ETL, a Novel Seven-transmembrane Receptor That Is Developmentally Regulated in the Heart

ETL IS A MEMBER OF THE SECRETIN FAMILY AND BELONGS TO THE EPIDERMAL GROWTH FACTOR-SEVEN-TRANSMEMBRANE SUBFAMILY*

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Using differential display of rat fetal and postnatal cardiomyocytes, we have identified a novel seven-transmembrane receptor, ETL. The cDNA-predicted amino acid sequence of ETL indicated that it encodes a 738-aa protein composed of a large extracellular domain with epidermal growth factor (EGF)-like repeats, a seven-transmembrane domain, and a short cytoplasmic tail. ETL belongs to the secretin family of G-protein-coupled peptide hormone receptors and the EGF-TM7 subfamily of receptors. The latter are characterized by a variable number of extracellular EGF and cell surface domains and conserved seven transmembrane-spanning regions. ETL mRNA expression is up-regulated in the adult rat and human heart. In situ hybridization analyses revealed expression in rat cardiomyocytes and abundant expression in vascular and bronchiolar smooth muscle cells. In COS-7 cells transfected with Myc-tagged rat ETL, rat ETL exists as a stable dimer and under goes endoproteolytic cleavage of the extracellular domain. The proteolytic activity can be abolished by a specific mutation, T455A, in this domain. In transfected mammalian cells, ETL is associated with cell membranes and is also observed in cytoplasmic vesicles. ETL is the first seven-transmembrane receptor containing EGF-like repeats that is developmentally regulated in the heart.

A complex series of events takes place during growth and maturation of cardiac myocytes. The proliferative growth of cardiac myocytes is primarily limited to fetal and early neonatal periods of development (1). Postnatal maturation of cardiac myocytes is marked by cellular hypertrophy and is also accompanied by ventricular remodeling of the nonmyocyte compartment, such as extracellular matrix formation and coronary angiogenesis (2). The signals that coordinate these processes in cardiac muscle are not well understood, but several growth factors and hormones have been shown to influence heart development (3, 4). It is becoming clear that developmentally regulated gene expression of specific extracellular factors and their cognate receptors contributes to cardiac muscle differentiation (4–9).

Complex cellular responses, such as proliferation and differentiation, are frequently modulated by external stimuli. Intra cellular signaling cascades, in turn, act as mediators to translate the stimulus into transcriptional activity. A large family of receptors involved in a broad spectrum of cell signaling is the G-protein-coupled seven-transmembrane (TM7) receptor (GPCR) family. This family of molecules mediates signals from hormones, cytokines, light, and odors (10). GPCRs, activated by humoral, endothelial, or platelet-derived factors, are also able to stimulate mitogen-activated protein kinase pathways (11, 12), signaling intermediates involved in cellular mitogenesis and proliferation.

GPCRs have a common topology characterized by an extracellular N terminus, seven membrane-spanning helices flanked by a cytoplasmic tail. A group of receptors that shares homology in the heptahelical region and activated by peptide hormones is the secretin receptor family (13, 14). Recently, a subfamily of secretins has emerged that exhibit cell-surface interaction and cell adhesion modules in unusually large extracellular domains (15–19). This novel subtype of GPCRs consists of a small number of EGF-TM7 receptors such as EMR1, a receptor of neuroectodermal origin (20), its mouse homolog, F4/80 (21), and CD97, a leukocyte-activating antigen (22). All three receptors contain EGF modules and mucin-like domains in the N terminus. The recently discovered Celsr1 gene also belongs to the EGF-TM7 group. In addition to EGF repeats, Celsr1 contains cadherin and laminin type repeats (16).

In an effort to pinpoint the stimuli and signal transduction machinery that regulate the transition of myocyte from hyperplasia to hypertrophy, coronary capillary formation, and extracellular matrix deposition in the mammalian heart, we conducted differential display analysis on mRNAs using fetal and adult cardiomyocytes. As a result, we have identified a new member of the EGF-TM7 receptor family named ETL (for EGF-TM7-latrophin-related protein). The large extracellular domain of rat ETL consists of EGF modules, a Ser/Thr rich linker region, and a Cys-rich proteolysis domain. A seven-transmembrane region is followed by a short cytoplasmic tail. Besides having structural homology to the EGF-TM7 family, ETL shares considerable similarity with closely related heptahelical receptors CL1 (calcium-independent receptor for late-
toxin (CIRL)/latrophilin 1), CL2, and CL3 (18, 19). The similarity with latrophilins includes a Cys-rich domain that may direct endoproteolytic cleavage of the extracellular domain. The expression of the ETL mRNA is developmentally regulated in the heart, suggesting that ETL seven-transmembrane receptors may be important in cardiac switching from fetal to adult phenotypes.

