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Cell Specific Processing and Release of the Hormone-Like Precursor and Candidate Tumor Suppressor Gene Product, Ecrg4

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Abstract

The human open reading frame C2orf40 encodes esophageal cancer related gene-4 (Ecrg4), a newly recognized neuropeptide-like precursor protein whose gene expression by cells *in vitro*, over-expression in mice *in vivo* and knock-down in zebrafish affects cell proliferation, migration and senescence, progenitor cell survival and differentiation and inflammatory function. Unlike traditionally secreted neuropeptide precursors however, we find that Ecrg4 localizes to the epithelial cell surface and remains tethered after secretion. Here, we used cell surface biotinylation to establish that 14kDa Ecrg4 localizes to the cell surface of prostate (PC3), or kidney (HEK) epithelial cells after transfection. Accordingly, this Ecrg4 is resistant to washing cells with neutral, high salt (2M NaCl), acidic (50 mM glycine, pH 2.8) or basic (100 mM Na_2CO_3, pH 11) buffers. Mutagenesis of Ecrg4 established that cell tethering was mediated by an NH_2-terminus hydrophobic leader sequence that enabled both trafficking to the surface and tethering. Immunoblotting analyses however showed that different cells process Ecrg4 differently. Whereas PC3 cells release cell surface Ecrg4 to generate soluble Ecrg4 peptides of 6 to 14 kDa, HEK cells do neither and the 14kDa precursor resembles a sentinel attached to the cell surface. Because a phorbol ester treatment of PC3 cells stimulated Ecrg4 release from, and processing at, the cell surface, these data are consistent with a multifunctional role for Ecrg4 that is dependent on its cell of origin and the molecular form produced.

Keywords

tumor suppressor gene; membrane protein; secretion; protein trafficking

INTRODUCTION

Esophageal cancer related gene-4 (Ecrg4) is a candidate tumor suppressor gene that has been implicated in the progression of cancer and metastasis, proliferation and migration of epithelial cells, induction of cell senescence and the survival and differentiation of neuroprogenitor and hematopoietic cells (Gonzalez et al., 2011; Gotze et al., 2009; Huh et al., 2009; Kujuro et al., 2010; Li et al., 2010a; Li et al., 2011; Ozawa et al., 2011; Podvin et al., 2011; Tadross et al., 2010). Unlike prototypic tumor suppressor genes (Lee and Muller, 2010; Rothenberg and Settleman, 2010), however, bioinformatic algorithms predict that Ecrg4 is not an intracellular cytoplasmic protein (Mirabeau et al., 2007; Schuster-Bockler and Bateman, 2007; Southey et al., 2009; Tegge et al., 2008). Instead, Ecrg4 resembles a ligand encoding a neuropeptide-like precursor characteristic of the human secretome (Clark

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et al., 2003; Hathout, 2007; Mustafa et al., 2011). In humans, the Ecrg4 open reading frame, C2orf40 (Yue et al., 2003), encodes a protein with a hydrophobic leader sequence that directs its secretion from cells and potential for proteolytic processing into smaller peptides by the identification of furin, protein convertase or thrombin processing consensus sequences (Mirabeau et al., 2007). Accordingly, several investigators (Huh et al., 2009; Kujuro et al., 2010; Mirabeau et al., 2007; Ozawa et al., 2011) localized tagged-Ecrg4 fusion proteins to intracellular secretory vesicles, identified Ecrg4 peptide processing in the conditioned media of Ecrg4-transduced cells and suggested that intracellular post-translational processing cleaved the Ecrg4 pre-pro-precursor neuropeptide into smaller secreted peptides before secretion.

In the course of immunostaining and immunoblotting mouse, rat and human choroid plexus tissues, we observed that a significant portion of the Ecrg4 immunoreactivity was relatively insoluble and localized to neuro-epithelial cell membranes (Gonzalez et al., 2011; Podvin et al., 2011). Furthermore, both immunolocalization and flow cytometry demonstrated that Ecrg4 is expressed on the surface of circulating leukocytes (Baird et al., 2012). Here, we used a combination of cell surface biotinylation, confocal microscopy and mutagenesis to show that the cell surface localization of Ecrg4 in epithelial cells precedes its release into conditioned media. This release is also cell specific suggesting that, like with many neuropeptide pre-pro precursor proteins, tissue specific processing may control different Ecrg4 activities in different tissues.

MATERIALS AND METHODS

Cell culture and chemicals

Human embryonic kidney (HEK) and prostate cancer PC3 epithelial cells were maintained in DMEM containing penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS) at 37°C in an incubator supplemented with 5% CO_2/95% air. Phorbol-12-myristate-13-acetate (PMA) and all other chemicals were from Sigma Aldrich (St Louis, MO) unless specified otherwise.

Antibodies

A polyclonal IgY antibody was raised in chickens against recombinant human Ecrg4(71–148) and antigen affinity purified by commercial contract with GenWay Biotech, Inc., (San Diego, CA). For immunoblotting conditioned media, a commercial rabbit anti-human Ecrg4 primary antibody (Sigma-Aldrich) was used and a goat anti-rabbit–horseradish peroxidase (HRP) conjugated secondary antibody (JacksonImmuno Labs, West Grove, PA) used to detect protein on nitrocellulose membranes.

