Epsin 3 Is a Novel Extracellular Matrix-induced Transcript Specific to Wounded Epithelia*

Received for publication, February 22, 2001, and in revised form, April 19, 2001
Published, JBC Papers in Press, May 18, 2001, DOI 10.1074/jbc.M101663200

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Using an in vitro model of keratinocyte activation by the extracellular matrix following injury, we have identified epsin 3, a novel protein closely related to, but distinct from previously described epsins. Epsin 3 contains a domain structure common to this gene family, yet demonstrates novel differences in its regulation and pattern of expression. Epsin 3 mRNA and protein were undetectable in keratinocytes isolated from unwounded skin, but induced in cells following contact with fibrillar type I collagen. The native triple helical structure of collagen was required to mediate this response as cells failed to express epsin 3 when plated on gelatin. Consistent with the reported function of other epsins, epsin 3 was evident in keratinocytes as punctate vesicles throughout the cytoplasm that partially co-localized with clathrin. In addition, epsin 3 exhibited nuclear accumulation when nuclear export was inhibited. In contrast to other known epsins, epsin 3 was restricted to keratinocytes migrating across collagen and down-regulated following cell differentiation, suggesting that expression was spatially and temporally regulated. Indeed, epsin 3 was localized specifically to migrating keratinocytes in cutaneous wounds, but not found in intact skin. Intriguingly, Northern hybridization and reverse transcriptase-polymerase chain reaction experiments indicated that epsin 3 expression was restricted to epithelial wounds or pathologies exhibiting altered cell-extracellular matrix interactions. Thus, we have identified a novel type I collagen-induced epsin that demonstrates structural and behavioral similarity to this gene family, yet exhibits restricted and regulated expression, suggesting that epsin 3 may serve an important function in activated epithelial cells during tissue morphogenesis.

The epidermis consists of a multilayered epithelial sheet that provides a physical barrier against the outside environment and heals in response to injury. In unwounded skin, basal keratinocytes reside on a basement membrane that physically separates these cells from the underlying dermal connective tissue rich in type I collagen. While in contact with this extracellular matrix (ECM), keratinocytes express a programmed subset of genes that promotes proliferation and differentiation. Following injury, however, keratinocytes from the surrounding tissue are activated by exposure to ligands released into the wound site and by contact with ECM macromolecules (1–3). Keratinocyte activation, which typically begins 18–24 h prior to the onset of migration, occurs as cells at the wound edge enhance the expression of genes that support repair of the tissue defect (4, 5). Many of the genes up-regulated in keratinocytes during healing, including secreted proteinases and integrin receptors, enable a fundamental shift in cell behavior that supports sustained and directed migration to re-establish the normal cytoarchitecture of the skin (3). The preponderance of studies to date attempting to identify signals that stimulate keratinocyte activation during wound healing have focused on soluble mediators, whereas the role that alterations in cell-ECM interactions play in this process has received relatively little attention.

Migration of keratinocytes from the wound edge occurs as the cells dissect under a provisional matrix of fibrin and fibronectin (6) and over or through a viable dermis, which includes structural molecules distinct from those in the basement membrane. Loss of contact with the basement membrane and subsequent exposure to the underlying dermal matrix may be a critical determinant that alters gene expression and induces the activated keratinocyte phenotype. Indeed, recent evidence supports this idea as the matrix metalloproteinase collagenase-1 is invariably expressed in keratinocytes not in contact with a basement membrane, but rather in cells migrating over the dermal matrix and in close apposition to collagen fibers (7–10). Moreover, expression of this enzyme is selectively induced in keratinocytes following contact with fibrillar type I collagen in vitro and its proteolytic activity is essential for cell migration across this matrix (10, 11). Together, these data suggest that keratinocyte contact with dermal ECM, and in particular fibrillar type I collagen, profoundly influences keratinocyte activation following injury by inducing the expression of transcripts required for efficient repair.

We used an in vitro model of keratinocyte activation by collagen to identify novel transcripts associated with migrating keratinocytes and to gain a better understanding of the role that changes in cell-ECM contacts play in keratinocyte behavior during wound repair. We report here the identification of a type I collagen-induced epsin that retains typical structural motifs and behavioral activity common to previously described epsins, epsin NH2-terminal homology; bp, base pair(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TRITC, tetramethylrhodamine B isothiocyanate.

* This work was supported by the National Institutes of Health (NIAMS) and the National Institutes of Health funded University of Texas Southwestern Skin Disease Research Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: ECM, extracellular matrix; dRT-PCR, differential display reverse transcription-polymerase chain reaction; ENTH, epsin NH2-terminal homology; bp, base pair(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TRITC, tetramethylrhodamine B isothiocyanate.
epsins. However, epsin 3 is profoundly divergent from other members of this gene family, as expression is limited to wounded or pathologic tissues with altered cell-ECM interactions, suggesting that epsin 3 may serve a role during repair of tissues that demonstrate a disrupted basement membrane. Furthermore, our findings underscore the importance of the ECM in stimulating keratinocyte activation following cutaneous injury and suggest that common mechanisms may influence cell activation in other wounded or pathologic epithelial tissues.

EXPERIMENTAL PROCEDURES

Materials—Bovine type I collagen (Vitrogen-100) was purchased from Colltix Laboratories (Palo Alto, CA). Leptomycin B was purchased from Sigma-Aldrich Chemical.

Isolation and Culture of Human Keratinocytes—Human keratinocytes were harvested from healthy adult skin as previously described (12, 13). Under the culture conditions used, keratinocytes proliferate, migrate, differentiate, and form similar to cells in vivo (12). Keratinocyte isolates were plated onto tissue culture dishes or coverslips coated with 100 μg/ml type I collagen, which is necessary for matrix-induced activation. Some cell suspensions were also plated onto dishes coated with 100 μg/ml gelatin (11) to serve as a negative control as this substrate supports attachment and spreading but does not stimulate activation.

