Differentiation of F9 Teratocarcinoma Stem Cells to Primitive Endoderm Is Regulated by the G_{ia2}/G_{sa} Axis via Phospholipase C and Not Adenylylcyclase*

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Morphogen-induced decline in G_s triggers F9 teratocarcinoma stem cells to progress to primitive endoderm via activation of protein kinase C and mitogen-activated protein kinase (Gao, P., and Malbon, C. C. (1996) J. Biol. Chem. 271, 9002-9008). Constitutive expression of G_{ia2} blocks, whereas expression of G_{sa} provokes, progression to primitive endoderm, permitting identification of the effectors of the response-utilizing chimera created between G_{ia2} and G_s. N-terminal substitution of G_s with G_{ia2} sequence to create chimera G_{ia2} (1-54/G_s) produced a chimera that activated adenylylcyclase but abolished progression to primitive endoderm and activation of phospholipase C. C-terminal substitution of G_s with G_{ia2} sequence to G_s/G_{ia2} (320-355) enhanced the ability of G_s to promote progression. The Q205L-activated mutant of G_{ia2} suppresses, whereas the G225T-activated mutant of G_s strongly activates phospholipase C and progression in these cells. The N-terminal region of G_s (residues 62-86) appears to act as a dominant switch for the G_s (activation) versus G_{ia2} (suppression) mediated control of phospholipase C and progression to primitive endoderm.

Embryonal carcinoma cells mimic the early embryo and have proven to be a useful model for study of development. These stem cells can be induced to differentiate in vitro into cell types that resemble those found at various stages of early mouse development (1). Progression of F9 teratocarcinoma embryonic stem cells can be divided into two separate events, the production of primitive endodermal and of parietal endodermal cells. When F9 cells are exposed to retinoic acid (RA) alone, cells become flat and show typical endodermal morphology, which is the functional equivalent of primitive endoderm (PE) of normal embryogenesis, and express the protease tissue plasminogen activator (tPA, a PE marker) as well as components of the basal lamina, such as type IV collagen and laminin B1 (2). Elevation of intracellular cyclic AMP levels in F9 cells treated in combination with RA promote primitive endodermal cells to a parietal endoderm-like phenotype in early mouse embryogenesis (1).

Many complex biological processes such as oncogenesis, differentiation, and early neonatal mouse development have been shown to be regulated by heterotrimeric G-proteins, such as G_s and G_{ia2}. In the F9 mouse teratocarcinoma model of early mouse development, G_{ia2} levels decline precipitously in response to the morphogen RA as the cells commit to PE (3). Constitutive expression of RNA antisense to G_{ia2}, but not to G_s, or G_{ia2}, induces progression to PE in the absence of RA (4). Overexpression of G_{ia2}, a G-protein that antagonizes G_s with respect to one known effector adenyllylcyclase, blocks stem cells from progression to PE even in the presence of RA (4). Expression of G_s or its constitutively active mutant form induced PE in the absence of RA (5). Furthermore, the RA-induced decline in G_{ia2} triggers F9 teratocarcinoma stem cells to progress to PE via activation of protein kinase C and mitogen-activated protein (MAP) kinase (6). These observations suggest that G_{ia2} suppresses the commitment of stem cells to primitive endoderm, while G_s expression somehow eliminates the suppression and/or induces differentiation itself.

In an effort to identify the effector for the G_{ia2}/G_s axis with respect to regulating development of PE from stem cells, we expressed chimera with N-terminal substitutions of G_{ia2} in the G_s molecule. Using stably transfected clones of F9 cells expressing elevated levels of the chimeric G-proteins, we probed the downstream events to the level of MAP kinase and progression to PE. The results identify a central role of phospholipase C in the regulation by G_{ia2}/G_s, via protein kinase C and MAP kinase activation. Additionally, substitution of region (55-64) of G_{ia2} for G_s and the C-terminal 36 amino acids of G_{ia2} for the corresponding 38 C-terminal residues of G_s promotes progression to PE in the absence of the morphogen and identifies these regions as important determinants for regulation of phospholipase C.