Immunostaining of cells for cell surface confocal microscopy

Non-permeabilized Ad-ECRG4-transduced HEK 293 were subjected to immunofluorescence staining following fixation in 2% paraformaldehyde in 2% glucose, 0.1 M sodium phosphate buffer, pH 7.2 for 20 minutes at room temperature. Cells were blocked with 5% normal donkey serum in 1% BSA for 30 minutes at room temperature. The affinity purified chicken anti-Ecrg4 IgY antibody (Genway, 1:2,000) was diluted in 1% BSA in PBS and incubated for one hour at room temperature. Following PBS washes, an Alexa 488-conjugated donkey anti-chicken secondary antibody (1:1000) was incubated for 1 hour at room temperature, washed in PBS and mounted in Slow fade for imaging. For the immunofluorescent analyses of cell surface staining in leukocytes, unpermeabilized cells were fixed and blocked as above, and incubated with chicken anti-Ecrg4 IgY (Genway, 1:2000) and goat polyclonal anti-CD14 (sc-6998, Santa Cruz, 1:250). Alexa-fluor-conjugated secondary antibodies were used (Molecular Probes, Eugene, OR, 1:700). All

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cells were counterstained with DAPI and imaged with an Olympus Fluoview 1000 (ASW 1.7b) laser scanning confocal microscope equipped with 10x/0.4N.A. or 20x/0.7N.A. dry objective lenses on a BX61 microscope (Olympus, Melville, NY). All images are x, y, or z projections from confocal stacks of 25–35 image slices.

Immunoblotting

Nitrocellulose membranes were blocked with PBS supplemented with 0.05% Tween 20 (PBS-T) containing 5% Bovine Serum Albumin (BSA) at RT for one hour. Rabbit anti-ECRG4 (Sigma) diluted into PBS-T containing 1% BSA at 1: 5000 was incubated with the membranes at 4°C for overnight with continuous shaking. Membranes were washed three times for 10 minutes each with PBS-T containing 1% BSA at RT. Goat anti-rabbit IgG-HRP (Horseradish peroxidase, BioRad) diluted into PBS-T containing 1% BSA at 1: 10,000 was incubated with the membranes for one hour at RT. The membranes were washed three times for 10 minutes each with PBS-T at RT. SuperSignal West Pico Chemiluminescent Substrate (Pierce) was added onto the membranes and incubated for 3 minutes at RT. The membranes were then processed to image immunoreactive bands using an IVIS® Lumina imaging system (Caliper Life Sciences, Hopkinton, MA, USA).

Transient transfection of HEK and PC3 cells

Cells were trypsinized and seeded at 8×10^5 per 60mm dish. The following day, cells were refreshed with DMEM containing 10% FBS without penicillin and streptomycin. Two hours later, cells were transfected with plasmid DNA using Lipofectamine2000 (Invitrogen) according to vendor's instructions. Briefly, 5.0 μl of the Lipofectamine2000 was added to 100 μl of OPTI-MEM, vortex mixed for a few seconds, and incubated at RT on the bench top for 5 minutes. Two μg of plasmid diluted into 100 μl of OPTI-MEM was mixed with 100 μl of this Lipofectamine2000 mixture, vortexed for a few seconds and incubated at RT for another 20 minutes. The plasmid-Lipofectamine2000 cocktail was then added drop-wisely onto target cells and incubated for a further 30 hours before cell surface labeling and lysis.

Adenovirus (Ad) Expressing Ecrg4

In some experiments, we used an Ad vector containing either a transgene for Ecrg4 (AdEcrg4) or GFP (Adgfp). The virus was prepared according to manufacturer's instructions using the AdEasy XL Adenoviral Vector System (Agilent Technologies, Santa Clara, CA, USA). Primers used to amplify the human Ecrg4 ORF were 5′-TATGTCGACCCGCCATGGCTGCC-3′ (forward) and 5′-TATAAGCTTAGTAGTCATCGTA-3′ (reverse). Adenovirus was produced in their proprietary AD-HEK293 cells optimized for virus production. Virus was purified and titered with the Adeno-X Virus Purification Kit (Clontech Laboratories, Inc., Madison, WI, USA).

GFP fluorescence microscopy

Cells in chamber slides were transfected as described above. Thirty hours after transfection, cells were fixed and localization of GFP was observed under confocal microscopy.

Ecrg4 plasmids and mutagenesis

Plasmids expressing full-length Ecrg4(1–148), Ecrg4(1–132) (CΔ16-Ecrg4), Ecrg4(1–140) (Δ8-Ecrg4), Ecrg4(1–144) (Δ4-Ecrg4), the $R^{67}A$ and $K^{69}A$ mutant ($Ala^{67,69}Ecrg4$) and $P^{131}A$ and $R^{132}A$ mutant ($Ala^{131,132}Ecrg4$) were amplified by standard PCR or PCR-mediated site-directed mutagenesis. All the fragments were purified and subcloned into pcDNA3. The templates used for all plasmids were the human C2orf40 cDNA (Origene, Cat# SC104814). The sense primer, hEcrg4U, carrying a HindIII restriction site was the

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same for all constructs Table 1 and the anti-sense primers for Ecrg4(1–148), CΔ16-Ecrg4, CΔ8-Ecrg4, CΔ4-Ecrg4, and $Ala^{131,132}$ all carried an in-frame stop codon and a BamHI restriction site. The two inner primers for $Ala^{67,69}$ carried the appropriate substituted codons to change the assigned amino acid and chimeric plasmids Ecrg4(1–30)-GFP and Ecrg4(16–30)-GFP were fused in-frame to express on the NH_2-terminus of GFP and from the backbone of pEGFP-N1 (Clontech, Mountain View, CA). The coding sequence of Ecrg4(1–30) was amplified by PCR with the sense primer hECRG4U and anti-sense primer carrying a BamHI restriction site. The coding sequence for Ecrg4(16–30) was generated by annealing their sense and anti-sense oligos that carry a HindIII restriction site on their 5′ and a BamHI restriction site on their 3′ respectively. All constructs were sequenced to confirm their identities (Retrogen, San Diego, CA) and the plasmids were used for transient transfection were prepared using EndoFree Plasmid Maxi Kit from Qiagen.

