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EEDA: A Protein Associated with an Early Stage of Stratified Epithelial Differentiation

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Abstract

Using suppressive subtractive hybridization, we have identified a novel gene, which we named EEDA (early epithelial differentiation-associated), which is uniquely associated with an early stage of stratified epithelial differentiation. In epidermis, esophageal epithelium, and tongue epithelium, EEDA mRNA and antigen was abundant in suprabasal cells, but was barely detectable in more differentiated cells. Consistent with the limbal location of corneal epithelial stem cells, EEDA was expressed in basal corneal epithelial cells that are out of the stem cell compartment, as well as the suprabasal corneal epithelial cells. The strongest EEDA expression occurred in suprabasal precortical cells of mouse, bovine and human anagen follicles. Developmental studies showed that the appearance of EEDA in embryonic mouse epidermis (E 15.5) coincided with morphological keratinization. Interestingly, EEDA expression is turned off when epithelia were perturbed by wounding and by cultivation under both low and high Ca^{2+} conditions. Our results indicate that EEDA is involved in the early stages of normal epithelial differentiation, and that EEDA is important for the “normal” differentiation pathway in a wide range of stratified epithelia.

Keywords

epidermis; corneal epithelium; hair follicle; wound repair

INTRODUCTION

There are many molecular markers indicating that a keratinocyte has transitioned from a proliferate state to a committed, differentiated state. One such group of markers are the “differentiation products”, many of which are structural proteins usually synthesized in large quantities. Examples of these include the K1/10 keratins and involucrin that are expressed in the suprabasal cell layer of the epidermis (Eichner et al., 1984; Rice and Green, 1979); the K3/K12 keratins, serine proteinase inhibitor PAI-2, and the surface mucin MUC1 that are expressed in the more differentiated corneal epithelial cell (Gipson and Inatomi, 1998; Schermer et al., 1986; Williams et al., 1999); keratins hHa5 and hHb5 that are markers of early follicular differentiation (Langbein et al., 1999); and trichohyalin, which is the first protein to

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appear in cells destined to become a portion of the inner root sheath (Fietz et al., 1993). Another group of markers are non-structural proteins that are mostly associated with the basal cell layer, that play a role in regulating keratinocyte growth. These include epidermal growth factor receptors (EGFR) which, when occupied stimulates keratinocyte growth (for reviews see Cabodi et al., 2000; Eckert et al., 1997; Fuchs, 1990).

Despite progress in characterizing genes encoding structural proteins and basal cell-associated, growth-related proteins, relatively little is known about genes involved in initiating the process of differentiation. Using a corneal epithelium-specific subtraction cDNA library, we have identified a novel cDNA, which encodes a molecule that we named early epithelial differentiation-associated (EEDA) protein. EEDA is expressed in cells that have just exited from the proliferative compartment in a wide range of stratified epithelia, and it becomes markedly diminished in cells that are further along the differentiation pathway. Our data suggest that EEDA maybe important for “normal” differentiation in a wide range of stratified epithelia, but it is turned off when differentiation is perturbed such as when the tissue undergoes wound repair or when the cells are placed in culture.

MATERIALS AND METHODS

Identification of corneal epithelium-specific molecules by suppression subtractive hybridization

Fresh bovine bladder, brain, eye, heart, liver, lung, stomach, and testis were obtained from a local slaughterhouse. The corneal, bladder and stomach epithelia were removed by scraping and frozen in liquid nitrogen. Portions of the other tissues were frozen in liquid nitrogen. Total RNA from these tissues was isolated using TRIzol reagent (Life Technologies, Inc.), and poly (A)+ mRNA was isolated from total RNA preparations using a QIAGEN OligotexTM mRNA kit (Sambrook et al., 1989).

Suppression subtractive hybridization was performed using a CLONTECH PCR-SelectTM cDNA Subtraction kit as previously described (Diatchenko et al., 1996; Sun et al., 2000; Sun et al., 1999). Differentially expressed clones obtained from the corneal epithelial subtractive cDNA library were subjected to DNA sequencing and GenBank analysis.

