Biological Activity of Ectodysplasin A Is Conditioned by Its Collagen and Heparan Sulfate Proteoglycan-binding Domains

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Mutations in the TNF family ligand EDA1 cause X-linked hypohidrotic ectodermal dysplasia (XLHED), a condition characterized by defective development of skin appendages. The EDA1 protein displays a proteolytic processing site responsible for its conversion to a soluble form, a collagen domain, and a trimeric TNF homology domain (THD) that binds the receptor EDAR. In-frame deletions in the collagen domain reduced the thermal stability of EDA1. Removal of the collagen domain decreased its activity about 100-fold, as measured with natural and engineered EDA1-responsive cell lines. The collagen domain could be functionally replaced by multimerization and engineered EDA1-responsive cell lines. The collagen domain decreased its activity about 100-fold, as measured with natural and engineered EDA1-responsive cell lines. The collagen domain could be functionally replaced by multimerization domains or by cross-linking antibodies, suggesting that it functions as an oligomerization unit. Surprisingly, mature soluble EDA1 containing the collagen domain was poorly active when administered in newborn, EDA-deficient (Tabby) mice. This was due to a short stretch of basic amino acids located at the N terminus of the collagen domain that confers EDA1 with proteoglycan binding ability. In contrast to wild-type EDA1, EDA1 with mutations in this basic sequence was a potent inducer of tail hair development in vivo. Thus, the collagen domain activates EDA1 by multimerization, whereas the proteoglycan-binding domain may restrict the distribution of endogenous EDA1 in vivo.

Tumor necrosis factor (TNF) family ligands spontaneously form homotrimers that can bind three individual receptors (1). For some family members, these trimeric complexes are biologically active. For example, trimers of TNF and TWEAK (TNF homologue with weak apoptosis-inducing activity) signal cell death in their respective target cells, and cross-linking of several trimers, via the action of an anti-Flag antibody, does not result in increased activity (2). For other ligands, the trimeric, soluble complexes are not or only poorly biologically active, but their membrane-bound forms are. This is the case, among others, for FasL and CD40L. These ligands can however become active in a soluble form if trimers are cross-linked, either with antibodies, or by fusion with oligomerizing proteins, such as the Fc portion of an immunoglobulin, or the collagen domain of ACRP/ adiponectin (2, 3).

Ectodysplasin A (EDA) is a TNF family ligand involved in the development of various structures derived from the ectoderm, such as hair, teeth, and sweat glands, and EDA loss of function in mouse and human is associated with X-linked hypohidrotic ectodermal dysplasia (OMIM 305100). Of the several EDA isoforms that have been described, two of which comprise the TNF homology domain, only EDA1 has been implicated in the development of ectodermal appendages (4, 5). Activation of the NF-κB transcription factor through the EDA1 receptor (EDAR) is a central signaling event as suggested by the ectodermal dysplasia phenotype of NF-κB-compromised mice (6), and by the fact that early events of placode formation can be initiated by EDAR-independent NF-κB signals (7). One relevant NF-κB target downstream of EDAR is CTGF/CCN2 (connexin growth factor/CCN family protein2) that locally counteracts inhibitors of placode fate, thus allowing placode formation at the site of EDAR signaling in embryonic skin (8, 9).

Several lines of evidence indicate that EDA1 trimers must probably be aggregated in order to signal. First, EDA1 possesses a collagen domain that may potentially aggregate the TNF homology domain (10). Second, inactive trimeric FasL is partially activated by fusion with the collagen domain of EDA1 (11). Third, a number of X-linked hypohidrotic ectodermal dysplasia (XLHED) patients have point mutations or in-frame deletions in the collagen domain that suggest this domain fulfills a functional role (11). Fourth, fusion of the EDA1 receptor-binding domain to the Fc portion of an immunoglobulin yields a biologically active ligand (12). However, it has never been formally demonstrated that EDA1 needs to be cross-linked to be active, and that the collagen domain of EDA1 fulfills this cross-linking function.