Analyses of conditioned media

Aliquots of the medium conditioned during the 30 hour transfection period were clarified by centrifugation and 1% of media (26 μl) used for immunoblotting. As indicated in the text, transfected cells were washed and incubated with buffer or 2.0 μg/ml PMA for an additional 30 minutes at 37°C prior to harvesting the conditioned media.

Analyses of cell lysates, biotinylated cell surface and released proteins

Cell surface biotinylation was performed as described (Trudel et al., 2000) using the Cell Surface Protein Isolation Kit (Pierce, CAT# 89881) with some modifications. Thirty hours after transfection (see above), cells were washed twice with ice-cold PBS and followed by two washes of ice-cold PBS. As indicated in the text, cells were also washed with either 50 mM Glycine (pH 2.8), ice-cold 2.0 M NaCl (pH7) or ice-cold 0.1 M Na_2CO_3 (pH11). The cells were then washed again with ice-cold PBS and incubated with 4 ml of ice-cold EZ-Link Sulfa-NHS-SS-Biotin for 30 minutes with continuous shaking on ice after which the labeling reaction was stopped by adding 200 μl of the quenching solution provided in the kit. Cells were scraped, harvested by centrifugation, washed with Tris Buffered Saline (TBS), and then lysed with 300 μl of RIPA buffer (50mM Tris-Cl pH 8.0, 150 mM NaCl, 0.1% SDS, 1.0 mM EDTA, 0.5% Na-deoxycholate, and 1.0% Triton X-100) containing protease inhibitors. The lysates were sonicated with seven 1 second bursts and incubated on ice for 30 minutes. The cell lysates were then cleared by centrifugation and $1/12^{th}$ of the supernatant (8.4%) saved while the remaining supernatant was incubated at RT on a rotator for one hour with 50 μl neutravidin slurry pre-equilibrated in RIPA. The resins were washed 3 times with ice-cold RIPA buffer, centrifuged, and biotinylated proteins eluted with 25 μl of 2 X SDS loading buffer at 90°C for 10 minutes. The 8% aliquot saved as starting material was not processed and used to assess total Ecrg4 in cell lysates, while 100% of the neutravidin bound fraction (cell surface) and 26ul (1.1%) of the corresponding conditioned medium were resolved on 4–12% NuPAGE (BioRad), transferred to nitrocellulose membranes, and processed for immunoblotting as described below.

Evaluating the effects of PMA on cell surface Ecrg4

Biotin-labeled cells were washed once with PBS at room temperature immediately after biotinylation and then incubated with PBS alone or PBS containing 2.0 μg/ml PMA at 37°C for 30 minutes. The cells were processed as described above to determine Ecrg4 levels in cell lysates and on cell surface and 26 μl of the conditioned PBS was used for immunoblotting to measure release.

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RESULTS

HEK translocate Ecrg4 to the surface but do not release protein into media

To confirm the cell surface localization of Ecrg4, we transduced HEK cells with $pcDNA3_{Ecrg4}$ under control of the CMV promoter to overexpress 14kDa Ecrg4. We found that while cells expressed 14kDa protein (Fig 1a, lane 2), none was detectable in cell media conditioned for 30 hrs (Lane 6). Instead, a robust signal was observed in the cell surface fraction after neutravidin precipitation of biotinylated cell surface proteins (Lane 4). To ensure specificity of this cell surface detection, we demonstrated that Ecrg4 was only detectable on the cell surface when cells were transduced and biotinylated (Fig 2b). As shown in Panel c, this cell surface biotinylated Ecrg4 was not removed by washing cells with PBS, high salt (2M), low pH 2.8 (glycine) or high pH 11 (Na_2CO_3) which has been shown elsewhere to remove low affinity, ionic, ligand and non-covalently bound proteins, respectively (Trudel et al., 2000). Only trypsin pre-treatment of cells removed Ecrg4 from the cell surface (Lane 5). Finally, we evaluated Ecrg4 staining by confocal microscopy (Fig 1d) of non-permeabilized cells. We observed a punctate and non-uniform immunoreactive Ecrg4 staining on the cell surface as observed in projected confocal microscopy stacks of the stained cells and alternatively, using z-stack optical reconstructions of the sections (labeled a and b). We conclude that Ecrg4 is a membrane protein and specifically noted the presence of foci of Ecrg4 immunoreactivity on the cell surface rather than diffuse uniform staining.

PC3 cells release and process Ecrg4

Several investigators (Gonzalez et al., 2011; Gotze et al., 2009; Huh et al., 2009; Kujuro et al., 2010; Li et al., 2010a; Li et al., 2011; Ozawa et al., 2011; Podvin et al., 2011; Tadross et al., 2010), including ourselves (Gonzalez et al., 2011; Podvin et al., 2011), have shown that transduced cells release of Ecrg4-derived peptides into conditioned media of transduced cells and biological fluids. Transduced prostate cancer PC3 epithelial cells have the ability to express the transfected gene (Fig 2a, lane 3) and then secrete Ecrg4 immunoreactivity (lane 8) into their conditioned media. Similar to HEK cells (Fig 1), a significant portion of the immunoreactive Ecrg4 expressed on PC-3 cells could also be biotinylated on the cell surface (Fig 2 Panel a, Lane 6). Interestingly, a second 6–8 kDa molecular form of Ecrg4 was also detected in conditioned media (lane 8) but was not detected on the cell surface. This suggested that Ecrg4 was processed and released at the surface. Like with HEK cells, Ecrg4 in PC-3 cells was a membrane protein (Fig 2, Panel b). It was efficiently expressed (lanes 2–5), but not removed by extensive washing of cells with PBS (lane 7), low pH (lane 8), high salt (lane 9), or high pH (lane 10). The signal in PC-3 cells and HEK cells was removed from the cell surface with trypsin (Figure 1c and data not shown). We concluded that Ecrg4 was a cell membrane protein on all of the cell types examined, however, its release and processing appeared to be cell-type specific.