Identification of Collagen-regulated Genes in Human Keratinocytes—Total RNA was isolated from keratinocytes immediately following cell harvest (0 h) or 24 h after plating on type I collagen by phenol-chloroform extraction (14). Any potential DNA contamination was removed by DNase (Promega Corp., Madison, WI) treatment as described (15). Differential display reverse transcription polymerase chain reaction (ddRT-PCR) was performed on samples using the RNAImage™ mRNA differential display system (GenHunter Corp., Nashville, TN) according to the manufacturer’s instructions. Amplification of the cDNAs was performed with 0.2 μM arbitrary primer 5'-AAGCTTACGATGC-3' and anchored primer H-T11G, the amplified samples were resolved on a 6% denaturing polyacrylamide gel, and differentially expressed products were identified. Differential expression was confirmed by Northern hybridization and selection of cDNAs for investigation was limited to genes up- or down-regulated >5-fold in duplicate RNA samples from two individual donors.

Identification and Cloning of Epsin 3—The 143 bp cDNA clone GAP4G1 was sequenced and compared to genes in public data bases using BLASTn through the NCBI. One resulting significant match, nucleotides 10237–10379 of Homo sapiens chromosome 17 (GenBank™ accession number AC004590), demonstrated 100% sequence identity. To determine if the chromosome 17 genomic clone sequence 5' of the GAP4G1 match coded for a novel gene we used the internet-based GenScan 1.0 program (16). To confirm the predicted gene sequence, the full-length cDNA clone GAP4G2 of epsin 3 was amplified by RT-PCR. Alignment of the epsin 3 amino acid sequence with other known epsins was accomplished using the ClustalW program (17) and the dendrogram was generated using results obtained from ClustalW and TreeView software (18).

Northern Analysis and Epsin 3 RT-PCR—Total RNA was harvested from primary keratinocytes, dermal papillae, or whole vein endothelial cells as described above. 10 μg/sample was denatured and resolved by electrophoresis through a 1% formaldehyde agarose gel. The RNA was then transferred and hybridized with a radiolabeled epsin 3 cDNA probe. To characterize epsin 3 expression in multiple tissues we probed Northern Territory™ Human Normal Tissue Blot II (Invitrogen Corp., Carlsbad, CA) and Human RNA Master Blot™ (CLONTECH Laboratories, Inc., Palo Alto, CA) with a radiolabeled epsin 3 cDNA probe according to the manufacturer’s instructions. RNA from both vendors was pooled from multiple individuals of varying sex and age and confirmed to be free of disease. Following hybridization, membranes were washed and exposed to x-ray film for an appropriate duration.

To detect epsin 3 mRNA in various normal, wounded, and pathologic specimens, tissue samples were obtained, immediately immersed in LiNa, and homogenized with a Polytron in TRIZol reagent (Life Technologies, Rockville, MD) for RNA isolation. DNase-treated total RNA (1.0 μg) was reverse transcribed with random hexamers using kit reagents and under the manufacturer’s recommended conditions (GeneAmp RNA PCR kit, PerkinElmer Life Sciences, Norwalk, CT). Epsin 3 cDNA was visualized by amplifying a 601-bp fragment of epsin 3 using the 3’-antisense primer, 5’-GGTCACAATCGGAGGT-3’, and the sense primer, 5’-ATGAGCAGCTCCGACT-3’. These primers are to adjacent exons, and thus, the 601-bp cDNA produced from epsin 3 mRNA would be easily distinguished from products amplified from contaminating DNA or pre-processed mRNA. In addition, the PCR product identity was verified by restriction digestion and sequence analysis. To determine equal loading of RNA we used an established method to amplify GAPDH by RT-PCR (11). PCR for epsin 3 was done for 23 cycles and 25 cycles for GAPDH. The resultant products were detected by Southern hybridization using radiolabeled oligonucleotides and visualized using a Typhoon 8600 variable mode PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Preparation of Antibodies and Immunoblotting Assay for Epsin 3—An 8-chain branching multiple antigenic peptide of 20 amino acids, KQGTTKEDPDGLLIGEAL, corresponding to a unique sequence of epsin 3 (amino acid residues 464–483, see Fig. 3A), was used as an antigen (Research Genetics, Huntsville, AL). Antibodies were collected as whole serum harvested at 10 weeks after primary injection and purified by affinity chromatography with the antigenic peptide coupled to NHS Sepharose-4B according to the manufacturer’s instructions (Amersham Pharmacia Biotech). For immunoblotting, keratinocytes were plated onto type I collagen-coated tissue culture dishes and cultured over a time course (0–5 days).

Total Cell Lysate Preparation—Total cell proteins were isolated by incubation in 200 μl of cell lysis solution (20 μm Tris, 2.0 mM sodium vanadate, 1.0 mM sodium fluoride, 100 mM sodium chloride, 1.0% Igepal, 0.5% sodium deoxycholate, 2.0 mM EDTA, 2.0 mM EGTA, and 25 μg each of apotinin, leupeptin, and pepstatin). Cell lysates were Dounce homogenized 50 times, centrifuged 14,000 × g for 10 min, and the supernatant was transferred to new microcentrifuge tubes.

