Evolution of Lutropin to Chorionic Gonadotropin Generates a Specific Routing Signal for Apical Release in Vivo*

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One of the fundamental differences among mammals is the mechanism of maintaining the corpus luteum of pregnancy. Placentation in primates is associated with the production of the glycoprotein hormone chorionic gonadotropin (CG), which is secreted into the maternal serum and stimulates progesterone synthesis from the corpus luteum, thereby allowing early implantation and development of the embryo (3, 4). CG and its pituitary counterpart, lutropin (LH), comprise a family of heterodimeric glycoprotein hormones, including follitropin and thyrotropin, that share a common α subunit, but differ in their hormone-specific β subunits (5). Although the β subunits determine biological specificity of each hormone, there is significant structural similarity between them. This is most evident for the LHβ and CGβ subunits, which share 85% amino acid identity in the first 114 amino acids (6). A major difference between the two subunits is the presence in the CGβ subunit of a 31-amino acid carboxyl-terminal extension (CTP) compared with a shorter 7-amino acid stretch in LHβ (see “Results”).

The CGβ subunit is specific to primates and evolved by a gene duplication event from the LHβ locus (7); functionally the two are interchangeable. Similar to other mammals, primates express LH during pregnancy, raising the question as to why primates require CG. When comparing the biosynthesis of LH and CG, two important differences emerge: the pathways of secretion and polarity of their release. LH is packaged into storage granules (8), subject to regulated exocytosis by secretagogues (9), and released via the basolateral surface of pituitary gonadotrope cells (10, 11). In contrast, CG is secreted by an apical route through the villous and directly into the intervillus space created by the implanted placenta (1, 12–14). The molecular basis by which the intracellular trafficking of CG is directed to exit the chorionic villi into the maternal lumen is unknown. To examine whether the unique circulation profiles exhibited by LH and CG in vivo are reflected in differences in polarized secretion, and to identify potential targeting sequences, we co-transfected the common α subunit and LHβ/CGβ genes into Madin-Darby canine kidney (MDCK) cells. This cell line is an excellent model to study polarized secretion of endogenous and exogenous proteins in culture (15, 16). The plasma membrane of these epithelial cells is divided into apical and basolateral domains by tight junctions (17). The apical side faces the luminal or exterior milieu, which is covered by microvilli and is involved in absorptive or secretory processes, whereas the basolateral side faces the serosal environment (16, 17). Here we examined the polarized secretion of LH and CG in transfected MDCK cells and show that apical targeting of CG is programmed by the unique CTP, a novel sorting signal that is generated by a single framenshift mutation in the ancestral LHβ gene.

The evolution of primates is associated with a fundamental change in the morphogenesis of the placenta (1, 2). Placentation in primates is coupled to the synthesis of glycoprotein hormone chorionic gonadotropin (CG), which is secreted into the maternal serum and stimulates progesterone synthesis from the corpus luteum, thereby allowing early implantation and development of the embryo. CG, two important differences emerge: the pathways of secretion and polarity of their release. LH is packaged into storage granules (8), subject to regulated exocytosis by secretagogues (9), and released via the basolateral surface of pituitary gonadotrope cells (10, 11). In contrast, CG is secreted by an apical route through the villous and directly into the intervillous space created by the implanted placenta (1, 12–14). The molecular basis by which the intracellular trafficking of CG is directed to exit the chorionic villi into the maternal lumen is unknown. To examine whether the unique circulation profiles exhibited by LH and CG in vivo are reflected in differences in polarized secretion, and to identify potential targeting sequences, we co-transfected the common α subunit and LHβ/CGβ genes into Madin-Darby canine kidney (MDCK) cells. This cell line is an excellent model to study polarized secretion of endogenous and exogenous proteins in culture (15, 16). The plasma membrane of these epithelial cells is divided into apical and basolateral domains by tight junctions (17). The apical side faces the luminal or exterior milieu, which is covered by microvilli and is involved in absorptive or secretory processes, whereas the basolateral side faces the serosal environment (16, 17). Here we examined the polarized secretion of LH and CG in transfected MDCK cells and show that apical targeting of CG is programmed by the unique CTP, a novel sorting signal that is generated by a single framenshift mutation in the ancestral LHβ gene.