The amino terminus tethers Ecrg4 to the cell surface

Although GFP is generally regarded as a cytoplasmic protein, cells traffic GFP differently and some can even release the protein, albeit at low efficiency, into conditioned media (El Meskini et al., 2001a; El Meskini et al., 2001b; Lemberg, 2011). We therefore evaluated whether the Ecrg4 leader sequence would alter the intra- and extra-cellular distribution of GFP in HEK and PC3 cells (Figure 3). HEK cells maintain GFP in the cytoplasmic compartment that is readily detected in transduced cells (Panel a). However, fusion of the Ecrg4(1–30) leader sequence with GFP leads to a secretory and vesicular distribution. A similar pattern was observed in PC3 cells (Panel b). When the presence of GFP in conditioned media was monitored, HEK cells did not release GFP but the sequence of Ecrg4(16–30) increased secretion and could serve as a secretory leader peptide (Panel c). In contrast to HEK cells, PC3 cells released GFP after over-expression of the GFP gene (Panel

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d, Lane 2). This release was blocked by the native amino-terminus Ecrg4(1–30) (lane 3), but secretion was enhanced by the same leader sequence Ecrg4(16–30) (lane 4) that promoted GFP release in HEK cells. Together, these data all suggested that Ecrg4 was retained at the cell surface by an unusually long (30 amino acid) hybrid NH_2-terminus hydrophobic sequence that served two functions: ER-Golgi secretion and cell membrane retention.

Mutagenesis prevents processing but not release

To distinguish cell surface tethering from cell surface processing, we evaluated the behavior of a series of Ecrg4 mutants in both HEK (Figure 4, Panel a) and PC3 (Panel b) epithelial cells. Of the many mutants examined, none conferred HEK cells with an ability to condition their media with Ecrg4 (lanes 17–23). At the same time, all peptides with the exception of the NΔ(1–30)Ecrg4 mutant (which lacks the canonical hydrophobic leader sequence) were detected on the surface, and importantly none were found to be processed. The NΔ(1–30)Ecrg4 mutant was poorly expressed, with little was detected in cell lysates, presumably because of protein instability and mis-folding.

The fate of these mutant proteins was also evaluated in PC3 cells to determine the role of processing in Ecrg4 release from the cell surface (Figure 4, Panel b). Whereas the mutants were expressed in cells (lanes 3–6) and localized to the cell surface as 14kDa proteins (lanes 9–12), mutagenesis of protein convertase/furin consensus sites at $Arg^{67}Lys^{69}$ eliminated any low molecular weight fragment of Ecrg4 in conditioned media (lane 17). This allowed us to tentatively identify the 6–8 kDa product as the result of cleavage at $Arg^{67}Lys^{69}$ of Ecrg4(1–148). We also noted a small increase in the apparent molecular weights of the $Ala^{132,133}$ Ecrg4(1–148) on the cell surface (lane 12) and in conditioned media (lane 18), compared to their respective Ecrg4(1–148) controls (lane 9 and 14 respectively). This suggested that the cell surface Ecrg4 was the CΔ16-Ecrg4(1–132) form of Ecrg4. Indeed MALDI-TOF sequencing of peptides in conditioned media by the UCSD peptide sequencing core facility unambiguously identified the CΔ16 peptide NH_2-SPYGFRHGASVNYDDY-COOH (not shown). Closer examination of the molecular weights of Ecrg4 and $Ala^{132,133}$ Ecrg4(1–148) present on the surface of HEK cell (Panel a, lanes 12 and 15) also suggested processing to Ecrg4(31–132) because the slightly higher size of $Ala^{132,133}$ Ecrg4(1–148). Together these findings suggested that the CΔ16 peptide was released into conditioned media by cultured cells.

PMA stimulates release of Ecrg4 from the cell surface

A short-term exposure of cells to the phorbol ester PMA activates cell surface proteases and induces the shedding of growth regulatory membrane proteins (Le Gall et al., 2003). Therefore, we investigated whether PMA would alter the distribution of Ecrg4 on the PC3 cell surface (Figure 5). Cells were washed with PBS and the conditioned media replaced with PBS containing PMA. Thirty minutes later, the conditioned PBS was collected, the cell surface biotinylated, and the cell lysates analyzed for Ecrg4 content. While the amounts in cell lysates were unchanged by PMA (lane 1 and 2), Ecrg4 was shed from the cell surface by incubating cells with PMA (lane 3 and 4), with a cognate 14 kDa Ecrg4 peptide detected in conditioned PBS (lane 5 and 6). We concluded that Ecrg4 was released from the cell surface and likely regulated by PMA-dependent cell surface proteases.

DISCUSSION

The data presented here establish that Ecrg4 is a cell membrane protein that can be stimulated with cell surface enzyme activators like PMA (Higashiyama and Nanba, 2005; Iwamoto and Mekada, 2000; Le Gall et al., 2003) for release from the cell surface. Contrary to bioinformatic predictions (Mirabeau et al., 2007), Ecrg4 does not behave like a traditional