Northern blot hybridization

Poly(A)+ RNAs (0.5 µg) from various bovine tissues were used as templates to synthesize double-stranded cDNA using a SMARTTM PCR cDNA Synthesis kit (CLONTECH). The synthesized cDNA was electrophoresed on a 1% agarose gel, transferred onto a positively charged nylon membrane (Roche Molecular Biochemicals), and UV-cross-linked (Sambrook et al., 1989). The membrane was prehybridized at 72 °C in ExpressHybTM hybridization solution (CLONTECH) for 1 h and hybridized in fresh buffer with denatured random primer-labeled probes at 72 °C overnight. After hybridization, the blot was sequentially washed in 2X SSC and 0.5% SDS for 20 min twice at 68 °C and in 0.2% SSC and 0.5% SDS for 20 min twice at 68 °C and exposed to a PhosphorImager screen overnight.

Rabbit polyclonal antibody preparation

An EEDA fusion protein construct was generated by inserting EEDA DNA fragments into the expression vector pRSET containing sequences encoding N-terminal polyhistidine (6xHis) tag for purification with Probond resin (Invitrogen). The EEDA fusion protein was expressed in *E. coli* by IPTG induction and purified by an Xpress protein purification system (Invitrogen) and this protein was used to immunize rabbits. We detected a single band at 12 kDa from human foreskin epidermal, human corneal epithelial, or mouse footpad epithelial protein extracts on

western blot using polyclonal antibodies against human or mouse EEDA (Fig. 4B), respectively.

Cell culture

Human neonatal foreskin keratinocyte cultures were initiated and propagated in MCDB 153 medium (Sigma, St Louis, MO) with 30 μ M CaCl₂ and the following additives: bovine pituitary extract, insulin, epidermal growth factor, hydrocortisone, and high amino acids as previously described (Jensen et al., 1997; Rodeck et al., 1997b). This medium is referred to as complete MCDB medium. Foreskin keratinocytes were used for experiments between the second and third passages.

Normal human corneas were obtained from the Delaware Valley Lions Eye Bank, and the epithelium was recovered as previously described (Williams et al., 1999). Keratinocyte cultures were initiated and propagated in complete MCDB medium with 30 μ M CaCl₂ as previously described (Risse-Marsh et al., 2002). Corneal keratinocytes were used for experiments between the second and fourth passages. In most experiments involving either human foreskin or corneal keratinocytes, when the cultures reached confluence, the Ca²⁺ concentration in the medium was elevated between 0.5 – 1.2mM in order to induce stratification and differentiation.

Tissue extraction

Human foreskins, corneas, and mouse footpads were immersed in 0.15M NaCl for 1 min at 56°C to separate the epithelium from the underlying stroma. Epithelia were homogenized on ice in RIPA buffer (50mM Tris-HCl, pH7.5; 150mM NaCl; 1.0% Triton X-100; 0.5 sodium deoxycholate; 0.1%SDS) After centrifugation (ca. 7000 xg for 10 min), the supernatant was collected and stored at –70°C. Extracts of cultured human foreskin epidermal keratinocytes and corneal keratinocytes were prepared by scraping the cells directly into RIPA buffer. After centrifugation (ca. 3000 xg for 10 min), the supernatant was collected. All samples were stored at –70°C.

Western Blotting

Proteins from the extracts were separated on 15% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech), and blocked in 5% milk for 1 h. Primary antibodies against EEDA were incubated at room temperature in 5% milk for 1 hour. An alkaline phosphatase-conjugated secondary antibody was incubated for 1 h at room temperature in 5% milk and detection was performed with enhanced chemiluminescence (ECL) reagents as described by the manufacturer (Amersham Pharmacia Biotech).