MATERIALS AND METHODS

Polyclonal antisera directed against the α and CGβ subunit were prepared in this laboratory. Purified hCG (CR-127; 14,900 IU/mg) was provided by Dr. A. F. Parlow (National Hormone and Pituitary Program, National Institutes of Health, Torrance, CA).

Cell Culture—MDCK cells (strain II) were a gift of Dr. Sharon Milgram (University of North Carolina). These cells were grown in DMEM/F-12 medium (for no more than 10–15 passages) supplemented with 2

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mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and containing 5% FBS (v/v) at 37 °C in a humidified 5% CO₂ incubator (18). The cells (3.5 × 10⁵ cells/ml) were seeded on 12-mm or 24-mm Transwell filters (0.4 μm pore size, Corning-Costar, Cambridge, MA) allowing separate access of media to the apical and basolateral faces of the membrane. Filter-grown cells were cultured for 3–4 days with changes of medium daily.

To measure the integrity of epithelial monolayer and formation of tight junctions (as an indicator of achieving polarization), electrical resistance was measured across the cell monolayer using the Millicell Electrical Resistance System (Millipore Corp., Bedford, MA) (18).

Transfection and Clone Isolation—Transfection was performed using the DNA-calcium-phosphate precipitation method as described previously (19). Cells grown on plastic dishes were stably transfected with pM2HA (20, 21) containing either the α, LHβ, CGβ, or CGβ144 subunit genes.

Metabolic Labeling—MDCK cells were labeled overnight with 25 μCi/ml [³⁵S]cysteine in DMEM/F-12 medium lacking cysteine and supplemented with 5% dialyzed fetal bovine serum, l-glutamine, and streptomycin (20). Labeling medium was added to the apical and basolateral compartments, and media from them were collected into separate tubes and were immunoprecipitated with antisera directed against α and CGβ subunits using Pansorbin. The reduced with tRNAM2HA (20, 21) and heated (4 min) proteins were resolved as on 15% SDS-polyacrylamide gels described previously (19).

RESULTS AND DISCUSSION

We first examined the secretion patterns of the LH and CG dimers from transfected MDCK cells grown in Transwells and labeled overnight with [³⁵S]cysteine. The media were immunoprecipitated with CGβ antisera, which reacts equally well with either the LHβ or CGβ subunits; co-precipitation of the α subunit is indicative of heterodimer formation. LH dimer released from the cells was primarily associated with the basolateral side, indicating that it is secreted in a polarized manner (Fig. 1A, lanes 1 and 2). The basolateral to apical ratio for LH was 3.1 (76.2 ± 1.6% and 23.8 ± 1.6%, respectively; n = 6; p < 0.05) (Fig. 1B). Control experiments demonstrated that when the integrity of the monolayer was disrupted with a cell scraper, determined by reduction in electrical resistance, LH dimer traversed the filter and distributed equally to both compartments (Fig. 1A, lanes 3 and 4). The preferential basolateral routing of LH is consistent with its known distribution and release from the pituitary (10, 11). LH-containing secretory granules redistribute subcellularly and become polarized to the side of the gonadotrope nearest to the vascular sinusoid during the preovulatory stage, and release of the granule content occurs in the basal region of the cell (10, 11).

In contrast to LH, CG was secreted preferentially into the apical compartment (Fig. 2A, lanes 1 and 2). Densitometric analysis of labeled bands demonstrated an apical to basolateral ratio of 2.7:1 (73 ± 1.5% versus 27 ± 1.5%, respectively; n = 6; p < 0.05) (Fig. 2B). These data reflect differences in the secretion behavior of CG compared with its pituitary homologue LH. The apical secretion of CG is consistent with its in vivo release, since CG enters the maternal blood lakes of the uterus via an apical route through the villous (1, 12–14).

