Morphogen-induced Decline in Giα2 Triggers F9 Teratocarcinoma Stem Cell Progression via Phospholipase C and Mitogen-activated Protein Kinase*

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The linkage between Giα2 and morphogen-induced promotion of F9 embryonic teratocarcinoma stem (F9 stem) cells to primitive endoderm was explored using probes of the mitogen-activated protein (MAP) kinase network. The morphogen-induced decline in Giα2 is shown to trigger activation of phospholipase C, thereby activating protein kinase C, MAP kinase, and cell progression to primitive endoderm. In the absence of retinoic acid, reduction-of-function mutants (Giα2-deficient) display the effects of morphogen, i.e. activation of phospholipase C, protein kinase C, MAP kinase, and progression to primitive endoderm. Gain-of-function mutants (expressing the Q205L activating-mutation of Giα2) displayed no activation of phospholipase C, protein kinase C, MAP kinase and no progression to primitive endoderm, even in the presence of retinoic acid. Selective inhibitors of protein kinase C, like the gain-of-function mutations, effectively block morphogen-induced progression to primitive endoderm. Morphogen triggers F9 stem cell progression by triggering Giα2 loss and thereby activation of downstream elements, including protein kinase C and MAP kinase.

Mouse F9 teratocarcinoma stem (F9 stem) cells provide a plentiful source of embryonic cells whose differentiation can be induced or influenced by exogenously added agents. F9 stem cells show little spontaneous differentiation in culture and can be induced to differentiate to primitive endoderm (PE)† by physiological concentrations of retinoic acid (RA) (1). The new cell type PE of F9 cells give rise to two different cell types, both of which are characteristic of the extraembryonic endoderm lineage in the mouse embryo; addition of dibutyryl cyclic AMP yields parietal endoderm (2), whereas aggregation yields cells characteristic of visceral endoderm (3). This differentiation process can be monitored by following the expression of several specific markers. The production of tissue plasminogen activator (tPA), Type IV collagen, laminin, and c-jun is induced with differentiation (4, 5), while the stage-specific embryonic antigen disappears from the cell surface (6) and the level of c-myc is dramatically decreased (7). Because of these distinct and versatile responses to differentiation, F9 cells have been widely used as a model for studying the mechanism of embryonic differentiation. Despite numerous studies, however, the molecular mechanism of differentiation, particularly the nature of the intracellular cascade leading to differentiation, remains poorly understood.

The G-proteins are a family of membrane-associated guanine nucleotide-binding proteins that transduce signals from cell surface receptors to intracellular effectors. Members of this family are heterotrimers composed of α, β, and γ subunits (8). The α subunit confers receptor and effector specificity on the heterotrimer. When the G-protein is activated by interaction with receptor, the α subunit exchanges bound GDP for GTP. The intrinsic GTPase activity of the α subunit restores it to the basal state in which GDP is bound. Giα subunits, too, modulate the activities of effectors units, including some K⁺ channels, PLCβ (forms 2 and 3), phosphoinositol action in budding yeast, and some adenylyl cyclase (for a recent review, see Ref. 9). This form of signal transduction is basic to the mechanisms that cells use in responding to hormones, neurotransmitters, and growth factors.

RA induces F9 stem cell progression to PE and a sharp decline in Giα2 (10). A decrease in Giα2 by expression of antisense Giα2 RNA (reduction-of-function) mutant induces PE-like phenotype in the absence of RA, while an increase in Giα2 by expression of a constitutively active (gain-of-function) mutant Giα2Q205L is sufficient to block differentiation (11). Pertussis toxin-sensitive G-proteins, like Giα2, have been implicated as potential mediators both of inhibitory phospholipase C (PLC) signaling and of mitogenic responses in several cell lines (12-19). Recently, suppression of Giα2 in cultured cells and adipocytes from transgenic mice has been shown to enhance PLC signaling, providing a possible link between Giα2 and upstream regulators of the mitogen-activated protein kinase (MAPK) network (20). We tested this linkage, exploring the PLC pathway.

EXPERIMENTAL PROCEDURES

Cell Growth and Transfection—The wild-type and transfected F9 stem cells were cultured on 0.1% gelatin-coated Falcon Petri dishes in Dulbecco's modified Eagle's medium supplemented with 15% of fetal calf serum (HyClone Laboratories). The retrovirus infected reduction-of-function mutants pNCX-ASGβ2 F9 cells (F33) and gain-of-function mutants pCW1GαQ205L F9 cells (FQ) have been previously described (11). Clones were selected in 500 μg/ml and maintained in 100 μg/ml (active form) of G418 sulfate (Life Technologies, Inc.).

*H]inositol 1,4,5-triphosphate (IP3) Mass Assay—Cells (10⁶) were cultured for 4 days in the presence and absence of RA (100 nm). PLC agonist was added for 10 s and the assay terminated by addition of perchloric acid. After neutralization, the mass of IP3 was determined by

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‡The abbreviations used are: PE, primitive endoderm; RA, retinoic acid; IP3, inositol 1,4,5-triphosphate; SHT, S-hydroxytryptamine; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein; CaM, calmodulin; DAG, diacylglycerol; PKC, protein kinase C; GM3, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; FPLC, fast protein liquid chromatography; MEK, mitogen-activated protein kinase.