Tissue preparation

Prior to biopsy, mice were killed by CO₂ asphyxiation. Skin biopsies were collected from the interscapular area of the back unless specified otherwise. Whole eye globes with surrounding conjunctiva and eyelids were excised. Fresh bovine skin was obtained from a local slaughterhouse. Tissues for immunohistochemical staining and in situ hybridization were fixed overnight in 4% paraformaldehyde/Dulbecco's phosphate-buffered saline (PBS; Life Technologies, Grand Island, NY), incubated in 80% ethanol for 24hrs, and processed for paraffin embedment as previously described (Risse et al., 1998).

In Situ Hybridization

A 1-kb fragment of mouse EEDA (Figure 1) was amplified by PCR and subcloned in pCRII. Digestion with *Xho*I and transcription with Sp6 RNA polymerase were utilized for antisense probes, and digestion with *Spe*I and transcription with T7 RNA polymerase were used for sense

probes. RNA probes were prepared using ^{35}S -labeled UTP. In situ hybridization was conducted as described (Jensen and Lavker, 1996; Sun et al., 2000; Sun et al., 1999).

Immunohistochemical staining

Paraffin embedded tissues were cut in 5 μm sections, briefly heated to 55°C, deparaffinized in xylene for 3 x 5 min, rehydrated through a graded ethanol series, and slides were treated for one minute in 10mM citrate, pH 6.0 in a microwave oven to retrieve the antigen. To exhaust endogenous peroxidase, sections were incubated in 1% H_2O_2 for 30 minutes. Blocking was done with 10% normal goat serum (NGS) in PBS for 1 hour at room temperature. Sections were incubated with IgG purified polyclonal antiserum against EEDA (5 $\mu\text{g}/\text{ml}$) for 1 hr at room temperature, followed by a biotin-conjugated goat anti-rabbit antibody (1:100). Bound antibodies were visualized using a horseradish-peroxidase-conjugated ABC (avidin-biotin-peroxidase complex) kit, according to the manufacturer's (Vector Laboratories, Burlingame, CA., USA) instructions.

Physical and chemical perturbation of the epidermis

SENCAR (NCI) and C57Bl/6 mice were used in compliance with protocols established by the University of Pennsylvania Animal Care and Ethics Committee.

Chemical stimulation—Groups of SENCAR mice (12–20 days old) were treated topically with 0.01% 0-tetradecanoylphorbol – 13- acetate (TPA; Sigma, St Louis, MO) in petrolatum as previously described (Wilson et al., 1994). Applications were made once daily to the intrascapular skin of the back for 5 days. Mice were sacrificed at 1 day and 5 days after treatment and skin was prepared for histology as described above.

Physical stimulation—Mice (C57Bl/6; 7-week-old) were anesthetized with gamma-hydroxybutyric acid (i.p. injection of 100 μl of 10% sol. in PBS). The hairs of the back skin were clipped carefully with a pair of scissors. The skin was folded and two neighboring, full-thickness wounds ca. 15 mm apart, were made with a 2-mm biopsy punch. Groups of mice (3) were sacrificed 1, 3, 5, and 7 days after wounding, the wound was excised and processed for histology and immunohistochemistry as described above.

RESULTS

Characterization of our bovine corneal epithelium-specific cDNA library led to the identification of 8 independent clones, all 450-bp in length and encoding a 12-kD basic protein (107 amino acids; P.I. 10.6), which we named EEDA (Fig. 1). Based on the available mouse genomic database (Ensemble), the EEDA gene resided on chromosome 2 and contained 4 exons spanning about 30 kb. The deduced amino acid sequence of bovine EEDA was 87% and 82% identical to those of certain human and mouse ESTs, respectively (Fig. 2). Interestingly, mouse EEDA showed a 47% similarity with, and a 34% identity to, a human “death-associated protein-1” (DAP-1; Fig. 3), which is a basic, proline-rich 15 kD protein that was expressed in HeLa cells undergoing γ -interferon-induced apoptosis (Deiss et al., 1995). To characterize EEDA, we generated a rabbit antiserum that recognized a single 12 kD protein in the total protein extracts of human corneal epithelium and epidermis (Figs. 4B, 10).

