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DOMAIN ANALYSIS OF HUMAN TRANSMEMBRANE GUANYLYL CYCLASE RECEPTORS: IMPLICATIONS FOR REGULATION

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1. ABSTRACT

In the human genome, sequence analysis indicates there are five functional transmembrane guanylyl cyclases, enzymes that synthesize the intracellular second messenger, cGMP. Two, GC-A and GC-B or NPR-A and NPR-B, are widely distributed receptors for atrial natriuretic peptide, brain natriuretic peptide and C-type natriuretic peptide, more commonly known as ANP, BNP and CNP, respectively. One cyclase, GC-C or StaR, is predominantly found in the intestinal epithelium and is the receptor for guanylin and uroguanylin, as well as for the bacterial pathogen, heat-stable enterotoxin (Sta). The remaining two cyclases, GC-E and GC-F or RetGC-1 and RetGC-2, are expressed in the retina and regulate the dark cycle of phototransduction. Unlike the other family members, GC-E and GC-F have no known extracellular ligands. Instead, they are activated under low calcium conditions by guanylyl cyclase activating proteins called GCAPs. All five members consist of an extracellular ligand binding domain, single transmembrane spanning domain, and intracellular kinase homology, dimerization and guanylyl cyclase catalytic domains. In the first part of this review, the tissue expression, ligands and "knockout" phenotypes of each receptor are summarized and individual domains are compared. In the second part, regulation by ATP, calcium, protein kinase C and phosphorylation is discussed.

2. INTRODUCTION

Enzymes that catalyze the formation of cGMP from GTP are called guanylyl cyclases (GCs), although initially and for many years thereafter, they were called guanylate cyclases. Early reports described activities present in both the soluble and particulate fractions of cells (1-3). Subsequent kinetic analysis suggested that these activities result from unique enzymes (4, 5). Purification (6-8) and cloning (9-18) studies verified these observations. The soluble forms are receptors for the membrane permeable gases, nitric oxide and carbon monoxide (19). The activity measured in particulate fractions results from the expression of a family of single membrane spanning proteins that are receptors for extracellular peptides or

Table 1. Comparison of tissue expression, activators, and mouse "knockout" phenotypes of human transmembrane guanylyl cyclase receptors. Abbreviations are: Sta, heat-stable enterotoxin; VSM, vascular smooth muscle

Receptor	Tissue Expression	Activator(S)	Mouse "Knockout" Phenotype
GC-A	Adrenal Brain, VSM Lung, Kidney Adipose, Heart	ANP, BNP	Hypertension Ventricular fibrosis Cardiac hypertrophy
GC-B	Chondrocytes Brain, Lung VSM, Uterus Fibroblast	CNP	Dwarf ism, Seizures Female sterility Decreased adiposity
GC-C	Intestine	Sta, Guanylin Uroguanylin	Resistance to Sta
GC-E	Retina, Pineal	GCAP1,GCAP2	Cone dystrophy
GC-F	Retina	GCAP1,GCAP2	Not reported

intracellular calcium-binding proteins. Thus, in contrast to the serpentine receptor/G protein/adenylyl cyclase paradigm, transmembrane guanylyl cyclases contain both ligand binding, signal transducing and cyclase catalytic domains in a single peptide. Seven cDNAs (guanylyl cyclase-A through G) coding for transmembrane guanylyl cyclases have been cloned from mammalian mRNA (20). However, this review will only discuss five members (GC-A, GC-B, GC-C, GC-E and GC-F) because the remaining two (GC-D and GC-G) are most likely pseudogenes in humans (21). The domains of all five members are compared, but the regulation of the natriuretic peptide receptors (GC-A and GC-B) is emphasized because they were the first mammalian guanylyl cyclases cloned and have become a paradigm for the study of this family.

3. LIGANDS, TISSUE EXPRESSION AND "KNOCKOUTS"

All five human transmembrane guanylyl cyclases have known activators (Table 1). Guanylyl cyclase-A (GC-A) is stimulated by the cardiac hormones, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) (22-26). Guanylyl cyclase B (GC-B) is activated by C-type natriuretic peptide (CNP) (22-26). Guanylyl cyclase C (GC-C) is activated physiologically by guanylin and uroguanylin, but is also the target for the bacterially produced heat-stable enterotoxin (Sta) (27, 28). No known extracellular peptide activators of GC-E and GC-F have been identified. Instead, in a low calcium environment, they are activated by guanylyl cyclase activating proteins or GCAPs (29, 30).

GC-A is highly expressed in vascular smooth muscle, lung, brain, heart, kidney, adrenal and adipose tissue (Table 1) (12, 31, 32). GC-A knockout animals display marked hypertension and cardiac hypertrophy (33, 34). GC-B is highly expressed in chondrocytes, lung, brain, vascular smooth muscle, uterus and fibroblasts (15, 31, 32, 35, 36). Disruption of the GC-B gene results in mice that display dwarfism, epileptic-like seizures, reduced adiposity and female sterility (David Garbers, 1st International Conference on cGMP, Leipzig, Germany, June, 2003). GC-C is primarily expressed in intestinal epithelium (14). GC-C knockout animals are resistant to heat stable enterotoxin, but otherwise appear normal (37, 38). GC-E is expressed in the cones and rods of the retina as well as the pineal gland (16, 18, 39). Disruption of the GC-E gene results in mice that lack cones after five weeks of age (40). GC-F is also expressed in the outer segment of the retina (13, 18). The disruption of the gene encoding GC-F has not been reported.

4. COMMON FEATURES OF TRANSMEMBRANE GUANYLYL CYCLASES

4.1. General topology and oligomeric state

Membrane-spanning guanylyl cyclases have a growth factor receptor like topology that consists of an amino-terminal extracellular domain, single membrane-spanning region and intracellular kinase homology, dimerization and carboxy-terminal guanylyl cyclase domains (figure 1). All indications are that the minimal catalytic unit for these enzymes is a homodimer, although higher ordered structures (trimers and tetramers) also have been reported and may be the physiologically relevant forms. With respect to GC-A, expression of a construct containing the carboxyl terminal 283 amino acids of the receptor (most of the dimerization and all of the cyclase domain, see figure 1) resulted in an active enzyme that migrated at the expected molecular weight of a dimer by gel filtration (41, 42). Deletion of an additional 58 amino-terminal amino acids, which included the dimerization domain, resulted in an inactive protein. Initial ^{125}I-ANP cross-linking of full-length GC-A, provided evidence for a disulfide-dependent homotetrameric form of the receptor (43). Immunoprecipitation and western blot experiments detected dimeric, trimeric and tetrameric complexes that were unaffected by ligand binding (44, 45). In contrast, a soluble extracellular version of GC-A exists as a monomer and dimer in the absence and presence of ANP, respectively, suggesting that ligand binding is required for extracellular domain dimerization and that the intracellular dimerization domain is required for hormone-independent oligomerization (46).