EXPERIMENTAL PROCEDURES

Stable Expression of Chimeras in F9 Stem Cells—Wild-type and stably-transfected clones of F9 mouse teratocarcinoma stem cells were cultured on 0.1% gelatin-coated 10-cm Falcon Petri dishes in Dulbecco’s modified Eagle’s medium supplemented with 15% of fetal calf serum (HyClone Laboratories). The expression vector pCW1 harboring cDNA constructs encoding either wild-type and constitutively activated mutants of G_s and G_{ia2} or chimera of G_{ia2}/G_s under the control of the SV40 early promoter were used to stably transfect F9 stem cells by calcium phosphate precipitation. Clones were selected (500 μg/ml) and maintained (100 μg/ml) in the presence of the active form of gentamicin analogue, antibiotic G418 sulfate (Life Technologies, Inc.).

Immunoblotting—Aliquots of crude membrane fractions (5 μg) from each subclone were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), the separated proteins were transferred to nitrocellulose, and the blots were stained with antibodies specific for either G_s (CM129) or to G_{ia2} (CM112). The immune complexes were made visible by staining with a second antibody (goat anti-rabbit IgG) coupled to calf alkaline phosphatase.

Cyclic AMP Accumulation—Cyclic AMP accumulation was deter-
mined by the competitive protein binding assay (7). Cell aliquots (10⁶) were washed and incubated in Krebs-Ringer phosphate buffer (2 × 10⁶ cells/ml) with the indicated agents for 15 min at 37°C. The reaction was terminated by the addition of ethanol maintained at ~70°C. Aliquots of cells were assayed in triplicate in either the absence or the presence of the β-adrenergic agonist (−)isoproterenol (100 μM) or the diterpene activator of adenylcyclase, forskolin (50 μM).

**Determination of PE**—The activity of tissue plasminogen activator (tPA) is the hallmark of the PE phenotype. Stem cells induced to PE produce and secrete tPA as well as assume a characteristic morphology, i.e. extended spindle shape with defined foci of growth (1). For production of PE, all-trans-RA (100 nM, Eastman Kodak) was added for 4 days to induce progression to PE (8). For tPA determinations, the culture medium of cells was assayed using the amidoanalytic assay (9). One unit of tPA is arbitrarily defined as that amount of tPA produced at a reaction rate of 10⁻³ ∆A₄₀₅ min⁻¹ (change in the optical absorbance at 405 nm divided by the square of the time, in min). For phase-contract microscopy, the cells were cultured for 4 days and then fixed with 3% (w/v) paraformaldehyde, viewed, and photographed by phase-contrast microscopy using a Zeiss Axioskop system.

**Quantitation of 1,2-Diacylglycerol (DAG) Mass**—Cells plated in 6-well plates (10⁶) were cultured in 6-well plates for 4 days in the presence and absence of RA (100 nM). Cells were harvested and subjected to treatment with perchloric acid. After neutralization, the mass of IP₃ was determined by a thin-layer chromatography to separate radioactivity incorporated into DAG contents was measured using the DAG kinase assay (11) employing the column, which was then washed with buffer B. The protein kinase C (PKC) activity was assayed using a commercially prepared assay (Life Technologies, Inc.) that utilizes a 50-μl reaction mixture containing 20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.5% Triton X-100, and 25 μg/ml each aprotinin and leupeptin. Homogenates were incubated on ice for 30 min, and supernatants were collected after microcentrifugation. Protein kinase C was partially purified by chromatography on a DEAE-cellulose column (0.2 g of DE52, Whatman equivalent buffer B (20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol) (12). The column was then washed with buffer B. The protein kinase C was eluted in buffer C (buffer B plus 0.2 M NaCl). Protein kinase C activity was assayed using a commercially prepared assay (Life Technologies, Inc.) that utilizes a 50-μl reaction mixture containing 20 mM Tris, pH 7.5, 20 mM MgCl₂, 1 mM CaCl₂, 20 μM [γ-³²P]ATP, 50 μM acetylated myelin basic protein substrate-(4–14) and activator phorbol myristic acid (10 mM) in combination with phosphatidyserine (0.28 mg/ml) or the presence versus absence of the pseudosubstrate inhibitor peptide, protein kinase C (C–19–36) (20 μM) (13). Reactions were initiated by the addition of 5 μl of the DEAE-purified homogenate and incubated at 30°C for 5 min. Twenty μl of the reaction sample were spotted onto a P-81 phosphocellulose filter, which was then washed with 1% (w/v) phosphoric acid. Protein kinase C activity is expressed as picomoles of [³²P] transferred to the myelin basic protein substrate (4–14)/min/10⁶ minus those transferred to the myelin basic protein in the presence of the pseudosubstrate inhibitor peptide, protein kinase C (19–36).