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pre-pro-neuropeptide protein precursor, but instead resembles a growth factor (Feige and Baird, 1995). Its ubiquitous distribution, cellular localization and pleiotropic activities are reminiscent of cytokines like tumor necrosis factors (Garcia et al., 2011; Horiuchi et al., 2010), chemokines like fractalkines (Proudfoot, 2006; Schafer et al., 2004) and growth factors like epidermal/transforming growth factors (Dempsey et al., 2003; Rall et al., 1985) that are cell membrane proteins that are released by cell surface processing. This should have significant ramifications in understanding the physiological function and tumor suppressor activity of Ecrg4. For example, the cell bound protein may have paracrine and/or autocrine functions in cell homeostasis while proteolytically processed peptides may have different functions altogether (Figure 6). To this end, it is interesting to note that the peptides responsible for Ecrg4 activities remain unknown. Ecrg4 gene overexpression modulates epithelial cell proliferation and migration, neuronal cell function and senescence and even progenitor cell survival and neuroepithelial function. Yet these experiments generally use over-expression of Ecrg4 gene *in vitro,* viral delivery of the Ecrg4 gene *in vivo* or gene knock-down with morpholinos (Gonzalez et al., 2011; Gotze et al., 2009; Huh et al., 2009; Kujuro et al., 2010; Li et al., 2010a; Li et al., 2011; Ozawa et al., 2011; Podvin et al., 2011; Tadross et al., 2010). Accordingly, the identity of the Ecrg4-encoded peptide(s) responsible for activity may be the cell surface bound peptide, or one of several peptides processed at and released from the cell surface. For example, one such peptide, Ecrg4(71–148), has a neuropeptide hormone-like function in the hypothalamus by stimulating the release of corticotrophin-releasing hormone (Tadross et al., 2010).

The first clue that Ecrg4 might be a cell membrane protein arose from an observation by one of us (SP) that Ecrg4 was resistant to detergent extraction. In the course of this work, we determined that the most reproducible technique for extraction, immunoblotting and tissue processing was enhanced in 4% SDS. A closer examination of immunohistochemical staining of Ecrg4 in leukocytes also pointed to a cell surface localization suggesting that Ecrg4 was a cell membrane protein (Baird et al 2012). This hypothesis was confirmed here by cell surface biotinylation (Figure 1) that unequivocally established the presence of Ecrg4 on the cell surface of transduced cells. Neither (1) high salt, which releases ionic interactions like those responsible for binding to cell surface proteoglycans, (2) low pH, which releases ligand-receptor interactions or (3) Na_2CO_3, which removes all non-covalently bound proteins from the cell surface was able to remove Ecrg4 from the cell surface. This points to a tight, likely covalent, and transmembrane tethering of Ecrg4 on the cell surface.

While we cannot conclusively point to a transmembrane domain, the data are all compatible with bioinformatic algorithms that recognize an unusually long hydrophobic leader sequence that could serve as a dual leader and trans-membrane domain for secretion and tethering. When this 30 amino acid hydrophobic peptide, Ecrg4(1–30), was fused in-frame to the NH_2-terminus of GFP, GFP was trapped in the secretory compartment of both HEK and PC3 cells (Figure 3, Panels a and c). In contrast, immunoblotting of conditioned media after transduction with the Ecrg4(16–30) promoted GFP secretion (Figure 3, Panels b and d) suggesting a dual function for the amino terminal Ecrg4(1–30) leader peptide. By inference, this points to Ecrg4 having a transmembrane tethering domain, but analogous experiments with the Ecrg4(1–15)-GFP fusions were equivocal (data not shown). We assume that cell surface tethering requires the secretory domain found in Ecrg4(16–30) for the protein to enter the secretory compartment after synthesis.

Two separate experiments point to there being cell-specific and dynamic control of Ecrg4 at the cell surface. First, mutagenesis of Arg^{67}/Lys^{69} to $Ala^{67,69}$ and of Pro^{131}/Arg^{132} to $Ala^{131,132}$ that target protein convertases and thrombin consensus sequences respectively, had no effect on the appearance of Ecrg4 onto the cell surface of either HEK or PC3 cells (Figure 4). While this suggested that proteolytic processing of Ecrg4 was extracellular, a 14

kDa Ecrg4 was detected in media conditioned by PC3 cells showing that processing was not required for release. Smaller peptides in PC3 conditioned media suggested proteolytic processing in media or at the time of release. As seen by treatment of PC3 cells with PMA, it was the 14 kDa peptide that was first released from the cell surface. This proteolytic processing may have a regulatory function in controlling Ecrg4 bioavailability.

On a final note, it is interesting to point out that there is a presumption that the down-regulation of Ecrg4 gene expression observed in cancer and after injury provides a growth advantage to tumor and repair cells respectively (Gotze et al., 2009; Li et al., 2010a; Li et al., 2011; Li et al., 2009; Li et al., 2010b; Mori et al., 2007; Yue et al., 2003). These data are also consistent with our observation that, while Ecrg4 is widely distributed in vivo, gene expression by cells in culture is low to non-detectable. We assume this is because cell culture reflects an injury phenotype. Still, the specific nature of the growth advantage conferred by Ecrg4 down regulation has not yet been identified. PMA, a well recognized transforming agent (Higashiyama and Nanba, 2005; Le Gall et al., 2003) that we show can strip the cell surface of Ecrg4 in vitro may thus abrogate its inhibitory activity at the cell surface if exposed in vivo. To this end, it has also not escaped our attention that Ecrg1, a gene that was co-identified with Ecrg4 by its association with esophageal cancer, is an extracellular enzyme that interacts with Ecrg4 (Li et al., 2011). Both factors may thus act in concert to regulate cell growth via Ecrg4 processing. If so, it underscores the possibility that Ecrg4, like most neuropeptide precursor-like proteins, plays multifunctional roles that are directly dependent on where it localizes, when it is expressed, how it is processed and the mechanisms that make it available to its target cells. In this way, it may be possible to ascribe a sentinel function to the cell surface 14 kDa precursor peptide that is distinct from the pro and/or anti-inflammatory signals that control cell growth, migration and differentiation after its release and processing (Baird et al 2012).

