the branch relaxation model as alternative versus the nearly neutral model as null model and a χ^2 distribution with 1 df for the test statistic distribution. We performed test 2 (Zhang et al. 2005) to test for positive selection, comparing the branch selection model (III) as alternative versus the branch relaxation model (II) as a null model. In this latter case, because the conservative procedure—use of a χ^2 distribution with 1 df—yields overestimated *P* values, we also calculated *P* values according to the exact null distribution for this test, which is a 50:50 mixture of point mass 0 and χ^2 with 1 df (Zhang et al. 2005). If test 1, test 1bis or test 2 rejected the null hypothesis, we identified sites under either relaxation or positive selection along the defined lineage using the posterior probabilities supplied by the empirical Bayesian procedure implemented in PAML (Yang et al. 2005).

Synteny Map

The synteny map for the *eda–xedar* region was generated from genome assembly data found in Ensembl. For each species, we walked on the chromosome (or scaffold) from the *xedar* gene or the *eda* gene and looked for genes described in Ensembl as orthologs of genes found in the human *xedar–eda* region. Only such genes were represented on the map.

Noncoding Sequence Alignment in the *edaradd* Gene

Ensembl genomes and Ensembl Trace were screened by BlastN with both a human and a *Monodelphis* probe spanning 1.4 kb around exons 1A and 1B of the *edaradd* gene (the sequence can be found in the supplementary table S1 [Supplementary Material online]). GenBank ESTs were screened with TBlastN for the coding sequence of exons 1A and 1B.

Trace data of *Spermophilus tridecemlineatus*, *Oryctolagus cuniculus*, and *C. porcellus* were compared in order to establish a consensus sequence for the region. These sequences were of poor quality and we used chromatogram data to check them manually (http://trace.ensembl.org/).

The EST spanning *edaradd* exon 1A of *Ovis aries* exhibited a sequence error introducing a frameshift. This error was confirmed by sequencing a small polymerase chain reaction fragment of genomic DNA (GenBank accession number: EU410408), and the corresponding sequence was corrected (supplementary table S1, Supplementary Material online). Large genomic regions spanning the *edaradd* gene were extracted from Ensembl for *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Canis familiaris*, *Bos taurus*, and *Monodelphis domestica*. They were aligned with *S. tridecemlineatus*, *O. cuniculus*, and *C. porcellus* short sequences using M-LAGAN (Brudno et al. 2003), and alignment was visualized using Vista (Mayor et al. 2000) and Seaview (Galtier et al. 1996).

Results

In our study, we considered the molecular evolution of the upstream genes of the EDA pathway, namely *eda*, *edar*, *xedar*, *troy*, and *edaradd*, which are specific to skin appendage development (fig. 1). We first produced an annotated data set for each gene (domains, exons, comparison with mutation in HED patients, and so on) by crossing data from EST sequences, complete genome sequence, and Trace Archive (supplementary figs. S1–S5 [Supplementary Material online], the data set is fully available in supplementary table S1 of Supplementary Material online or on the first author Web site: http://igfl.ens-lyon.fr/Members/spantala). Then, we studied conservation and clade specificities in these genes, with 2 questions: Can we detect evolutionary shifts that may be related to the remarkable diversification of vertebrate appendages? How far can we expect to extrapolate our knowledge of the pathway in human and mouse to other species—especially our knowledge of ligand–receptor relationships? At this point, it is important to note that, by encoding 2 alternatively spliced ligands with only 2 amino acid differences but with 2 different receptor specificities (fig. 1), the *eda* gene should coevolve with receptors of both ligands (i.e., *edar* and *xedar* for human and mouse).

In this result section, we will review conserved and clade-specific traits in the functional domains of these genes