EEDA expression was associated with an early stage of keratinocyte differentiation

A 500-bp EEDA mRNA was abundant in corneal epithelium; only a trace was detected in the liver, bladder, brain, heart, and stomach (Fig. 4). This EEDA mRNA was expressed in a differentiation-dependent fashion in many stratified squamous epithelia (e.g., corneal epithelium, epidermis, tongue epithelium) in association with early stages of epithelial differentiation (Figs. 5A, B, E, 6A, B, C, 7A, B, D, E). It was not detected in conjunctival

epithelium, urothelium, and small intestinal epithelium, indicating that EEDA expression is limited to a subgroup of stratified squamous epithelia.

In the eye, the EEDA mRNA and protein was virtually undetectable in the limbal basal cell, the site of stem and early precursor cells for the corneal epithelium (Cotsarelis et al., 1989; Schermer et al., 1986); Figs 5A, C). Message and protein were detected in central corneal epithelial basal and suprabasal wing cells (Figs. 5B, D), but were barely detectable in superficial cells (Figs. 5B, D), indicating that EEDA is associated with an intermediate stage of corneal epithelial differentiation. In adult mouse lens, the EEDA mRNA and protein were not detected in the central epithelial cells, but was strongly detected at the equatorial zone, where lens epithelial cells first differentiate into the fiber cells (data not shown). EEDA antigen was also detected in the non-pigmented, superficial cells of the ciliary epithelium of the adult mouse eye, but not in the retinal epithelium (data not shown).

Of all the epithelia surveyed, the largest amounts of EEDA mRNA and antigen were detected in the precortical zone of the growing (anagen) hair follicle (Figs. 6A–D). EEDA was absent in the undifferentiated, lower matrix but was heavily expressed in matrix keratinocytes immediately above the “line of Auber” that underwent intermediate stage of cortex-type of differentiation (Fig. 6D). In the nail, EEDA was similarly associated with the intermediate cell layer of the nail matrix, which has many developmental and structural similarities to the hair follicle (Fig. 6E). In the dorsal tongue epithelium, EEDA was again expressed in the intermediate cell layers (Fig. 6F). However, EEDA expression was mainly confined to the posterior portion, which contains the skin-and hair-related keratins (Dhouailly et al., 1989; Heid et al., 1988). In the epidermis (Figs. 7A, B, C), and foot pad epithelium (Figs. 7D–G), the EEDA mRNA and antigen were associated mainly with the intermediate spinous cell layers.

During development, EEDA antigen was weakly expressed in the outer cells of the two cell-layered ectoderm at embryonic day 14.5 (Fig. 8A). At E15.5 (Fig. 8B), EEDA was detected only in the intermediate cells, and was undetectable in the basal or outermost epidermal cells or the occasional follicular buds. In E17.5 and 18.5 epidermis (Figs. 8C, D), which contained granular and enucleated horny cells, EEDA expression was restricted to a single layer of suprabasal cells and was absent in the granular cell layer. EEDA was also present in the differentiated follicular cells at the apex of the “horseshoe-shaped” matrix.

Although EEDA was detected throughout the limbal and corneal epithelium at day 1 of post-natal life (Fig. 9A), at day 5 of post-natal life it was no longer detected in the limbal epithelium (Figs. 9B, C). By day 11 (Figs. 9D, E), EEDA attained the mature expression pattern in that it was detected in the basal and wing cells of the central and peripheral corneal epithelium, but not in the superficial cells, nor in the limbal cells (Figs. 9F, G).

EEDA expression was associated with normal keratinocyte differentiation

EEDA expression appeared to be tightly coupled with normal epithelial differentiation, as it ceased to express when epithelial differentiation was perturbed by various experimental manipulations. First, EEDA antigen was not expressed by cultured corneal or epidermal cells grown in either low or high Ca^{2+} (Fig. 10). Second, we stimulated mouse epidermis to undergo hyperplasia by topical application of 0.01% TPA. Twenty-four hours post-TPA application (Fig. 11B), epidermal expression of EEDA was drastically decreased compared to the petrolatum control (Fig. 11A). EEDA expression remained high in the hair follicle (Fig. 11B), possibly due to a lower accessibility to topically applied TPA. Third, we induced skin-wound healing by making 2-mm diameter full-thickness wounds in adult mouse skin (Fig. 12). Twenty-four hours later, the epithelial cells of the newly formed epidermis (“leading edge”; Fig. 12C) and its adjacent, thickened epidermis (“near edge”; Fig. 12B) became EEDA-negative. At five days post-wounding, EEDA was observed in some of the uppermost cells of

the newly formed epithelium (Fig. 12I). By day 7 post-wounding, the normal EEDA expression had returned (Figs. 12L, J).