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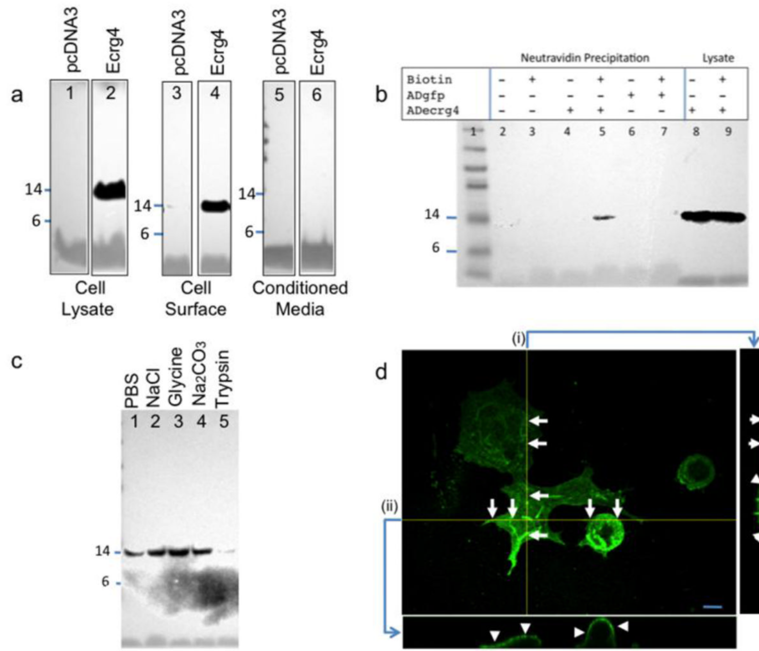

Figure 1. Ecrg4 encodes a 14 kDa protein that is tethered to the surface of HEK cells

Panel a: Expression of full-length human Ecrg4 cDNA in HEK cells produces a 14 kDa augurin that is detectable in the total cell lysates (lane 2) and on the surface (lane 4) after cell surface biotin labeling combined with Neutravidin pull-down. No peptide is detected in conditioned media (lane 6) or when cells are not transduced (lanes 1, 3, 5). **Panel b:** cell surface localization by biotinylation is specific and a signal is only detected when cells are transduced with Ecrg4 (plasmid or Adenovirus) and biotinylated. **Panel c** The cell surface-bound, biotin-labeled Ecrg4 stays on the cell surface after washing with PBS (lane 1), 2.0 M NaCl (lane 2), 50 mM glycine pH 2.8 (lane 3), or 0.5 M Na_2CO_3 pH 11(lane 4) but is digested off with trypsin (lane 5). **Panel d** HEK cells were transfected with AD_{Ecrg4} and stained with an anti-Ecrg4 antibody then viewed and imaged by fluorescent confocal microscopy. Bar = 10 μm. Arrows indicate X and Y cross-sectional planes shown as i and ii that were used to obtain the z-stacks and establish the uneven, focal and punctate distribution of immunoreactivity on the cell surface

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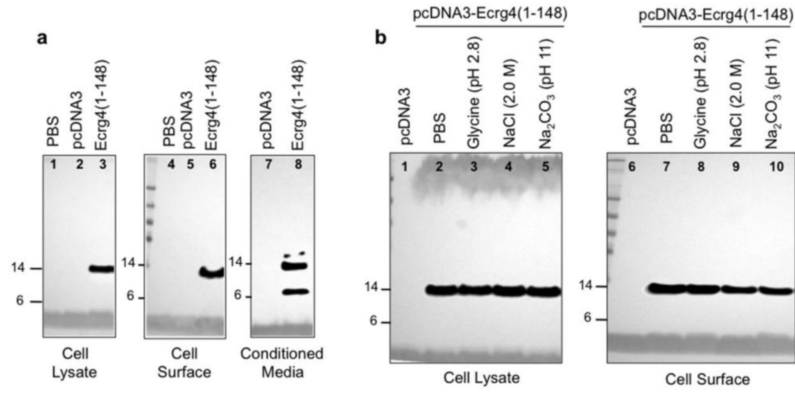

Figure 2. Ecrg4 encodes a 14 kDa protein that is tethered to but released and processed from the surface of PC3 epithelial cells

Panel a: Expression of full-length human Ecrg4 cDNA in PC3 cells produces a 14 kDa augurin that is detectable in the total cell lysates (lane 3) and on the surface (lane 6) after cell surface biotin labeling combined with Neutravidin pull-down. Two peptides are detected in conditioned media (lane 8) but when control or mock-transfected cells are examined no protein is detected (lanes 1, 2, 4, 5 and 7). **Panel b** Regardless of the presence of Ecrg4 in conditioned media, the intracellular and cell surface-bound/biotin-labeled Ecrg4 remain after washing with PBS (lane 2 and 7), 50 mM glycine pH 2.8 (lane 3 and 8), 2.0 M NaCl (lane 4 and 9) or 0.1 M Na_2CO_3 pH 11(lane 5 and 10). Mock-transfected cells have no detectable Ecrg4 (lanes 1 and 6)

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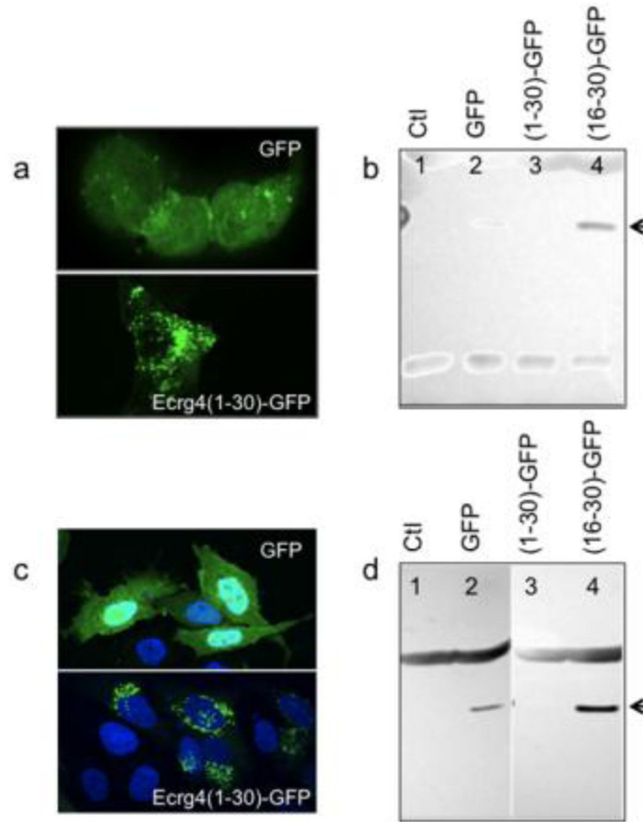