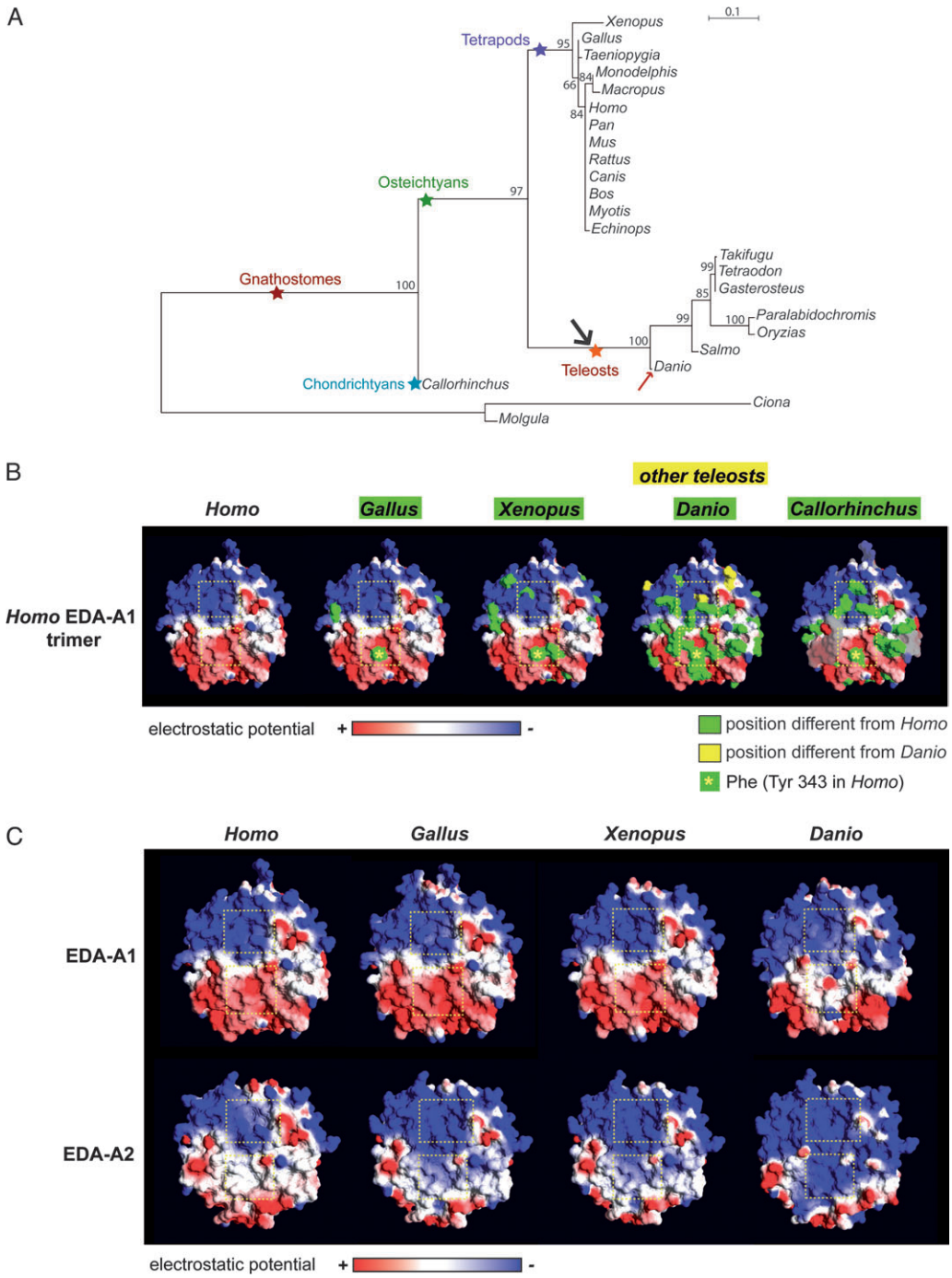

FIG. 2.—Conservation and divergence of the TNF domains of EDA-A1 and EDA-A2. (*A*) Phylogenetic relationships between EDA TNF domains of different vertebrate species as inferred by ML analysis (Phyml). Only a subset of species found in supplementary figure S1 (Supplementary Material online) were included. Tunicate sequences were used to root the tree. The alignment (143 positions) used to build the tree is available in supplementary table S1 (Supplementary Material online). The scale (mean number of amino acid substitution per site) as well as the bootstrap values superior to 60 are indicated. Note the long branch leading to teleosts (black arrow). (*B*) Location on the human EDA-A1 surface of positions that differ in other species. The surface of the human EDA-A1 and its electrostatic potential were calculated with the PDB software from the 3D structure obtained in Hymowitz et al. (2003). The surface was then colored according to the calculated electrostatic potential (positive charge in red, negative charge in blue, and neutral in white). All panels show the human EDA-A1 trimer, but the residues shown in green are those that differ radically from human in the above mentioned species (except for those that differ in teleost species other than *Danio*, which are shown in yellow). Valine/leucine/isoleucine and aspartate/glutamate substitutions were not considered as radically different and thus ignored. Missing amino acids of the incomplete *Callorhinchus* TNF domain were shaded with gray. Please note that in *Gallus* and *Xenopus*, very few amino acids differ from human. However, a phenylalanine (shown with a yellow star) is found in place of the tyrosine 343 in the lower of the 2 receptor-binding surfaces (outlined in yellow). In *Danio*, a lot of positions are different from human and they are preferentially gathered around the lower binding surface. Comparatively, fewer positions differ in the more distantly related species, *Callorhinchus*. Furthermore, note that differences that are found only in other teleost species are out of the receptor-binding surfaces. (*C*) Surface of the human EDA-A1 and EDA-A2 trimers and of the corresponding trimers as modeled in 3 other species. For each species mentioned above, the molecular surface of the EDA-A1 trimer (upper panel) or the EDA-A2 trimer (lower panel) is colored according to the calculated electrostatic

by running through the pathway, that is, starting with the ligand and the ligand-binding part of the 3 receptors and going on with intracellular signal transduction, that is, the cytoplasmic tail of the 3 receptors and the EDARADD adapter.

Two Cleaved EDA Ligands, EDA-A1 and EDA-A2, Are Found At Least in All Osteichthyans

In human and mouse, alternative splicing of the *eda* transcript generates 2 major isoforms, EDA-A1 and EDA-A2, differing by only 2 amino acids in the TNF domain (lacking in EDA-A2, fig. 1). These 2 transmembrane proteins are cleaved by a furin protease to free the 2 ligands, EDA-A1 and EDA-A2, which form homotrimers through their collagenous domain. Ultimately, in mammals, EDA-A1 and EDA-A2 trimers each bind a different receptor, EDAR and XEDAR, respectively, through their trimerized TNF domain.

From our data set, it appeared that any gnathostome EDA ligand should be cleaved and should be able to trimerize because all *eda* genes contain a consensus site for furin cleavage and a very well conserved collagenous domain (supplementary fig. S1, Supplementary Material online). The EDA-A1 isoform is predicted in all examined gnathostome genomes and ESTs and can be found in various clades (supplementary fig. S1, Supplementary Material online; see various ESTs in supplementary table S1, Supplementary Material online). The shorter EDA-A2 isoform is also predicted in all examined gnathostomes, but in its case, the prediction is more problematic. Indeed, the A2 alternative splicing event corresponds to the use of an internal donor splice, formed by the first 2 nt encoding Val308 of the EDA-A1 isoform (fig. 1). Consequently, at the genomic level, it is not possible to distinguish between constraints to maintain this valine or the donor splice site (or both). We found EST sequences corresponding to EDA-A2 in *Xenopus* and *Gallus* (supplementary table S1, Supplementary Material online) but none in fish. The existence of EDA-A2 splice variant outside tetrapods has been provided by Colosimo et al. (2005) who reported its detection in the stickleback *Gasterosteus aculeatus* by reverse transcriptase–polymerase chain reaction (RT–PCR).

Therefore, a cleaved collagenous EDA-A1 ligand is obviously a common feature of gnathostomes. It remains to be confirmed for the EDA-A2 isoform, which is, however, at least a common feature of osteichthyans (including both tetrapods and teleosts).

Another EDA Ligand with Lower Affinity, EDA-A5, Is Expressed in Various Tetrapods

In humans, another alternative splicing variant, called EDA-A5, is missing 3 amino acids in the very beginning of the TNF domain (supplementary fig. S1, Supplementary Material online). Few things are known about this isoform, except that it binds EDAR with a lower affinity than EDA-A1 (Hashimoto et al. 2006). Interestingly, this third isoform is also found in *Xenopus* and *Gallus* ESTs (see supplementary table S1, Supplementary Material online), suggesting that its presence is a functional feature of all tetrapods.

The Receptor-Binding Surface of the EDA Ligands Changed At Least Twice: Once Subtly in Therians and Once Radically in Teleosts

We have seen that the major EDA-A1 and EDA-A2 ligands are found in most vertebrates. However, biochemical data are only available in human and mouse, in which EDA-A1 binds to EDAR and EDA-A2 binds to XEDAR. Whether these relationships can be generalized to other species is currently unknown, but a comparative study of the TNF domain evolution in vertebrate species could provide useful insights into this question. As a first step, we performed an ML analysis, which revealed 2 trends (fig. 2*A*). First, the domain evolved at an extremely slow rate in tetrapods. Second, the branch leading to teleosts is almost twice longer than the branch leading to therian mammals (from the actinopterygian/sarcopterygian split, arrow and thick lines in fig. 2*A*), despite the fact that the divergence of the herein represented teleosts is equivalent to that of the represented therian mammals (150–165 MYA vs. 125–140 MYA, after Benton and Donoghue 2007). Interestingly, this long branch was related to nonconservative substitutions at positions otherwise conserved in other gnathostomes (i.e., *Callorhinchus* + tetrapods) (dark gray in supplementary fig. S1, Supplementary Material online). Such kind of substitutions were 2.8 more frequent for teleosts versus other gnathostomes than for therian mammals versus other gnathostomes. From this analysis, we thus expected that EDA TNF domain evolution is conservative in tetrapods, whereas a shift occurred in the course of teleost evolution. Because 3D data are available for both EDA-A1 and EDA-A2 TNF domains (Hymowitz et al. 2003), we had the opportunity to further characterize these trends.

As already mentioned, the functional units binding the receptors are trimers of EDA-A1 and trimers of EDA-A2. These EDA trimers display a bipartite receptor-binding surface: The upper part is similar in both trimers, whereas the lower part differs and thus confers receptor specificity (see fig. 2*C*) (Hymowitz et al. 2003). In order to evaluate the possible consequences of species differences in amino acid sequence, we located these differences on the human EDA-A1 and EDA-A2 trimers. We first noticed that the backbone of the trimer was very well conserved and that differences were almost exclusively found at its surface.

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potential. Concerning *Homo*, the surface and its electrostatic potential were calculated with the PDB software from the 3D structure obtained in Hymowitz et al. (2003). Concerning *Gallus*, *Xenopus*, and *Danio*, the 3D structure was first modeled on either the EDA-A1 or the EDA-A2 trimer in the PDB software; Then, the surface and its electrostatic potential were calculated with the same parameters used for *Homo*. Note that whereas the EDA-A1 and EDA-A2 modeled trimers of *Gallus* and *Xenopus* are very similar to their human counterpart, the *Danio* trimer differs radically in terms of both surface and electrostatic potential.

We thus examined them in more details for a few representative species (fig. 2*B*).

Unexpectedly, one of the very few differences found in *Gallus* and *Xenopus* (a phenylalanine at position 343 instead of a tyrosine) was located just in the lower part of the binding surface (yellow star in fig. 2*B* and supplementary fig. S1 [Supplementary Material online]). Nevertheless, our modeling showed that *Gallus* and *Xenopus* EDA-A1 and EDA-A2 trimers are similar to their human counterparts, in terms of surface and electrostatic potential (fig. 2*C*). The Tyr343Phe substitution, which occurred specifically in therian mammals versus all other vertebrates, thus did not induce a drastic change of the receptor-binding surface. However, given its crucial position, this therian-specific substitution is probably important for the fine-tuning of ligand–receptor assembly of one or both ligands in therians versus other tetrapods.