Full length GC-C also is a homooligomer in the absence and presence of ligand, and complexes with molecular weights of dimers and trimers have been reported for this receptor (47, 48). In the absence of ligand, GC-C mutants lacking intracellular domains migrate as

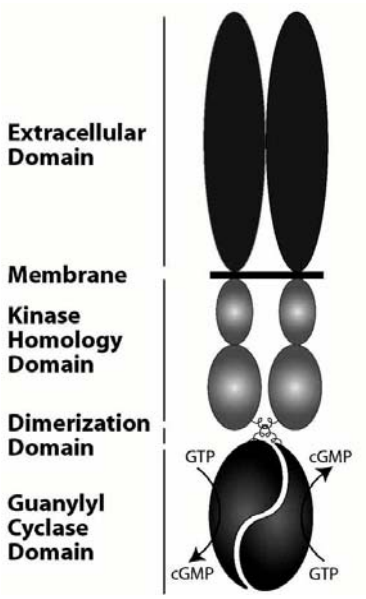

Figure 1. Schematic Representation of the Domains of a Human Transmembrane Guanylyl Cyclase Receptor. For GC-A, GC-B and GC-C the extracellular domain binds the respective activator. In other words, it is the ligand binding domain.

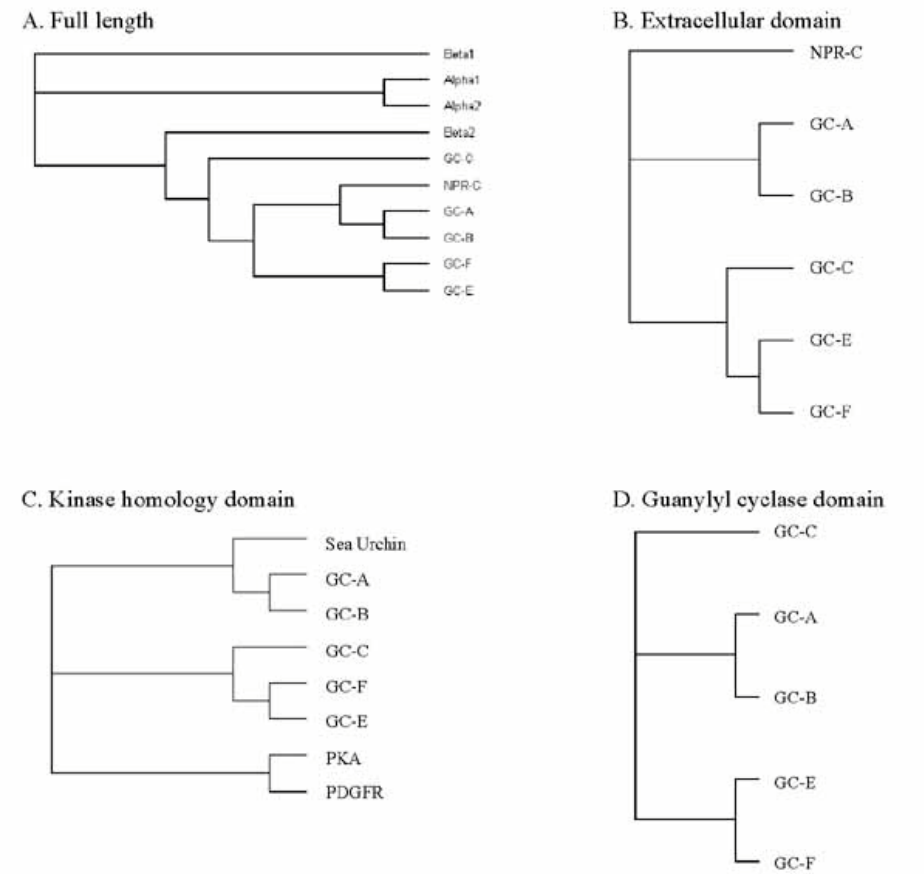

Figure 2. Dendrogram of Human Transmembrane Guanylyl Cyclases and Related Proteins. A. Full-length proteins. B. Extracellular domains. C. Kinase homology domains. D. Guanylyl cyclase domains. Abbreviations are: alpha1, soluble guanylyl cyclase alpha1 subunit; alpha2, soluble guanylyl cyclase alpha2 subunit; beta1, soluble guanylyl cyclase beta1 subunit; beta2, soluble guanylyl cyclase beta2 subunit; PKA, cAMP-dependent protein kinase; PDGFR, platelet-derived growth factor receptor; sea urchin, guanylyl cyclase from *Strongylocentrotus purpuratus*. Unless otherwise noted all sequences are human.