DISCUSSION

We describe here a novel gene, EEDA, that is expressed in cells immediately above the proliferative compartment in a variety of epithelia and we suggest that EEDA plays a role in the early stages of epithelial differentiation. The appearance of EEDA in the basal layer of the corneal epithelium at first sight may seem inconsistent with the idea that this protein is associated with cells in an early stage of differentiation. However, when compared with the adjacent limbal epithelial basal cell, the corneal epithelial basal cell is known to be more differentiated. For example, the K3/K12 keratin pair, a marker of advanced corneal epithelial differentiation, is not expressed by limbal epithelial basal cells but is expressed in corneal basal cells (Chaloin-Dufau et al., 1990; Chen et al., 1994; Liu et al., 1993; Schermer et al., 1986). A recently described protein CLED (calcium-linked epithelial differentiation protein) that appears to be involved in Ca^{+2} -mediated events occurring during an early stage of epithelial differentiation is not expressed in limbal basal cells but is expressed in corneal epithelial basal cells (Sun et al., 2000). Similarly, S100A12, involved in Ca^{2+} -dependent signal transduction events associated with differentiated cells, is expressed suprabasally in the limbal epithelium but is expressed throughout the corneal epithelium (Ryan et al., 2001). Taken together, these findings indicate that corneal epithelial basal cells are biochemically equivalent to the suprabasal, partially differentiated cells of other, more conventional stratified squamous epithelia. Therefore, EEDA expression in corneal epithelial basal cells, and not in limbal epithelial basal cells, is entirely consistent with the idea that this protein is expressed in early differentiating cells and serves to distinguish further the adjacent limbal and corneal basal cell populations.

While the expression of EEDA in cells immediately adjacent to the proliferative compartment suggests that this protein plays a role in differentiation, EEDA does not appear to directly affect the ability of cells to proliferate. For example, EEDA is expressed in the basal layer of peripheral and central corneal epithelium containing young and old transient amplifying (TA) cells, respectively (Lavker and Sun, 2000; Lehrer et al., 1998); both of these TA cells are capable of significant cell division (Ebato et al., 1987; Kruse and Tseng, 1991; Pellegrini et al., 1999). Thus, the presence of EEDA in corneal basal cells indicates a commitment to differentiation rather than a post-mitotic state. This interpretation is consistent with our other observations that EEDA mRNA and antigen are strongly expressed in the hyperproliferative neonatal epidermis (Figs. 4A, C), compared with the adult (Jensen and Lavker, 1996; Jensen and Lavker, 1999; Wilson et al., 1994). Furthermore, transfection of COS cells with EEDA did not affect the cell cycle time (data not shown).

Our in vivo and in vitro data indicate that EEDA expression is strictly linked to a normal pathway of epithelial differentiation. For example, EEDA protein cannot be detected in human foreskin or corneal keratinocytes that are cultured even in medium containing a high calcium concentration allowing stratification (Fig. 10). While keratinocytes within these cultures stratify and undergo terminal differentiation, they fail to elaborate many of the normal differentiation products including K1 and K10 keratins and filaggrin (Asselineau et al., 1986; Fuchs and Green, 1980; Stanley and Yuspa, 1983; Sun and Green, 1978). EEDA expression also ceases in epidermis and corneal epithelium that have been induced to undergo hyperplasia by wounding or by topical TPA treatment. Topical treatment of in vivo epidermis and corneal epithelium with TPA, a tumor promoter, results in marked hyperplasia (Cotsarelis et al., 1989; Lavker et al., 1998; Marks et al., 1978; Raick, 1973; Wilson et al., 1994).