Using an EDA1-dependent NF-κB activation assay in HaCat keratinocytes and a specific biological assay to measure oligomerization of EDA1 trimers, we demonstrate here that the
Collagen Domain of EDA1

![Collagen Domain of EDA1](image_url)

Collagen domain is required for EDA1 to signal efficiently through EDAR. This function of the collagen domain can be mimicked by antibody-mediated cross-linking, or by fusion of the TNF homology domain of EDA1 to the Fc portion of human immunoglobulin G1. In vivo, the biological activity of exogenously administered EDA1 is also dependent on multimerization, but to a lesser extent than predicted by in vitro results, and is additionally negatively regulated by a newly identified, evolutionarily conserved heparan sulfate proteoglycan (HSPG)-binding region that may restrict biodistribution of the protein.

MATERIALS AND METHODS

Cells—293T cells were grown in DMEM supplemented with 10% of heat-inactivated fetal calf serum (FCS). HaCat cells were grown in DMEM:NutMix-F12 (1:1, v/v), 5% FCS, 50 units/ml penicillin and streptomycin, 9 ng/ml of cholera toxin (Sigma), 5 μg/ml of insulin (Sigma), 24.3 μg/ml of adenosine (Sigma), 10 ng/ml of epidermal growth factor (Sigma), and 0.5 μg/ml of hydrocortisone (Sigma). Jurkat and Jurkat-EDAR:Fas cells were grown in RPMI supplemented with 10% FCS.

Cells were passaged twice weekly. HaCat cells were trypsinized. When cells were analyzed by FACS, however, they were detached with PBS, 1 mM EDTA.

Generation of EDAR:Fas Jurkat Cells—Retroviruses were produced essentially as described previously (13). Briefly, 293T cells were transiently transfected with pMSCVpuro-EDAR:Fas and co-transfected with the pHIT60 and VSV-G plasmids, containing the sequences for gag-pol and VSV-G, respectively. pMSCVpuro-EDAR:Fas encodes the extracellular domain of human EDAR (amino acids 1–183), amino acids VD and the transmembrane and intracellular domains of human Fas (amino acids 169–335). After transfection, 293T cells were incubated for 24 h in RPMI supplemented with 10% FCS. Fas-deficient Jurkat-JOM2 cells were a kind gift of Olivier Micheau (University of Dijon, France). Jurkat-JOM2 cells (10⁶ cells in 1 ml) were mixed with virus-containing supernatants (3 ml) supplemented with 8 μg/ml of polybrene, left for 15 min at 37 °C, and centrifuged for 1 h at 37 °C and at 450 × g (1500 rpm). Cells were selected with 5 μg/ml of puromycin and cloned. About 40 clones were tested for their sensitivity to Fc-EDA1 (12, 14), and one of the sensitive clones (Jurkat-2199 clone 23) was selected for further experimentation. HaCat IκBα-DN cells were generated in a similar way using pMSCVpuro-1κBα-S32G vector, except that cells were not cloned but used as a population.

Expression Constructs—Expression constructs were cloned into the PCR3 mammalian expression vector (Invitrogen) according to standard molecular biology techniques. Vector for expression of Flag-tagged ligands, ACRP-ligands, Fc-ligands, receptors-Fc, receptors-GPI, and receptors-COMP-Flag have been described previously (3, 14–16). Fc-ligands with a Prescision protease cleavage site were constructed by insertion of the Precission site in the Fc-ligand vector. Details regarding plasmids used in this study and the proteins they encode are provided in supplemental Table S1 and Fig. 1, respectively.

Calcium Phosphate Transfection of 293T Cells—10⁶ 293T cells were seeded in 8 ml of medium in a 10-cm diameter dish and transfected ~8 h later. The transfection was performed by mixing 7 μg of plasmid of interest and 1 μg of EGFP tracer plasmid with 50 μl of 2.5 M CaCl₂. Sterile water was then added to 500 μl. While vortexing the DNA mix, 500 μl of 2× HeBS buffer was added dropwise (16.4 g of NaCl, 11.9 g of Hepes, acid form, 0.21 g of Na₂HPO₄, 800 ml of H₂O, adjusted to pH 7.05 with NaOH, and finally brought to 1 liter and filtered). The transfection mix was added to cells within 1 min after mixing. The next day, cells were washed with PBS and 8 ml of fresh medium was added (DMEM, 10% fetal calf serum or serum-free Opti-MEM medium).