As the ML analysis indicated that the *Danio* sequence accumulated very few amino acid replacements after its divergence from the ancestral sequence of all teleosts (see the very short branch in fig. 2*A* highlighted by a small red arrow), we considered it as a representative of the shift that occurred at the basis of teleosts. Most of positions that differ between *Danio* and *Homo* were found in and around the lower part of the receptor-binding site (fig. 2*B*). Comparison with positions that differ in the more distantly related species *Callorhinchus* (fig. 2*B*) showed that most of them are in fact positions that specifically changed in teleosts (represented by *Danio*) versus other gnathostomes. Our observation thus suggested that the lower part of the binding surface, which is responsible for receptor specificity, could have specifically changed early in teleost fish evolution. The modeling of both *Danio* EDA-A1 and EDA-A2 confirmed that the lower part was actually very different from its human counterpart, whereas the upper part was still quite similar (fig. 2*C*). Indeed, the *Danio* EDA-A1 surface was globally more apolar than its human counterpart, and the *Danio* EDA-A2 surface was strongly positively charged instead of being apolar as in human. Nonetheless, like in tetrapods, *Danio* EDA-A1 and EDA-A2 trimers differed from one another only in their lower part (fig. 2*C*). All together, these observations strongly suggest that the EDA-A1 and EDA-A2 surfaces responsible for receptor specificity experienced an acceleration before the diversification of teleosts and were subsequently fixed in teleosts. Indeed, variable positions in teleosts (yellow in fig. 2*B*) are out of these surfaces.

In conclusion, the receptor-binding surfaces of the EDA ligands changed at least twice during vertebrate diversification: once during therian evolution and once more drastically during teleost evolution. An important issue is whether we can correlate these changes with changes in the ligand-binding domain (TNFR domain) of the 3 receptors, EDAR, XEDAR, and TROY. Of note, these 3 domains are phylogenetically related, with XEDAR and TROY being closer to each other than they are to EDAR (Cui and Schlessinger 2006, see also fig. 4). Nevertheless, in human and mouse, EDAR is the exclusive EDA-A1 receptor, XEDAR is the exclusive EDA-A2 receptor, and TROY is to date considered an orphan receptor (Bossen et al. 2006). We then characterized the evolution of all 3 receptors.

xedar Evolved Much Faster Than *xedar* and *troy* in Gnathostomes

As compared with *edar* and *troy*, bioinformatic identification of *xedar* genes was trickier. Up to now, the *xedar* gene was only known in amniotes (human, mouse, and chicken) and had not been detected in teleost fishes (Knecht et al. 2007). Nevertheless, in our analysis, we found EST sequences from *Xenopus* and *Danio* that we interpreted as clear *xedar* orthologs for 3 reasons: 1) in an ML analysis of the TNFR domain (supplementary fig. S6, Supplementary Material online), these genes grouped with the XEDAR–TROY family and are clearly excluded from the robustly supported TROY monophyletic group that already contains orthologs in the same species; 2) their cytoplasmic tail includes a short region containing a TRAF6-binding site, with high similarity to the unique intracellular region conserved between human and chicken *xedar* genes (see supplementary fig. S3, Supplementary Material online); 3) these genes are located in chromosomal regions that are clearly syntenic with the region encompassing *xedar* in human (supplementary fig. S7, Supplementary Material online). In addition, we also found sequences from *Squalus* (EST) and *Callorhinchus* (genomic prediction), which also clustered in the *xedar* group (supplementary fig. S6, Supplementary Material online) and were thus very likely to be *xedar* orthologs. All together, these data strongly suggested that we actually identified *xedar* orthologs in *Xenopus*, *Danio*, *Squalus*, and *Callorhinchus*. Now comparing all *xedar* gnathostomes genes, it appears that the TNFR domain of *xedar* genes evolved much faster as compared with the ones of *edar* and *troy* (compare branch lengths on fig. 3).

The *xedar* TNFR Domain Evolved Rapidly in Amniotes, Raising Questions about the Evolution of Specificity to the EDA Isoforms

The previous observation holds true among amniotes: in our ML analysis, we found long branches separating therians (placentals + marsupials) and birds from their common ancestral node (marked with a star on fig. 3). Close examination of amino acid sequences revealed that several residues specifically changed in therians (see yellow with red circle residues in supplementary fig. S3, Supplementary Material online). The third cysteine-rich repeat, which has been shown experimentally to be responsible for specific binding to EDA-A2 versus EDA-A1 (Hymowitz et al. 2003), is also concerned (note at position 89 the substitution of a glycine with an arginine, whereas this glycine was conserved in all non-therian XEDAR and even EDAR and TROY proteins, supplementary fig. S3 [Supplementary Material online]). Importantly, this pattern of evolution thus leaves open the possibility that the function known in mammals (EDA-A2 binding) only arose with the recent changes that we observed in therian mammals. Moreover, it is tempting to relate these observations to the Tyr343Phe substitution specifically found at the receptor-binding surface of EDA ligands in therians (fig. 2*B*).

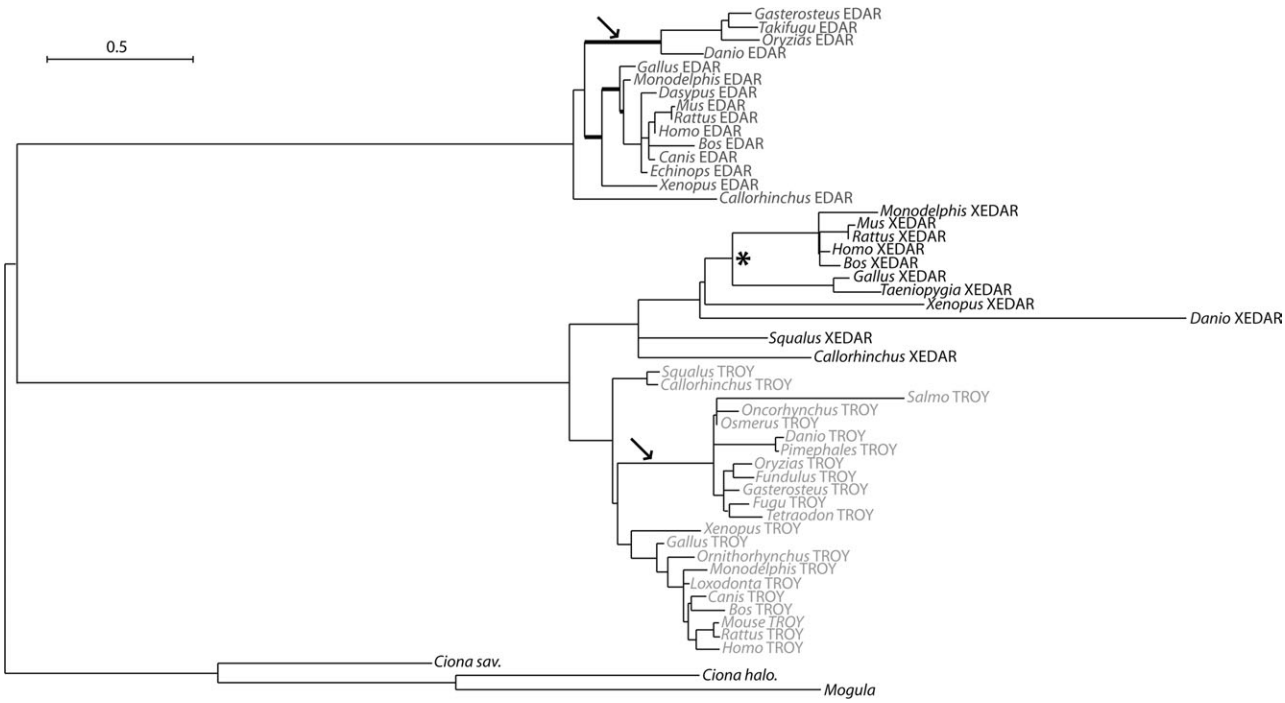


FIG. 3.—Rate of substitutions in EDAR, XEDAR, and TROY TNFR domains as inferred by ML analysis. After we had established phylogenetic relationships between the 3 receptors of various vertebrate species (see supplementary fig. S6, Supplementary Material online), a phylogenetic tree was reconstructed from the sequence data using the Phyml program and a predefined tree topology (based on species phylogeny). For clarity, only a subset of species found in supplementary figures S2–S4 (Supplementary Material online) were included. The tunicate sequences were used to root the trees. The alignment used is available in supplementary table S1 (Supplementary Material online). Note the long branches leading to teleost EDAR and TROY sequences (arrows) and to the bird and the mammal *xedar* sequences (star).