Since mouse EEDA showed a 47% similarity and 34% identity with human death-associated protein-1 (DAP-1), which is a basic, proline-rich 15 kD protein that is expressed in HeLa cells following gamma-interferon-induced apoptosis (Deiss et al., 1995), it is possible that EEDA plays a role in epithelial apoptosis or terminal differentiation. Similar to EEDA, DAP-1 is localized to the cytoplasm and has a potential site for phosphorylation by cyclin-dependent kinases. DAP-1 is believed to have a death-promoting effect since its over-expression increases the killing of cells by IFN- γ (Kimchi, 1998). EEDA expression is absent in epithelial cells just before they become terminally differentiated. For example EEDA is absent in: (i) the epidermal granular layer, where the transition from a synthetic keratinocyte into a terminal horny cell begins; (ii) the superficial cells of the corneal epithelium; (iii) the follicular cells above the precortical zone; and (iv) the fiber cells in the deeper part of the lens where degradation of the nucleus, endoplasmic reticulum, and mitochondria occur. These regions are all associated with advanced terminal differentiation, which has been suggested to be the counterpart of apoptosis in other cell types (Gandarillas, 2000; Lavker and Matoltsy, 1970; Zelenka et al., 1997). Since EEDA expression is detected just below this level it is possible that EEDA may function in the early stages of apoptosis (Jost et al., 2001; Rodeck et al., 1997a; Stoll et al., 1998).

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1  CTCAGCCTCCAGAGCACCGGCACTGCCACTGGCCTTGGCACACACTATGGCAAATGAAGT 60
    M A N E V 5
61  GCAAGTCCAGCTCTCCCCACTGAAAGGGGGCATCCTCCTGCAGTAAAAGCTGGAGGAAA 120
    Q V Q L S P L K G G H P P A V K A G G K 25
121  GCGAATTTCCAAAAACAGGAAATTGGCATCTTGGAGAGACACACCAAGAAAACAGGGTT 180
    R I S K K Q E I G I L E R H T K K T G L 45
181  AGAGAAAACAAGTGCCACTGCAAATGTTGCCAAAATACAGACGATGGATGCCCTGAATGA 240
    E K T S A T A N V A K I Q T M D A L N D 65
241  CACGCTGGAGAAGCTCAGCCATAAATTTCCAGCAGTAGCGCACATGGCACATCAAAAACC 300
    T L E K L S H K F P A V A H M A H Q K P 85
301  CAGACCTGCTCTGGAGAAGGTCACTCCACTGAAAAGGATCTACATTATTCAGCAGCCTCG 360
    R P A L E K V T P L K R I Y I I Q Q P R 105
361  AAAGTGTAAAGCTGGGACATAAAACAGCCGTCTAGCCAAGTGCCTCGAATGTCTGAGAGC 420
    N C * 107
421  TTAGCAAAAAGGACCACACTTTGCATAGGCCTAGTGT 457

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Figure 1.

The partial nucleotide and deduced amino acid sequences of the cDNA encoding bovine EEDA, an early epithelial differentiation-associated protein. The stop codon is marked with an asterisk.

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1                               50
h  MANEVQDLLS PRNGGHPPAV KAGGMRIKK QEIGTLERHT KKTGFECTSA
m  MANEVQVLPS PLKGRYAPAV KAGGMRIKK QEMGVLERHT KKTGLEKTS
b  MANEVQVQLS PLKGGHPPAV KAGGKRISK QEIGILERHT KKTGLEKTS
      *****
      (PKC)                               (CKII)

51                               100
h  IANVAKIQTL DALNDTLEKL NYKFPATVHM AHQKPTPALE KVVPLKRIYI
m  ITNVAKIQML DALTDTLDKL NHKFPATVHT AHQKPTPALE KAAPMKRAYI
b  TANVAKIQTM DALNDTLEKL SHKFPAAVHM AHQKPRPALE KVTPLKRIYI
      *****
      (N-Gly)