Soluble Protein Preparation—Soluble cell proteins were isolated by scraping cells into 500 μl of HB buffer (10 mM HEPES, 1 mM EDTA, 0.32 M sucrose, and 25 μg each of apotinin, leupeptin, and pepstatin). Cells were harvested by repeatedly passing through a 27-gauge needle, centrifuged 70,000 rpm for 30 min at 4 °C, and the supernatant was transferred to new microcentrifuge tubes. 4.0 μg of total protein per sample, as determined by Bradford protein assay (Bio-Rad), was mixed to the manufacturer’s instructions. For negative controls, blots were incubated with secondary IgG alone, or specific/nonspecific peptide-adsorbed epsin 3 antibodies.

Localization of Epsin 3 in Collagen-activated Keratinocytes and Cutaneous Wounds by Immunofluorescence—All protocols of keratinocyte cell culture and collagen-coated coverslips were followed for days. For localization in ex vivo cutaneous wounds, punch biopsies of normal human skin (6 mm) were obtained and grown as explant cultures in serum-containing Dulbecco’s modified Eagle’s medium for 3 days. Following culture, samples were washed, fixed in 4% paraformaldehyde (Polysciences, Inc., Warrington, PA), and stored in phosphate-buffered saline containing 0.1% sodium azide until staining. For cryosectioning, biopsy sections were immersed in 30% sucrose overnight at 4 °C, embedded in Tissue-Tek™ O.C.T. compound (Sikura Finetek, Torrance, CA), and 6-μm thick sections were cut using a Reichert-Jung cryostat.

For immunolocalization of epsin 3, coverslips and explant sections were permeabilized and incubated with 2.5 μg/ml affinity-purified epsin 3 antibodies overnight at 4 °C followed by incubation with TRITC-conjugated AffiniPure donkey anti-rabbit IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA). To visualize epsin 3 and clathrin within the same cells, coverslips were treated with clathrin monoclonal antibody X22 (1:1000 dilution) after incubation with anti-epsin 3 antibodies. Clathrin antibody localization was visualized using AlexaFluor™ 488-goat anti-mouse IgG (Molecular Probes, Eugene, OR). For negative control sections, samples were incubated with preimmune serum, secondary IgG alone, or specific/nonspecific peptide-adsorbed epsin 3 antibodies. Digital light photomicrographs were captured with a Polaroid DMC1 camera connected to a Nikon Eclipse E400 microscope. Whole mount immunofluorescence images were captured using a Hamamatsu C4742–9S digital camera connected to a Zeiss Axioskop 2 microscope using OpenLab scientific imaging software (Improvement,
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GAP4G1

- 3.9 kb

GAPDH

0 24

Time, hr

FIG. 1. Identification of differentially expressed cDNA clone GAP4G1 in keratinocytes following contact with type I collagen.

Primary human keratinocytes were isolated from intact skin and dRT-PCR was performed on RNA obtained from cells immediately following isolation (0 h) or 24 h after plating on type I collagen (100 μg/ml). One resultant cDNA exhibiting significant up-regulation (clone GAP4G1) was selected and used to probe a Northern blot containing total RNA exhibiting significant up-regulation (clone GAP4G1) was selected and used to probe a Northern blot containing total RNA (10 μg/lane) from 0- and 24-h collagen-activated keratinocytes. The Northern blot confirmed that this cDNA represented an mRNA that was significantly induced in cells following contact with type I collagen. The blot shown is representative of data obtained from at least four separate experiments using RNA from individual donors.

RESULTS

Keratinocyte Contact with Collagen Induces Epsin 3—Because keratinocyte contact with type I collagen specifically induces collagenase-1, a marker of the activated keratinocyte phenotype (10, 11, 19), we wanted to determine if novel activation-specific transcripts were induced by this ECM protein. dRT-PCR was used to compare transcripts expressed in keratinocytes freshly isolated from intact skin or after plating onto fibrillar type I collagen. cDNA clone GAP4G1, an unknown transcript potently induced in keratinocytes following collagen contact, was selected for characterization. As determined by Northern hybridization, cDNA GAP4G1 hybridized to a single 3.9-kilobase mRNA species that was induced >20-fold in collagen-activated keratinocytes when compared with cells freshly isolated from unwounded skin (Fig. 1). Sequence analysis revealed 100% identity to nucleotides 10237–10379 of human chromosome 17-clone hCIT.22_K.21 (accession number AC004590). Because this genomic clone had no association with a known gene, we used the internet-based GenScan 1.0 program to determine if the sequence surrounding GAP4G1 contained an open reading frame. A putative gene was identified containing an initiation codon, nine exons, and in-frame termination codon that, when translated, coded for a novel 332-amino acid protein with a calculated molecular mass of 68 kDa. To confirm the predicted gene structure, we amplified the full-length coding sequence using multiple pairs of gene specific inter-exonic primers. Sequencing of the resultant cDNA demonstrated a 9 exon, 1896-bp open reading frame as predicted (Fig. 2). Comparison with known proteins in the public data bases using a BLASTp search revealed that the encoded protein had not been previously reported and that it demonstrated remarkable similarity to, but was distinct from, members of the epsin protein family (Figs. 2 and 3A). In addition, and to confirm our initial Northern results (Fig. 1), we also amplified a 400-bp fragment unique to exon 7 of epsin 3 and used this cDNA to probe total RNA from freshly isolated and collagen-activated keratinocytes. As before, strong hybridization to a 3.9-kilobase mRNA was seen only in collagen-activated keratinocytes (not shown).