xedar Is Highly Divergent or Lost in Teleost Fishes, Whereas the TNFR Domain of Both *edar* and *troy* May Have Experienced an Evolutionary Shift at the Base of Teleosts

As judged by our ML calculations, the *xedar* TNFR domain of the teleost *Danio rerio* was much more divergent than that of any other species. From then on, one could expect difficulties in detecting divergent *xedar* genes by TBlastN searches in the 4 acanthopterygian genomes. Indeed, despite close scrutiny, we did not find any ortholog of *xedar* in the well assembled *Gasterosteus*, *Takifugu*, and *Tetraodon* genomes. However, we could find 2 exons of a highly divergent *xedar* homolog in the *Oryzias* genome. This divergent gene is located on the same chromosome as *eda* (supplementary fig. S7, Supplementary Material online), but the synteny relationship of neighboring genes found between *Danio* and human is scrambled in *Oryzias*. Still, the most likely hypothesis is that it is actually a *xedar* gene even more divergent than the one in *Danio*. We concluded that in teleosts, the *xedar* gene was either highly divergent (e.g., in *Danio* and *Oryzias*) or lost (maybe the case of *Gasterosteus*, *Takifugu*, and *Tetraodon*).

Interestingly, this high divergence/loss of *xedar* correlated with an evolutionary shift on *edar* and *troy* TNFR domain at the base of teleosts. Indeed, for both *edar* and *troy* TNFR domain, we noticed in our ML analysis the aberrant position of teleost sequences outside the gnathostome clade represented by the *Callorhinchus* plus tetrapod sequences (see arrows on supplementary fig. S6, Supplementary Material online). This topology was typical of a long-branch attraction artifact and, indeed, recalculation of branch lengths on an imposed species phylogeny resulted in long branches leading to teleost fishes (arrows on fig. 3). As for *eda*, we quantified teleost- and therian-specific substitutions at sites otherwise conserved in gnathostomes. For both *edar* and *troy*, we found, respectively, 2 and 1.8 more substitutions for teleosts than for therian mammals.

Close examination of the EDAR TNFR domain sequence (supplementary fig. S2, Supplementary Material online) revealed 7 positions for which tetrapod sequences share the same amino acids as *Callorhinchus* sequences, whereas teleost sequences diverged. These 7 positions are albeit identical within teleosts (colored in dark gray in supplementary fig. S2 [Supplementary Material online], positions Glu54, Gln85, Glu94, Gly95, Phe96, Phe97, and Pro143, by reference to the human sequence). Together, this sequence pattern suggested an ancient case of positive selection at the base of teleosts. Interestingly, 6 positions out of these 7 teleost-specific positions were clustered in the second and third cysteine-rich repeats of the TNFR domain, which are known to mediate interactions with the ligand in mammals (Hymowitz et al. 2003). These data suggest that the EDAR TNFR domain, and more specifically its ligand-binding part, underwent an evolutionary shift at the base of teleosts.

The TNFR domain of *troy* shows a similar sequence pattern (dark gray residues in supplementary fig. S4, Supplementary Material online), whereas the divergence observed inside teleost species is low and affected sites

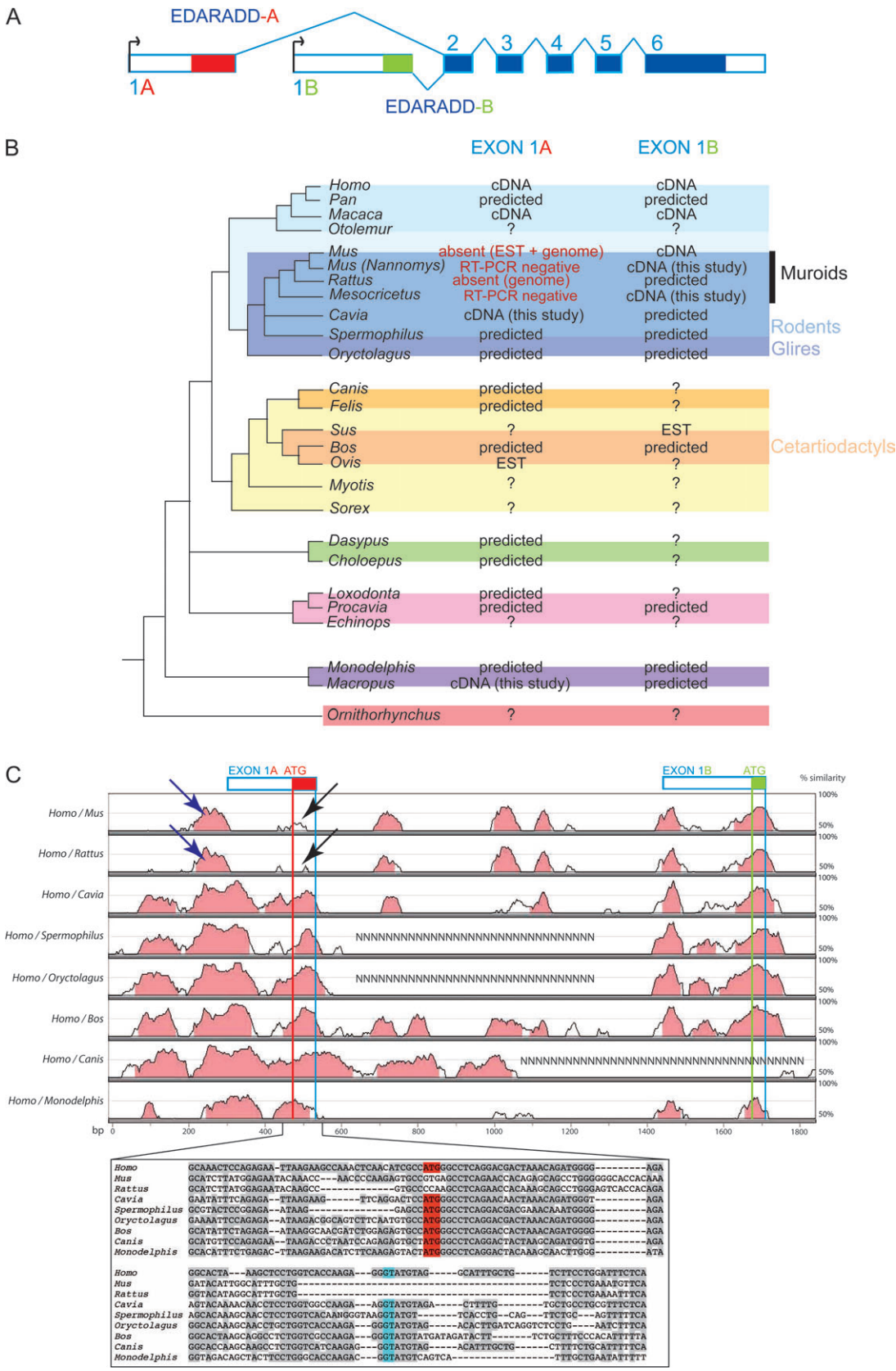


FIG. 4.—EDARADD-A and EDARADD-B isoforms are conserved among mammals, but isoform-A is lost in the *Mus*/*Rattus* lineage. (*A*) In human, the 2 isoforms EDARADD-A and EDARADD-B are produced through 2 alternative first coding exons (called 1A and 1B), each one having probably its own promoter. (*B*) Exons 1A and 1B are found in the major clades of therian mammals. Assumptions rely on bioinformatic prediction (predicted), EST data (EST), or cDNA data (among which cDNA cloned for the purpose of this study). "RT–PCR negative" means that no transcript

likely to evolve neutrally (supplementary fig. S4, Supplementary Material online). This pattern suggests that the TNFR domain of TROY, like the one of EDAR, underwent a single shift at the base of teleosts.

