The ectodermal dysplasia receptor (EDAR) is a recently isolated member of the tumor necrosis factor receptor family that has been shown to play a key role in the process of ectodermal differentiation. We present evidence that EDAR is capable of activating the nuclear factor-κB, JNK, and caspase-independent cell death pathways and that these activities are impaired in mutants lacking its death domain or those associated with anhidrotic ectodermal dysplasia and the *douless* phenotype. Although EDAR possesses a death domain, it did not interact with the death domain-containing adaptor proteins TRADD and FADD. EDAR successfully interacted with various TRAF family members; however, a dominant-negative mutant of TRAF2 was incapable of blocking EDAR-induced nuclear factor-κB or JNK activation. Collectively, the above results suggest that EDAR utilizes a novel signal transduction pathway. Finally, ectodysplasin A can physically interact with the extracellular domain of EDAR and thus represents its biological ligand.

Anhidrotic (or hypohidrotic) ectodermal dysplasia is a disorder of ectodermal differentiation characterized by a triad of signs consisting of sparse hair, abnormal or missing teeth, and an inability to sweat (1). A similar phenotype is seen in mice with mutations involving the *douless* locus, suggesting the existence of a common underlying genetic defect. Recently, mutations in EDAR, a novel receptor of the TNFR family, were found in several families with autosomal dominant and recessive forms of anhidrotic ectodermal dysplasia and in *douless* mice (2, 3). Although the above studies established the role of EDAR in ectodermal differentiation, the signaling pathways activated by this receptor remain to be elucidated.

Death domain-containing receptors of the TNFR family are believed to activate two main signaling cascades: a kinase cascade leading to NF-κB and JNK activation and a caspase cascade leading to cell death (4). As the cytoplasmic domain of EDAR was reported to contain a region with partial homology to the death domain, we decided to test its abilities to activate the above signaling cascades (2, 3). In this report, we present evidence that, like the classical death domain-containing receptors, EDAR is capable of activating the NF-κB, JNK, and cell death pathways. However, EDAR does not interact with the death domain-containing adaptor proteins TRADD or FADD and does not activate the caspase cascade. Our results suggest the existence of a novel signaling pathway utilized by EDAR.

**MATERIALS AND METHODS**

*Cell Lines and Reagents*—293T and MCF7 cells were obtained from Dr. David Han (University of Washington, Seattle, WA). 293 EBNA cells were obtained from Invitrogen. Rabbit polyclonal antibodies against FLAG, Myc, and hemagglutinin tags were obtained from Santa Cruz Biotechnology. FLAG beads and control beads were obtained from Sigma. The pull-down kinase assay kit for JNK was obtained from New England Biolabs Inc., and the constructs for the Pathdetect luciferase reporter assay were purchased from Stratagene. YOPRO-1 was obtained from Molecular Probes, Inc. Synthetic caspase inhibitors (Z-VAD-fmk and t-butoxycarbonyl-Asp-fmk) were purchased from Calbiochem.

**Expression Constructs**—EDAR cDNA encoding amino acids 22–448 was amplified by reverse transcription-polymerase chain reaction using normal prostate RNA as a template. The primers used for amplification also carried restriction enzyme sites at their 5′ termini. The amplified product was subsequently cloned in a modified pSecTagA vector carrying a FLAG epitope tag downstream of the murine Igκ signal peptide. The above FLAG-EDAR construct was used to generate deletion mutants EDARΔC223, EDARΔC164, and EDARΔC94 by taking advantage of the naturally occurring *Sma*I, *Sal*I, and *Xho*I sites in the EDAR cytoplasmic domain, whereas the deletion mutant EDARΔC58 was generated using a custom primer containing an *Xho*I site. Site-directed mutagenesis was carried out using the Quick-Change mutagenesis kit (Stratagene) and using the FLAG-EDAR plasmid as a template. To generate the EDAR immunoadhesin (EDAR Fc), the 5′-fragment of the FLAG-EDAR construct containing the signal peptide, the FLAG epitope, and nucleotides encoding the extracellular domain of EDAR (amino acids 21–177) was amplified using custom primers. The resulting product was cloned upstream of the Fc portion (amino acids 236–462) of the murine Ig heavy chain. This construct also carried a C-terminal FLAG tag. A murine TAJ immunoadhesin construct (*mTAJ*-Fc) was generated similarly by fusing the extracellular ligand-binding domain of murine TAJ protein (amino acids 1–172) to the Fc portion of the murine Ig heavy chain. To construct Myc-EDA1 the nucleotide sequence corresponding to amino acids 134–391 of the EDAR-A1 isoform...
was amplified by reverse transcription-polymerase chain reaction using total RNA derived from human prostate gland as a template. The upstream primer used for amplification was 5'-CGCGGGATCCCGCCCT-ATGGATTTCCTC-3' and the downstream primer used was 5'-CGC- GGTGACCTAGGATGCAGGGGCTTCAC-3'. The amplified product was digested with BamHI and SalI enzymes and subsequently cloned into a modified pFastBac1 vector (Life Technologies, Inc.), which contained a Myc epitope tag downstream of a baculovirus gp67 signal peptide. The sequences of all constructs were confirmed by automated fluorescent sequencing. Constructs encoding NIK and its mutants (5), the IKK2 mutant (6); IκB-αS32/S36A (7); and murine TRAF1, murine TRAF2 and its mutant, TRAF3, I-TRAF, FADD, DR5, CD40, and NF-κB/luciferase reporter constructs have been described previously (8–10) and were obtained from the indicated sources. The dominant-negative IKK1 mutant was kindly provided by Dr. Richard Gaynor.