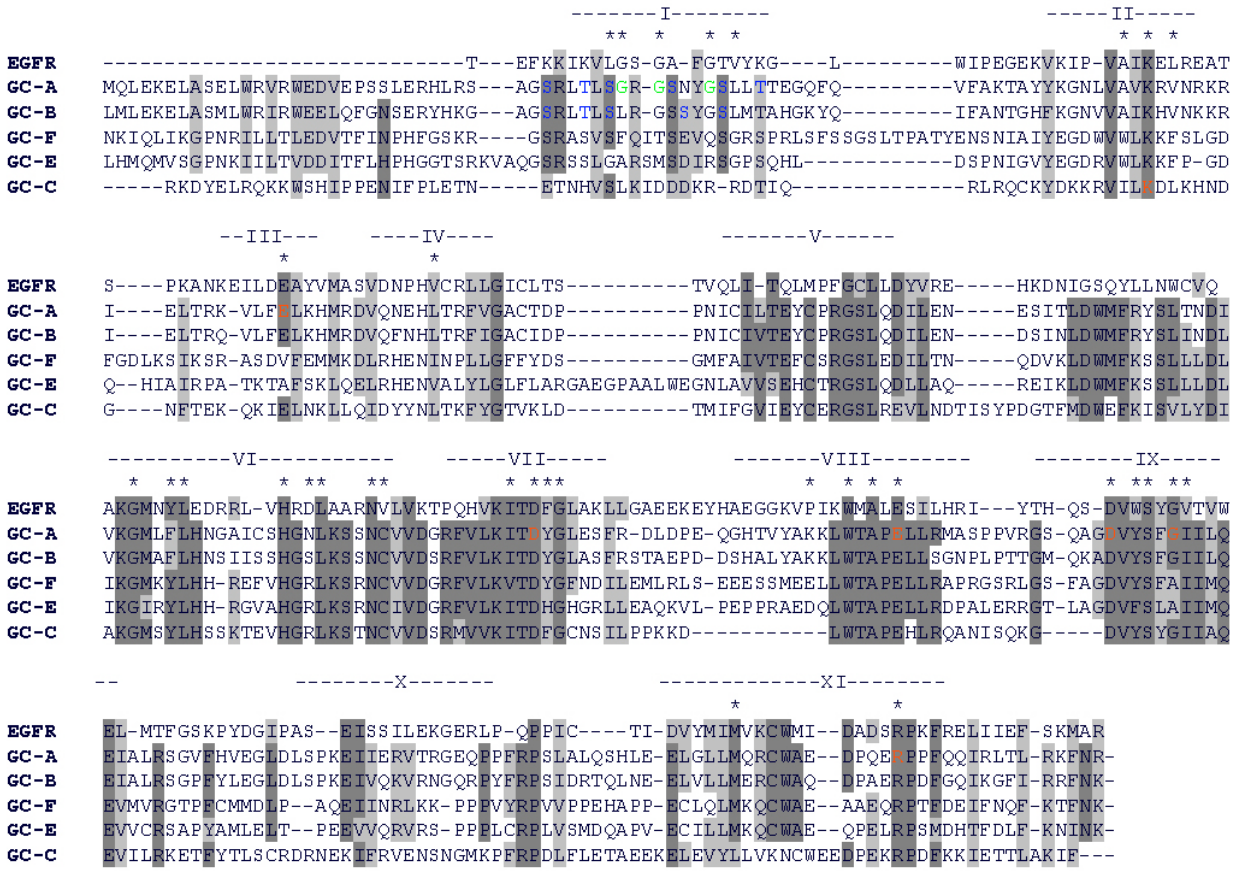

Figure 3. Comparison of the kinase homology domains of human transmembrane guanylyl cyclases with the catalytic domain of the human epidermal growth factor receptor (EGFR). The Roman numerals bracketed by dashes indicate the 11 protein kinase subdomains as originally identified by Hanks et al [66]. The astricks indicate the 33 invariant or highly conserved residues within protein kinases. Residues conserved in 4 or more proteins are shaded in dark grey. Positions where structurally similar residues are conserved in four or more proteins are shaded light grey. Structurally similar grouping used for this purpose are those originally described by Hanks and colleagues [66]: nonpolar R groups (M, L, I, V and C); aromatic or ring-containing R groups (F, Y, W, and H); small R groups with near neutral polarity (A, G, S, T and P); acidic and uncharged polar R groups (D, E, N, and Q); and basic polar R groups (K, R and H). Red residues indicate inactivating mutations. All mutations indicate single alanine substitutions except for the D and G in subdomain of IX of GC-A, which is a double mutant. All three green residues were mutated to alanine in the same receptor and resulted in little or no effect on GC-A activity or phosphorylation state. Blue residues indicate known phosphorylation sites. All sequences are human. The alignment was performed with Clustal.

trimers on gel filtration columns, but in the presence of ligand, they form disulfide-stabilized dimers (48). Hence, full length GC-C may exist as a homotrimer under basal conditions that undergoes an intermolecular rearrangement to a homodimer upon receptor occupancy.

In the absence and presence of GCAP, GC-E is a monomer and homodimer, respectively (49). Therefore, GCAP-dependent activation of GC-E may result from GCAP facilitated oligomerization. Sequential coimmunoprecipitation-western blot assays identified the presence of heterooligomeric complexes between the retinal cyclases, although the vast majority of the GC-E and GC-F were self-associated (50). Similar heteromeric complexes between GC-A and GC-B have been detected in overexpressing cells (44). The physiologic significance of either heteromeric complex is unknown.

4.2. Homology of full-length receptors

Comparison of the primary amino acid sequences of the full-length proteins indicates that the natriuretic peptide receptor-linked cyclases (GC-A and GC-B) and retinal cyclases (GC-E and GC-F) are more closely related to each other than to other family members (figure 2A). The extracellular domain of the cyclase deficient natriuretic peptide clearance receptor (NPR-C) is about 35% identical to the extracellular domains of GC-A and GC-B at the primary amino acid level. GC-C is the lone member of its subfamily but is surprisingly similar to the beta2 subunit of the soluble guanylyl cyclase.

4.3. Extracellular domain

The extracellular domain of each family member consists of roughly 500 amino acids. It is the least homologous of the five domains. There is higher sequence

Figure 4. Comparison of the primary amino acid sequences of the dimerization domains of human transmembrane guanylyl cyclases. The numbering for GC-E and GC-F is taken from Lowe et al [13]. Identical residues in 4 or more proteins are shaded in dark grey. Positions where structurally similar residues are conserved in four or more proteins are shaded light grey. Grouping of structurally similar amino acids is based on rules described in figure 3. Red residues are mutated in human disease. The blue residue indicates an inactivating mutation in GC-A. The alignment was performed with Clustal.