Comparison of the epsin 3 amino acid sequence with human epsins 1 and 2 demonstrates an identical tripartite domain structure common to this protein family (Figs. 2 and 3A). Conserved domains include a COOH-terminal consensus sequence of three NPF repeats that bind Eps15 (20), multiple DPW motifs that bind clathrin AP-2 (21, 22), and a 150-amino acid protein module, the epsin NH2-terminal homology domain (ENTH domain) (22). The region of epsin 3 demonstrating the highest sequence identity is the ENTH domain that contains multiple regions of 100% conservation and 80–82% sequence identity with human epsins 1 and 2 (Fig. 3A). Although the DPW and NPF domains of epsin 3 possess significantly less sequence identity when compared with epsins 1 and 2 (11–28 and 28–34%, respectively), the three NPF motifs are 100% conserved. Furthermore, epsin 3 contains 4 conserved and 5 total DPW motifs, which is consistent with the variability demonstrated among each family member.

Intron/exon junctions of the epsin 3 gene conforming to the GT/AG rule for splice sites (23) were mapped by comparing cDNA and genomic sequences (Fig. 2). Exon-intron boundaries and the sizes of exons and introns are summarized in Table I. The most notable structural feature of the epsin 3 gene is that exon 1 codes for the 150-amino acid ENTH domain in its entirety. In contrast, the DPW domain encompasses exons 5–7 and the three NPF repeats are found in the distal sequence of exon 8 and throughout exon 9.

In addition to binding Eps15 and AP-2, epsins 1 and 2 exhibit type I cathepin-binding consensus sequences (257LMDLADV and 289LDDMDAL, respectively) proximal to the DPW domain. An additional motif (LV/D/N/LD) was recently identified within the carboxyl-terminal segment of rat epsins 1 and 2 that acts cooperatively with the type I consensus to bind clathrin at an independent site (24). Epsin 3 contains a distal 506LVNDDL low affinity clathrin-binding sequence as well as 306ILLDLADIF proximal to the second DPW repeat, which is reminiscent of a type I consensus as there are two intervening hydrophobic residues between two acidic amino acids (Figs. 2 and 3A).

Construction of a phylogenetic tree comparing human epsin 3 with 3 other known epsins (Fig. 3B) indicates that epsin 3 is most closely related to human and rat epsin 1 (47 and 46%, respectively) and Xenopus Mp90 (44%). In contrast, epsin 3 sequence identity is most divergent from human epsin 2b (39%) and mouse intersectin-binding protein 2 (37%), although a significant amount of sequence identity is retained.

Collagen-mediated Induction of Epsin 3 Is Transient and Requires the Native Triple Helical Substrate—To determine the molecular mechanisms regulating epsin 3, we assessed the expression of this transcript in keratinocytes at multiple time points following collagen contact. Northern blotting showed that epsin 3 mRNA was absent in keratinocytes freshly isolated from intact skin (Fig. 4A, 0 h). Expression was stimulated between 8 and 12 h after contact with the collagen matrix and became most prominent at 20 h (Fig. 4A). This collagen-mediated effect was transient, however, as epsin 3 mRNA signal progressively decreased to almost undetectable levels at 72 h, coincident with cells reaching confluence in culture.

The native, triple helical conformation of collagen is requisite for induction of collagenase-1 in keratinocytes as cells plated on gelatin express significantly lower levels of collagenase-1 mRNA and protein, although keratinocyte attachment and spreading is supported by this substrate (11). Keratinocytes were plated on fibrillar type I collagen or gelatin to determine if induction of epsin 3 was dependent on the native substrate.

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As assessed by Northern hybridization, epsin 3 mRNA was reduced by 85% in cells cultured on gelatin when compared with that expressed in cells plated on collagen (Fig. 4B).

To determine if epsin 3 mRNA was translated, we generated polyclonal antibodies directed against a unique 20-amino acid sequence located between the epsin 3 DPW and NPF domains. Keratinocytes were harvested from normal skin and cultured on collagen over a time course. Total or soluble cell proteins were isolated and epsin 3 was visualized by immunoblotting with affinity-purified antibodies. An immunoreactive 68-kDa species, the predicted molecular mass for epsin 3, was evident in keratinocytes 1 day after plating onto collagen and became

**Fig. 2. Nucleotide sequence of the human epsin 3 cDNA and its deduced amino acid sequence.** The entire coding sequence for human epsin 3 was amplified by RT-PCR from collagen-activated keratinocytes. Sequencing of the resulting cDNA and translation revealed an 1896-bp open reading frame that codes for a 632-amino acid protein. The deduced amino acid sequence is shown under the DNA sequence and exon borders are noted.

As assessed by Northern hybridization, epsin 3 mRNA was reduced by 85% in cells cultured on gelatin when compared with that expressed in cells plated on collagen (Fig. 4B). To determine if epsin 3 mRNA was translated, we generated polyclonal antibodies directed against a unique 20-amino acid sequence located between the epsin 3 DPW and NPF domains. Keratinocytes were harvested from normal skin and cultured on collagen over a time course. Total or soluble cell proteins were isolated and epsin 3 was visualized by immunoblotting with affinity-purified antibodies. An immunoreactive 68-kDa species, the predicted molecular mass for epsin 3, was evident in keratinocytes 1 day after plating onto collagen and became...
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### FIG. 3. Comparison of the epsin 3 amino acid sequence with other human epsins.

A. peptide sequences for human epsins 1, 2a, and 2b were retrieved from GenBank™ and aligned with epsin 3 using the ClustalW program. Identical amino acid residues are highlighted in **black**. Corresponding epsin 3 DPW motifs are **underlined**, NPF motifs are indicated by **asterisks**, and putative clathrin binding consensus sequences are **bracketed**.