Electrophoretic Mobility Shift Assay—293T cells (3 × 10⁵) were transfected with 2 μg of various constructs in each well of a six-well plate. After 36 h, nuclear extracts were prepared, and electrophoretic mobility shift assay was performed essentially as described previously (9).

Luciferase Reporter Assays—The NF-κB reporter assay was performed essentially as described previously (9). Briefly, 293T cells were transfected in duplicate in a 24-well plate with the various test plasmids along with an NF-κB/luciferase reporter construct (75 ng/well) and a Rous sarcoma virus promoter-driven β-galactosidase reporter construct (pBgal-luc) (500 ng), as well as a reporter plasmid encoding the yeast Gal4 upstream activating sequence (pFR-luc) (500 ng) along with a fusion transactivator plasmid containing the yeast Gal4 DNA-binding domain fused to transcription factor c-Jun (pBac-c-Jun) (50 ng), a reporter plasmid encoding the luciferase gene downstream of the Gal4 upstream activating sequence (pFR-luc) (500 ng), as well as a Rous sarcoma virus promoter-driven β-galactosidase reporter construct (75 ng). Cells were lysed 24 h later, and luciferase activity was normalized to β-galactosidase activity to control for the difference in the transfection efficiency.

For the c-Jun transcriptional activation assay, 293 EBNA cells (1.2 × 10⁵) were transfected in duplicate with various expression constructs (500 ng) along with a fusion transactivator plasmid containing the yeast Gal4 DNA-binding domain fused to transcription factor c-Jun (pFA-c-Jun) (50 ng), a reporter plasmid encoding the luciferase gene downstream of the Gal4 upstream activating sequence (pFR-luc) (500 ng), as well as a Rous sarcoma virus promoter-driven β-galactosidase reporter construct (75 ng). Cells were lysed 24 h later, and luciferase activity was performed essentially as described previously (10).

Coinmunoprecipitation Assays—For studying in vivo interaction, 2 × 10⁵ 293T cells were plated in a 100-mm plate and cotransfected 18–24 h later with 5 μg of each epitope-tagged construct by calcium phosphate coprecipitation. A hemagglutinin-tagged green fluorescent protein-encoding plasmid was also included in some experiments. Twenty-four hours post-transfection, cells were lysed in 1 ml of lysis buffer containing 0.1% Triton X-100, 20 mM sodium phosphate (pH 7.4), 150 mM NaCl, and one EDTA-free mini-protease inhibitor tablet (Roche Molecular Biochemicals)/10 ml. Cell lysates (500 μl) were incubated for 1 h at 4 °C with 10 μl of FLAG or control mouse Ig beads precoated with a supersaturated casein solution. Beads were washed twice with lysis buffer; twice with wash buffer containing 0.1% Triton X-100, 20 mM sodium phosphate (pH 7.4), and 500 mM NaCl; and again with lysis buffer. Bound proteins were eluted by boiling, separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and analyzed by Western blotting. Essentially a similar procedure was used for experiments involving coimmunoprecipitation of TRADD and TRAFs, except cells were lysed in a modified radiolabelling precipitation assay buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 1% Nonidet P-40, 0.1% sodium deoxycholate, and 1 mM EDTA, and the beads were washed extensively in the above buffer containing 1 mM NaCl.