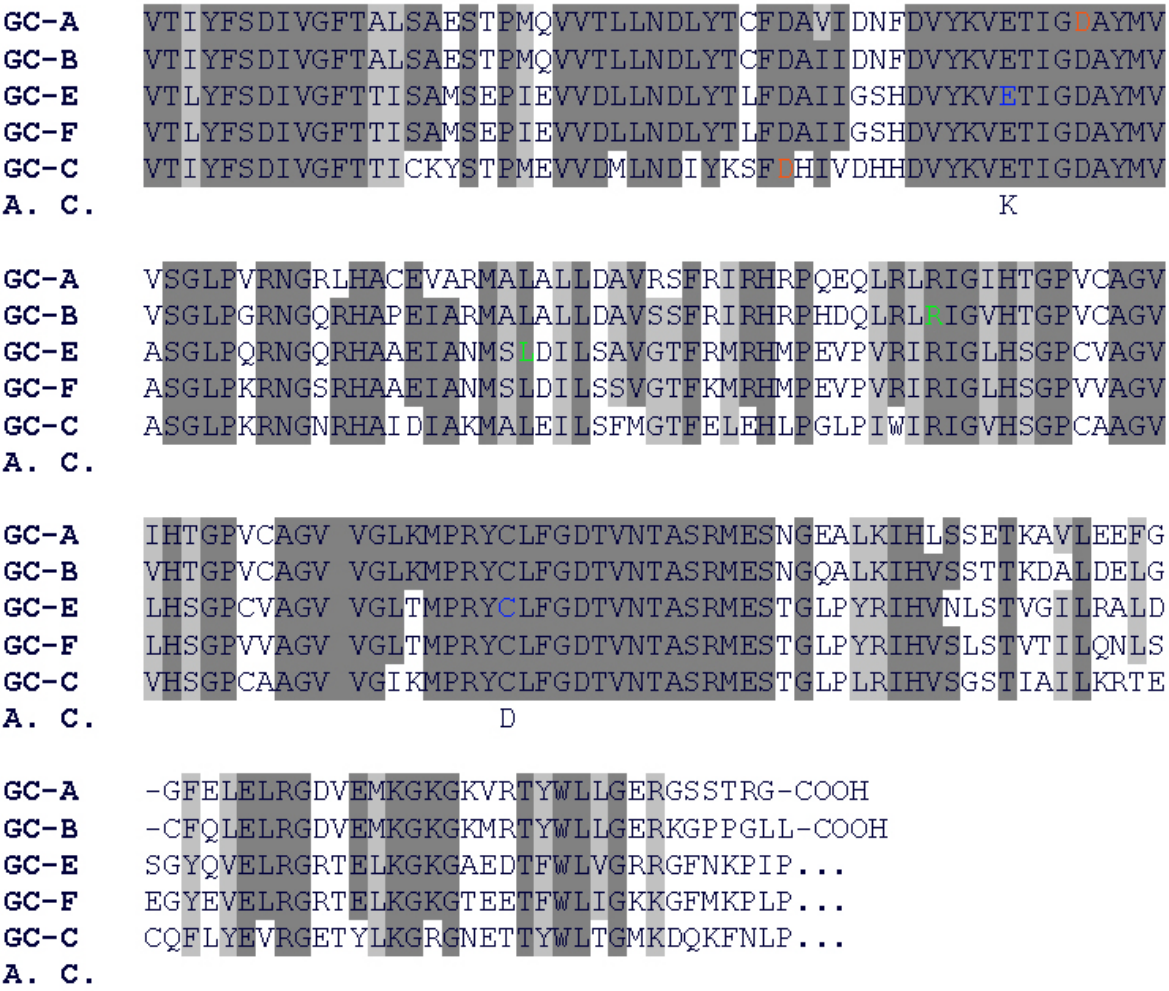

Figure 5. Comparison of the primary amino acid sequences of the catalytic domains of human transmembrane guanylyl cyclases. Red letters indicate residues that result in loss of function-dominant-negative mutations when mutated to alanine. Blue residues indicate residues that determine substrate specificity in GC-E. Green residues represent presumed loss of function mutations identified in human patients. The K and D beside A. C. (adenylyl cyclase) are invariant residues that control purine specificity. The alignment was performed with Clustal.

conservation (about 35% identity) within subfamilies (between GC-A, GC-B and NPR-C or GC-E and GC-F) (figure 2B). The extracellular domain of GC-C is more similar to the retinal cyclases than the natriuretic peptide receptors.

Since all family members are targeted to membranes, each contains an amino-terminal signal peptide that is cleaved from the mature protein. In three cases the start of the mature proteins have been determined by Edman degradation. For human GC-A, human GC-B and bovine GC-E the

initial polypeptide sequences are GXLTVAVVLPLAXT, VARPPGARXLTLAVVLPEHNLSXAWAWPR, and AVFTVGVLGPWA, respectively, where X indicates that no amino acid was determined in that particular Edman cycle (51, 52). For GC-A the peptide sequence corresponds exactly to the sequence of the mature protein that was predicted from cDNA sequence. Both Xs are predicted to code for asparagine and were subsequently shown to be glycosylated (see below). For GC-B, the peptide sequence started 6 amino acids before the first predicted residue. In this sequence the first X codes for an asparagine and was shown to be glycosylated, whereas the second X codes for a tyrosine. For GC-E the Edman sequence from the bovine receptor corresponded exactly to the amino-terminal sequence that was predicted from the human cDNA (52, 53).

4.3.1. Glycosylation

All five transmembrane receptor guanylyl cyclases display size heterogeneity when fractionated by SDS-PAGE, which is primarily due to glycosylation of extracellular asparagine residues. Sequencing of the amino termini of human natriuretic peptide receptor-IgG fusion proteins purified from Chinese hamster ovary cells indicated that Asn-2 and Asn13 of GC-A and Asn-2 of GC-B are glycosylated (51). The extracellular domain of rat GC-A overexpressed in Cos-1 cells was shown to be glycosylated on Asn-13, Asn-180, Asn-306, Asn-347 and Asn-395 by a combined Edman degradation/mass spectrometry approach (54). Most of these sites were also observed in the crystal structure of the extracellular domain of GC-A (55). Asn-2 is not conserved in the rat version of GC-A. Some asparagines contain complex forms of glycosylation (Asn-180, Asn-306 and Asn-347), whereas other residues (Asn-13) contain high mannose forms. The role of glycosylation in the regulation of guanylyl cyclase-linked natriuretic peptide receptors is controversial. Two reports suggest that terminal glycosylation of GC-A affects ligand binding (56, 57) whereas three reports indicate that the incompletely glycosylated versions of GC-A bind ligand similarly to the wild type receptor (54, 58, 59). Mutational studies were used to address the role of glycosylation in the regulation of GC-B. Five of the seven extracellular asparagines were suggested to be glycosylated based on this approach. The mutation of Asn-24 to Asp resulted in a 90% loss in CNP binding, which probably results from improper folding or cellular targeting of the receptor (60).

The extracellular domain of human GC-C contains ten potential N-linked glycosylation sites, but a chemical determination of the exact modification sites has not been reported. A mutational analysis of the predicted N-linked glycosylation sites of porcine GC-C indicated that the loss of any single site does not significantly affect its affinity for ligand (61). On the other hand, the mutation of asparagine 379 to alanine reduced maximal binding in a manner that was not explained by decreased protein expression, suggesting that glycosylation of this residue may be required for the structural stability of GC-C. In a separate study, GC-C expressed in HEK293 cells was shown to be differentially glycosylated (62). A 145 kDa form containing sialic acid and galactose residues was found in the plasma membrane fraction, whereas a 130 kDa form containing high mannose and was found primarily in the endoplasmic reticulum fraction. Ligand binding affinities for both forms of GC-C were similar, but only the higher molecular weight form was activated by ligand. Interestingly, the enzymatic deglycosylation of GC-C had no effect on its ability to respond to ligand. Hence, terminal glycosylation appears to be required to achieve a hormonally responsive state, but is dispensable after that state is obtained.