101
h  IQQPRKC
m  IQQPRKC
b  IQQPRNC

```

Figure 2.

Alignment of EEDA sequences. Numbers indicate the amino acid positions among the b: bovine; m: mouse; h: human EEDAs. Asterisks denote the following motifs: PKC: protein kinase C phosphorylation site; CKII: casein kinase II phosphorylation site; and N-Gly: N glycosylation site.

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EEDA: 13 KGRYAPAVKAGGMRI SKKQEMGVLERHTKKTGLEK--TSAITNVAKIQMLDALDTLDK 69
          K + PAVKAGGMRI +K          HT T EK          + + + ++ + +
DAP-1: 12 KAGHPPAVKAGGMRI VQKHP-----HTGDTKEEKDKDDQEWESPPPKPTVFISGVIA R 65

EEDA: 70 LNHKF-PATVHTAHQKPTPALEKAAPMKRAYIIQQPRK 106
          + F PA AHQKP +++K P R IQQPRK
DAP-1: 66 GDKDFPPAAQVAHQKPHASMDK-HPSPRTQHIQQPRK 102

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Figure 3.

Comparison of mouse EEDA with human death-associated protein-1 (DAP-1). Note the high degree of homology between mouse EEDA and human DAP-1.

Figure 4. Northern blot (A) analysis of EEDA mRNAs from various bovine tissues. (Upper panel) mRNAs from various bovine tissues were hybridized with a cDNA fragment of EEDA. An intense signal is observed in the corneal epithelium and a weak signal is seen in the stomach. No signal is seen in the bladder, brain, heart, or liver. (Lower panel) The probe on the same blot was stripped off and rehybridized with a glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA probe as a control

Western blot (B) analysis of a human corneal epithelial extract. Human corneal epithelium was extracted with Tris/Triton-X buffer, resolved on SDS-PAGE, transferred to nitrocellulose membranes, immunoblotted with rabbit polyclonal anti-EEDA antibody and detected with a ECL. A single band is obtained at 12 kD, which is consistent with the molecular weight determined from the Northern blots and confirms the presence of EEDA in human corneal epithelium.

Figure 5. EEDA is expressed in Corneal but not Limbal Epithelium

EEDA mRNA (A, B) and antigen (C, D) were localized in mouse peripheral (A, C) and central (B, D) corneal epithelia. EEDA mRNA and antigen are observed in the basal and wing cells of mouse corneal (co) epithelium, but not in the superficial cells (arrows). Little, if any EEDA can be detected in the limbal (lim) epithelium (A, C); however, in the peripheral corneal (pc) epithelium immediately adjacent to the limbal epithelium EEDA expression is distinct.

Figure 6. EEDA is Expressed Immediately above the Proliferative Zones of the Hair Follicle, Nail Matrix and Dorsal Tongue Epithelium

EEDA mRNA (A, B, C) and antigen (D, E) are localized in bovine (A), human (B) and mouse (C, D) hair follicles and in mouse (E) nails. Within the follicular epithelium (AD), EEDA is detected in the cells immediately above the line of Auber (dashed line), and is of significance in that the bulk of the mitotic activity that gives rise to the hair shaft and inner root sheath occurs in the matrix keratinocytes (m) below this level (Lavker et al., 2003). Within the nail (E) EEDA is seen in the intermediate cells of the nail matrix (nm) but not in the cells of the nail plate (np). Dp, dermal papilla. EEDA antigen (F) is present in the intermediate cells of the posterior (p) portion of the dorsal tongue epithelium. Note lack of EEDA expression in the anterior (a) portion of the epithelium.

Figure 7. EEDA is Expressed Immediately above the Proliferative Zones of Epidermis and Footpad Epithelium

Mouse epidermis (A, B) and footpad epithelium (D, E) were processed for in situ hybridization with ^{35}S -labeled sense (A, D) and antisense (B, E) probes to EEDA. A strong signal for EEDA mRNA is observed in the suprabasal cells immediately above the basal (b) layer in epidermis (B) and footpad epithelium (E). Little if any signal for EEDA mRNA is seen in the granular (g) cells. Inset (E) is a higher magnification of the footpad epithelium showing EEDA mRNA associated mainly with the intermediate cell layers. Mouse epidermis (C) and footpad epithelium (F, G) were processed for immunohistochemical staining using polyclonal rabbit anti-mouse EEDA IgG, biotinylated secondary IgG, the avidin-biotin-peroxidase complex, and