Receptor-Ligand Interaction Assays—293T cells were transfected with expression plasmids encoding EDAR and TAJ immunoadhesins, and 12 h post-transfection, the medium was changed to 293FSM (Life Technologies, Inc.). Supernatants containing the secreted immunoadhesin were collected 48 h later and stored in aliquots at −70 °C until use. Myc-EDA I protein was produced by infection of Sf9 insect cells with the corresponding baculovirus construct following the manufacturer's instructions (Life Technologies, Inc.). Supernatant containing the secreted protein was collected 48 h post-infection, filtered, and stored at −70 °C until use.

For coimmunoprecipitation assay, equal volumes (500 μl) of the immunoadhesin and Myc-EDA supernatants were mixed in a buffer containing 50 mM Tris (pH 7), 150 mM NaCl, and 0.1% Triton X-100 and incubated at 4 °C overnight with gentle shaking. Supernatants were subsequently divided into two halves and immunoprecipitated with goat anti-mouse IgG1 beads (Sigma) or control antibody beads precoated with supersaturated casein solution. After extensive washing, the bound proteins were eluted by boiling, separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and analyzed by Western blotting.

For enzyme-linked immunosorbent assay, 5 μl of a control supernatant or the supernatant containing Myc-EDA I were immobilized overnight at 4 °C in the wells of a microwell plate in Na₂CO₃/NaHCO₃ buffer (pH 9.6). After washing with Tris-buffered saline containing 0.05% Tween (TBST), 5 μl of supernatants containing FLAG-tagged immunoadhesins (or a control supernatant) diluted in Tris-buffered saline were added to the wells and incubated for 4 h at room temperature. After washing, goat anti-mouse peroxidase (1:2000 in TBST) was added for 1 h. Color was developed using o-phenylenediamine dihydrochloride (Sigma), and the absorbance was measured at 490 nm.
RESULTS

EDAR Activates the NF-κB Pathway—We began by testing the ability of EDAR to activate an NF-κB-driven luciferase reporter construct upon transient transfection in 293T cells. As shown in Fig. 1A, transfection of EDAR in these cells led to significant activation of the NF-κB pathway that was comparable in magnitude to that induced by TNFR1. In comparison, TAJ/TROY, another TNFR family member that is highly expressed in skin during embryonic development (11, 12), failed to activate the NF-κB pathway. NF-κB activation by EDAR was further confirmed using an electrophoretic mobility shift assay (Fig. 1B).

Mechanism of EDAR-induced NF-κB Activation—TRAF2 has been known to mediate NF-κB activation by various TNFR family members, and TANK/I-TRAF has been known to regulate this process (13). Therefore, we investigated the roles of TRAF2 and TANK/I-TRAF in the activation of the NF-κB pathway by EDAR. An N-terminal deletion mutant of TRAF2 (14) that could effectively block NF-κB activation by CD40 was ineffective in blocking NF-κB induction by EDAR (Fig. 2A).

Similarly, EDAR-induced NF-κB activation was not affected by coexpression of TANK/I-TRAF (Fig. 2A). Collectively, these results suggest either that TRAF2 and TANK/I-TRAF are not involved in NF-κB activation via EDAR or that they play a functionally redundant role in this process. We have also tested the ability of dominant-negative mutants of the receptor-interacting protein and TRADD to block EDAR-induced NF-κB, but have failed to observe any significant inhibitory effect (data not shown).

The NIK and IKK serine/threonine kinases have been shown to be involved in the activation of the NF-κB pathway by the members of the TNFR and interleukin-1 receptor families (15). To determine the role of these proteins in EDAR-induced NF-κB activation, we took advantage of the dominant-negative inhibitors of these kinases. As shown in Fig. 2B, a C-terminal deletion mutant (NIK-2101) and a catalytic site mutant (NIK-K429R) could effectively block NF-κB induced by EDAR. However, a dominant-negative mutant of MEKK1 (MEKK1-D1369A), a related MAPK, failed to block EDAR-, CD40-, or TNFR1-induced NF-κB, suggesting the specificity of the ob-
served effect. EDAR-induced NF-κB activation was also effectively blocked by dominant-negative mutants of IKK1 (IKK1-K44M) and IKK2 (IKK2-K44M), respectively (Fig. 2C).