In summary, although XEDAR strongly diverged or was even lost in some teleost species, it seems that there was a correlated shift on the TNFR domain of the remaining receptors: EDAR and TROY, which is consistent with the shift observed on the EDA-A1 and EDA-A2 receptor-binding surfaces.

All the Domains Allowing EDAR Intracellular Signaling Are Very Well Conserved in Gnathostomes, Whereas TROY and XEDAR Signaling Domains Changed At Least Twice: Once for Birds (XEDAR) and Once for Teleost Fishes (TROY)

In mammals, EDAR signaling is mediated by TRAF proteins. EDAR can recruit these proteins directly through specific binding motifs found in its cytosplasmic tail. Alternatively, EDAR can recruit EDARADD through heterotypic interactions of their respective death domains, and EDARADD in its turn allows further signal transduction by recruiting TRAF proteins (Thesleff and Mikkola 2002).

In the cytoplasmic tail of EDAR, the TRAF2- and the TRAF6-binding sites are identical in all vertebrates and even the backbone supporting them is well conserved (see black residues in supplementary fig. S2, Supplementary Material online). The death domains of both EDAR and EDARADD are extremely well conserved in vertebrates (see black residues in supplementary figs. S2 and S5, Supplementary Material online). Moreover, in EDARADD, the 2 different TRAF-binding sites (1 for TRAF6 1 one for TRAF1, 2, 3, and 5) are conserved in all vertebrates (supplementary fig. S5, Supplementary Material online). These observations suggest that the EDAR–EDARADD signaling through different TRAFs is conserved in all gnathostomes.

As compared with EDAR, the signaling through XEDAR and TROY has been less studied. In mammals, XEDAR allows TRAF recruitment (Sinha et al. 2002) and indeed a TRAF6-binding site is conserved in all osteichthyans. More interestingly, chicken *xedar* has a supplementary C-terminal part encoding a death domain (Drew et al. 2007). We found this C-terminal death domain in the bird *Taeniopygia*, but neither in *Xenopus*, nor in any mammalian genome. Thus, at least for birds versus amphibians and mammals, XEDAR signaling can in principle have different signaling outputs.

TROY signaling domains have not been mapped precisely in mammals. However, our alignment revealed that a small region of the cytoplasmic tail is conserved in both chondrichthyans and amniotes. This motif thus probably plays a role in signaling. Importantly, this region is divergent in teleost fishes, suggesting that a shift in the downstream signaling abilities of TROY correlated with the changes seen at the level of the ligand binding.

Two Alternative EDARADD Isoforms Are Conserved in Mammals

In humans, 2 major EDARADD isoforms differing in their very N-terminal ends have been described: EDARADD-A and EDARADD-B (fig. 4*A*). Examination of the extensive set of ESTs available for both isoforms suggested that they were transcribed through alternative promoter usage at 2 alternative exons: exons 1A and 1B (fig. 4*A*). We could predict bioinformatically the presence of both exons in most mammals while EST data were supporting their expression in cetartiodactyls (fig. 4*B*). In addition, we experimentally cloned an *edaradd-A* transcript in a marsupial (*M. eugenii*), for which bioinformatic prediction of exon 1A was questionable. We thus concluded that both *edaradd-A* and *edaradd-B* transcripts are expressed in marsupial and placental mammals. We could not find any similar exons in the *Ornithorhynchus* genome, but because this genome is still incomplete, their absence remains to be confirmed. In chicken, the first known exon has similarities with the short exon 1B at the protein level (supplementary fig. S5, Supplementary Material online), and no sequence reminiscent of exon 1A was detected. The isoform 1B might thus be considered closer to the ancestral form of the EDARADD protein, whereas the isoform 1A might have been gained later during mammalian evolution.

EDARADD-A Isoform Was Selectively Lost in the *Mus*/*Rattus* Lineage

Despite the large number of mouse ESTs available in public databases, we could only find mouse ESTs for the 1B isoform and we could not find an exon 1A by TBlastN searches in the mouse or rat genomes (fig. 4*B*). This raised the possibility that exon 1A could have been lost in the *Mus*/*Rattus* lineage. In the assembled genomes of *Homo*, *Canis*, *Bos*, and *Monodelphis*, a small region of 2 kb contains conserved sequences corresponding to exons 1A and 1B as well as their respective promoters (as visualized with vista, fig. 4*C*). In the orthologous region of mouse and rat, we

←

could be amplified in RT–PCR using degenerated primers. Question marks stand for an absence of conclusive data. (*C*) Vista analysis of the region encompassing exons 1A and 1B of the *edaradd* gene in therian mammals. Plots show percent identity between aligned regions of different species couples (alignment with M-LAGAN). Relative position (base pairs) is given on the *x* axis, and conservation (between 50% and 100%) is given on the *y* axis. Exons 1A and 1B are symbolized above the plots, with the coding part colored in red (1A) or green (1B) and the 5′ untranslated regions in white. Conserved peaks greater than 75% identity on a 30-bp sliding window are shaded pink. *Spermophilus*, *Oryctolagus*, and *Canis* genomic sequences were incomplete: The unknown sequence was symbolized with "NNNNN" to distinguish this lack of sequence from a lack of similarity. The box at the bottom of the plots shows the corresponding multiple alignments around the coding part of exon 1A, with the 1A initiator codon shaded red and the 1A splice donor shaded blue. Note that those 2 features, and more generally the whole exon (black arrow), are not conserved in mouse and rat. The blue arrows point mouse and rat sequences that display similarities with the promoter region of exon 1A of other mammals.

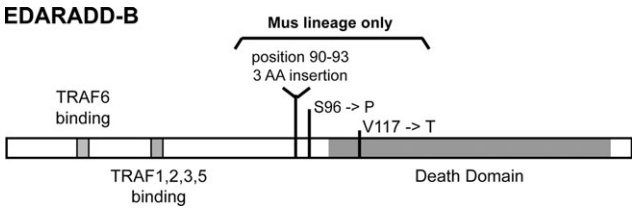


FIG. 5.—Three amino acid insertion and cases of positive selection in the EDARADD protein of the *Mus* lineage. The EDARADD-B protein is shown with its functional features (death domain, binding sites for TRAFs) and the location of the differences observed in the *Mus* lineage (represented by species *Mus (Mus) musculus* and *Mus (Nannomys) musculoides*). Note the 3 amino acid insertion and 2 lineage-specific substitutions as compared with other rodents (or even mammals). These residues are circled in red in supplementary figure S5 (Supplementary Material online). Detailed results are presented in supplementary figure S8 (Supplementary Material online).

detected a sequence with similarities to the promoter region of exon 1A of other mammals (blue arrow in fig. 4*C*) but we failed to detect the exon 1A-coding region (black arrow in fig. 4C). Closer examination revealed that the sequence aligned with the exon 1A-coding region of other mammals is highly divergent in mouse and rat: The initiator codon is missing, as well as the splice donor site (fig. 4*C*). By screening Trace data with the 2-kb human region, we could find orthologous regions for 2 rodents (the squirrel *S. tridecemlineatus* and the guinea pig *C. porcellus*) and a lagomorph (*O. cuniculus*). Clearly, exon 1A is present in all species, an observation confirmed by our experimental cloning of *edaradd-A* isoform in *Cavia* (fig. 4*B* and *C*). All together, these data indicated that the EDARADD-A isoform was specifically lost in a sublineage of rodents including *Mus* and *Rattus*. Because we were unable to clone an *edaradd-A* transcript by RT–PCR in the hamster *M. auratus*, the loss may have occurred during the early evolution of the muroid family, which includes hamster, rat, and mouse but excludes ground squirrel and guinea pig (fig. 4*B*).