**EXPERIMENTAL PROCEDURES**

**Differential mRNA Display**—Purified preparations of cardiomyocytes were generated as described (23, 24) from 50 embryonic day 16, postnatal day 1, day 3, day 5, and day 12 rat hearts. Total cellular RNA was carried out using a RNAimage kit (GenHunter) according to the manufacturer’s instructions. Differentially displayed bands were excised from the polyacrylamide gels and reamplified. The resultant PCR amplicons were tested for differential expression on Northern blots using whole heart poly(A\(^+\)) RNA and then used as probes to screen cDNA libraries for full-length transcripts.

**cDNA Library Construction and Screening**—Double-selected poly(A\(^+\)) mRNAs from rat heart and total RNAs from lung were obtained using Messagemaker (Life Technologies, Inc.). The cDNA libraries were constructed using a Superscript cDNA synthesis kit. The cDNAs were size-fractionated, adapted with linkers, ligated into ZipLox arson (Life Technologies, Inc.), and packaged using GigapackIII Gold (Stratagene). Approximately 30 clones were incorporated into the ETL cDNA contig. The GenBank\(^\text{TM}\) accession number for rat ETL is AF192401, and the accession number for the type II isof orm is AF192402.

**Nucleotide Sequencing and Analysis**—Clones obtained from cDNA library were sequenced and a contig was compiled using Sequencer 3.0 software. The consensus sequence was analyzed by Blast for homologies and ExPaSy tools for protein motifs and patterns.

**HR Mapping**—Primers RP29.2A–RP29.2B, that amplify a genomic Hs human Bac RP11–29e12 (Research Genetics), were used in PCR with DNAs from medium resolution (G3) and high resolution (TG3) radiation hybrid panels (Research Genetics). Results were analyzed using the Stanford RH mapping database (available on the World Wide Web).

**RT-PCR of hETL** (Human ETL)—Four human-specific primer pairs were used to amplify hETL: 29.13A-B, 29.18A-11B, 29.8A-B, and 29.7A-B. The GenBank\(^\text{TM}\) accession number for a partial hETL cDNA sequence is AF192403.

**Human ETS Incorporated into the Contig of ETL**—Incorporated ESTs had the following GenBank\(^\text{TM}\) accession numbers: AA64110, W72803, H000259, AA487872, T10363, AI024874, AI024852, AA266994 (EST 112146), AI429173, AW60713, W65553, AI802994, AW20465, AI241562, AA478797, AI093076, AA730682, and AA724866.

**Northern Blot Analyses**—Equal amounts of RNA were run on formaldehyde gels, blotted on nylon membranes (GeneScreen) and UV-cross-linked. Membranes were stained with methylene blue for visualization. Hybridizations were performed in Express hybridization solution (CLONTECH) followed by washes and autoradiography. For normalization of poly(A\(^+\)) RNAs, blots were probed with GAPDH and quantified by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA).

**Construction of Expression Vectors**—The cDNA sequence corresponding to the ETL open reading frame was amplified by RT-PCR from rat heart using primers 69F50XH and 69XBB, containing 5' overhangs. The amplified product was cloned into pBluescript, and 10 clones were sequenced. Clone 1 contained no mutations, while clone 3 overhangs. The amplified product was cloned into pBluescript, and 10 clones were sequenced. Clone 1 contained no mutations, while clone 3 contained a T455A substitution. These were selected and subcloned in 28.7A-B. The GenBank\(^\text{TM}\) accession number for a partial hETL cDNA sequence is AF192403.