As IKKs function by mediating inducible phosphorylation and degradation of IκB proteins, we tested the ability of a dominant-negative mutant of IκBα (IκBα-S32A/S36A) to block NF-κB induction by EDAR. This mutant contains serine-to-alanine substitutions at amino acids 32 and 36, respectively, and is resistant to phosphorylation-induced degradation of IκBα (16). As shown in Fig. 2D, NF-κB induction by EDAR was effectively blocked by IκBα-S32A/S36A. Taken together, the above results suggest that EDAR activates NF-κB by NIK- and IKK-induced phosphorylation and degradation of the IκBα protein.

**Mutagenesis Analysis of EDAR-induced NF-κB Activation**—We used C-terminal deletion mutagenesis to map the domain of EDAR responsible for NF-κB activation. Deletion mutants EDARΔC38 and EDARΔC94, which are missing the C-terminal 38 and 94 amino acids, respectively, were only minimally effective in NF-κB activation (Fig. 3, A and B). The EDARΔC38 mutant possesses a partial death domain, whereas the EDARΔC94 mutant is missing it entirely (Fig. 3A). These results suggest that the death domain plays a crucial role in NF-κB activation by EDAR. A complete lack of NF-κB activation was also observed upon expression of mutants EDARΔC164 and EDARΔC223, which are missing additional C-terminal sequences of the cytoplasmic domain (Fig. 3, A and B).

We next tested whether two EDAR mutations seen in association with anhidrotic ectodermal dysplasia could affect the ability of EDAR to activate the NF-κB pathway. The E379K mutation is an autosomal recessive mutation in the death domain of murine EDAR and is responsible for the spontaneous Jackson phenotype, whereas the R420Q mutation has been detected in the death domain of human EDAR in a family with autosomal dominant anhidrotic ectodermal dysplasia (2, 3). We used site-directed mutagenesis to generate the corresponding mutants of the human EDAR gene. As shown in Fig. 3 (B and C), whereas the E379K mutant retained significant residual ability to activate the NF-κB pathway, the R420Q mutant demonstrated a more severe loss of this activity. These results suggest that the recessive phenotype of the E379K mutant may be due to the need for two mutant alleles to significantly influence NF-κB signaling. We would like to further point out that, in addition to the R420Q mutation, a nonsense mutation in the cytoplasmic domain of EDAR has also been detected in a family with autosomal dominant anhidrotic ectodermal dysplasia (2). This mutation (R358ter) results in the production of a truncated protein that is missing the C-terminal 90 amino acid residues and that closely resembles the deletion mutant EDARΔC94, which failed to activate the NF-κB pathway (Fig. 3, A and B). Taken together, the above results suggest that the impaired ability to activate NF-κB may be a key determinant in the pathogenesis of anhidrotic ectodermal dysplasia.

**EDAR Activates the JNK Pathway**—In addition to NF-κB activation, different members of the TNFR family are also known to activate the JNK pathway. Therefore, we tested the ability of EDAR to activate this pathway using a luciferase-based c-Jun transactivation activation assay. In this assay, luciferase expression is driven by JNK-mediated phosphorylation of the activation domain of transcription factor c-Jun that is fused to the Ga4 DNA-binding domain. As shown in Fig. 4A, expression of EDAR in the 293 EBNA cells led to modest activation of the JNK pathway. In contrast to the situation with NF-κB activation, the JNK-inducing ability of EDAR was relatively weak as compared with that of TAJ/TROY. Activation of the JNK pathway by EDAR was further confirmed using a pull-down kinase assay based on in vitro phosphorylation of GST-c-Jun (Fig. 4B).
We used deletion and point mutagenesis to map the region of the EDAR cytoplasmic domain responsible for JNK activation. These studies revealed that, although the EDAR DC38 and EDAR DC94 deletion mutant have some residual JNK activation ability, almost a complete lack of this ability is present in deletion mutants EDAR DC164 and EDAR DC223 (Fig. 4C). Thus, the putative death domain of EDAR is essential for both NF-κB and JNK activation.