HaCat Cell Stimulation Assay—Two days before stimulation, cells were seeded in 24-well plates (10⁴ cells/well). The day before stimulation, cells were starved overnight in serum-free DMEM medium, 50 units/ml penicillin, and streptomycin. On the day of stimulation, medium was removed, and 0.5–1 ml of fresh DMEM (no serum) was added that contained the ligand of interest either in a purified form (between 0.05 and 1 μg/ml) or as Opti-MEM supernatants.

Immunoprecipitations of Recombinant EDA1 from Cell Culture Supernatant—Conditioned Opti-MEM supernatant was concentrated 20 times in Amicon Ultra filter devices (molecular mass cutoff of 10,000 Da). Immunoprecipitations were performed on 200–400 μl of Opti-MEM supernatant (or 10–20 μl of concentrated Opti-MEM) with 10 μl of protein A-Sepharose beads. 1 μg of hEDA1-Fc was added if the target EDA1 protein did not contain an Fc moiety. PBS was added to a final volume of 400 μl, and the mixture was incubated for 1 h at 4 °C on a
rotating wheel. Beads were washed twice with 1 ml of PBS, and transferred into a mini column made of a 200-μl tip plugged with a 1-mm diameter “stopper,” eluted with 15–20 μl of 100 mm sodium citrate pH 4, and neutralized with the appropriate volume of 1 M Tris-HCl, pH 9.

For heparin-Sepharose pull-downs (GE Healthcare), 10 μl of beads freshly rehydrated in PBS were mixed with 750 μl of Opti-MEM supernatant in PBS (1:1 v/v), incubated for 1 h at 4 °C on a rotating wheel, transferred into a mini column, washed with PBS, and eluted with 15 μl of PBS supplemented with 0.8 M NaCl.

*Cell Lysis—HaCat cells were harvested at defined time points. Plates were put on ice, cells were washed once with ice-cold PBS and lysed in 50 μl of lysis buffer (1% Triton X-100, 5 mm 2-glycerophosphate, 10 mm NaF, 1 mm Na<sub>3</sub>VO<sub>4</sub>, 1 mm dithiothreitol, and one tablet/50 ml of Complete protease inhibitor (Roche, Basel, Switzerland)). Plates were briefly vortexed, cells were scraped from the plate, and the lysates were centrifuged for 5 min at 4 °C and 13,000 rpm (16,000 × g) in a tabletop centrifuge. Protein concentration in cell lysate supernatants was determined with the BCA assay (Pierce).

*SDS-PAGE and Western Blotting—Proteins were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membranes. The following primary antibodies were used: rabbit anti-EDA1 serum AL166 (11), mouse monoclonal antibody anti-Flag (Sigma), polyclonal rabbit antibody anti-1xBo (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibody anti-α-tubulin (Sigma), M2 mouse monoclonal antibody anti-Flag (Sigma), and horseradish peroxidase-coupled antibody anti-human IgG (Jackson ImmunoResearch). Blots were revealed with ECL (GE Healthcare).

*Purification of Flag-tagged Proteins—Various Flag-tagged EDA1 constructs were purified from supernatants of transiently transfected 293T cells using anti-Flag M2-agarose (Sigma). Flag-EDA1 were eluted with citrate-NaOH, pH 3 and neutralized with Tris-HCl, pH 9. Buffer was exchanged for PBS, and the protein was stored at −70 °C until use.

*Purification of Fc-tagged Proteins—Fc-PreScission-EDA1-E245, Fc-EDA-A238, and hEDAR-PreScission-Fc proteins were purified from culture supernatants of stably transfected CHO cell clones by protein A affinity chromatography (15).