**RESULTS AND DISCUSSION**

**Stable Expression of Chimeric G₁₂α/Gₛα Subunits**—The cDNAs encoding the chimeras (Fig. 1) were inserted in the pCW1 expression vector driven by the SV40 early promoter and harboring the neomycin resistance gene. N-terminal substitutions of Gₛα by analogous sequences of G₁₂α were employed (15), as was the substitution of the C-terminal 38 residues of Gₛα with the corresponding C-terminal 36 residues of G₁₂α (16). Similarly, wild-type and the constitutively active G₂₂₅T mutant of Gₛα and wild-type and the constitutively active Q₂₀₅L mutant of G₁₂α were expressed also. Clones selected in the presence of the gentamicin analogue G418 displayed elevated expression of the chimera (Fig. 2). Immunoblots of chimeras with Gₛα C-terminal sequences were stained by a specific antibody raised against the decapeptide C-terminus of Gₛₚ (CM112; Fig. 2, top panel). Chimera with G₁₂α sequences expressed in the C terminus were stained with antibody specific to the decapeptide C-terminus of G₁₂α (CM129; Fig. 2, bottom panel). Expression of each of the chimeras ranged from 1- to 2-fold over endogenous levels of the G-protein subunits expressed by F9 clones stably transfected with the empty vector pCW1 alone.

**Activation of Adenylyl cyclase**—The ability of the chimeras to...
activate adenylyl cyclase was explored by measuring cyclic AMP accumulation of the clones in response to stimulation either by the β-adrenergic agonist isoproterenol or by the diterpene forskolin. The patterns for cyclic AMP accumulation by clones expressing the various subunits and their respective chimeras in F9 stem cells were similar for the basal, isoproterenol-stimulated accumulation by clones expressing the various subunits and chimeras, and forskolin-stimulated (50 μM) conditions. These data demonstrate that, when stably expressed in the context of F9 teratocarcinoma cells, Gs stimulates, Gia2 inhibits, and substitution of Gs with increasing N-terminal regions of Gia2 attenuates activation of adenylyl cyclase. Thus, the G-proteins expressed have retained the ability to recognize receptor, bind and exchange GTP, and to activate one known effector, adenylyl cyclase.

**Progression of Stem Cells to PE**—Secretion of tPA is a hallmark for stem cell differentiation to PE (1). Using the sensitive amidolytic assay (9), we measured the ability of clones expressing Gs, Gia2/G225T, and chimeras of Gs with increasing substitution of Gia2 to progress to PE, both in the absence and presence of the morphogen RA (Fig. 4). Expression of either Gs/G225T or wild-type Gs promoted progression to PE in the absence of morphogen, as evidenced by expression of the tPA marker. In combination with RA, expression of Gs markedly potentiated (3-fold), whereas expression of Gia2 suppressed (>50%), the progression to PE heralded by tPA production. Substitution of Gs N-terminal sequence with increasing amounts of Gia2 sequence attenuated the effect on progression. The markedly elevated response to Gs expression was reduced to that of the empty-vector control by substitution in the N terminus with as little as seven residues of Gia2 and reduced progressively below the control by expression of Gia2 (1–54) in Gia2 (1–212) and Giα2 (1–225) chimeras. The Gia2 (1–54) construct, which displayed the highest activation of adenylyl cyclase (Fig. 3), strongly inhibited stem cell progression, as measured by tPA production.