GC-E and GC-F are predicted to have one or none N-linked glycosylation sites, respectively, based on sequence analysis. Experimentally, bovine GC-E was shown to contain high mannose or hybrid oligosaccharide chains (53). The enzymatic deglycosylation of the receptor had no effect on its basal or stimulated guanylyl cyclase activity, which is reasonable since GCAP activation of this receptor is independent of its extracellular or transmembrane domains (63, 64).

4.3.2. Extracellular disulfide bonds

Three intramolecular disulfide bonds were identified between Cys-60 and Cys-86, Cys164 and Cys-215, and Cys-423 and Cys-432 in a secreted extracellular only version of rat GC-A (54). Mutational analysis of extracellular cysteines in GC-B is consistent with the conservation of the amino-terminal and juxtamembrane intramolecular disulfide bonds in this receptor (65). No data are available on the disulfide bonding patterns of GC-C, GC-E or GC-F. However, since the amino-terminal and juxtamembrane cysteine pairs are conserved in all five family members, similar disulfide bonds may form in these receptors as well.

4.4. Transmembrane domain

All five members of the human particulate guanylyl cyclase family have a stretch of 21 to 25 hydrophobic amino acids followed by at least two basic residues that are predicted to span the plasma membrane. Little amino acid identity is observed within this region between family members. Whether specific residues are required for proper signaling or whether any hydrophobic residues will suffice has not been reported. The exact length of the membrane-spanning region of any mammalian transmembrane guanylyl cyclase is currently based solely on hydrophathy analysis and has not been confirmed experimentally.

4.5. Kinase homology domain

Each member of the human transmembrane, but not soluble, guanylyl cyclase family contains a region of approximately 300 amino acids that possesses similarity to proteins with demonstrated protein phosphotransferase activity called the kinase homology domain (KHD). The KHDs of GC-A and GC-B are most similar to each other but are also highly related to the KHD of the guanylyl cyclase receptor for speract in the sea urchin species *Strongylocentrotus purpuratus* (figure 2C). The homology between cyclase-linked natriuretic peptide receptors and sea urchin cyclases is expected because the rat versions of the former were identified in a low stringency library

screen using the cDNA from *Arbacia punctulata* as a probe (9, 15). The KHDs of GC-E and GC-F resemble each other more than any other KHD-containing protein. The KHDs of GC-C is more similar to the retinal cyclases than to the natriuretic peptide receptors. The KHDs from enzymes with demonstrated protein kinase activity, cAMP-dependent protein kinase and the platelet derived growth factor receptor, display a high degree of similarity to each other but only limited similarity to receptor guanylyl cyclases. Nonetheless, human GC-A, GC-B, GC-C, GC-E and GC-F contain 30, 28, 24, 24, and 23, respectively, of the 33 highly conserved or invariant residues originally identified in proteins kinases (66) if conservative substitutions are taken into account (figure 3). Of the 11 original protein kinase subdomains, all five receptor cyclases contain at least some of the highly conserved or invariant residues in subdomains II, IV, VI, VII, VIII, IX and XI. In addition, GC-A and GC-B contain some of the conserved residues in subdomain I as well as the highly conserved glutamate in subdomain III. The highest degree of homology between the cyclases and protein kinases is in subdomains VI, VII, VIII and IX. Of particular note is the conspicuous absence of the invariant glutamate in subdomain VI in all five cyclases. Since this residue is likely the catalytic base that accepts the proton from the attacking substrate hydroxyl group, these receptors are either devoid of protein kinase activity or have evolved a unique mechanism for phosphate transfer. Although the KHDs of GC-A and GC-B are highly phosphorylated, only GC-E has been reported to possess intrinsic protein kinase activity (see below). Interestingly, alanine substitutions for highly conserved residues within the KHD of GC-A resulted in receptor mutants that were incompletely glycosylated and not phosphorylated, which is consistent with the mutated residues playing similar functional roles in GC-A and known protein kinases (58).

4.6. Dimerization domain

A region of approximately 50 amino acids that bisects the kinase homology and guanylyl cyclase domains of these receptors is known by many names including the hinge region, coiled-coil domain and dimerization domain. Based on its primary amino acid sequence, it is predicted to from an amphipathic alpha helix, which mediates protein-protein interactions. Wilson and Chinkers determined that the region between L781 and H823 is required for the dimerization of the intracellular domain of GC-A (67). They also found that this region is necessary and sufficient for dimerization in a yeast two-hybrid assay and that deletion of this region results in intracellular mutants of GC-A that lack cyclase activity and migrate as monomers on size exclusion columns. The dimerization domain is conserved within all five family members and is almost invariant within the natriuretic peptide receptor and retinal receptor subfamilies, having only one or two substitutions, respectively (figure 4). This is consistent with the notion of this region being highly conserved because it serves an essential function; the mutation of L817 to R was shown to abolish catalytic activity of GC-A, although the basis for the loss of activity was not determined (68).

Mutations within the putative dimerization domain of GC-E were identified that cause retinal disease in a dominant manner (69). One of these mutations, R838C, (red R in figure 4) was biochemically characterized and found to allow GC-E to be stimulated by GCAP-1 at higher than normal calcium concentrations (70). Hence, it was concluded that a single mutant allele causes cone-rod dystrophy not by failing to be activated, but by failing to shut down cGMP production during conditions when calcium levels are elevated. Another mutation in GC-E, proline 858 to serine, occurs at the carboxyl terminus of the dimerization domain and causes a recessive form of retinal dystrophy called Leber's Congenital Amaourosis (71).

4.7. Catalytic domain

Adenylyl and soluble guanylyl cylase are heterodimers that contain one active site per dimer. In contrast, the transmembrane forms of guanylyl cyclases are homodimers that contain two active sites per dimer. It is hypothesized that the two catalytic subunits of the dimer are arranged in an antiparallel fashion (figure 1). The substrate for purine cyclases is a metal-purine complex. The metal required for physiologic activation is magnesium, but in the presence of nonionic detergents, manganese can be used to activate guanylyl cyclases to very high levels in a ligand-independent manner. The presence of more than one binding site per catalytic unit is consistent with the positive cooperative kinetics observed when GC-A, GC-C and GC-E are assayed in the presence of nonionic detergent and manganese (6, 72, 73). However, linear kinetics are observed when assayed under physiologic conditions (no detergent and magnesium-GTP as substrate).