**Transfection into COS-7 Cells and Western Blotting**—1.0 × 10\(^6\) COS-7 cells were seeded into six-well dishes and transfected with constructs using LipofectAMINE reagent (Life Technologies, Inc.) and 1 µg of plasmid DNA purified with a Qiagen column. Proteins were harvested 48 h after transfection in 300 µl of triple detergent solution (25). Ten µl of total protein extracts and 10 µl of a molecular weight ladder (New England Biolabs) were heated in the presence of 1% β-mercaptoethanol and resolved in Laemmli buffer (25) on 4–20% SDS-polyacrylamide gel electrophoresis gradient gels (Bio-Rad) or 7.5% non-gradient gels with 8 µm urea. Rat ETL (ETL)–Myc was visualized using mouse monoclonal anti-Myc antibody (Oncogene Research Products) and the appropriate secondary antibody for ECL detection (Amersham Pharmacia Biotech). Peroxidase-labeled goat anti-human antibody (Kirkegaard & Perry Laboratories), followed by ECL detection, was used for ETL–Fc protein visualization. Membrane preparations were processed as described (17). Conditioned medium from COS-7 cells, transiently transfected with the 1–455-Myc extracellular domain of ETL was concentrated using a Centricon-3 spin column and loaded on SDS-gels. Proteins were electrophoresed and transferred to nitrocellulose, and hybridized with a DIG RNA labeling kit (Roche) and used in hybridization at 1 ng/µl. Paraffin-embedded 2-week-old rat lung and heart tissues were sectioned, 8–9 µm thick, deparaffinized in Hemo-DE, hydrated through a series of graded ethanol and water, and treated with proteinase K at 6 µg/ml for 90 min. Sections were hybridized overnight at 60 °C in a buffer containing 50% deionized formamide, 10% dextran sulfate, 1× Denhardt’s solution, 100 µM dithiothreitol, 20 µM Tris–HCl, pH 7.5, 5 µM EDTA, 300 mM NaCl, 1% SDS, 1× NTE, and 50% formamide, 2× SSC, 20 µM dithiothreitol for 30 min at 50 °C; 1× NTE for 15 min at 37 °C. Immunological detection was performed using a DIG nucleic acid detection kit (Roche Molecular Biochemicals). Washes consisted of 2× SSC for 5 min, 0.1× SSC for 15 min, buffer 1 (Roche) for 15 min, buffer 2 containing 20% sheep serum for 30 min. Washes were followed by incubation with anti-DIG-AP conjugate, 1.500 dilution, for 2 h. Post incubation washes were performed in buffer 1 (Roche Molecular Biochemicals) three times for 10 min and buffer 3 for 5 min, followed by incubation with the substrate, 1% nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate in buffer 3 (Roche Molecular Biochemicals). Development was carried out for several hours and terminated for both antisense and sense slides at the same time. Slides were counterstained in 0.5% methyl blue, washed in water and 100% butanol, dehydrated in Hemo-DE, mounted, and photographed using a Zeiss microscope.

**Immunofluorescence and Confocal Microscopy**—COS-7 cells were seeded at a density of 4 × 10\(^4\) on chamber slides, transiently transfected with an ETL–Myc expression construct, allowed to recover for 24–48 h, serum-starved for 4 h where indicated, and stained for immunofluorescence using a rabbit polyclonal antibody to ETL (Vector Laboratories) per-mevalinized in 0.2% Triton X-100, and blocked in 1% nonfat milk. Cells were then incubated with anti-Myc antibody (Oncogene Research Products) followed by appropriate secondary IgG conjugated to biotin and Alexa-594 conjugated to streptavidin (Molecular Probes, Inc., Eugene, OR). Cells were examined by fluorescent microscopy using a Texas Red filter or by confocal microscopy at 584-nm wavelength.

**Primers**—The following primers were used: 29.13A, GAAATTTAAC- TCACTGCTTGGG; 29.13B, GTCCCAAACACTAAGTGATCC; 29.18A, GCTGATCATCTTCTTACAGTT; 29.11B, AGTAAATCTTAAAAATGCACATAGACT; 29.2B, AGGCACACAGGCCTACA; 29.2B, ATAGGAGATGGTTGATAATGC; 29.2A, CCCACCCATATACGACATGGTA; 29.8B, GGCAATGCCTGCCATATGATAAGGCT; 29.7A, GCAGATCAGCT- TATCTCATTGT; 29.7B, TCAAGGTCGCAACCGAGTCACAGA; 69F50XH, CGTTCAGGAATAAAGGGGACTCTCCTCTTCTCT; 69CFB, GGAT- CCTCTTAAACATTTCAACCAACAGCAG; 69F50B, GGATACATCAATAAT- GGACCTCCCTCCTGGCTTCT; 69CFB, GGATACATCAATAAT- GGACCTCCCTCCTGGCTTCT; 69CFB, GGATACATCAATAAT- GGACCTCCCTCCTGGCTTCT; 69CFB, GGATACATCAATAAT- GGACCTCCCTCCTGGCTTCT; 69CFB, GGATACATCAATAAT- GGACCTCCCTCCTGGCTTCT.