*Cytotoxicity Assays—The cytotoxicity assay using EDAR-Fc or Fas-Fc (Alexis, Lausen, Switzerland) were coated at 1 μg/ml to capture the ligands, and biotinylated anti-Flag M2 antibody (Sigma) followed by horse radish peroxidase-coupled streptavidin were used for revelation. The Superdex 200 column was calibrated with the following standard proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa) ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

*Flow Cytometry Analysis—HaCat cells were detached with PBS, 1 mm EDTA, and washed once with FACS buffer (PBS, 1% FCS). Staining was performed in 96-well plates with 0.5 × 10<sup>6</sup> to 1 × 10<sup>6</sup> cells/well, using 10 μl of Fc-PS-EDA1-E245 Opti-MEM supernatant and 40 μl of FACS buffer, for 20 min at 4 °C. In some instances, FACS buffer was replaced by 20-fold concentrated Opti-MEM supernatant containing the soluble receptors EDAR-COMP-Flag or BCMA-COMP-Flag. After washing with FACS buffer, Fc-EDA1 was revealed with phycocyanin (PE)-coupled anti-human Fc antibodies (Southern Biotech, Birmingham, AL).

*293T cells co-transfected with EDAR-GPI (or XEDAR-GPI control) and EGFP, and Jurkat cells transfected with syndecan-1, syndecan-2, or glypican-1 expression constructs, were stained with various Fc-ligands or Flag-ligands as described previously (14).

*Intraperitoneal Injections in Newborn Tabby Mice—Tabby mice (Jackson Laboratories, Bar Harbor, ME) were handled according to institutional and Swiss Federal Veterinary Office guidelines, with the authorization of the Office vétérinaire cantonal du canton de Vaud.

Pups were labeled by puncture of a footpad with a 30-gauge needle dipped in Aramis tattoo ink (Braintree Scientific, Braintree, MA). Intraperitoneal injections of EDA1 proteins were performed within 24 h after birth with a maximal volume of 15 μl using 0.5 ml of U-100 insulin syringe (Becton Dickinson, Franklin Lakes, NJ). When two injections were required, Fc-EDA1 and Fc-EDA-Fc were each administered in 7.5 μl. Examination and photography of tail hairs were performed 3–4 weeks postinjection.

*Genotyping—TNF<sup>−/−</sup> mice (17) were kindly provided by Dr. Fabienne Tacchini-Cottier. The following pairs of oligonucleotides were used for genotyping: TNE<sup>−/−</sup>; 5'-AGGGCTGTGGGACCTAAATGTC-3' and 5'-TTTGAAGCGTGGCAAGAGGCACTTAATGTC-3' and 5'-TTTGAAGCTTGGAGGACGTGGACCTAATGTC-3' and 5'-TTGAGTTTGGAGGACGTGGACCTAATGTC-3'.

**RESULTS**

*HaCat Cells Express EDAR and Activate NF-κB in Response to EDA1 Stimulation—HaCat cells have been shown previously to express messenger RNA for EDAR (18). We have used a fusion protein, in which the receptor-binding portion of EDA1 was fused to the Fc portion of human immunoglobulin G1 (Fc-EDA1) (12), as a probe to detect surface expression of EDAR in HaCat cells (Fig. 2A). As expected for a specific interaction, this staining was dose-dependent (supplemental Fig. S1) and could be competed by preincubation of Fc-EDA1 with a soluble form...
of recombinant EDAR, but not by preincubation with an irrelevant receptor (Fig. 2A). We conclude that HaCat cells express endogenous EDAR at the cell surface.

To determine whether EDA1 or TNF could activate NF-κB signaling in HaCat cells, the phosphorylation and degradation of the NF-κB inhibitor IκBα (19) was assessed following EDAR or TNF receptor engagement. HaCat cells were starved in serum-free medium to down-regulate spontaneous NF-κB activation, and subsequently stimulated with TNF as a positive control for NF-κB activation. TNF induced robust phosphorylation of IκBα within 5 min. This event was followed by the degradation of IκBα, as observed after 15 and 30 min, and its resynthesis after 1 and 4 h (Fig. 2B). Discrete upper bands of phospho-IκBα detected at 5 min most probably represent ubiquitin derivatives of IκBα that are targeted to degradation (19). Compared with untreated control cells, biologically active Fc-EDA1 (12) also induced some IκBα phosphorylation, although the response was weaker and slower than with TNF, and the disappearance of IκBα was at best partial at later time points (Fig. 2B). These results indicate that HaCat cell can respond to biologically active EDA1.