To date, no structure of a guanylyl cyclase catalytic domain has been reported. Hence, our current understanding of the architecture of their catalytic domains is based on modeling experiments using coordinates from the weakly homologous type II adenylyl cyclase. The adenylyl cyclase active site is located at the dimer interface and contains three acidic residues that bind magnesium ions that are hypothesized to participate in the cyclization reaction. Because the residues that interact with the ribose, triphosphate and magnesium ions in adenylyl cyclase are conserved in guanylyl cyclases, these acidic residues are assumed to play similar roles in both enzymes. In support of this idea, alanine substitutions for the highly conserved aspartate at position 893 in GC-A (42) or 834 in GC-C (74) abolish the catalytic activity of these receptors without affecting their ability to oligomerize (red Ds in figure 5).

In contrast, the residues that interact with the purine base determine substrate specificity and differ between adenylyl and guanylyl cyclases. Consistent with this model, when E925 and C997 in GC-E were mutated to K and D, the corresponding residues in adenylyl cyclase, the substrate specificity of the mutant enzyme changed from GTP to ATP (75). Similar mutations in the soluble guanylyl cyclase changed its substrate preference as well (76).

Finally, mutations within the catalytic domains of human transmembrane guanylyl cyclases have been identified that are associated with human diseases (figure 5). The conversion of the highly conserved arginine at

position 957 in GC-B to cysteine causes a severe form of dwarfism called Acromesomelic Dysplasia, Type Maroteaux (77), whereas the mutation of leucine 954 to proline in GC-E causes Leber's Congenital Amaurosis (71).

5. REGULATION

5.1. Role of ATP

ATP has been shown to increase the guanylyl cyclase activity of GC-A, GC-B, GC-C and GC-E. However, the exact mechanism(s) involved in this process has not been clearly delineated. Kurose and colleagues first reported that magnesium-bound, but not free, ATP enhances maximal velocities of ANP-dependent GC-A activity in membranes from various rat tissues (78). ATP also is required to maximally activate GC-B (79). Since AMPPNP, an ATP analog that presumably cannot be used as a substrate by protein kinases, increases activity, Kurose and coworkers suggested that ATP directly binds GC-A and causes it to undergo an allosteric change that increases its catalytic activity. Other groups made similar observations (80, 81), but concluded that ATP binds an unidentified GC-A activating protein because purified receptor preparations or washed membranes were less responsive to ATP than unpurified or unwashed preparations. Foster and Garbers discovered that membranes incubated with ATPgammaS were sensitized to subsequent stimulation by ANP and AMPPNP, which suggested that ATP was serving both as a substrate for the GC-A kinase (see below) as well as an allosteric activator of the receptor.

ATP enhances the guanylyl cyclase activity of GC-C as well (48, 82, 83), although ATP is not absolutely required for ligand-dependent stimulation as has been suggested for GC-A (84). The stimulation does not result from a direct activation of the enzyme, but rather from the stabilization of an active state or an inhibition of the deactivation process (48).

ATP increases GC-E cyclase activity in the presence and absence of GCAPs and calcium. Whether this effect is mediated by direct activation, decreased inactivation, phosphorylation (see below) or a combination of events is unclear (64, 85-87). GC-E purified from bovine retina was cross-linked to 8-N_3(a-^{32}P)ATP (88). ATP binding was not magnesium-dependent or competitive with GTP. Interestingly, this same purified GC-E preparation contained a protein kinase activity that was able to phosphorylate GC-E as well as exogenous substrates (88). Hence, the authors concluded that GC-E is an autophosphorylating protein kinase. However, since the stoichiometry of phosphorylation was only about 5%, the possibility that the preparation was contaminated with an unidentified protein kinase cannot be ruled out. To date, this observation has not been confirmed.

The deletion of the KHD from GC-A or GC-B results in mutant receptors with constitutively elevated guanylyl cyclase activities that are not further elevated by natriuretic peptides and/or ATP (89, 90). The effect of deleting the GC-C KHD is controversial. One group found that it causes constitutive activation (74), whereas another observed basal or repressed activities (91). A theory that is congruent with the majority of the data for the extracellular-ligand-activated receptors (GC-A, GC-B and GC-C) suggests that hormone binding disrupts the KHD-dependent inhibition of the catalytic domain. In contrast, deletion of the KHD from GC-E does not result in dramatically increased basal guanylyl cyclase activity. The KDH is required for GCAP activation of GC-E, but whether GCAPs directly interact with the KHD or the cyclase domain is unclear.

Consistent with the idea of direct ATP binding is the sequence GXGXXXG within KHD of GC-A that resembles the canonical ATP binding motif GXGXXG found in subdomain I of most bona fide protein kinases. Because mutations within this region decreased hormone responsiveness of GC-A and GC-B, it was termed the ATP regulatory domain or ARM domain (92). However, a subsequent study found that the reduced activity associated with these mutations results from receptor dephosphorylation, not reduced ATP binding (93). In support of this region not binding ATP, the mutation of all 3 glycines within the putative ARM had little or no effect on the ANP-dependent cyclase activity of GC-A (58) (green residues in figure 3). Furthermore, although ATP regulates all members of this family, only GC-A contains the GXGXXXG sequence. GC-B is missing the first G, and GC-E-the only member of the family that has been cross-linked to an ATP analog-lacks this sequence all together. Additionally, since this glycine-rich region is highly phosphorylated in GC-A and GC-B (see below), one might expect that ATP binding would be inhibited due to electrostatic repulsion. I believe it is unlikely that ATP binds to this highly negatively charged putative ARM domain that is only present in one family member. In contrast, the lysine in subdomain II (red K in GC-C in figure 3) is conserved in all family members, and the mutation of this residue (K516) to alanine in GC-C markedly reduces its ligand-dependent cyclase activity (94). It will be informative to see if similar mutations disrupt signal transduction in other family members.

Although the exact nature of ATP-dependent regulation of these receptor cyclases is not known, analysis of the literature supports a few general conclusions. First, although the KHD domain is required for ATP-dependent regulation, no data definitively show that ATP binds the KHD. GC-E has been cross-linked to an ATP analog, but the binding site was not determined. Similarly, ATP and its analogs have been shown to modulate the ligand binding (95) and cyclase activities (73) of very highly purified GC-A preparations, which is consistent with a direct binding model. On the other hand, the modulatory effect of ATP is always reduced in purified preparations compared to its affect in membranes. One explanation for the reduced responsiveness is the presence of nonionic detergent in the purified samples, which may disrupt interactions that are required to repress basal cyclase activities. However, another plausible explanation is that the purified preparations are contaminated with a very small amount of an ATP-binding regulatory protein. A second consistently

observed finding is that the rank order of activation is always ATPgammaS>ATP>AMPPNP. Since thiophosphorylated proteins are resistant to dephosphorylation and because AMPPNP is presumably not a substrate for protein kinases, a portion of the effect may involve phosphorylation of the receptors or regulatory proteins. A related observation is that ATP mediates two effects. One involves direct participation in the activation process, whereas another prevents the inactivation of an activated receptor. This latter function may involve receptor dephosphorylation, which may explain why ATPgammaS is a better activator than ATP or AMPPNP. A definitive determination of the ATP binding site in one of these receptors would clarify this issue immensely.