Further analysis of the N and C termini of Gs by substitution with Gia2 sequences identified two regions of the molecule critical to progression of stem cells to PE in either the absence (Fig. 5) or the presence (not shown) of RA. Although expression of Gia2 (1–54) strongly activates adenylyl cyclase (Fig. 3) and inhibits progression (Fig. 4), expression of Gia2 (1–64) strongly promoted progression of stem cells to PE with respect to expression of Gs, although both chimeras were equivalent with respect to activation of adenylyl cyclase. Substitution of Gs C-terminal domain with that of Gia2, Gia2 (120–355), enhanced the progression to PE in the absence of morphogen for Gs and the Gia2 (1–64), but not for either the Gia2 (1–54) or Gia2 (1–122) chimera (not shown).
The inability of agents that elevate intracellular cyclic AMP accumulation, like cholera toxin, pertussis toxin, and forskolin, as well as the addition of dibutyryl cyclic AMP itself to induce differentiation of F9 stem cells (1–5) are consistent with the lack of correlation observed between activation of adenylylcyclase and promotion of PE observed herein. The differential effects of expression of G\textsubscript{i2} (1–54/s) on cyclic AMP accumulation (strongly stimulated) versus tPA secretion (strongly inhibited) highlight this point.

The recent demonstration that suppression of G\textsubscript{i2} leads to increased phospholipase C activity in a variety of cells (17), prompted us to evaluate the PLC response by measuring IP\textsubscript{3} and DAG generation (Fig. 6). Expression of G\textsubscript{i2} and G\textsubscript{s225T} increased IP\textsubscript{3} accumulation in the absence of morphogen. Substituting G\textsubscript{i2} N-terminal sequence from 7 to 212 residues for G\textsubscript{s} attenuated IP\textsubscript{3} accumulation. This trend was broken, however, by clones expressing the G\textsubscript{s225T} chimera, which displayed nearly a 4-fold increase in IP\textsubscript{3} accumulation over that observed in stem cells transfected with the empty vector alone (Fig. 6, left panel). These stimulated levels of IP\textsubscript{3} generated in clones promoted to PE through expression of the G\textsubscript{i2} (1–64/s) chimera are in excess of those in F9 cells promoted to PE by RA itself. Expression of the G\textsubscript{s225T} (1–380) chimera substituted in the N-terminus with G\textsubscript{i2} also promoted markedly enhanced PLC activity. The PLC activity of the clones expressing G\textsubscript{s225T} G\textsubscript{i2} (1–64/s) and G\textsubscript{i2} (1–320–350) chimeras remained elevated even when the clones were induced with RA (Fig. 6, center panel).

DAG levels, explored in the absence of morphogen, confirmed the observations derived from IP\textsubscript{3} mass measurements. Although equivalent with respect to adenylylcyclase activation (Fig. 3), the G\textsubscript{i2} (1–54/s) and G\textsubscript{i2} (1–64/s) chimeras can be differentiated by the ability of the latter to increase IP\textsubscript{3} accumulation, to increase DAG accumulation (Fig. 6, right panel), and to promote conversion of stem cells to PE in the absence of morphogen (Fig. 5).

**Role of Protein Kinase C—Activation of PLC activity, evidenced by elevated IP\textsubscript{3} and DAG levels, implices protein kinase C in the signaling by the chimera. Analysis of protein kinase C activity in the absence of morphogen revealed a pattern of stimulation of protein kinase C (Fig. 7, left panel) not unlike that of IP\textsubscript{3} and DAG accumulation (Fig. 6). Expression of G\textsubscript{s}G225T and G\textsubscript{s} itself markedly activated protein kinase C, while substitution of G\textsubscript{i2} sequence in the N-terminus of G\textsubscript{s} attenuated the elevated activity. The G\textsubscript{i2} (1–64/s) chimera, in contrast to the other members of the array of chimeras with increasing G\textsubscript{i2} substitution, displayed activation of protein kinase C similar to that of G225 G\textsubscript{s} the constitutively active mutant of G\textsubscript{s}. Expression of the G\textsubscript{s} (1–320–350) C-terminal substituted chimera also strongly activated protein kinase C. The rank order and relative scale for protein kinase C activation...
and progression to PE, measured by tPA secretion (Fig. 7, right panel), is remarkably similar.