To examine the possibility of nonspecific transfer of CG and LH across the MDCK cell monolayer, conditioned media containing [³⁵S]labeled CG or LH from the MDCK cell-line was added to nontransfected MDCK cells grown on Transwell filters. After overnight incubation lysates and media from the apical and basolateral compartments were immunoprecipitated. No transfer of protein from one compartment to the other was detected (data not shown), indicating direct and specific sorting of CG and LH into the apical and basolateral compartments, respectively. These data demonstrate that the MDCK model recapitulates the secretion polarity for pituitary LH and placental CG as seen in vivo.

Numerous studies have shown that the uncombined α subunit is primarily secreted constitutively (22, 23). To assess the specificity of the MDCK model, cells expressing only the α subunit were examined (Fig. 3). In this case the secretion pattern was random, i.e. the α subunit was observed equally in both compartments (Fig. 3A, lanes 1 and 2). Presumably, the differential sorting exhibited by LH and CG is due to the β subunit, since the α subunit is the same for both hormones. To test this prediction, and to address the question if dimer formation is a prerequisite for sorting, MDCK cells expressing either the LHβ or CGβ subunits were grown on Transwells (Fig. 3, lanes 3–6). The β subunits exhibited the same polarity as the corresponding dimers, indicating that the sorting signals are encoded in the hormone-specific β subunit, and heterodimer formation is not required for the secretion polarity.

Based on their extensive sequence identity, it was suggested that CGβ evolved from an ancestral LHβ gene (7). The sequences of the carboxyl ends of the LHβ and CGβ termini are shown in Scheme 1. (The shaded areas indicate identical sequence, and the asterisks show the O-linked serine acceptor site in the CGβ subunits.) A single base pair deletion at codon 114 in the ancestral gene lead to generation of an mRNA encoding the CGβ subunit composed of 145 amino acids com-
The seven amino acids at the carboxyl terminus of LH\(\beta\)H9252 is very hydrophobic; in contrast, the CG\(\beta\) carboxyl-terminal sequence is hydrophilic containing several serine residues, of which four are O-linked carbohydrate acceptor sites in the CTP sequence. Thus, the apparent major evolutionary change resulting in a truncated protein of 114 amino acids (CG\(\beta\)114) was secreted basolaterally (68.9% \(\pm\) 1.7; \(n = 6\); \(p < 0.05\)) (Fig. 2B). These results confirm that the carboxyl-terminal sequence of the CG\(\beta\) subunit is a major routing signal for the apical secretion of CG dimer.

![Diagram](http://www.jbc.org/)

**Fig. 2. Secretion of CG and CG114 dimers.** A, MDCK cells expressing wild type CG or the truncated carboxyl-terminal mutant CG114 dimer were metabolically labeled overnight with \(^{35}\)S-cysteine. The media from the apical (Ap) and basolateral (BL) compartments were immunoprecipitated with CG\(\beta\) antiserum and analyzed as described for Fig. 1. The migration of CG\(\beta\)114 species bearing two (N2) or one (N1) asparagine-linked oligosaccharides and the \(\alpha\) subunit are indicated by arrows. N2 indicates the presence of both of the CG\(\beta\) and CG\(\alpha\) sequences, and N1 is a minor form bearing only one Asn-linked carbohydrate unit. CG\(\beta\)114 co-migrates with the \(\alpha\) subunit on SDS gels. That the N2 form of CG\(\beta\)114 and \(\alpha\) subunit reflect heterodimer formation was confirmed by nondenatured Western blots, which demonstrated a molecular mass of 35 kDa, corresponding to the intact dimer (data not shown). B, the relative percent secretion of CG and CG114 dimers was quantitated by densitometry. Results are shown as the mean \(\pm\) S.E. (\(n = 6\)–8). Asterisks indicate significant differences, \(p < 0.05\).