Figure 3. The amino terminus of Ecrg4 directs secretion and tethering

Panel a: Fluorescence microscopy of HEK cells transduced with GFP or Ecrg4(1–30)-GFP fusion show dramatically different patterns of green fluorescence that are uniform or punctate-perinuclear respectively. **Panel b** Immunoblotting of conditioned media for GFP using anti-GFP antibodies reveals that GFP is not released by HEK cells (lanes 1–3) but that the Ecrg4(16–30) sequence can deliver GFP outside of the cell (lane 4). **Panel c** Fluorescence microscopy of PC3 cells transduced with GFP or Ecrg4(1–30)-GFP fusion show similarly different patterns of green fluorescence that are also uniform or punctate-perinuclear respectively. Nuclei are identified with DAPI and shown in blue. **Panel d** Immunoblotting of conditioned media for GFP reveals a low level of GFP release by PC3 cells (lane 2) that is blocked by the Ecrg4(1–30) sequence (lane 3) but enhanced by the Ecrg4(16–30) sequence which delivers GFP outside of the cell (lane 4).

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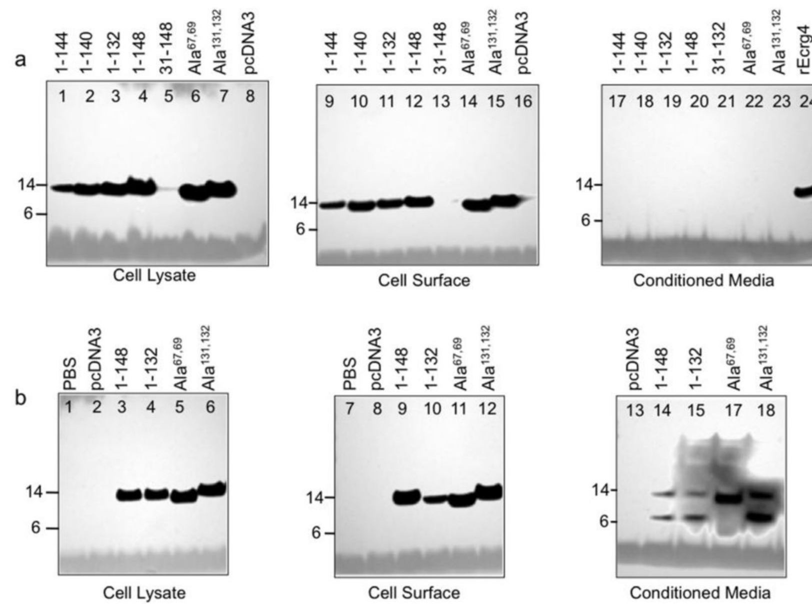

Figure 4. Structural basis for augurin tethering to the surface in HEK and PC3 cells

Panel a Cell surface tethering is not dependent on the tyrosine sulfation consensus sequences at the C-terminus (Δ4 and Δ8), the thrombin consensus sequence (Δ16 and Ala^{131,132}) or the furin cleavage site (Ala^{67,69}) because Ecrg4 was detected in HEK cell lysates 30 hours after transduction (Lanes 1–4 and 6, 7). A cognate signal of biotinylated Ecrg4 mutants is detected on the cell surface (Lanes 9–12, 14,15) and no signal was detected in conditioned media (lanes 17–23). Note that cells do not express the NΔ30 mutant that lacks the Ecrg4(1–30 sequence). Recombinant (r) Ecrg4(31–148) (Lane 24) was expressed in *E coli*. **Panel b:** Cell surface tethering is not dependent on the thrombin consensus sequence (Δ16 and Ala^{131,132}) or the furin cleavage site (Ala^{67,69}) because Ecrg4 was detected in PC3 cell lysates 30 hours after transduction (Lanes 3–6) and a cognate signal of biotinylated Ecrg4 mutants is detected on the cell surface (Lanes 9–12) and no signal was detected in control lysates or cell surface (lanes 1, 2 and 7, 8). The Ecrg4 in media however is dependent on processing as reflected by (1) the single band in (Ala^{67,69}) mutant (lane 17) (2) an apparent increase in molecular weights of Ala^{131,132} mutants that suggest a loss of the thrombin CΔ16 peptide and the equivalent molecular weight in the CΔ16 and Ecrg4(1–148) mutants.