B. a dendrogram was generated using pairwise sequence alignments of known epsins using TreeView. Numerical data in the **right-hand column** represent percent sequence identity when aligned with human epsin 3.
increasingly stronger on day 3 (Fig. 4C). In contrast, epsin 3 was absent in cells freshly isolated from intact skin (day 0) and after 5 days on collagen, which is consistent with the transient epsin 3 mRNA expression noted in collagen-activated keratinocytes by Northern hybridization (Fig. 4A). A second, smaller unknown immunoreactive band of ~50-kDa was visualized in keratinocyte whole cell lysates, but its expression was not affected by contact with collagen, suggesting that either the antibody bound a nonspecific protein or a currently uncharacterized species (Fig. 4C, left panel). When epsin 3 present in keratinocyte-soluble proteins was visualized, however, only the immunoreactive 68-kDaepsin 3 band was evident. Immunoblots performed following preadsorption of the epsin 3 antibody with antigenic peptide showed no immunoreactive bands (Fig. 4C, Ab peptide), whereas preadsorption with a nonspecific epsin 3 peptide had no effect (Fig. 4C, Ab + N.S. peptide).

Collagen-activated Epsin 3 Partially Co-localizes with Clathrin and Shuttles to the Nucleus—To assess the subcellular distribution of epsin 3, keratinocytes were plated onto collagen-coated coverslips and processed for immunofluorescence stain-
Nuclei were visualized by staining with Vectashield™-DAPI mounting shuttles to the nucleus. A-E, cytops, partially co-localizes to clathrin-coated structures, and B, D). Clathrin expression was not re-

Resolution of the epsin 1 ENTH domain crystal structure has revealed remarkable structural similarity to β-catenin armadillo repeats (25). In addition to its involvement in endocytosis, epsin 1 is capable of interacting with the transcription factor promyelocytic leukemia Zn2+ finger protein and translocating to the nucleus where it may regulate transcription (25). Because the epsin 3 ENTH domain is highly conserved, we hypothesized that epsin 3 may exhibit similar behavior. To determine the ability of epsin 3 to shuttle between the nucleus and cytoplasm, collagen-activated keratinocytes were treated with leptomycin B, an antifungal antibiotic that blocks the Crm1-dependent nuclear export pathway and induces the nuclear accumulation of proteins that have shuttling activity (26–28). As shown in Fig. 5F, epsin 3 in keratinocytes treated transiently with leptomycin B accumulated in the nucleus (arrowheads), whereas control cells treated with vehicle alone exhibited prominent cytoplasmic localization (not shown).

Epsin 3 is Expressed by Migrating Keratinocytes in Vitro and ex Vivo—Keratinocytes grown on collagen under high calcium (1.8 mM) conditions recapitulate the epidermal wound healing response by forming subpopulations of migrating, proliferating, and differentiating cells (12). When viewed from above, differentiating keratinocytes are seen as islands of confluent cells (asterisks, Fig. 6, B and C) surrounded by hyperproliferative cells. Bordering the hyperproliferative cells, and often detached from them, are migrating keratinocytes (arrows, Fig. 6, B and C). Because collagen-induced epsin 3 mRNA and protein expression by keratinocytes was transient (Fig. 4, A and C), we examined whether expression of this protein was spatially restricted. Keratinocytes were isolated from intact skin and suspensions of low or high cell density were plated onto collagen-coated coverslips. The low density cell suspension prolonged individual migrating keratinocytes for the duration of the experiment, whereas the higher cell density suspension promoted cell-cell contact that resulted in the formation of differentiating islands. In cultures predominantly made up of individual, migrating keratinocytes epsin 3 was prominently expressed in all cells examined (Fig. 6A). In differentiation promoting cultures, however, epsin 3 was spatially confined to keratinocytes migrating away from and bordering islands of differentiating cells (Fig. 6, B and C, arrows). Clathrin expression was not restricted to a particular cell population as positive staining was noted in both migrating and differentiating keratinocytes (Fig. 6D). Co-localization of epsin 3 with clathrin was evident in the migrating cells as seen previously (Fig. 6, C and D, arrowheads).

Because epsin 3 was predominantly expressed by migrating keratinocytes in vitro, we assessed if this protein was expressed during cutaneous repair. Punch biopsies of normal human skin were cultured ex vivo over a time course and processed for immunofluorescence staining using affinity purified epsin 3 antibodies (Fig. 5C) and monoclonal antibody X22 directed against the clathrin heavy chain (Fig. 5D). Consistent with the behavior of epsins 1 and 2, merging of the epsin 3 and clathrin images indicated that a subpopulation of epsin 3 co-localized to clathrin-coated structures (Fig. 5E, arrows). Vesicles that exhibited double labeling were predominantly located at the cell periphery where clathrin-coated pits would be assembling, whereas epsin 3 immunoreactivity was absent from a pool of clathrin located near the nucleus (Fig. 5, C-E, arrowhead).

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FIG. 5. Epsin 3 is expressed by collagen-activated keratinocytes, partially co-localizes to clathrin-coated structures, and shuttles to the nucleus. A-E, keratinocytes were isolated from normal, intact skin and cultured on collagen-coated coverslips for 3 days. Following fixation, cells were processed for immunofluorescence staining using affinity-purified epsin 3 antibodies (A and C), preimmune serum (B), or monoclonal antibody X22 directed against clathrin heavy chain (D). Panel E represents the merged epsin 3 and clathrin images. Nuclei were visualized by staining with Vectashield™-DAPI mounting medium. Keratinocytes grown on collagen displayed prominent staining for epsin 3 that was visualized as punctate vesicles throughout the cytoplasm and concentrated around the nucleus. Double labeling with the clathrin antibody revealed that epsin 3 partially co-localized to clathrin-coated structures at the cell periphery (arrow), whereas a pool of clathrin located near the nucleus (arrowhead) was devoid of epsin 3 immunoreactivity (C-E). No specific immunoreactivity was noted in keratinocytes processed with preimmune serum (B). F, collagen-activated keratinocytes were treated for 3.5 h with vehicle control (not shown) or leptomycin B (10 ng/ml, F), an inhibitor of Crm1-dependent nuclear export. Following treatment, cells were processed for epsin 3 immunofluorescence staining. Treatment with leptomycin B induced an accumulation of epsin 3 in the nucleus (F, arrowheads), whereas control cells exhibited prominent cytoplasmic staining (not shown), illustrating the ability of epsin 3 to shuttle between the cytoplasm and the nucleus. Bars, 15 μm (A and F), 10 μm (B), 5 μm (C-E).
membrane and onto the denuded dermis (Fig. 7A, arrowheads). We have shown in previous studies that expression of collagenase-1 in this model mirrors that seen in vivo (15).