**MAP Kinase Activation**—The MAP kinase regulatory network is an important conduit for signaling from the cell surface to the nucleus. We explored MAP kinase activity only in clones expressing chimeras that induce progression to PE in the absence of retinoic acid (Fig. 8). The peak values of MAP kinase activity were resolved on Mono-Q FPLC separations and assayed with the EGF receptor peptide. In close agreement with the effects of expression of the Gs\(_{\alpha}\) and Gi\(_{\alpha2}\) (1–64)/s chimeras uniquely on protein kinase C and differentiation, MAP kinase was strongly activated. Expression of the constitutively active form of Gi\(_{\alpha2}\) Q205L, providing a useful control, inhibits MAP kinase activation, much like it inhibited PLC activation, and the activation of protein kinase C and progression to PE. Immunoblotting of fractions from Mono-Q FPLC reveal multiple, phosphorylated forms of MAP kinase in extracts from the cells stably expressing the Gi\(_{\alpha2}\) (1–64)/s chimera that progress to PE in the absence of RA as compared with the MAP kinase forms in the F9 stem cells (Fig. 8B). These changes in MAP kinase activity and profiles on FPLC separations were noted previously in a comparison of extracts prepared from F9 stem cells with cells promoted to PE by treatment with RA (6). The similarities between the rank orders for protein kinase C activation (Fig. 7, left panel), MAP kinase activity (Fig. 8), and progression to PE (Fig. 7, right panel) implicate both protein kinase C and MAP kinase in the regulation of stem cell conversion to PE.

**Control of Stem Cell Progression via the Gs\(_{\alpha}/Gi_{\alpha2}\) Axis**—The progression of F9 stem cells to PE is induced by the morphogen RA. Early gene products include Gs\(_{\alpha}\) (18), ERA1/Hox1.6 (19), and RARs,\(\beta\) (20, 21), which are synthesized within the first day of induction by RA. Gs\(_{\alpha}\) expression falls precipitously (3), early in RA-induced progression. Mimicking the decline in Gs\(_{\alpha}\) via expression of antisense RNA promotes progression to PE in the absence of RA, while constitutive expression of Gi\(_{\alpha2}\) blocks the ability of RA to induce progression (4). Likewise, enhanced expression of either wild-type Gs\(_{\alpha}\) or the G225T-activated mutant provokes progression in the absence of RA, mimicking the
A

pCW1 Vector
s

s/(38)

i(64)/s

i(64)/i(38)
iQ205L

F9 STEM

MAPK p42 (pmol/min/mg)

0 10 20 30 40 50 60

Fraction (ml)

B

Stem

\(i(64)/s\)–

Fig. 8. Analysis of MAP kinase activity of F9 cells stably expressing \(G_{a\alpha}\) chimeras with C- and N-terminal substitutions of \(G_{a\alpha}2\). Panel A, MAP kinase (MAPK) activity was measured after separation on Mono-Q FPLC. Cells (5 × 10^6) cultured for 4 days were harvested and lysed. Soluble extracts (1 ml) were fractionated onto a Mono-Q FPLC column with a 28-ml linear 0–350 mM NaCl gradient, collecting 1-ml fractions. The enzyme activity was quantified by measurement of the phosphorylation of EGF receptor-(62–86) peptide, which contains the consensus MAP kinase phosphorylation site, PXXTP. The data represent the peak of MAP kinase activity of the p42 form. Panel B, immunoblot analysis of Mono-Q FPLC fractions obtained from F9 stem cells (Stem) and clones expressing the \(G_{a\alpha}2(1–64)/G_{a\alpha}\) chimera that progress to PE in the absence of RA. Column fractions were precipitated with trichloroacetic acid/sodium deoxycholate, and subjected to SDS-PAGE on 10% gels. The blots were probed with a monoclonal antibody to MAP2 kinase. The blots reveal the multiple phosphorylated forms of MAP kinase for the cells expressing the \(G_{a\alpha}2(1–64)/G_{a\alpha}\) chimera that progress to PE in the absence of RA compared with the forms observed in the F9 stem cells.

rise in \(G_{a\alpha}\) induced early by RA (18).