the substrate 3,3'-diaminobenzidine. Control experiments were performed using normal rabbit IgG (F). Expression pattern for EEDA antigen is similar to that observed for EEDA mRNA.

Figure 8. EEDA is expressed during the Later Stages of Epidermal Development

EEDA antigen is faintly detected in the two cell epithelium that comprised the E 14.5day (A) epidermis. At embryonic day 15.5 (B) EEDA is easily detected in the cells immediately above the basal (b) layer, but is absent in basal cells and follicular buds (fb). By embryonic day 17.5 (C) EEDA expression is observed above the basal (b) layer and can not be detected in the prominent granular (g) layer. Within the follicular epithelium, EEDA can be observed in an occasional cell (arrow) located at the apex of the matrix (m). EEDA expression pattern in the embryonic 18.5 day skin (D) is similar to that seen in the adult, with strong staining in the suprabasal cells and little if any staining the granular (g) or horny (h) cells. Only those cells (arrow) at the apex of the follicular matrix (m) express EEDA at this time. Dp, dermal papilla

Figure 9. EEDA Expression Becomes Compartmentalized During Neonatal Corneal Epithelial Development

At post-natal day 1(A) EEDA is observed in the single cell limbal (l) peripheral (pc) and central (cc) corneal epithelia. At post-natal day 5 (B, C) EEDA expression is already compartmentalized as evidenced by its absence in the limbal (l) epithelium, faint presence in the peripheral (pc) corneal epithelium and distinct expression in the central (cc) corneal epithelium. At 9 days of age (D, E) EEDA is expressed in the basal and wing cells, but not in the superficial cells of the corneal epithelium (cc). EEDA expression is also easily observed in the peripheral corneal (pc) epithelium, but not in the limbal (l) epithelium. This pattern of EEDA expression is maintained in the adult (F, G).

Figure 10. EEDA is not expressed in Cultured Keratinocytes

Western blot analysis of epidermal (epi) extracts and extracts of epidermal and corneal keratinocytes cultured under low ($0.3\mu\text{M}$) and high ($1.2\mu\text{M}$) Ca^{2+} conditions. All lanes were loaded with $20\mu\text{g}$ total cellular protein. Epidermal EEDA is detected as a single 12kD band, whereas EEDA can not be detected in any of the keratinocyte extracts either before (0 hrs) or after (24, 48, 72 hrs) the addition of Ca^{2+} .

Figure 11. Phorbol Ester Reduces EEDA Expression in the Epidermis

A marked reduction in EEDA expression is observed after 1 (B) day of topical application of 0.01% TPA in petrolatum to the backs of mice compared to topical application of petrolatum (A). Interestingly EEDA expression within the precortical follicular epithelial cells (arrows) is not affected by topical TPA treatment.

Figure 12. Wounding Reduces EEDA Expression in the Epidermis

Penetrating wounds were created on the back skin of adult (7 wk) C57Bl/6 mice. These mice were sacrificed 1 (A, B, C), 3 (D, E, F), 5 (G, H, I) and 7 (J, K, L) days after wounding, and the skin was processed for immunohistochemistry. Immediately following wounding, EEDA expression is markedly reduced in the newly formed epithelium (leading edge; C) and epidermis proximal to the leading edge (near edge; B). As reepithelialization progresses, EEDA expression is initially observed in a patchy pattern, appearing first in the more superficial cells (arrows; E). By 7 days post-wounding EEDA expression in the newly re-epithelialized tissue (L) and the adjacent epidermis (K) appears similar to the unwounded epidermis (J).