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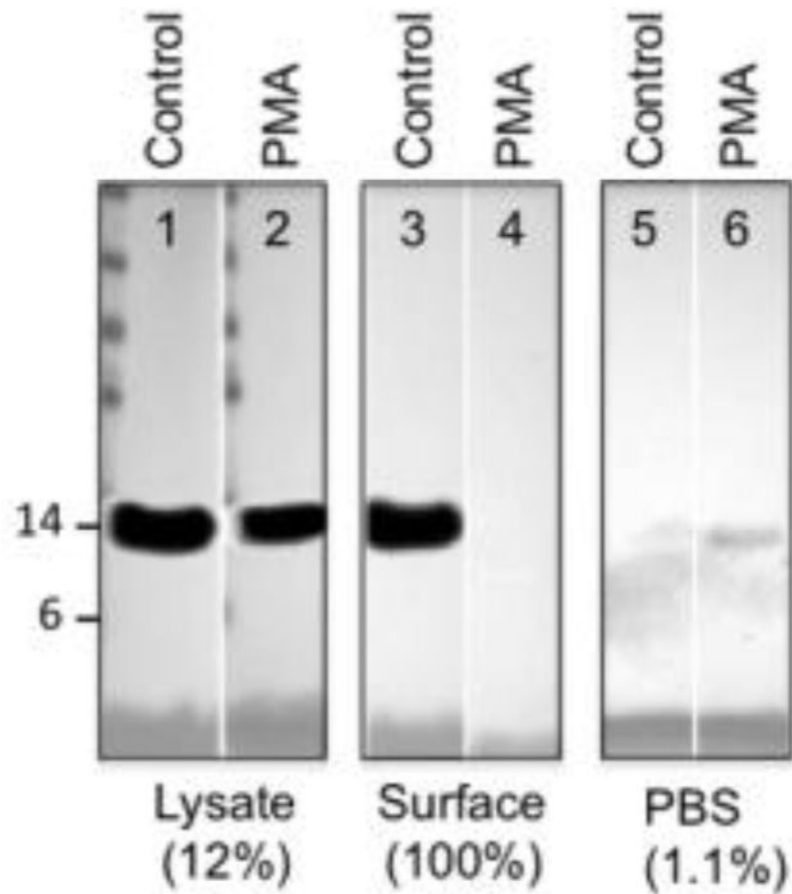

Figure 5. PMA stimulates release from the PC3 cell surface

Ecrg4 transfected PC3 cells were washed with PBS and left untreated (control) or treated for 30 minutes with 2.0 μg/ml the phorbol-12-myristate-13-acetate (PMA) and Ecrg4 in cell lysates (lanes 1 and 2) and on the cell surface (lanes 3 and 4) PBS (lane 5 and 6) determined by immunoblotting.

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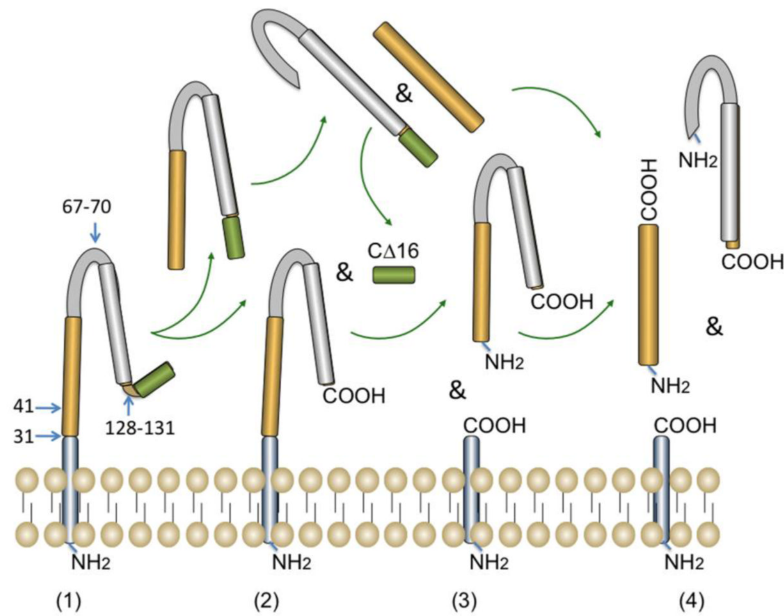

Figure 6. Model for Ecrg4 on the epithelial cell surface

Ecrg4 encodes a sentinel-like pre-pro- precursor protein that (1) localizes to the cell surface via its NH_2- terminus. There, it can generate multiple peptide products depending on how it is processed. Immediate processing of Ecrg4(1–148) at a thrombin-like Pro^{131}-X-Arg^{132} consensus sequence (2) releases the COOH peptide CΔ16 product leaving Ecrg4(1–132) at the cell surface but constitutive and stimulated proteases at the cell surface release the protein by targeting the cell surface interface (3) or via protein convertases (4) targeting the dibasic cleavage sites at Arg^{67}-X-Lys^{69} generate several smaller peptides.

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Table 1

Primer sequences used for analyses

hEcrg4U	5′-TCCTTGAAGCTTGCCCCGCCGCCATGGCTG-3′
Ecrg4(1–148)	5′-ATTCGGATCCATGGTTAGTAGTCATCGTA-3′
CΔ16-Ecrg4	5′-ATTCGGATCCTTACCGGGGACCAATTGCAGA-3′
CΔ-8-Ecrg4	5′-ATTCGGATCCTTATCCATGCCTAAAGCCGTA-3′
CΔ-4-Ecrg4	5′-ATTAGGATCCTTAGTTGACGCTGGCTCCATG-3′
Ala^{131,132}	5′ATTCGGATCCTTAGTAGTCATCGTAGTTGACGCTGGCTCCATGCCTAAAGCCGTAGGGGCTCGCGGCACCAATTGCAGAGTCTTC-3′
Ala$^{67, 69}$	5′-TTCCTTGGCAGCCTGAAGGCCCAGGCGCGGCAGCTGTGGGACCGG-3′ 5′-CCGGTCCCACAGCTGCCGCGCCTGGGCCTTCAGGCTGCCAAGGAA-3′
Ecrg4(1–30)	5′-ACTTGGATCCCGACTTATGCCACCTGGGCC-3′
Ecrg4(16–30)	5′TCCTTGAAGCTTCTGGCGCTGCTCCTGCTCCTGTGCTGGGGCCCAGGTGGCATAAGTCGGGATCCACCGA-3′