**EDAR Does Not Signal Cell Death in HaCat Cells and Does Not Rescue Putative TNF-induced Cell Death during Embryonic Development**—Mouse embryos with impaired NF-κB signaling suffer from massive liver damage and die at around gestational day 15, but can be rescued by TNF or TNF-R1 inactivation, indicating that TNF is the cause of liver damage (20, 21). When NF-κB is compromised, TNF-R1 signals apoptosis through a death domain-dependent secondary signaling complex (22). As EDAR resembles TNF-R1 not only by its ability to activate NF-κB, but also by the presence of an intracellular death domain, we wondered whether EDA1 could negatively affect viability of HaCat cells with compromised NF-κB signaling. HaCat cells expressing a super-repressor of NF-κB (non-degradable IκBα) died as expected in response to TNF, but remained completely resistant to EDA1, suggesting that EDAR does not transmit death signals similar to TNF-R1 (supplemental Fig. S2A).

Nevertheless, cells involved in early placode development could be killed in a TNF-dependent manner in the absence of protective, EDAR-mediated NF-κB signals, as observed for fetal liver cells in response to TNF (20, 21). In this scenario, EDA deficiency should be rescued by concomitant inactivation of TNF. This was however experimentally not the case, as EDA-deficient mice expressing TNF or not both displayed identical phenotype of ectodermal dysplasia (supplemental Fig. S2B).

**The TNF Homology Domain of EDA1 Is a Poor NF-κB Agonist Compared with Longer Forms of EDA1**—Although the THD of EDA1 (EDA-E245) is both required and sufficient to bind EDAR (11) (supplemental Fig. S3B), high concentration of this ligand was required to induce only a weak NF-κB signal in HaCat cells (Fig. 3A). In contrast, a longer form of EDA1 starting at the furin cleavage site (EDA1-S160) and comprising the

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**Collagen Domain of EDA1**

**FIGURE 2.** EDA1 activates NF-κB in HaCat keratinocytes. Panel A, HaCat keratinocytes were stained with Fc-EDA1-E245 (EDA1), in the presence or absence of EDAR-COMP-Flag (sol. EDAR) or BCMA-COMP-Flag (sol. BCMA), and bound Fc-EDA1 was monitored by FACS. Panel B, HaCat cells were stimulated with 200 ng/ml of Fc-TNF (TNF) or 200 ng/ml of Fc-EDA1-E245 (EDA1), for the indicated period of time. Cell extracts were analyzed by Western blotting for the presence of total IκBα (IκBα), phosphorylated IκBα (P-IκBα), or the loading control tubulin.

**FIGURE 3.** The collagen domain confers signaling capacity to the TNF domain of EDA1. HaCat cells were stimulated for 20 min with various EDA1 constructs, and levels of phospho-IκBα, IκBα and tubulin were visualized by Western blotting. Panel A, cells were stimulated with the indicated EDA1 proteins, which were obtained from Fc-PreScission-EDA1 that had been cleaved with PreScission protease (see supplemental Fig. S3). Panel B, cells were stimulated with supernatants of 293T cells transfected with full-length EDA1, Flag-tagged soluble EDA1, and deletion mutants thereof (see supplemental Fig. S3). The stimulation was performed in the presence or absence of anti-Flag antibody. Anti-Flag alone had no effect in this assay (data not shown). Panel C, cells were stimulated with Fc-EDA1-E245 or Fc-PreScission-EDA1-E245 that had been treated or not with PreScission protease (see supplemental Fig. S4).
The collagen domain of EDA1 is important for the activation of the collagen domain contains short in-frame deletions. The presence of the collagen domain increases the biological activity of the protein in this assay (Fig. 3, white symbols). Constructs with in-frame deletions in the collagen domain were as efficient as the wild-type EDA1 constructs were generated in which the collagen domain was replaced with the Fc portion of human IgG1, with or without insertion of a PreScission viral protease site between the Fc and THD domains. Fusion of the dimeric Fc fragment to trimeric EDA1 is predicted to yield a hexameric protein containing 3 Fc moieties and 2 EDA1 trimers moieties, or even higher oligomers (supplemental Fig. S3A, scheme). Fc-EDA1-E245 indeed eluted with high apparent molecular weight by size exclusion chromatography, consistent with an oligomeric form of the protein (supplemental Fig. S4B), whereas treatment of the protease-sensitive protein with PreScission protease resulted in a quantitative release of EDA1-E245 that eluted at the position expected for a trimer (supplemental Fig. S4, A and C).