Finally, we tested the ability of the two EDAR mutants known to be associated with anhidrotic ectodermal dysplasia to activate the JNK pathway. As in the situation with NF-κB activation, the E379K mutant was almost half as effective as the wild-type protein in JNK activation, whereas a more severe impairment of JNK activation was seen with the R420Q mutant.

TRAF2 has been shown to play an essential role in JNK activation via various members of the TNFR family (17, 18). However, as shown in Fig. 4D, a dominant-negative mutant of TRAF2 that could effectively block CD40-induced JNK activation failed to block JNK activation via EDAR. These results suggest either that TRAF2 is not involved in JNK activation via EDAR or that it plays a functionally redundant role in this process. Finally, EDAR-induced JNK activation was effectively blocked by the JNK-binding domain of JIP1 (Fig. 4E), a recently described inhibitor of the JNK pathway (19).

EDAR Induces Caspase-independent Cell Death—As discussed above, EDAR is known to possess a region in its cytoplasmic domain with partial sequence homology to the “death domain” present in the apoptosis-inducing members of the TNFR family. Previous studies have demonstrated that transient transfection of EDA, the putative ligand for EDAR, in MCF7 cells leads to cellular rounding and detachment, which are not inhibited by the caspase inhibitor Z-VAD-fmk (20, 21). Consistent with these results, transient transfection of EDAR in 293T, 293 EBNA, or MCF7 cells led to cellular rounding and detachment, two features suggestive of cell death (Fig. 5, A–C). However, unlike TNFR1-transfected cells, those cells transfected with EDAR failed to demonstrate membrane budding, a feature associated with caspase-dependent cell death (Fig. 5A).

We were next interested in testing whether EDAR-expressing cells actually undergo cell death. To test this hypothesis, we used nuclear staining with YOPRO-1, a cell-impermeable DNA-intercalating dye. As shown in Fig. 5D, although the majority of vector-transfected cells failed to show nuclear staining with this dye, a large number of EDAR-transfected cells stained positively, suggesting a lack of membrane integrity indicative of cell death. However, unlike the TNFR1-trans-
EDAR Activates NF-κB/JNK/Cell Death Pathways and Binds EDA

EDAR activates NF-κB/JNK/c-Jun N-terminal kinase (JNK) pathways in this process (data not shown). EDAR successfully coimmunoprecipitated FADD and TRADD when overexpressed with them in 293T cells (Fig. 7, A and B). Control experiments, performed in parallel, confirmed successful coimmunoprecipitation of FADD with Fas and TRADD with TNFR1, thereby demonstrating the validity of the assay.

In addition to the death domain-containing adaptor proteins, different members of TRAF family have been shown to interact with various members of the TNFR family (13). Therefore, we tested the ability of these proteins to interact with EDAR using a coimmunoprecipitation assay. EDAR failed to coimmunoprecipitate FADD and TRADD when overexpressed with them in 293T cells (Fig. 7, A and B). Control experiments, performed in parallel, confirmed successful coimmunoprecipitation of FADD with Fas and TRADD with TNFR1, thereby demonstrating the validity of the assay.

Caspase-3 is one of the executioner caspases of the caspase cascade and is activated during apoptosis induced by death receptors belonging to the TNFR family (22). We used a chromogenic assay, based on caspase-3-mediated cleavage of the chromogenic substrate Z-DEVD-p-nitroanilide, to test the activation of caspase-3 during EDAR-induced cell death. As shown in Fig. 6D, cell lysates from TNFR1- or DR4-transfected cells demonstrated caspase-3 activation, whereas EDAR-transfected cells failed to do so. Collectively, the above results suggest that cellular rounding and eventual cell death induced by EDAR overexpression are not mediated by a caspase-dependent mechanism.

We used deletion and point mutagenesis to map the region in the EDAR cytoplasmic domain responsible for induction of cell death. As shown in Fig. 6E, the EDARΔC38 deletion mutant, which retains a partial death domain, demonstrated significant residual ability to induce cell death, whereas the EDARΔC94 mutant, which completely lacks the death domain, possessed only a minor cell death-inducing ability. However, a complete lack of this activity was present in the deletion mutants lacking the death domain, such as EDARΔC164 and EDARΔC232. Finally, the point mutants E379K and R420Q demonstrated significant residual cell death-inducing ability.