\(G_{a\alpha}2\) has been shown to tonically inhibit PLC activity, and elimination of \(G_{a\alpha}2\) de-represses basal PLC as well as potentiates hormonal stimulation of PLC (17). Recently, we have shown that the decline in \(G_{a\alpha}2\) triggers activation of PLC, protein kinase C, and MAP kinase as stem cells are induced to PE (6). The \(G_{a\alpha}/G_{a\alpha}2\) axis is shown in the present work to control stem cell progression via this pathway. Using activated mutants of these G-protein \(\alpha\)-subunits and chimeras with various N- and C-terminal substitutions of \(G_{a\alpha}2\) in \(G_{a\alpha}\) revealed several important insights. Comparison of the effects of expressing the activated mutants and chimeras on progression with those on cyclic AMP accumulation clearly eliminates adenylyllyclease as the effector for progression, although it is the most prominent effector controlled by the \(G_{a\alpha}/G_{a\alpha}2\) axis.

Based upon the concordance of the data for the activation of downstream elements by the activated subunits and chimeras, the target for regulation of progression appears to be PLC. Activation of PLC and protein kinase C by \(G_{a\alpha}\) chimeras with N-terminal sequence of \(G_{a\alpha}2\) demonstrated high sensitivity to even the smallest substitution, \(G_{a\alpha}2(1–7)/G_{a\alpha}\) (Figs. 6, 7). Substitution of \(G_{a\alpha}2\) sequence at the C terminus, in contrast, enhanced the activation of PLC, protein kinase C, and MAP kinase, and progression to PE. Unexpectedly, substitutions in the N-terminal region of \(G_{a\alpha}\) residues–(62–86) (corresponding to \(G_{a\alpha}2\) sequence–(55–64)) were found to have a profound effect on PLC regulation, suggesting that this region of the \(G_{a\alpha}\) molecule, when aligned with the crystal structure of other heterotrimeric G-protein \(\alpha\)-subunits (22, 23), is involved in regulation of PLC (Fig. 9, highlighted in blue). This region of the molecule is the site of the insertion of the intervening peptide sequence GGEDPQAAARSNSDG that distinguishes the long and short forms of \(G_{a\alpha}\), a product of differential splicing of mRNA (24).

Although speculation, this intervening sequence may act as an inhibitor of PLC activation in the long form of \(G_{a\alpha}\), providing an explanation for the profound increase in PLC activation occurring in the \(G_{a\alpha}2(1–64)/G_{a\alpha}\) chimera that lacks this short region (14–16). The –(62–86) region of \(G_{a\alpha}\) is also topographically distinct from the region (Fig. 9, highlighted in yellow) implicated in the control of adenylyllyclease (25), as well as from the regions on the aligned sequence of \(G_{a\alpha}\) (α-helix 3) recently shown to be involved in PLC activation, as deduced by ala

n scanning mutagenesis (26). More detailed analysis of this interesting region of \(G_{a\alpha}\) and its influence on PLC activation will be required. Our results do suggest that signaling via PLC, regulated via the \(G_{a\alpha}/G_{a\alpha}2\) axis, is critical to induction of stem cell progression to PE, and presumably mammalian embryogenesis.

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FIG. 9. Mapping of region –(62–86) of \(G_{a\alpha}\) onto the deduced crystal structure of \(G_{a\alpha}\), and identification of regions regulating adenylyllyclease (yellow) and phospholipase C (blue). The landmarks for \(G_{a\alpha}\) were projected upon the deduced crystal structure of \(G_{a\alpha}1\) in which the space-filling model is initially red, with the region participating in the control of stem cell progression via phospholipase C colored blue and the region controlling adenylyllyclease colored yellow.
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