**RESULTS**

**ETL Is Up-regulated in the Heart after Birth**—To identify genes involved in heart development, we conducted a differential display of fetal and postnatal mRNAs isolated from purified cardiac cardiomyocytes using fetal, day 1, day 3, day 5, and day 12 rat hearts. Several genes were identified by RT-PCR as up-regulated or down-regulated during heart development. Here, we describe clone 69, subsequently named ETL, that is up-regulated postnatally during cardiomycocyte development (Fig. 1A). The differentially expressed PCR amplicon was sub-
The blot was probed with human ETL cDNA. total RNA from human fetal (3 months) and adult heart, as designated. differential expression of rETL were reproduced using three different probe for GAPDH was used as a normalization control. The data on stage embryonic day 16 through postnatal day 12, as designated. A marked lane subsequently excised from the polyacrylamide gel and used for Northern blot analyses.

consequently spliced exon of 234 bp contains a TAA termination codon upstream of the Met. Exon 1a was revealed by a high homology to the rat protein, yet is it spliced out in several tissues we examined and in EST 112416. Exon 13 carries a conserved translational stop codon and 3'-untranslated region sequences. Pairwise comparisons of human ETL cDNA sequence and predicted peptide sequence against the rat homolog indicated 80% identity at the nucleotide and 87% similarity at the amino acid level. In obtained 3'-untranslated region sequences, 234 bp show striking interspecies identity (87%). This untranslated region is located —200 bp from the conserved stop codon and potentially represents a regulatory element for mRNA expression or stability.

**Human ETL Maps to Chromosome 1**—Using 29.2A-2B primers, we amplified a genomic STS from BAC RP11–29e12 and DNAs from medium and high resolution Radiation Hybrid panels (Research Genetics). We linked hETL to two chromosome 1 markers, SHGC-21318 within 8 centirays and to SHGC-57820 within 15 centirays with LOD scores of 8 and 11, respectively (data not shown). These markers, although not ordered on the chromosome 1 map, are tightly linked to the D1S500, a GDB marker, which is mapped by fluorescent in situ hybridization analysis on mouse chromosome 3, H3-H4. This mouse genomic region is syntenic with human chromosome 1p32-p33.

**rETL Is a Predicted Seven-transmembrane Receptor with EGF Modules in the Extracellular Domain**—The novel rETL cDNA sequence codes for a 738-amino acid protein. The molecular masses of the deduced ETL precursor and mature peptides are 82.5 and 80.3 kDa. As determined by hydropathy and motif analyses, rETL peptide consists of three regions: a large extracellular domain of 481 aa, a seven-transmembrane region of 291 aa, and a short intracellular cytoplasmic tail of 26 aa (Fig. 1).
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TABLE I

Exons of human ETL are contained within three contigs of BAC RP11-29e12

| Exon no.\(^a\) | Size  | Splice acceptor | Splice donor | Intron size\(^b\) | Exon location\(^c\) |
|-------------|-------|-----------------|--------------|----------------|-------------------|
| 1           | 153   | tattgcacagAGTATAATGAAA | CTCCTGATAGgtgaagtctggt | 6744 | 139560–139712 |
| 1\(^a\)     | 150   | agtatttcacagAGTATAATGAG | TTAATGGCAAAgtgtaatatcagtc | 140188–140337 |
| 2           | 71    | 117             | TGGCTGATAGgtggaagctttt | 908  | 147707–147868 |
| 3           | 180   | TGGCTGATAGgtggaagctttt | TCAATGGCATAgttgaatctggtt | 147396–148179 |
| 4           | 194   | TGGCTGATAGgtggaagctttt | AAGGCTATGAtggaagaacaa | 1398  | 149575–149681 |
| 5           | 117   | TGGCTGATAGgtggaagctttt | TTAGTTCAATAGgtggaagctttt | 75200–75252 |
| 6           | 206   | TGGCTGATAGgtggaagctttt | GTCTCAGAAAgtgctgttgtt | 35099 | 80325–80498 |
| 7           | 174   | TGGCTGATAGgtggaagctttt | GTCCTGATAGgtgtaaatt | 21232 | 84061–84291 |
| 8           | 204   | TGGCTGATAGgtggaagctttt | GCTCTGTACATgtggaatat | 1226  | 81725–81928 |
| 9           | 220   | TGGCTGATAGgtggaagctttt | ACACCAAGggtagtgtttaa | 2352  | 84411–84477 |
| 10          | 67    | TGGCTGATAGgtggaagctttt | GTATGTGCATgtggaatat | 2352  | 84411–84477 |
| 11          | 92    | TGGCTGATAGgtggaagctttt | GAAACATATGgtgctgcttt | 9180  | 65053–65962 |
| 12          | 169   | TGGCTGATAGgtggaagctttt | TAMTCTATAGgtggaatat | 1665  | 63555–63387 |
| 13\(^*\)    | 713   | TGGCTGATAGgtggaagctttt | ATCCATAGAAGgtgaaaccactttt | 308  | 63079– |

\(^a\) 13 exons span 2689 nucleotides, excluding exon 1a.
\(^b\) Intron sizes were determined precisely only for exons contained within one contig.
\(^c\) Exon location is shown in genomic sequences of BAC RP11 29e12. Exons 1–5, 6–10, and 11–13 fall into three unordered contigs.
\(^*\) Size of exon 13 is at least 713 bp, since we were unable to determine splice donor, it contains stop codon TAA and 3' untranslated region.