Uncleaved Fc-EDA1 activated NF-κB in HaCat cells regardless of the presence or absence of the protease site (Fig. 3C). This biological effect was lost by physical separation of the Fc domain from the EDA1 domain by cleavage with PreScission protease, but, as expected, the same treatment did not affect the protease-resistant Fc-EDA1 (Fig. 3C).

Taken together, these data are consistent with the notion that the collagen domain renders EDA1 active by oligomerization. This function can be mimicked by other oligomerization means, such as Flag plus anti-Flag or fusion to the Fc portion of IgG1.

The Collagen Domain Potentiates the Ability of EDA1 to Deliver Oligomerization-dependent Signals in an Engineered Cell Death Assay by More than Three Orders of Magnitude—The HaCat assay provided qualitative rather than quantitative information on EDA1 activity and unfortunately lacked robustness due to the relatively weak NF-κB activation. We therefore thought to adapt a well-characterized FasL-dependent cell death assay to the purpose of measuring the functional effect of EDA1 oligomerization. Thus, Fas-deficient Jurkat T cells were transfected with a chimeric receptor consisting of the extracellular domain of EDAR fused to the transmembrane and intracellular domains of Fas, and clones undergoing apoptosis in an EDA1-sensitive manner were selected.

Purified Flag-EDA1-S160, with or without in-frame deletions in the collagen domain, killed Jurkat EDAR:Fas cells with an IC_{50} of about 1 ng/ml, and was further activated only about 2-fold in the presence of anti-Flag antibodies (Fig. 4 and supplemental Fig. S5). Flag-EDA1-E245 displayed no activity by itself in this assay, but this latency was overcome in the presence of anti-Flag antibodies (Fig. 4 and supplemental Fig. S3A). Flag-EDA1 was either used directly (squares) or preincubated for 3 days at 50 °C prior to the assay (circles). Cell viability was monitored with the PMS/MTS assay. Of note, EDA1-S160 failed to activate NF-κB when cross-linked with anti-Flag antibodies (Fig. 3B). The same was true of these data are consistent with the notion that the collagen domain renders EDA1 active by oligomerization. This function can be mimicked by other oligomerization means, such as Flag plus anti-Flag or fusion to the Fc portion of IgG1.

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obtained for NF-κB activation in HaCat cells, we conclude that the collagen domain of EDA1 fulfills a prominent role to stimulate the signaling ability of EDA1 trimers by oligomerization. Although deletions in the collagen domain have no or little impact on the activity of the protein, they decreased heat stability of the protein. Taken together, these in vitro results demonstrate that the collagen domain is a positive regulator of EDA signals.