Following 3 days after wounding, epsin 3 immunoreactivity was predominantly localized to basal keratinocytes within the migrating epithelial tongue (Fig. 7, B and C, arrowheads). Paralleling our in vitro data, epsin 3 was specifically localized to basal keratinocytes at the cell-ECM interface, whereas suprabasal differentiating cells in the migrating tongue exhibited only background staining (Fig. 7, B and C). Epsin 3 signal progressively diminished away from the wound edge and was detectable at low levels in only a few basal cells of intact epidermis (Fig. 7B). Immunoreactivity was also noted in a subpopulation of cells scattered throughout the dermis, most of
TABLE II
Expression of epsin 3 mRNA in cultured human cells and tissues

| Cell/tissue examined              | Level of expression |
|-----------------------------------|---------------------|
| Collagen-activated keratinocytes  | ++ + + +            |
| Chronic wound                     | ++                  |
| Basal cell carcinoma              | ++                  |
| Ulcerative colitis                | ++ +                |
| Unwounded skin                    | Negative            |
| Stomach                           | +/-                 |
| Dermal fibroblasts (± PMA, IL-1β) | Negative            |
| Endothelial cells (± PMA, IL-1α, TNF-α) | Negative |
| Brain and spinal cord (fetal and adult) | Negative |
| Heart and aorta (fetal and adult)  | Negative            |
| Skeletal muscle and adipose       | Negative            |
| Esophagus, duodenum, appendix, colon | Negative |
| Liver, pancreas, spleen, thymus   | Negative            |
| Lung and trachea                  | Negative            |
| Kidney, prostate, bladder         | Negative            |
| Uterus, placenta, ovary, testis   | Negative            |
| Pituitary, thyroid, adrenal, salivary, and mammary gland | Negative |
| Peripheral leukocyte, lymph node, bone marrow | Negative |

which had a fibroblast-like appearance. No specific staining was detected in samples processed with preimmune serum (Fig. 7D) or epsin 3 antibodies preadsorbed with antigenic peptide (not shown).

**Epsin 3 Expression Is Restricted to Epithelial Wounds and Pathologies with Altered Cell-Extracellular Matrix Interactions**—Our findings demonstrate the spatially confined expression of epsin 3 in migrating keratinocytes suggested that, unlike the previously described members of this protein family, epsin 3 was expressed in a restricted manner. To analyze expression in various tissues, we hybridized total RNA from multiple tissues using an epsin 3 cDNA probe. Surprisingly, epsin 3 was not expressed in multiple fetal or most adult tissues examined (summarized in Table II). A low level of epsin 3 mRNA (0.3-fold over background) was detected in adult stomach, but only after extended exposure of the x-ray film (Fig. 8A). This finding was variable, however, as hybridization was not found in stomach total RNA obtained from a separate pool of RNA donors (not shown). We also screened dermal fibroblasts and endothelial cells, two additional cell types activated during wound repair. Epsin 3 was not detected in total RNA harvested from resting or activated cells (not shown).

Because epsin 3 was not expressed in any resting tissues or cultured cells examined (with the exception of collagen-activated keratinocytes), we hypothesized that expression may be specific to wounded or pathologic tissues in which epithelial cells undergo altered cell-ECM interactions. This may explain our results demonstrating variable and low levels of epsin 3 mRNA in stomach, as the pooled samples may have been contaminated with wounded tissue (i.e. gastric ulceration). Expression of epsin 3 in wounded or pathologic tissues was determined by amplifying a 601-bp fragment of epsin 3 by RT-PCR. Interestingly, epsin 3 mRNA was expressed in total RNA isolated from chronic cutaneous wound, basal cell carcinoma, and ulcerative colitis, all wounds or pathologies that undergo altered cell-ECM interactions (Fig. 8B). In contrast, expression was not found in normal, unwounded skin (Fig. 8B).

**DISCUSSION**

The epsins are constitutively expressed genes that demonstrate a wide tissue distribution and are purported to provide a role in mediating the assembly and internalization of clathrin-coated pits (21, 22). Originally identified by screening for proteins that interact with the EH (Eps15 homology) domains of Eps15 (20, 29) and clathrin AP-2 (30), epsin functions as a molecular bridge to facilitate the ordered assembly of these molecules into the clathrin lattice at the plasma membrane. In addition, epsin contains two clathrin binding consensus sequences that have been shown to act cooperatively in clathrin mediated endocytosis by coordinating multiple molecular interactions (24). Together, these data suggest that epsin is a requisite for coordinating a specific molecular architecture prior to coated pit invagination. In fact, perturbation of epsin function in fibroblasts by overexpression or antibody injection potently inhibits this process (21, 22).