5.2. Phosphorylation and homologous desensitization

The fundamental role of phosphorylation in the regulation of natriuretic peptide receptor-linked guanylyl cyclases has been well established, although many questions remain to be answered. Phosphorylation-dependent regulation of GC-A was first correctly characterized in stably expressing HEK293 cells (96). In resting cells GC-A is highly phosphorylated, but ligand exposure results in receptor dephosphorylation and desensitization. *In vitro* treatment with a purified protein phosphatase also results in receptor dephosphorylation and inhibition. Although the exact stoichiometry of phosphorylation has never been determined, it is at least 1:1 because dephosphorylation resulting from ligand exposure or phosphatase treatment increases the electrophoretic mobility of GC-A (96). GC-B is highly phosphorylated in resting cells and dephosphorylation as a result of ligand binding or phosphatase treatment inhibits this enzyme as well (97). These studies led to the "desensitization by dephosphorylation" hypothesis, which is exactly the opposite of the prevailing desensitization theory for G protein coupled receptors. Subsequent studies confirmed and extended these observations. Koller and colleagues identified point mutations (red residues in figure 3) within the KHD GC-A that result in incompletely glycosylated and completely dephosphorylated receptors that were unresponsive to ANP (58). Jourbert and colleagues determined that ANP-dependent dephosphorylation primarily results from reduced phosphorylation, not increased dephosphorylation of the receptor (98). Interestingly, the tryptic phosphopeptide maps of receptors isolated from cells incubated in the presence or absence of natriuretic peptide are qualitatively similar (97, 99). In other words, the maps from desensitized cells do not lack any specific phosphopeptide even though the receptors are clearly dephosphorylated. At least two explanations are consistent with these results. One, there are two populations of receptors in cells. The first is completely dephosphorylated in response to hormone binding and the second is not dephosphorylated at all. A second possibility is that a specific phosphorylation site(s) is dephosphorylated in response to ligand binding, but the phosphopeptide that contains this site(s) is lost during the purification, and therefore, does not show up in maps of GC-A or GC-B isolated from either control or desensitized cells.

GC-B and GC-A contain five or six known phosphorylation sites within the amino-terminal portion of their KHDs, respectively (93, 100). The phosphorylated amino acids are the blue residues within subdomain I in figure 3. The mutation of any one site to alanine results in decreased natriuretic-dependent guanylyl cyclase activity, reduced receptor phosphorylation and changes in the two dimensional tryptic phosphopeptide maps of the mutated receptor compared to maps from wild type receptors. When multiple phosphorylation sites within GC-A and GC-B were mutated to alanine, these resulting proteins were unresponsive to natriuretic peptides. These data indicated that phosphorylation is absolutely required for the activation of GC-A and GC-B.

To directly test the desensitization by dephosphorylation hypothesis, a mutant version of GC-A was constructed where five (5E) or all six (6E) of its known phosphorylation sites were converted to glutamate. Like phosphate, glutamate residues are negatively charged at physiologic pH and have been shown to functionally substitute for phosphorylated serines and threonines in proteins (101). Membranes from cells expressing the GC-A-5E could be activated 6 to 7 fold whereas GC-A-6E was activated almost 10 fold by ANP and ATP (102). Although these receptors are not as responsive as the wild type receptor, which can be activated more than 40 fold, they provided an excellent system to directly assess the contribution of receptor dephosphorylation to the desensitization process. The idea being that if dephosphorylation mediates desensitization, then a receptor that cannot be dephosphorylated should be resistant to desensitization. This is what was observed because the rate of cGMP formation in membranes or whole cells expressing GC-A-6E was linear for a much longer period of time compared to activities determined in membranes or cells expressing the wild type receptor (102, 103). Furthermore, although the protein phosphatase inhibitor, microcystin, markedly increased cyclase activity in membranes containing the wild type receptor, it had no effect on membranes containing the NPR-A-6E, consistent with GC-A being the sole target of microcystin in this assay.

The kinase or kinases that phosphorylate GC-A and GC-B have not been identified, but they presumably must be constitutively active and widely expressed. In addition, the possibility of sequential phosphorylation has not been investigated, although it remains a distinct possibility with the high number of phosphorylation sites contained in GC-A and GC-B. Initial characterization of the phosphatases the dephosphorylate GC-A identified two separate activities (103). One did not require a divalent metal ion for activity and was inhibited by the protein phosphatase inhibitor, microcystin, whereas the other was magnesium- or manganese-dependent and insensitive to microcystin. Additionally, protein phosphatase 5 was shown to bind the KHD of GC-A in a yeast two-hybrid screen (104). The dephosphorylation of GC-A by PP5 in whole mammalian cells has not been reported.

The homologous desensitization of GC-C is clearly apparent in the colonic cell lines T84 or CaCo2, but not in overexpressing HEK293 cells, suggesting the involvement of a cell specific factor (105). Phosphorylation

does not appear to be involved in the homologous desensitization of GC-C. However, one report (106), but not another (105), observed GC-C degradation in response to long-term (12 h) ligand exposure, suggesting that ligand-dependent degradation may be involved in the desensitization of GC-C.

As described above, GC-E was reported to be an autophosphorylating kinase. Phosphoamino acid analysis of *in vitro* phosphorylated GC-E revealed only phosphoserine. No effect of phosphorylation was detected on GC-E cyclase activity. This may be due to the presence of detergent in the assay or perhaps due to the low stoichiometry of phosphorylation. The sites of phosphorylation have not been identified. In a separate study, ATP was shown to increase the activity of GC-E by two-fold in a manner requiring the presence of an enzyme with properties similar to PKC (107). GC-E isolated from metabolically labeled HEK 293 cells is phosphorylated primarily on serine and to a lesser extent on threonine (L. R. Potter and J. B. Hurley, personal communication). No phosphotyrosine was detected. The functional effects of this modification have not been fully explored.