EDA Oligomerizes via Its Collagen Domain—To determine the oligomerization status of EDA, its size was estimated by gel permeation chromatography (Fig. 5 and Table 1). The TNF domain only (EDA1-E245) eluted at a size 3.2× that of the monomer, in line with the trimeric structure determined by crystallography (23). EDA1-S160, EDA1-S160 with mutation KKKGKK → SASGAS in front of the collagen domain and ACRP-EDA1-E245 (with the collagen domain of ACRP30) had apparent multiplicities of 10.4, 14.4, and 16.4, respectively, suggesting that they are bigger than trimers. However, size exclusion chromatography overestimates the size of asymmetric proteins, such as a protein containing an elongated collagen domain. Constructs of another TNF family ligand, FasL, were therefore analyzed in parallel. The TNF homology domain of FasL taken alone eluted as expected as a timer. FasL preceded by the collagen domain of EDA1 and ACRP-FasL eluted with apparent multiplicities of 9.7 and 12.7. As ACRP-FasL was previously characterized as a hexamer by electron microscopy (3), it can be concluded that the presence of a collagen domain roughly doubles the molecular weight determined by size exclusion chromatography. When taking into account this correction factor, estimated multiplicities of EDA1-S160 and EDA1-S160 KKKGKK → SASGAS are about 5.2 and 7.2. This is in any case bigger than a trimer and re-enforces the assumption that EDA with a collagen domain (from EDA or ACRP) forms multimers, probably hexamers.

Less than 4-h Exposure to Active EDA1 Is Sufficient to Induce Tail Hair Formation in Newborn, EDA-deficient Tabby Mice—Tabby mice have no hair on the tail, a defect that can be corrected by ip administration of Fc-EDA1-E245 in newborn mice (12). As expected, tail hair formation induced by a dose of 2 μg (2 mg/kg) Fc-EDA1-E245 was abrogated by co-injection of an excess (37.5 μg) of the decoy receptor EDAR-Fc. Interestingly, delayed administration of EDAR-Fc for up to 1 h prevented tail hair induction, but failed to do so after 3.5 or 18 h (Fig. 6). This demonstrates that less than 4 h exposure to active EDA1 is sufficient to induce tail hair formation and suggests that this experimental system should be relatively insensitive to the half-life of EDA1.

EDA1 S160 Is Less Active than EDA1 E245 in Vivo, in Contrast to in Vitro Results—From the combined in vitro results, we predicted that EDA-S160 and EDA-E245 would be, respectively, active and inactive when administered in vivo. To test this hypothesis, newborn Tabby mice were treated ip with increasing doses of EDA1-E245 or EDA1-S160, and examined 3–4 weeks later for the presence of hair on the tail. To our surprise, Flag-EDA1-E245 was relatively efficient at inducing development of tail hair in this setting, whereas Flag-EDA1-S160 only induced few tail hairs, even at the highest dose that we could test (6 mg/kg) (Fig. 7 and data not shown). This sug-
The HSPG-binding Domain of EDA1 Attenuates Its Activity in Vivo—We wondered whether the poor activity of EDA1-S160 in vivo after ip administration could be explained by its interaction with ubiquitously expressed HSPGs that would sequester EDA1 away from developing skin. We found that EDA1-S160 with a mutated HSPG binding site (KKKGKK → SASGAS) induced numerous tail hair in Tabby mice very similar in density to those of a wild-type mouse. Such a phenotype was never achieved with wild-type EDA1-S160, containing the basic sequence, but not EDA1-E245 in which this region is absent or EDA1-S160 KKKGKK in which the basic site was mutated, interacted specifically with Jurkat cells transfected with syndecan1, syndecan2, or glypican1, but not with mock-transfected cells, in a heparin-inhibitable manner (supplemental Fig. S6). These results indicate that the basic region of EDA1 mediates binding to heparin and proteoglycans.

The HSPG-binding Domain of EDA1 attenuates its activity in vivo...
must be cleaved in order to exert its activity (11, 26). A similar proportion of patients display point mutations or in-frame deletions in the collagen domain, pointing to its essential but poorly characterized function.

We demonstrate that the collagen domain of EDA functionally acts as a cross-linker of the receptor-binding, trimeric THD domain. This cross-linking function of the collagen domain can be mimicked in several ways: antibody-mediated cross-linking, fusion with the Fc portion of IgG1 or fusion with the collagen domain of ACRP/adiponectin. All of these means rendered the ligand as much as 1000-fold more active than the THD alone in a surrogate assay measuring activation of the oligomerization-dependent Fas pathway. Size estimation of the EDA1 proteins suggests that the collagen domain may serve as a scaffold to bring at least two trimeric TNF homology domains into the same molecule.