Fig. 2. rETL is a putative seven-transmembrane receptor with extracellular EGF-like domains. To derive a predicted structure of rETL, we used motif and homology searches. From top to bottom, the rETL N-terminal extracellular domain, followed by a transmembrane domain crossing the lipid bilayer seven times and a short cytoplasmic tail. A putative signal peptide cleavage site and an endoproteolytic domain crossing the lipid bilayer seven times and a short cytoplasmic tail. A putative signal peptide cleavage site and an endoproteolytic domain crossing the lipid bilayer seven times and a short cytoplasmic tail. A putative signal peptide cleavage site and an endoproteolytic domain crossing the lipid bilayer seven times and a short cytoplasmic tail.

2). The N-terminal region begins with a 19-aa signal peptide, followed by a short domain (~26 aa) related to a lectin-type domain (27), one EGF-like domain, and the presence of both Cys binding EGF domains (~91 aa). A Ser/Thr-rich linker region of ~297 aa follows the EGF domains and precedes a conserved Cys-rich proteolysis domain (~50 aa). The latter has recently been described in a small number of transmembrane proteins and is also referred to as the GPCR proteolysis site (GPS) (19, 28, 29). This region, together with the short stretch of the Ser/Thr linker and the adjacent transmembrane and cytoplasmic domain, shows the highest conservation between rat and human proteins (Fig. 3B).

Analyses of the putative hETL protein revealed only one Ca\(^{2+}\)-EGF binding domain. This domain is potentially encoded by exon 1a. However, in human EST 112416, encompassing exons 1–3, as well as in our RT-PCR experiments using human heart, placenta, lung, and kidney RNAs, exon 1a is spliced out. Since this exon shows a high degree of identity in humans and rats (80% of 150 bp) and codes for a potentially important functional EGF domain, we cannot rule out the existence of isoforms carrying exon 1a.

The extracellular domain of rETL also contains a potential Asn hydroxylation site within each Ca\(^{2+}\) binding EGF-like domain. One O-linked putative glycosylation site is found at Ser\(^{188}\) in the Ser/Thr rich domain. The extracellular domain also carries nine potential N-linked glycosylation sites, mostly within the Ser/Thr-rich linker and GPS domain, and all but one conserved in both species. Rat and human ETLs also possess cAMP- and cGMP-dependent protein kinase phosphorylation sites and several common potential protein kinase C and casein kinase II phosphorylation sites.

The hydropathy and homology analyses of the rETL transmembrane segment predicted seven helices and a class II/secretin G-protein-coupled receptor signature sequence. Fig. 3A displays the alignment of rat ETL and a human homolog in the heptahelical domain along with several members of the secretin family, EGF-TM7 subfamily members, and several receptors with large extracellular domains. The overall structure is most similar to the EGF-TM7 family, with 30% identity to EMR1 and CD97 in pairwise comparisons (data not shown). The ETL TM7 segment together with the adjacent Cys-rich proteolysis domain and the Ser/Thr linker, displays significant homology to the three related heptahelical receptors, CL1, CL2, and CL3, with 40% identity. Based on these homologies, we designated our protein ETL (for EGF-TM7-latrophilin-related protein). A comparison of rETL and rat CL1–3, revealed conservation of the proteolysis domain, with 60% identity in the region (Fig. 3B). CL1 is endoproteolytically cleaved at the end of this domain (18), and cleavage of CL2 and CL3 has been referenced as well (19). The proteolysis domain is characterized by several invariant amino acids; most numerous among them are Cys residues. Several recently discovered TM7 receptors with large extracellular domains also exhibit this motif (Fig. 3B and Ref. 19), but cleavage has not been documented.

The short cytoplasmic tail carries a tyrosine kinase phosphorylation site that could play a role in desensitization of the receptor (30) or coupling to a tyrosine kinase signaling pathway (11). A putative tyrosine phosphorylation site, preserved in both rat and human ETLs, could be involved in cross-talk.
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between signal transduction modules employing tyrosine kinases such as a MAP kinase pathways (11) and could represent a scaffold for the assembly of a phosphotyrosine-dependent complex. The overall structure of rETL suggests that the protein might participate in both cell surface events such as cell-cell recognition and adhesion and in signal transduction cascades.