EDAR interacts with TRAFs and NIK, but fails to interact with FADD or TRADD—The death domain-containing adaptor proteins TRADD and FADD have been shown to play an essential role in signaling via various death domain-containing receptors of the TNFR family (4). Therefore, we tested the ability of these proteins to interact with EDAR using a coimmunoprecipitation assay. EDAR failed to coimmunoprecipitate FADD and TRADD when overexpressed with them in 293T cells (Fig. 7, A and B). Control experiments, performed in parallel, confirmed successful coimmunoprecipitation of FADD with Fas and TRADD with TNFR1, thereby demonstrating the validity of the assay.

In addition to the death domain-containing adaptor proteins, different members of TRAF family have been shown to interact with various members of the TNFR family (13). Therefore, we tested the ability of these proteins to interact with EDAR using a coimmunoprecipitation assay. EDAR successfully coimmunoprecipitated murine TRAF1, murine TRAF2, and TRAF5 when these proteins were coexpressed in 293T cells (Fig. 7, C–F). Deletion mutagenesis revealed that the C-terminal 94 amino acids, encoding the death domain, were not essential for interaction of EDAR with murine TRAF1. Consistent with this hypothesis, the death domain point mutants E79K and R420Q were as effective as the wild-type protein in coimmunoprecipitating murine TRAF1. Finally, EDAR successfully coimmunoprecipitated with NIK, a protein known to be involved in NF-κB activation by various members of the TNFR family (Fig. 7G). However, we have so far failed to detect an interaction between a GST fusion protein containing the EDAR cytoplasmic domain and in vitro transcribed and translated TRAF2 or NIK (data not shown). Similarly, no interaction has been detected between the cytoplasmic domain of EDAR and TRAF2 or NIK using a yeast two-hybrid assay (data not shown). Collect-
tively, the above results suggest that the interaction between EDAR and TRAFs or NIK might be facilitated by the presence of intermediate bridging proteins present in the 293T cells.

**EDA Is the Ligand for EDAR—**
EDA is believed to be the ligand for EDAR based on the similarity in the clinical features of genetic disorders resulting from the mutations in these genes (2, 23, 24). However, EDA has never been shown to bind to EDAR. To test the ability of EDA to bind to EDAR, we generated a baculovirus construct containing the extracellular receptor-binding domain of EDA fused to an N-terminal Myc epitope tag. As shown in Fig. 8A, the Myc-EDA I construct contained the second and third collagenous repeats of the EDA-A1 isoform, in addition to its TNF homology domain. Myc-tagged soluble EDA proteins was collected from the supernatant of baculovirus-infected insect cells and tested for its ability to bind to EDAR-Fc or mTAJ-Fc in a coimmunoprecipitation assay. As shown in Fig. 8B, EDAR-Fc successfully coimmunoprecipitated the Myc-EDA I protein, whereas mTAJ-Fc failed to do so. The ability of Myc-EDA I to bind to EDAR was further confirmed using enzyme-linked immunosorbent assay (Fig. 8C).

**DISCUSSION**
During the process of skin differentiation, the mitotically active cells of the basal epithelium cease proliferating and then migrate outwards and undergo terminal differentiation (25). The NF-κB proteins are initially present in the cytoplasm of basal cells, but later migrate to the nuclei of suprabasal cells, suggesting a role for NF-κB activation in the switch from proliferation to growth arrest and differentiation (25). This hypothesis is supported by the result of a recent study involving a functional blockade of NF-κB via the expression of a dominant-negative mutant of IκBα in transgenic murine and human epidermis. This mutant resulted in the production of hyperplastic epithelium due to increased thickness of the suprabasal squamous layer (25). More recently, targeted disruption of the IKK1 gene has resulted in a similar phenotype (26–28). As the IKK1-deficient keratinocytes exhibited near normal IKK activation in response to a number of pro-inflammatory stimuli, such as TNF-α, interleukin-1, and lipopolysaccharide, these results have led to the suggestion that IKK1 is critical for IκB-dependent activation of NF-κB in response to an
Fig. 7. EDAR coimmunoprecipitates TRAFs and NIK, but fails to coimmunoprecipitate TRADD or FADD. 293T cells were transfected with the indicated plasmids, and cell lysates (L) were immunoprecipitated (I.P.) with FLAG beads (F) or control mouse IgG beads (C). Coimmunoprecipitated proteins were detected by Western analysis with the indicated antibodies. A and B, lack of interaction of EDAR with TRADD or FADD. C and D, interactions of full-length EDAR (EDAR-FL) and its deletion and point mutants with murine TRAF1 (mTRAF1). Western analysis of total cell lysates was used to confirm equivalent expression of various EDAR constructs. Lack of coimmunoprecipitation of cotransfected hemagglutinin (HA)-tagged green fluorescent protein (GFP) in C indicates the specificity of the interaction. E–G, EDAR interacts with murine TRAF2, TRAF3, and NIK. HVEM, herpes virus entry mediator.