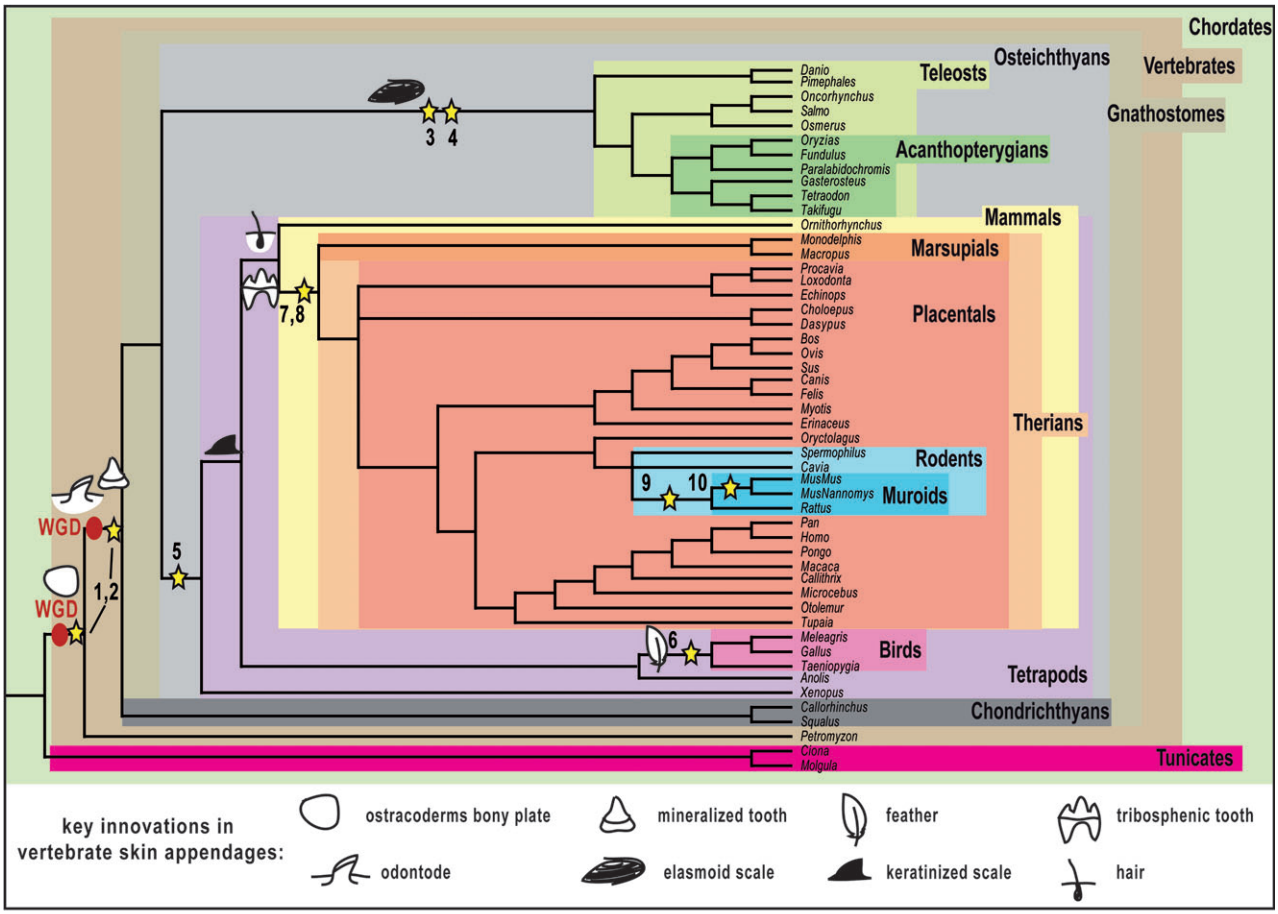
EDARADD-B Underwent Positive Selection in the *Mus* Lineage

By analyzing rodent EDARADD sequences, we noticed an insertion next to the death domain in the *M. musculus* EDARADD protein but not in the *R. norvegicus* one. We cloned EDARADD in 2 other muroid species (*M. auratus* and another *Mus* species belonging to the subgenus *Nannomys*: *Mus (Nannomys) musculoïdes*. We found that this insertion was absent from the hamster sequence but present in *Mus Nannomys*, confirming that it was acquired in the *Mus* lineage, after the *Mus*/*Rattus* split. Interestingly, 2 substitutions were fixed in the vicinity of this insertion along the *Mus* stem lineage (fig. 5, see also in red supplementary fig. S5 [Supplementary Material online]). This observation prompted us to determine the mode of evolution of both sites: neutral versus positive selection. We used the tests proposed by Zhang et al. (2005) and Yang et al. (2005) to determine first, if positive selection was acting in the branch leading to the 2 *Mus* species, and second, which amino acids were concerned. The test favored the hypothesis of positive selection (instead of relaxation) operating along the branch (see supplementary fig. S8 [Supplementary Material online] and its legend for detailed results). Only 2 positions were found under positive selection, but both were in the vicinity of the newly fixed insertion in the *Mus* species: the Thr residue found in the death domain at position 117 and the Pro residue at position 96 (fig. 5, see supplementary figure S8 [Supplementary Material online] and its legend for detailed results). In summary, in 2 species within *Mus*, we identified a specific insertion and 2 residues under positive selection in its vicinity. These findings are strongly indicative of a localized evolutionary shift in this region of the EDARADD protein. Interestingly, the new threonine residue in helix1 of the death domain presents a consensus for phosphorylation by ProteinKinaseC (as predicted with NetPhos2.0 server, Blom et al. 1999) that provides a perspective to understand the functional significance of this shift.

Discussion

In this paper, we studied the evolution of the EDA pathway genes in vertebrates. We focused on the 5 upstream genes of the pathway, that is, *eda*, encoding the TNF ligands EDA-A1 and EDA-A2; *edar*, *xedar*, and *troy*, each encoding a TNFR type receptor; and lastly, *edaradd*, encoding an adapter to the TRAF/NF-κB pathway. Our study highlights several critical points in the evolution of these proteins during vertebrate diversification (fig. 6). Of note, in most cases, we only used the term "evolutionary shift" for what we presume will be a "functional shift" when functional data will be available (for a discussion on this terminology, please refer to Levasseur et al. [2006]).