rETL Forms a Stable Receptor-Dimer in COS-7 Cells—We expressed rETL protein tagged with a Myc epitope on the C terminus in COS-7 cells and identified an ~85-kDa protein using anti-Myc antibody on total protein lysates by Western blot analyses. This 85-kDa band was not present in vector alone transfections (Fig. 4A). The observed mass of 85 kDa closely correlated with the expected mass of the mature rETL-Myc-His protein (83.7 kDa). We also observed a broad intense band at ~175 kDa. This band probably represents an rETL dimer with additional post-translational modifications that is stable in the presence of most reducing agents and boiling. Extraction of total proteins with 6 M guanidine hydrochloride led to nearly complete disappearance of the 175-kDa band (Fig. 4B, ETL lane).

rETL Is Cleaved within the Putative Extracellular Domain—Conservation of the rETL Cys-rich domain, a domain known to undergo cleavage in CL1, CL2, and CL3 receptors (18, 19), prompted us to test whether rETL also undergoes proteolytic processing. Previously, the cleavage of CL1 was only demonstrated in the presence of 8 M urea, both in gel and sample buffer (18). When total protein extracts from COS-7 cells transfected with the rETL construct were subjected to these conditions, a band of ~35 kDa appeared, and the 85-kDa band became less intense (Fig. 4D, ETL lane). This observation correlated with the predicted products of cleavage between Leu454 and Thr455 of a 48-kDa peptide (untagged) and a C-terminal 35.7-kDa peptide (tagged with Myc) (Fig. 4D, ETL lane). The 85-kDa doublet in rETL probably represents the mature and precursor rETL proteins (Fig. 4D, ETL lane). The fact that only certain chaotropic agents allowed us to observe the cleaved products suggests that these cleaved peptides stay bound as it has been shown for CL1 receptor (18). This proteolytic processing may play an important role in the formation of a functional receptor.

To further analyze the processing of rETL, we used a mutant clone, rETL*T455A. This clone, generated as a PCR cloning artifact, carries a mutation at the conserved Thr residue previously determined to be at the processing site in latrophilin (18). The mutated protein shows resistance to cleavage as assessed by the failure to detect a 35-kDa band in the presence of 8 M urea (Fig. 4D, ETL*T455A lane). This suggests that Thr455 is required for proteolytic processing. Interestingly, 8 M urea did not lead to the disappearance of the 175-kDa band, implying strong modifications after receptor-dimer formation.

In 6 M guanidine HCl preparations, rETL*T455A displays a

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Fig. 3. ETL is related to the secretin peptide hormone receptor family and has a conserved G-protein-coupled receptor proteolysis domain. A, rat and human ETL transmembrane domains were aligned with several members of the secretin receptor family. GenBank accession numbers are given in parentheses. h, r, or m, sequence from human, rat, or mouse species. The aligned proteins are as follows: rCL1/latrophilin, receptor for latrotoxin; rCL2; rCL3; epidermal growth factor module-containing mucin-like receptor 1 (hEMR1); leukocyte antigen (hCD97); vasoactive intestinal peptide receptor (hVIPR); secretin receptor (hSECR); glucagon receptor (hGLUCR); and corticotropin-releasing factor receptor (hCTRFR). Transmembrane domains are shown in blocks and designated as TM1 to TM7; identical amino acids are shown in white on dark gray background and designated by an asterisk. Conservative (colon) and semiconservative (parentheses) amino acid changes are shown in light gray columns. Alignments were built using ClustalW. B, amino acids in the Cys-rich proteolysis domain of rat and human ETLs were aligned with several transmembrane molecules carrying this domain. Aligned proteins included seven transmembrane receptors CL1, CL2, CL3, bCD97, lectomedin-1 (hLec1), brain-specific angiogenesis inhibitor 3 (mFlamingo), Flamingo seven-pass transmembrane cadherin (mFlamingo), brain-specific angiogenesis inhibitor 3 (hBAI3), and serpentine receptor (mCT5S). Other molecules, such as KIAA0729, h287, hR29368_2, hMEGF2, and hTM7X1, are putative TM7 receptors. Cel0026 is a Caenorhabditis elegans putative G-protein-coupled receptor; SuREJ is a Strongylocentrotus purpuratus sperm receptor for egg jelly. GenBank accession numbers are given in parentheses. The putative proteolytic processing site is shown by an arrow, and the location of the mutation in rETL*T455A is indicated by a white letter above the arrow.
rETL forms a dimer and undergoes endoproteolytic cleavage in transfected COS-7 cells. A–F, Western blot analysis of total proteins obtained from transiently transfected COS-7 cells, lysed as indicated below. Proteins were resolved on gradient gels, stained with monoclonal anti-Myc antibody followed by ECL detection. A, cells were transfected with pcDNA-ETL-Myc constructs or control vector as indicated and lysed in triple detergent. Note that both rETL and rETL*T455A display 175- and 85-kDa proteins, suggesting dimer formation and cleavage. B, cells were transfected as in A and lysed with 8 M guanidine hydrochloride, precipitated, and dissolved in 8 M urea. Note that under highly denaturing conditions, the ratio of 175- to 85-kDa protein is much higher for the mutant clone than for wild type. C, membrane proteins from COS-7 cells, transfected as in A, were lysed in Triton detergent. Note that sizes of the detected rETL and rETL*T455A peptides correspond to the cleaved and uncleaved dimers, respectively. The 85-kDa species were not detected in these experiments. The faint band between the two arrows is ~120 kDa and may correspond to a dimer of cleaved and uncleaved rETL. D, cells were transfected and lysed as in A and resolved on 7.5% SDS-polyacrylamide gel containing 8 M urea in the gel and sample buffer. Note that these denaturing conditions revealed a proteolytic cleavage of rETL. As predicted, mutant rETL resisted cleavage. E, cells on the left were transfected with the construct of extracellular domain of rETL (aa 1–483), fused to Fc. On the right, cells were transfected with the construct of extracellular domain of rETL N-terminal to the cleavage site (aa 1–455 including Thr455) at the cleavage site), fused to Fc. Anti-Fc antibody and subsequent ECL were used in detection. Note that amino acids 456–483 are required for cleavage. As predicted, mutant rETL resisted cleavage and was observed as a 68-kDa doublet, approximately corresponding to the predicted mass of a cleaved dimer (Fig. 4C, ETL455-Fc lane), 71.4 kDa. In these experiments, rETL was not detected as an 85- or 175-kDa protein species, corresponding to the rETL uncleaved monomer or uncleaved dimer, respectively. The mutation T455A did not affect the membrane association of the rETL*T455A protein. The cleavage-resistant ETL*T455A protein was also detected in dimer-only cleavage, as revealed by the presence of a 175-kDa band on the Western blot (Fig. 4C, ETL455-A lane). In addition to the rETL membrane association, these results suggest that rETL is cleaved during intracellular processing because the membrane preparations included both the ER and plasma membranes.