**Fig. 3. Secretion of \(\alpha\), LH\(\beta\), and CG\(\beta\) subunits.** A, cells expressing CG\(\alpha\), cells expressing LH\(\beta\) or CG\(\beta\) subunits grown on Transwell filters were metabolically labeled overnight with \(^{35}\)S-cysteine. Media from the apical (Ap) and basolateral (BL) compartments were immuno-precipitated with \(\alpha\) or CG\(\beta\) antiserum. B, the relative percent secretion of \(\alpha\), LH\(\beta\), and CG\(\beta\) subunits was quantitated by densitometry as described for Fig. 1. Results are shown as the mean \(\pm\) S.E. (\(n = 5\)). Asterisks indicate significant differences, \(p < 0.05\).

**Scheme 1. Sequences of the carboxyl ends of the LH\(\beta\) and CG\(\beta\) termini.**

It has been reported that N- and O-linked oligosaccharides are associated with apical sorting signals for some glycoproteins. The O-glycosylated stalked region of the neurotrophin receptor has been implicated in its sorting to the apical membrane, since deletion of this region leads to a mistargeting of the mutant protein to the basolateral membrane in MDCK cells (27). Moreover, eliminating or reducing the number of O-glycans affects the polarity of intestinal brush border enzymes released from MDCK cells (28, 29). However, deleting the O-linked oligosaccharides from aminopeptidase N had no effect on its apical secretion pattern from MDCK cells (28). Because there are four O-linked oligosaccharides in the CTP sequence, it is unclear whether the targeting function of CTP is encoded in the protein sequence and/or the carbohydrate. To address whether the O-linked carbohydrate in the CTP contributes to the apical secretion of CG, we constructed a CG\(\beta\) mutant (CG\(\beta\)-Odg) (Fig. 4A) in which the four serine acceptor sites at residues 121, 127, 132, and 138 in the CTP were changed to alanine. (The Ser at position 130 was also mutated in this variant because we observed that some alternative O-glycosylation occurred at this site when the mutant was expressed in CHO cells.)

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Fig. 4. Secretion of CG dimer bearing the CGβ subunit devoid of O-linked carbohydrate (CG-Odg) and uncombined mutated subunit (CGβ-Odg). A, MDCK cells expressing CG-Odg dimer and CGβ-Odg subunit were metabolically labeled overnight with [3H]glucosamine. The media from the apical (Ap) and basolateral (BL) compartments were immunoprecipitated with CG antisera and analyzed as described in the legend to Fig. 1. Dimerized CG-Odg dimer and CGβ-Odg subunit contain two units. Thus, while the CTP is sufficient to route CG apically, we cannot exclude that the observed sorting is achieved in concert with the additional N-linked oligosaccharide on the CGβ subunit. There is evidence that N-glycans can act as apical signals for some secretory and integral membrane proteins (30), but their exact role is unclear (31), since many glycoproteins do not display glycan-dependent apical targeting (32). This indicates that the contribution of N-glycans to apical targeting is protein specific. It has been suggested that N-glycans do not function as sorting signals directly but rather play an accessory role necessary for the function of an apical determinant (31,33).

There are several reports showing that motifs consisting of a tyrosine residue near at least one hydrophobic amino acid, or a motif bearing a leucine/leucine or a leucine/isoleucine pair are involved in basolateral sorting of membrane proteins (34–36). It is intriguing that the unique carboxy-terminal heptapeptide in the LHβ subunit contains the dileucine motif. Why only the CGβ subunit among the glycoprotein hormone family contains CTP is unclear. Expression of CG in the placenta suggests that this extension is a critical factor in the gestational role of CG. A major function of the placenta during pregnancy is the exchange of metabolic products between the fetal and maternal blood streams (1). Thus, the CTP directs CG apically from the fetally derived placenta into the maternal interstitial spaces; these are luminal compartments filled with maternal blood derived from pressure gradients generated in the spiral arteries. Our data reveal that LH, which is elaborated by the pituitary directly into a single vascular compartment, is distinct from the production of humoral agents in the fetoplacental unit. The use of the MDCK model has permitted the identification of a novel targeting domain that reversed the polarity of two evolutionarily related heterodimers, which activate the identical receptor. The appearance of CTP represents not only an adaptive response to maintain a high level of circulating gonadotropin, but also a targetting signal redirecting a pituitary function to the placenta, thus ensuring a successful implantation and development of the primate embryo.

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