Some TNF family ligands such as TNF or TWEAK are active as soluble trimeric ligands that do not require further oligomerization to signal through (at least one of) their receptors. In contrast, membrane-bound ligands such as FasL or CD40L are inactive in a soluble form, unless oligomerized to meet the requirements of their oligomerization-dependent receptors (2, 3, 27, 28). EDA appears to be a “mixture” of both cases: although it is released in a soluble form, it can nevertheless activate its oligomerization-dependent receptor because of a built-in oligomerization domain.

In line with the hypothesis that the signaling pathway downstream of EDAR is oligomerization-dependent, activation of NF-κB in EDAR-positive HaCat cells required the collagen domain of EDA1, and oligomerization of EDA1 was also required for its full activity when administered in newborn Tabby mice. However, although trimeric EDA1 was essentially inactive in vitro assays, it displayed some activity in vivo, suggesting that the requirement of EDAR for oligomerized EDA1 in the context of hair development induced by a recombinant ligand may not be as stringent as initially thought.

It only takes a few hours of exposure to EDA to induce hair (and sweat gland, data not shown) formation. However, as different ectodermal appendages can develop at distinct time points, agonists with longer half-lives should be able to induce development of a wider spectrum of structures. In this respect, agonist anti-EDAR antibodies may prove to be particularly useful.

A short stretch of basic amino acids, mostly encoded by the 23 bp exon 4 of EDA, precedes the collagen domain. It is striking that this feature, like the furin site and the collagen domain, is evolutionarily conserved between mammals, birds, amphibians, and fishes. We demonstrate here that this sequence is an HSPG-binding site that impairs in vivo activity of recombinant EDA. For exogenously added EDA, this domain probably results in the scavenging of EDA before it reaches its target organ, an effect that can be partially overcome by increasing the dose. For endogenous EDA, HSPG binding probably restricts diffusion and fine tunes its effects. HSPG binding generally allows to establish concentration gradients for proteins such as chemokines that coordinate immune responses (29), or mor-
can wonder why it was never found mutated in XLHED patients. First, exon 4 cannot be deleted in-frame. Second, mis-sense mutation of just one of the basic residues is probably not sufficient to completely abolish HSPG binding, and may not translate into a phenotype severe enough to be noticed. Alternatively, decreased binding to HSPG may promote EDA signaling rather than hampering it, by allowing an easier access to the receptor. Interestingly, mutation of the basic site not only abolished proteoglycan binding but also decreased heat stability, suggesting that the basic sequence may also help stabilizing the multimeric structure of EDA1.

In-frame deletions in the collagen domain that deactivate EDA in XLHED patients have apparently no impact on the cross-linking potential of the collagen domain. Apart from the expected slight reduction of MW by SDS-PAGE, these in-frame deletions affected neither expression levels upon transient transfection of the full-length or soluble proteins, nor proteolytic processing, nor gel filtration elution profiles, nor the activity in vitro, nor proteoglycan binding. It did however display a modest effect on heat stability of the purified proteins. It is uncertain whether this partial stability defect is sufficient to explain why patients harboring these mutations are as severely affected as those with non-sense mutations resulting in total loss of EDA1 (11). Endogenous levels of EDA1 are probably limiting, and a decrease of EDA1 protein levels as a result of in-frame deletions in the collagen domain, which could be due to impaired binding to an unidentified cofactor or to impaired stability of the protein, may severely compromise formation of ectodermal appendages. Recombinant proteins lacking this domain are probably active because they saturate the system, thus masking subtle regulatory mechanisms.

These findings on the collagen and HSPG-binding domains of EDA1 are relevant to the choice of recombinant EDA that may potentially be used for the early treatment of XL-HED: so far, Fc-EDA1 remains the best agonist tested in vivo, probably because of a numbers of reasons. Indeed, the Fc favorably impacts the protein half-life in addition to providing the pro-
tein with the potential to be carried through maternal Fc-receptors. Also, the Fc provides the necessary cross-linking and alleviates the potentially deleterious effect of the HSPG-binding domain. Finally, Fc-EDA1 is easier to produce than proteins containing the collagen domain of EDA.

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