ETL Detected in Membrane Preparations—Both rETL and rETL*T455A were detected in membrane preparations of COS-7 cells transfected with these constructs (Fig. 4C). Interestingly, no 85-kDa protein species were observed in the Triton-extracted membrane proteins. When proteins were resolved under regular Laemmli conditions, wild type rETL appeared as a 68-kDa doublet, approximately corresponding to the predicted mass of a cleaved dimer (Fig. 4C, ETL lane), 71.4 kDa. In these experiments, rETL was not detected as an 85- or 175-kDa protein species, corresponding to the rETL uncleaved monomer or uncleaved dimer, respectively. The mutation T455A did not affect the membrane association of the rETL*T455A protein. The cleavage-resistant ETL*T455A protein was also detected in dimer-only cleavage, as revealed by the presence of a 175-kDa band on the Western blot (Fig. 4C, ETL455-A lane). In addition to the rETL membrane association, these results suggest that rETL is cleaved during intracellular processing because the membrane preparations included both the ER and plasma membranes.

ETL Transmembrane Domain Is Required for Dimerization but Not Cleavage—To determine whether the transmembrane domain is required for endoproteolytic processing, we constructed a fusion protein consisting of the rETL exodomain up to the Thr455 residue, including ETL455-Fc, and observed no 30-kDa species (Fig. 4E, ETL455-Fc lanes). These observations demonstrate that the amino acids immediately following the processing site play an important, most likely conformational, role in cleavage.

rETL Is a Plasma Membrane-associated Protein—The predicted amino acid sequence of rETL and the substantial structural homology to known receptor families suggested that rETL localized to the plasma membrane. Further data supporting a plasma membrane localization for rETL were obtained using Western analyses. As noted above and in Fig. 4C, rETL was detected in membrane preparations. We also generated a construct encoding only the extracellular domain of rETL. When the exodomain of rETL, aa 1–455, tagged C-terminally with Myc epitope, was transiently expressed in COS-7 cells, it was detected as a soluble protein in both conditioned medium (Fig. 4F, ETL455Fc sup) and whole cell lysates (Fig. 4F, ETL455 cell). Taken together, these data are highly suggestive that rETL is a plasma membrane protein.