The findings described in this report identify a novel epsin that retains the conserved structural features common to this gene family. Epsin 3 exhibits a significant degree of sequence identity when compared with other epsins and contains the ENTH protein module that is highly conserved from yeast to humans. Epsin 3 demonstrates greater sequence diversity distal to the ENTH domain, yet multiple DPW and three NPF motifs shown to be required for Eps15 and AP-2 binding (21, 22), respectively, are fully conserved. We therefore hypothesize that epsin 3 provides a similar function in activated epithelial cells that is consistent with previously described members of this gene family.

The assumption that epsin 3 functions similarly to other epsins is strengthened by our in vitro data demonstrating co-localization with clathrin and shuttling between the cytoplasm and the nucleus. When collagen-activated keratinocytes were double-labeled with epsin 3 and clathrin antibodies, we...
Activated keratinocytes engaged in re-epithelialization of damaged tissue in migrating cells, a gene expression pattern identical to that noted in keratinocytes following contact with fibrillar type I collagen. Epsin 3 expression was not expressed in keratinocytes isolated from normal, unwounded skin, but induced specifically and invariantly in migrating cells following contact with fibrillar type I collagen. Previous studies from our group and others have shown that keratinocyte contact with collagen induces collagenolytic activity in migrating cells, a gene expression pattern identical to that reported for keratinocytes engaged in re-epithelialization in vivo (11, 31). The results reported here further underscore the profound influence that collagen contact has on keratinocyte behavior during wound healing and supports the hypothesis that in addition to soluble factors, contact with dermal ECM induces keratinocyte activation by stimulating the expression of transcripts during repair.

In addition to collagen-induction of epsin 3 in keratinocytes, we further determined that expression of this transcript is restricted to tissues that demonstrate alterations in cell-ECM contacts as occurs during wound healing and tissue morphogenesis. In an exhaustive tissue survey we found that epsin 3 was not expressed in resting, homeostatic tissues, but rather in epithelial wounds or pathologies that typically exhibit a disrupted basement membrane. This is in marked contrast to other epsins, which are widely distributed in resting tissues, and suggests that epsin 3 may play a role in regulating the function of epithelial cells in which cell-ECM interactions have been altered.

During the preparation of this manuscript, a full-length cDNA was identified in human colon (accession number AK000785) coding for a putative protein having nearly 100% sequence identity to epsin 3 described here. In addition, following the release of our sequence (accession number AF324241), a coding sequence predicted from cDNA AK000785 was submitted and named human epsin 3 (accession number NM_017957). Interestingly, cDNA NM_017957 was cloned from colon total RNA, yet our probing of multiple tissue blots for epsin 3 only produced faint and variable hybridization in the stomach (Fig. 8A). Furthermore, we were unable to verify hybridization in total RNA from colon, except in samples obtained from ulcerative colitis (Table I). We hypothesize that the variable expression of epsin 3 found in stomach resulted as a consequence of wounded epithelium present in the donor tissue used. Pooled RNA from multiple donors is blotted onto each tissue Northern in used in our studies and RNA from an injured epithelial lining of a single donor would produce a faint band due to dilution of the transcript. Perhaps the colon sample used to generate cDNA NM_017957 included RNA from wounded tissue, which would explain our inability to identify epsin 3 in normal colon.

As keratinocytes complete re-epithelialization they restore cell-cell contacts, establish an intact basement membrane, and revert to a gene expression program that supports proliferation and differentiation. Interestingly, we found that epsin 3 expression was limited to keratinocytes actively involved in migration and absent in differentiating cells in vitro and ex vivo. The formation of polarized cell-cell contacts and synthesis of a new basement membrane are the most likely candidates that down-regulate epsin 3 expression in collagen-activated keratinocytes. Because we assessed expression in higher cell density cultures over 3 days, a time in which both cell-cell contacts are established and basement membrane proteins are synthesized, we cannot yet separate the effects of either in determining how down-regulation of epsin 3 expression is mediated. Because epsin 3 expression parallels the spatial and temporal pattern of collagenase-1 in migrating keratinocytes, we speculate that both phenomena contribute to epsin 3 down-regulation in confluent cells and in intact skin after healing has completed.

The spatially and temporally distinct expression pattern of epsin 3 noted in keratinocytes in vitro and ex vivo indicates that this protein serves an important function in activated, migrating cells. The ability of keratinocytes to respond to injury requires the processing of multiple extracellular signals, including soluble ligands binding to cognate receptors and exposure to new ECM. Signaling through receptor tyrosine kinases by growth factors such as epidermal growth factor, keratinocyte growth factor, and transforming growth factor-α elicits a number of transient cell responses including enhanced cell proliferation and migration (2). As healing progresses, however, epidermal cells lose their responsiveness to these signals and revert to a gene expression program that promotes differentiation. Activated growth factor receptors, such as the epidermal growth factor receptor (32) and keratinocyte growth factor receptor (33), are rapidly internalized via endocytosis as cell migration ceases and this pathway serves as an important regulatory mechanism to control cell surface receptor expression and downstream signaling events. A mechanism by which activated receptors are internalized via epsin 3-enhanced endocytosis would have profound effects on the ability of keratinocytes to respond to extracellular stimuli and mediate the wound healing response.

In summary, our results demonstrate that epsin 3 is induced in wounded or pathologic epithelial tissues exhibiting altered cell-ECM interactions as occurs following basement membrane disruption and contact with underlying collagen. Epsin 3 demonstrates a regulated and specific pattern of expression, observed for any other known epsin, and indicates that the cell machinery required for epithelial repair differs fundamentally from that required to maintain tissue homeostasis. Although a function for epsin 3 has yet to be determined, its novel temporal and spatial expression pattern suggests a role in regulating cell responses to the extracellular environment during morphogenetic events.