5.3. Regulation by protein kinase c and calcium

Cross-talk between a transmembrane receptor guanylyl cyclase and other signaling pathways has been most often observed for pressor hormone-dependent inhibition of natriuretic peptide receptors. Examples of hormones or factors that inhibit GC-A and/or GC-B are arginine-vasopressin (108, 109), angiotensin II (110), endothelin (111), histamine (112), lysophosphatidic acid (113), sphingosine-1-phosphate (114, 115), platelet-derived growth factor and basic fibroblast growth factor (36). All of these agents bind serpentine receptors or tyrosine kinase receptors that stimulate phospholipase C (PLC). This family of enzymes produces diacylglycerol, a direct activator of the classic forms of protein kinase C, and inositol triphosphate, which binds to receptors that elevate intracellular calcium concentrations. Several studies have implicated protein kinase C activation in the heterologous desensitization process because phorbol 12-myristate 13-acetate (PMA), a direct activator of protein kinase C, mimics the ability of the vasoconstrictory hormones to desensitize natriuretic peptide receptors. In addition, protein kinase C inhibitors have been reported to block angiotensin II-dependent (110) and endothelin-1-dependent (116) desensitization of GC-A and GC-B, respectively, which is highly suggestive of protein kinase C participating in these processes.

Although early studies found that protein kinase C phosphorylates GC-A *in vitro* (117, 118), *in vivo* labeling experiments indicated that protein kinase C activation causes GC-A and GC-B dephosphorylation (99, 119). In contrast to the natriuretic peptide-dependent desensitization process, where the tryptic phosphopeptide mapping patterns are unchanged by ligand exposure, PKC activation results in the dephosphorylation of a single site or a small subset of the total phosphorylation sites (99, 119). Tryptic phosphopeptide mapping studies of GC-B expressed in HEK293 cells indicated that S523 is dephosphorylated, while phosphorylation of S518 is increased by PKC activation (119). Consistent with the notion of dephosphorylation of S523 being the mechanism for the desensitization, the mutation of S523 to alanine or glutamate resulted in less responsive receptor that could not be further inhibited or dephosphorylated by PMA treatment. A version of GC-B containing glutamate substitutions at all five phosphorylation sites was resistant to PMA-dependent desensitization as well. GC-A is also selectively dephosphorylated and desensitized by PMA exposure, but the specific residue(s) that is targeted by this process has not been identified (99).

Although the protein kinase C-dependent dephosphorylation of Ser-523 is a proven inhibitory mechanism for GC-B, no physiologic treatment has been shown to use it. In fact, based on studies with the protein kinase C inhibitor, GF-109203X, and/or the chronic down regulation of protein kinase C, with the treatments (arginine-vasopressin, lysophosphatidic acid, sphingosine-1-phosphate and hyperosmolarity) and cell lines (A10 smooth muscle, NIH3T3 fibroblasts and human embryonic kidney cells) that we have tested, protein kinase C contributes little if any to the inhibitory process. These observations suggest that the inositol triphosphate-calcium arm of the phospholipase C pathway plays the dominant role in the desensitization of GC-B. Consistent with this hypothesis, AVP, S1P and hyperosomotic conditions elevate intracellular calcium concentrations in these cells (R. Potthast and L. Potter, unpublished data) (109). Furthermore, ionomycin, a calcium-ionophore, mimics the inhibitory effects of these conditions, whereas a cell-permeable calcium-chelator blocks all or most of these responses. Together, these data are consistent with calcium elevations, not protein kinase C activation, mediating the cross-talk between GC-B and various antagonistic pathways or environmental conditions.

The mechanism for the calcium-dependent inhibition is not completely understood, but involves receptor dephosphorylation because agents that inhibit GC-B through calcium dependent processes (hyperosmotic medium, ionomycin, sphingosine-1-phosphate, fetal bovine serum), reduce the amount of phosphate associated with the receptor (R. Potthast, S. Abbey-Hosch, D. Smirnov and L. Potter, manuscripts submitted). Furthermore, a receptor containing glutamates in place of its known phosphorylation sites is completely or partially refractory to the inhibitory effect of these agents. Interestingly, although dephosphorylation is involved in both processes, the mechanisms associated with each are unique. The protein kinase C-dependent process primarily decreases the affinity of GC-B for CNP and GTP, whereas, the calcium-dependent process primarily reduces the maximal velocity of cGMP synthesis (S. Abbey-Hosch, D. Smirnov and L. Potter, submitted).

In contrast to the natriuretic peptide receptors, GC-C activity is increased by phorbol esters in T84 cells (120). Metabolic labeling studies indicated that GC-C is phosphorylated in resting cells and that phorbol esters increase its ^{32}P-content two-fold (121). The stoichiometry

of phosphorylation was not determined. Purified rat brain protein kinase C phosphorylated immunoprecipitated GC-C and the phosphorylation was associated with the doubling of its ligand-dependent guanylyl cyclase activity. Serine1029 was hypothesized to be the protein kinase C phosphorylation site. However, data proving that this site is phosphorylated *in vivo* or indicating that the specific mutation of this site blocks the phorbol ester-dependent augmentation of cyclase activity has not been reported.

Finally, as mentioned above, an enzyme with properties similar to protein kinase C was shown to stimulate GC-E two-fold in the presence of ATP (107). Direct phosphorylation of GC-E by protein kinase C has not been reported.

6. PERSPECTIVE

Over the past 20 years a tremendous amount of data has been generated regarding transmembrane guanylyl cyclases. Much of this information results from the purification and/or cloning of GC-A, GC-B, GC-C, GC-E and GC-F. However, based on sequence analysis, all active guanylyl cyclases have been identified in the human genome. Thus, future advances will likely stem from the identification of new functions, forms of regulation or therapeutic uses of known enzymes. Interestingly, the number of individual transmembrane guanylyl cyclases per genome appears to be inversely related to the species' rung on the evolutionary tree, since worms, rodents and humans express 26, 7, and 5 transmembrane guanylyl cyclases, respectively. One possible consequence of the decrease in the total number of receptors is the consolidation of regulatory functions within the remaining family members. Thus, GC-A, GC-B, GC-C, GC-E and GC-F may have adopted some signaling roles that were once mediated by ancestral family members. A corollary of this prediction is that the phenotypes of knock out mice may not be absolutely predictive of all the human functions of these receptors. Consistent, with this scenario is the ability of ANP to stimulate lipolysis in primates, but not rodents (122). Future studies will likely reveal additional intriguing functions and modes of regulation for transmembrane receptor guanylyl cyclases.

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