We used confocal microscopy to determine a subcellular localization of C-termally Myc-tagged rETL in COS-7 cells. Rat ETL was observed in the perinuclearity of plasma membrane and intracellular vesicles in permeabilized cells only (Fig. 5A). Transient transfection allowed us to control the specificity of indirect immunofluorescence, since only a percentage of cells receive the plasmid and fluoresce (data not shown). The vesicles most likely represent endoplasmic reticulum, Golgi apparatus, and cytoplasmic transport vesicles involved in process-
ETL, Novel Member of the Secretin and EGF-TM7 Receptor Family

In this study, we have isolated and characterized a novel cDNA clone, ETL, encoding a new member of the EGF-TM7 subfamily of receptors. Similar to all members of the EGF-TM7 receptor family, ETL has a tripartite domain structure consisting of a large extracellular domain, a seven-membrane-spanning domain, and a short cytoplasmic tail. A characteristic feature of the EGF-TM7 protein group, an unusually large exodomain, incorporates cell surface interaction modules, such as EGF-like motifs, lectin-like motifs, and a Ser/Thr rich domain, with numerous sites for N- and O-linked glycosylation. Rat ETL has several EGF domains, which are often found in extracellular portions of a large number of proteins, including fibrillin, fibulin, entactin, tenascin, and thrombospondins (31), and are functionally associated with protein-protein interactions. The evolutionary implications of the addition of cell surface modules, a feature previously found only in single membrane-spanning molecules, to the GCPR's signal transduction domain remains obscure. One possibility is that these modules are essential for ligand specificity or presentation.

Ca^{2+}, potentially bound to an Asn β-hydroxylation site inside EGF domain, is likely to stabilize protein-protein interactions of rETL (32). Most of the members of the EGF-TM7 family display this Ca^{2+}-binding feature, suggesting a common mechanism for ligand binding. To date, the only known ligand for the EGF-TM7 receptor family is CD55, or decay-accelerating factor, a complement component. CD55 interacts with one of the isoforms of the CD97 receptor that is expressed on the cell surface of leukocytes (33). CD97 carries several tandem EGF repeats, and deletion analyses showed that binding requires both Ca^{2+}-EGF domains and Ca^{2+} for ligand binding (34). In several human tissues examined, the Ca^{2+}-binding EGF domain of hETL appears to be spliced out. However, protein motif analyses detected another potential Ca^{2+}-binding domain in hETL, an EF-hand signature sequence. This human-specific domain is present in a large family of calcium-binding proteins (35).

As noted in Fig. 3B, we found a significant conservation between TM7 regions of secretin receptors and ETL. The importance of this homology is unknown but may indicate a conservation of critical residues for G-protein coupling to intracellular loops and/or ligand-binding determinants within the extracellular loops of GCPR transmembrane domains (36–38).

We show here that ETL carries a Cys-rich proteolysis domain with a high degree of identity to CL1, CL2, and CL3 heptahelical receptors. CL1, also known as latrophilin, is shown to interact with α-latrotoxin, a toxin from the black widow spider (39). Recent studies showed that latrophilin tethers α-latrotoxin to the membrane, initiating Ca^{2+} channel formation (39). The proteolysis domain shared with CL1–3 receptors is present in all EGF-TM7 receptor family members. Recently, this motif has been identified in other transmembrane molecules, such as polycystin-1 (PKD1) (28), the protein defective in ADPKD polycystic kidney disease (40), and two other PKD1-related proteins, sea urchin egg jelly receptor, REJ, and its human homolog, PKDREJ (41, 42). The latter three proteins exhibit a different number of membrane helices, from 1 to 11. However, similar to CL1, these proteins support extracellular Ca^{2+} influx after activation, functional characteristics thought to be due to the common proteolysis domain and first transmembrane domain (28). This commonality supports the hypothesis that ETL, upon activation, is involved in cation influx.

Cleavage within the proteolysis domain of CL1 (18, 19) and rETL, shown here, suggests that other proteins carrying the conserved domain may also be cleaved. The detection of rETL in cleaved dimer conformation in membrane preparations suggests that the proteolytic activity is an intracellular event. The 68-kDa doublet may imply that cleavage of the extracellular domain precedes the cleavage of N-terminal signaling peptide. The detection of rETL cleavage with a limited number of chelotropic agents suggests that the cleaved N-terminal domain of
rETL remains tightly tethered to the transmembrane domain, as has been shown for CL1 receptor cleavage product (18). Interestingly, the mutation T455A in rETL that we have shown completely abolishes cleavage is present in the native EMR1 receptor, but there is no biochemical data available on EMR1 processing. Based on the high degree of homology in the proteolysis domain of rETL and CL1 and on the size of rETL cleaved product that we observed in our experiments, it is likely that rETL utilizes the same site for proteolytic processing. Therefore, the T455A mutation most likely resides directly at the cleavage site, as is the case for CL1 (18). The ETL*T455A clone will be useful for determining if post-translational processing is important for receptor function when an ETL ligand is present at the cleavage site, as is the case for CL1 (18). The ETL*T455A clone will be useful for determining if post-translational processing is important for receptor function when an ETL ligand is present.

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