Point 1: A pathway signaling through EDA-A1, EDAR, and EDARADD was very likely present and implicated in skin appendage development since early gnathostome evolution. The functional domains of *eda*, *edar*, and *edaradd* display high amino acid similarity at the gnathostome level. The EDA-A1 isoform is conserved in all gnathostomes, and the *eda-A1* transcript is found in embryonic ESTs of the major osteichthyan clades. As a result, the EDA-A1/EDAR/EDARADD pathway probably worked in much the same way since early gnathostome evolution. An EDA-A1 ligand with both a TNF and a collagenous domain is cleaved at a conserved furin site and interacts with EDAR TNFR domain. Then, signal transduction can occur in multiple ways: through direct TRAF recruitment (with conserved TRAF2- and TRAF6-binding sites in EDAR) and/or through EDARADD recruitment (conserved death domain). EDARADD itself allows recruitment of TRAFs through its conserved binding sites for TRAF1/2/3/5 and TRAF6. In addition, we know that *edar* loss of function impairs appendage development in mammals (tooth, hair, glands; Mikkola and Thesleff 2003), chicken (feather; Drew et al. 2007), and teleost fishes (tooth and scales in zebrafish; Harris MP, Rohner N, Konstantinidis P, Schwarz H, Nüsslein-Volhard C, unpublished data). It would be surprising if it was not also involved in tooth development in chondrichthyans because the teeth of all gnathostomes are generally



Common vertebrate features

1- EDA-A1/EDAR/EDARADD pathway
2- EDA-A2 splicing

Species specificities and evolutionary shifts

3- loss/divergence of xedar in teleost fish
4- Co-evolution of eda/edar/troy in teleost fish
5- Another alternative isoform in tetrapods
6- Gain of a Death Domain in bird XEDAR
7- Change in EDA-A2 receptor specificity in therians?
8- two alternative isoforms, EDARADD-A and B, conserved in therians
9- EDARADD-A lost in Muroids rodents
10- positive selection in EDARADD in the Mus lineage

FIG. 6.—Major events of the evolution of EDA pathway genes in chordates as studied in this article. See Discussion for detailed explanation of points 1–10 relating major events in EDA pathway genes evolution. These events might have participated in the evolution of vertebrate skin appendages (some key innovations were figured on the tree).

considered as homologous (Reif 1982; Gillis and Donoghue 2007). In summary, both biochemically and developmentally, the EDA-A1 pathway has been remarkably conserved since early gnathostome evolution. An interesting question is whether this pathway arose with vertebrates or earlier, for example, in the ancestor of all extant chordates. Of note, clear *eda* and *edar*/*xedar*/*troy* orthologs are found in invertebrate chordate genomes (*Ciona* and *Branchiostoma*; Pantalacci S, unpublished data), but their developmental role as well as their biochemical function is totally unknown. However, we did not find orthologous domains for the death domains of EDAR and EDARADD in invertebrate chordate genomes, whereas we found them in the lamprey (Pantalacci S, unpublished data). The ancestral chordate receptor, if able to activate the NF-κB pathway, should have done it by direct recruiting of TRAF proteins to the receptor (as still possible for *edar* and *xedar* vertebrate receptors, fig. 1). The EDA/EDAR/EDARADD/TRAF/NF-κB pathway as known in vertebrate skin appendages is thus probably an innovation of early vertebrates. It is tempting to speculate that this innovation has something to do with the origin of the first mineralized skin appendages, such as dermal plates and odontodes found in early vertebrate fossils. Whether the ancestral chordate pathway might already have been involved in epithelial morphogenesis and was later co-opted for those vertebrate-specific epithelial appendages will be investigated in our laboratory in a near future using amphioxus as a model system.

Point 2: Both EDA isoforms, EDA-A1 and EDA-A2, were likely already present in early gnathostome evolution. Actually, we showed that both isoforms can formally be encoded from every gnathostome *eda* gene and that

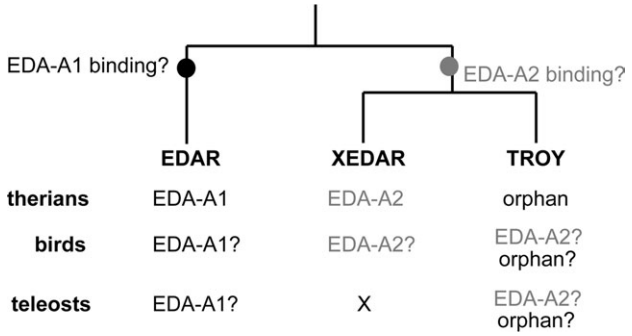


FIG. 7.—Hypothetical relationships between EDA pathway ligands and receptors in vertebrates. Experimentally, biochemical relationships between ligands and receptors of the EDA pathway were only established in mouse and human. We propose that these relationships can be generalized to all therian mammals. However, for birds and teleosts, biochemical experiments are needed. XEDAR and TROY are recent duplicates, leaving the possibility that TROY could also be an EDA-A2 receptor in these clades. In teleosts, XEDAR is either highly divergent or even lost. We thus consider it unlikely that it could still bind an EDA ligand.

both splicing forms are experimentally found in several major osteichthyan clades (namely mammals, birds, amphibians, and teleost fishes). EDA-A2 splicing should, however, be confirmed in chondrichtyes. Interestingly, this alternative splicing is not possible in invertebrate chordate *eda*-like genes, where we accordingly found a single receptor as a homolog for the 3 vertebrate receptors (Pantalacci S, unpublished data). The most likely evolutionary scenario would thus be that after a first round of duplication of the ancestral chordate receptor, 2 receptors specialized in the binding of one isoform: EDAR binding EDA-A1 and XEDAR/TROY binding EDA-A2 (fig. 7). After the second round of duplication, both XEDAR and TROY could bind EDA-A2 but then, as we will discuss it later, they might have evolved different binding specificities during the evolution of the major vertebrate clades. An important consequence of this hypothesis would be that the 3 receptor genes and the *eda* gene coevolved to maintain individual ligand–receptor interactions from early vertebrate evolution.

Point 3: and 4 A major shift occurred in the EDA pathway at the base of teleost fish evolution. We showed that the receptor-binding domains of the EDA-A1 and EDA-A2 ligands, and probably also the ligand-binding domains of both EDAR and TROY receptors, underwent a shift at the base of teleost evolution. Moreover, the *xedar* gene is either highly divergent or lost in all examined teleost species. All together, these data suggest that a major shift occurred at the base of teleosts evolution. Because EDA-A2 is still expressed in *Gasterosteus* where *xedar* is lost, it seems obvious that the binding relationships in teleosts are different from those known in mammals. The most likely hypothesis would be that EDAR binds EDA-A1 and TROY (which is more closely related to XEDAR) binds EDA-A2, but this should be confirmed by biochemical experiments. If this is confirmed, then the loss of 1 of the 3 coevolving receptors, namely *xedar*, could be sufficient to explain the shift that we observed, by punctually relaxing the selective pressure on the ligand *eda* and the 2 remaining receptors *edar* and *troy*. Interestingly, we also observed a shift on the cytoplasmic tail of TROY, suggesting that the TROY pathway also changed at the intracellular signaling level. All together, it seems that whereas the EDA/EDAR/EDARADD pathway was strongly conserved in teleost fishes, the accessory pathways (TROY and XEDAR) were deeply reorganized at the base of teleost fishes.

Point 5: The low affinity EDAR-binding isoform EDA-A5 is conserved in various tetrapods. Up to know, very little attention has been paid to this isoform, probably because it had only been described in human. However, it is well known that ligands with different affinities can induce different level or type of intracellular signaling (Pires-daSilva and Sommer 2003); thus, the EDA-A5 isoform may have specific roles in the regulation of the EDAR signaling pathway, and by consequence, in the development of skin appendages. The finding that EDA-A5 is conserved in various tetrapods prompts to investigate this possibility.

Point 6: A recent functional shift in XEDAR intracellular signaling occurred in the bird lineage. Indeed, in birds, the cytoplasmic tail of XEDAR includes a death domain, which cannot be found in any other gnathostome species. As a consequence, the intracellular XEDAR signal transduction is predicted to be different between birds and mammals. The most parsimonious hypothesis would be that the *xedar* gene gained this death domain by exon shuffling in the course of avian evolution (in accordance with this view, the death domain is found as a supplementary last exon). Of note, the protein interacting with this death domain is unknown. Could EDARADD bind this death domain as it binds the one of EDAR? We do not favor this hypothesis because we failed to detect traces of an evolutionary shift on the bird *edaradd* gene as it would probably have been the case if EDARADD had gained this capacity. Finally, it is tempting to speculate that this change in *xedar* signaling might be linked to feather acquisition in birds, and it will thus be interesting to test this hypothesis by specifically investigating the role of this death domain in chicken.