Fig. 8. EDA binds to EDAR. A, a schematic representation of wild-type EDA-A1 and the Myc-tagged baculovirus construct used in this study. TM, transmembrane region; CR, collagenous repeat domain; Sig. Pep., signal peptide. B, EDAR-Fc coimmunoprecipitates Myc-EDA I. The experiment was performed as described under "Materials and Methods." The blot was reprobed with a goat anti-mouse IgG (GaM) to demonstrate the expression and immunoprecipitation of EDAR and murine TAJ immunoadhesin. mTAJ-Fc comigrated with the heavy chain of the control antibody; S, supernatant; C, control antibody beads; aM, goat anti-mouse IgG1 beads. C, interaction of EDAR-Fc with Myc-EDA I using enzyme-linked immunosorbent assay. The values shown are means ± S.E. of an experiment performed in duplicate.
EDAR may be involved in NF-κB and does not interact with either TRADD or FADD. There are domains present in the known apoptosis-inducing death receptors only a weak sequence homology to the classical death pathways. However, the putative death domain of EDAR plays a key role in the activation of the NF-κB pathway. Therefore, we tend to favor the hypothesis that the death domain of EDAR may be involved in NF-κB, JNK, and cell death pathways. This is because EDAR could interact with various TRAF family members and NK in the cainunprecipitation assay in 293T cells, as we have so far failed to detect an interaction between these proteins in mammalian cell-free systems, suggesting that the interaction between EDAR and TRAFs or NK might be facilitated by the presence of intermediate bridging proteins. Future studies aimed at isolation of the adaptor proteins that directly bind to the cytoplasmic domain of EDAR will greatly enhance our understanding of EDAR signaling in the process of ectodermal differentiation.

Previous studies have demonstrated that transfection of an EDA expression construct in MCF7 cells led to cell rounding and detachment, resembling the morphology of cells undergoing cell death (20, 21). However, these morphological changes could not be blocked by the cell-permeable caspase inhibitor Z-VAD-fmk, suggesting the lack of a role for caspase activation in this process. In the present study, we have similarly demonstrated that transfection of EDA in 293T, 293 EBNA, and MCF7 cells also leads to cell rounding and detachment, followed by cell death. Cells dying in response to EDAR overexpression do not show any morphological or biochemical features of caspase activation, suggesting that EDAR induces cell death by using a caspase-independent mechanism. Such a caspase-independent form of cell death has been described previously for several death domain- and non-death domain-containing members of the TNFR family, and it remains to be seen whether EDAR shares a common mechanism of cell death induction with them (11, 32–39). Several potential mediators of caspase-independent cell death have been recently described, such as Bax, nitric oxide, and apoptosis-inducing factor (40–44). It will be interesting to test the involvement of these proteins in EDAR-induced cell death. It is also conceivable that cell death induced by EDAR is a consequence of cellular detachment from the plate.

Finally, in this report, we demonstrate for the first time that extracellular domains of EDAR and EDA can physically interact with each other. EDA is unique among the ligands of the TNF family in possessing three collagenous repeat domains, in addition to a TNF homology domain (20, 21, 24, 45, 46). Like other ligands of the TNF family, EDA is expressed in a trimeric form, and it is conceivable that the collagenous repeats of EDA help in this process (45). Several alternatively spliced isoforms of EDA have been described recently (20, 24, 46). Future studies should address the role of each of these subdomains of EDA in its interaction with NF-κB.

Acknowledgments—We thank Drs. Richard Gaynor, David Han, Hiroyaus Nakano, Roger Davis, Gioachino Natoli, Vishva Dizit, Edward Clark, and Melanie Cobb for various expression plasmids.

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J. Biol. Chem. 2001, 276:2668-2677.
doi: 10.1074/jbc.M008356200 originally published online October 16, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M008356200

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