Acknowledgments—We thank Dr. R. G. W. Anderson for providing the clathrin X22 monoclonal antibody, Dr. Mel Vaughan, Troy Gor, and Amanda Ackermann for technical assistance, and Drs. William Snell, Fred Grinnell, Scott Brady, and Gerardo Morifini for the critical evaluation of this manuscript and meaningful suggestions. We also thank Drs. Lyman Billhartz, Spencer Brown, Jim Chau, and Stan Taylor for providing clinical tissues used in these studies.

REFERENCES

1. Grinnell, F. (1990) J. Trauma 30, S144–S149
2. Clark, R. (1996) in The Molecular and Cellular Biology of Wound Repair (Clark, R., ed) 2nd Ed., Plenum Press, New York
3. Coulombe, P. A. (1997) Biochem. Biophys. Res. Commun. 236, 231–238
4. Mansbridge, J. N., and Knapp, A. M. (1987) J. Invest. Dermatol. 89, 253–263
5. Paladini, R. D., Takahashi, K., Bravo, N. S., and Coulombe, P. A. (1996) J. Cell Biol. 132, 381–397
6. Clark, R. A. F., Lanigan, J. M., Delapelle, P., Manseau, E., Dvorak, H. F., and Colvin, R. B. (1982) J. Invest. Dermatol. 79, 264–269
7. Inoue, M., Kratz, G., Hasegawa, A., and Stahle-Backdahl, M. (1995) J. Invest. Dermatol. 104, 479–483
8. Saarialho-Kere, U. K., Chang, E. S., Welgus, H. G., and Parks, W. C. (1992) J. Clin. Invest. 90, 1952–1957

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9. Saarialho-Kere, U. K., Kovacs, S. O., Pentland, A. P., Olerud, J., Welgus, H. G., and Parks, W. C. (1993) J. Clin. Invest. 92, 2858–2866
10. Pilcher, B. K., Dumin, J. A., Sudbeck, B. D., Krane, S. M., Welgus, H. G., and Parks, W. C. (1997) J. Cell Biol. 137, 1445–1457
11. Sudbeck, B. D., Pilcher, B. K., Welgus, H. G., and Parks, W. C. (1997) J. Biol. Chem. 272, 22103–22110
12. Pentland, A. P., and Needleman, P. (1986) J. Clin. Invest. 77, 246–251
13. Sudbeck, B. D., Parks, W. C., Welgus, H. G., and Pentland, A. P. (1994) J. Biol. Chem. 269, 30022–30029
14. Checzeny, P., and Sczepaniak, N. (1997) Anal. Biochem. 162, 156–159
15. Pilcher, B. K., Dumin, J., Schwartz, M. J., Mast, B. A., Schultz, G. S., Parks, W. C., and Welgus, H. G. (1999) J. Biol. Chem. 274, 10372–10381
16. Burge, C., and Karlin, S. (1997) J. Mol. Biol. 268, 78–94
17. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–80
18. Page, R. D. M. (1996) Comp. Appl. Biosci. 12, 357–358
19. Pilcher, B. K., Sudbeck, B. D., Dumin, J. A., Welgus, H. G., and Parks, W. C. (1998) Arch. Dermatol. Res. 290, (suppl.) S37–46
20. Salcini, A. E., Confalonieri, S., Doria, M., Santolini, E., Tassi, E., Minenkova, O., Cesareni, G., Pelicci, P. G., and Di Fiore, P. P. (1997) Genes Dev. 11, 2239–2249
21. Chen, H., Fre, S., Slepnev, V. I., Capua, M. R., Takei, K., Butler, M. H., Di Fiore, P. P., and De Camilli, P. (1998) Nature 394, 793–797
22. Rosenthal, J. A., Chen, H., Slepnev, V. I., Pellegrini, L., Salcini, A. E., Di Fiore, P. P., and De Camilli, P. (1999) J. Biol. Chem. 274, 33959–33965
23. Breathnach, R., and Chambon, P. (1981) Annu. Rev. Biochem. 50, 349–383
24. Drake, M. T., Downs, M. A., and Traub, L. M. (2000) J. Biol. Chem. 275, 6479–6489
25. Hyman, J., Chen, H., Di Fiore, P. P., De Camilli, P., and Brunger, A. T. (2000) J. Cell Biol. 149, 537–546
26. Nishii, K., Yoshida, M., Fujisawa, D., Nishikawa, M., Horinouchi, S., and Beppu, T. (1994) J. Biol. Chem. 269, 6320–6324
27. Kudo, N., Matsumori, N., Taoka, H., Fujisawa, D., Schreiner, E. P., Wolf, B., Yoshida, M., and Horinouchi, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9112–9117
28. van Hengel, J., Vanhoven, P., Staes, K., and van Roy, F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7980–7985
29. McPherson, P. S., de Heuvel, E., Phillie, J., Wang, W., Sengar, A., and Egan, S. (1998) Biochem. Biophys. Res. Commun. 244, 701–705
30. Wang, L. H., Sudhof, T. C., and Anderson, R. G. (1996) J. Biol. Chem. 271, 10079–10083
31. Woodley, D. T., Kalebec, T., Baines, A. J., Link, W., Prunieras, M., and Liotta, L. (1986) J. Invest. Dermatol. 4, 418–423
32. Sorkin, A., and Waters, C. M. (1993) Biosciences 15, 375–382
33. Marchese, C., Mancini, P., Bussotti, F., Felici, A., Gradini, R., Sansolli, T., Frati, L., and Torrisi, M. R. (1998) J. Cell Sci. 111, 3517–3527