Point 7: Could XEDAR and TROY receptor specificity as known in human and mouse be a recent acquisition of therians? In mammals, XEDAR is known as the exclusive receptor of EDA-A2, whereas TROY does not bind EDA-A2 or EDA-A1 (fig. 7). We showed that the XEDAR TNFR domain (including the part responsible for EDA-A2-specific binding) displays important differences in amino acid composition, even when comparing birds with therians. This finding was all the more curious because substitutions at sites otherwise conserved in tetrapods or even in gnathostomes are found in the TNFR domain of TROY (4 substitutions, yellow with red circle in supplementary fig. S4 [Supplementary Material online]) and in EDA ligands (a subtle change from phenylalanine to tyrosine at the receptor-binding surface, fig. 2*B*). Thus, it seems that a shift occurred concomitantly on both XEDAR,

TROY TNFR domains and EDA in therians. In these conditions, could exclusive binding of EDA-A2 to XEDAR and absence of binding to TROY have been set only recently, in the course of therian evolution (fig. 7)? Importantly, some data obtained by Drew et al. (2007) by manipulating TROY and XEDAR functions during in vivo feather development are not easily explained by transposing to chicken what is known from mammals. Moreover, they showed that EDA-A2 splicing is found well before XEDAR is expressed, at a time when only TROY and EDAR are expressed. The authors suggested that the splicing may be constitutive but an alternative explanation would be that TROY could be an EDA-A2 receptor in *Gallus*. Even if our proposition is speculative in absence of biochemical data, we think that the pattern of evolution of *xedar* and *troy* calls for the necessity to perform biochemical experiments in non-therian species to establish the exact relationships between the EDA ligands, XEDAR, and TROY.

Point 8: Two EDARADD isoforms are conserved in mammals, which differ only by their very N-terminal part, and use 2 different promoters. Interestingly, for both isoforms, we found a strong conservation of both the N-terminal peptide and the predicted promoter region. Beside the potential functional differences in the proteins themselves (of note, the N-terminal sequence is just found 6 amino acids away from the TRAF6-binding site and could thus influence TRAF6 binding), the use of 2 promoters offers many possibilities to achieve different regulations at different developmental times or in different tissues. It will be very interesting to see what are the specificities of those isoforms and their transcriptional regulation in skin appendage development. Indeed, because those 2 isoforms seem to be an acquisition of mammals, it is tempting to speculate that they brought new levels of regulation that helped the evolution of new mammalian-specific skin appendages like hair, mammary, or sebaceous gland.

Point 9: One of the 2 EDARADD isoforms (isoform-A) was lost in a lineage of rodents including mouse and rat. Importantly, whereas the possibility to encode the isoform-A is clearly lost, the predicted promoter region of the isoform-A is still partly conserved in mouse and rat. Thus, the regulatory information associated to isoform-A was not fully lost but may have been partly reorganized in the remaining promoter. The guinea pig is not concerned by this loss and, as a laboratory animal, will provide an interesting outgroup to study the nature of this change and its consequences for skin appendage development.

Point 10: In the *Mus* lineage, a positive selection event together with a 3 amino acid insertions occurred in the EDARADD protein. Interestingly, these changes are gathered just before and within the first α-helix of the death domain. It is unclear at present when precisely these changes occurred and thus if they can be correlated to specific morphological or physiological changes within the *Mus* lineage. Biochemical tests will be required to see in which way the *M. musculus* EDARADD protein could be functionally different from its closest counterpart in *R. norvegicus*. In particular, it will be interesting to test the phosphorylation status of both proteins because the *Mus* EDARADD proteins gained a threonine with a good phosphorylation consensus.

In conclusion, we provide the first overview of the evolution of a signaling pathway in vertebrates that is summarized in figure 6. By using a large set of species, we could emphasize both conserved and clade-specific traits in this pathway. We are confident that by simply enlarging the data set, we would be able to point out even more clade-specific traits and to fully take the measure of how different and divergent can be a conserved signaling pathway in different species. Already, we found that clade specificities are found at all levels, whether one considers the different levels of the pathway (from the ligand–receptor relationships to the intracellular signal transduction) or whether one considers the different taxonomic levels (from the deep actinopterygian/sarcopterygian split to the recent split of the *Mus* genus).

More specifically, our study establishes the EDA pathway as a paradigm for the evolution of appendages. Indeed, the high conservation of the EDA-A1/EDAR/EDARADD module among gnathostomes (point 1) is presumably associated to a conserved function related to the individualization of the organ at the very early stages of its development and thus could participate in the similarities noticed between vertebrate appendages in their early development (Sharpe 2001; Mikkola 2007). In contrast, not only subtle species specificities displayed by this module but also species specificities in the satellite and obviously more flexible XEDAR and TROY pathway may have participated in skin appendage diversity (fig. 6), both at a macroevolutionary (see points 3–8) and a microevolutionary scale (point 9). Further evolutionary developmental studies will shed light on how these species specificities may be related to morphological evolution (fig. 6).

Concluding Remarks

Our study highlighted the need to explore receptor specificities of the EDA-A1 and EDA-A2 ligands outside mammals. This is in contrast with developmental studies on the EDA pathway (e.g., in chicken; Drew et al. 2007), which rely on the implicit idea that the receptor specificities found in mammals could be generalized. Such implicit conclusions of conservation between species are common in developmental biology, probably due to a habit to emphasize conservation of developmental mechanisms. A careful phylogenetic study like the present one may thus help to orientate comparative biochemical studies and in the end may help to more objectively interpret data obtained in one species by comparison to others. More generally speaking, our study provides a typical case in which proteins that looked "conserved" at first glance in fact exhibit species specificities when looked into detail. Indeed, comparisons of developmental genes are often done with a few species and, as a consequence, they only put the stress on conservation. In contrast, in our case, we used a wide range of species and, as a consequence, we could point out several species specificities. We wonder if the lack of this type of approaches may have contributed, at least in the

evolutionary developmental biology (evo-devo) field, to underestimate the importance of evolutionary changes in coding sequences. Indeed, the current view tends to minimize the role of coding sequences and, by opposition, to set the evolution of *cis*-regulatory sequences as the main force driving morphological evolution (see e.g., Prud'homme et al. 2007; and for the specific case of the EDA pathway, Colosimo et al. 2005; Knecht et al. 2007). However, recently Hoekstra and Coyne argued that this view relies on several a priori and that, for the moment, it is not possible to determine whether, of *cis*-regulatory or coding sequences, one plays a more important role (Hoekstra and Coyne 2007)—both probably playing an important role (Oakley 2007). Our findings, by demonstrating that species specificities in signaling proteins are found even at a low taxonomic level, provided that we look for them, also supports this idea. For this reason, we think that this comparative approach could be applied with large benefits to other developmental signaling pathways.

Supplementary Material

Supplementary table S1 and figures S1–S8 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Note

A new study has just been published showing that, in mouse, the orphan receptor TROY actually binds a TNF-type ligand known as Lymphotoxin α (LTα), which was already known to bind three other TNF receptors (Hashimoto et al. 2008). These biochemical data are in contradiction with previous biochemical experiments (Bossen et al. 2006) but fit well with the involvement of LTα in hair formation. Of note, LTα is not directly related to EDA according to published phylogenies of TNF ligands (Collette et al. 2003). There are several examples of a TNF ligand binding several TNF receptors and reciprocally of a TNF receptor binding several TNF ligands and this may be variable from one species to another. For this reason, we think that this new finding leaves open a wide range of possibilities (as discussed in our manuscript and more) and emphasizes again the need of biochemical experiments in other key model species.

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