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a competitive protein binding assay of \(^{3}H\)IP₃ using rabbit cerebellar membrane as the IP₃ receptor (21). Binding assays contained cell extracts, 2.5 mM \(^{3}H\)IP₃ and 100 μM of binding protein in 50 mM Tris-HCl, 1 mM EDTA, pH 8.3. Assays were incubated 10 min at 4°C followed by centrifugation at 10,000 x g for 5 min. Pellets were counted for radioactivity determination.

Determinated PE—Production of PPA is the hallmark for PE. Stem cells induced to PE produce and secrete PPA as well as assume a characteristic morphology, i.e., extended spindle shape with defined focal adhesions (growth). To induce PE, RA was added for 4 days at 100 nM (22). For TPA determinations, the culture medium of cells was assayed using the amidolytic assay (23). One unit of TPA is arbitually defined as that amount of TPA that results in a reaction rate of 10\(^{-7}\) ΔAₕₐₘ₄/min (change in the optical absorbance at 405 nm divided by the square of the time, min). For phase-contrast microscopy, the cells were cultured for 4 days and then fixed with 3% (w/v) paraformaldehyde. Fixed cells were photographed for phase-contrast microscopy with a Zeiss Axiphot.

HPLC Analysis of Metabolically Labeled \(^{3}H\)Inositol Phosphates—
Confluent cells (10⁶) were labeled with myo-[\(^{3}H\)]inositol (90 μCi/mL) at day 3 of culture for 24 h. The incubation was terminated at day 4 by addition of perchloric acid, and inositol phosphates were extracted with fluorotrichloromethane and tri-n-acytelylamine. The extent of metabolic labeling was similar for all clones tested. Equivalent amounts of label, \(^{3}H\)inositol phosphates, were subjected to analysis by ion-exchange HPLC using a Whatman Partisil-10 SAX column and a linear gradient of 0-50% (24). Inositol phosphates were identified by reference to the standards of inositol 1-phosphate, inositol 1,4-bisphosphate, inositol 1,3,4-triphosphate, inositol 1,4,5-triphosphate, and inositol 1,3,4,5-tetrphosphate under the same separation conditions. Radioactivity was quantitated by liquid scintillation counting.

Immunoblotting—Aliquots of crude membrane fractions (100 μg/lane) from each subclone were subjected to sodium dodeyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the separated proteins transferred to nitrocellulose, and the blots stained with antibodies specific to G₁α₂, G₁α₃, Gα₁b, and G₁α₁ (10, 11). The immune complexes were made visible by staining with a secondary antibody goat anti–rabbit IgG coupled to calf alkaline phosphatase.

GTPγS-Dependent Protein Kinase II (CaM Kinase II) Assay—CaM kinase II activity was assayed by incorporation of \(^{3}H\)ATP (at 30°C for 2 min) into the peptide substrate syntide-2 in the presence and absence of GTPγS (CaM (25)). Reactions were terminated by spotting the reaction mixture on P-81 phosphocellulose papers, and quantified in a scintillation counter after washing in 1% (v/v) phosphoric acid. CaM kinase II activity is defined as activity sensitive to the CaM kinase-specific inhibitor KN-62.

In Situ Kinase Assay in Renatured SDS Gels—20 μg of protein from cell lysates were subjected to SDS-PAGE on an 10% acrylamide gel with myelin basic protein (MBP, 0.1 mg/mL) polymerized in the running gel (26). After electrophoresis, the proteins in the gel were successively denatured, renatured, and subjected to in situ phosphorylation by \(^{3}H\)ATP (at 30°C for 1 h). The reaction was terminated and washed extensively by immersing the gel in 5% trichloroacetic acid and 10 mM sodium pyrophosphate.

Quantitation of Diacylglycerol (DAG) Mass—Cells (10⁶) were grown to confluence for 4 days, and lipids were extracted into chloroform. DAG was measured using a protein kinase assay (27), followed by thin layer chromatography to separate radioactivity incorporated into phosphatidic acid generated from the reaction. The DAG mass was calculated from a standard curve.

Protein Kinase C (PKC) Assay—PKC was assayed in DEAE-cellulose-purified cell homogenates (28) by incorporation of \(^{3}H\)ATP (at 30°C for 10 min) into acetylated peptide substrate MBP(14–34) in the presence and absence of PKC activator phorbol 12-myristate-13-acetate (PMA) and phosphotidylserine (0.28 mg/ml) or pseudosubstrate inhibitor peptide PKC (19–36) (20 μM), respectively (29). Reaction samples were spotted onto P-81 phosphocellulose filters, which were then washed with 1% (v/v) phosphoric acid and counted. PKC activity is expressed as picomoles of \(^{3}H\)ATP transferred to the peptide substrate MBP(14–34)/min/10⁶ cells. Kinase activity was measured both in the presence and absence of PMA and phosphatidylserine. In all cases, PKC activity is defined as activity sensitive to the pseudosubstrate inhibitor peptide PKC(19–36).

Northern Blot—Total cellular RNA was prepared using RNA-MAX 60 reagent (Tel-Test, Friendswood, TX) from cells grown for 4 days with or without treatment by RA. A 40-μg aliquot of total RNA was subjected to separation on 1.2% formaldehyde-containing agarose gel, transferred by capillary to Nytran membrane, cross-linked by ultraviolet radiation (Stratalinker), and then probed by hybridization with a \(^{3}P\)-labeled mouse PKCα cDNA probe. The cDNA probe was labeled by random

RESULTS AND DISCUSSION

We explored the relationship between G1α₂ and F9 stem cell progression to PE using stem cells, loss-of-function, and gain-of-function mutants. G1α₂ is deficient in the F33 lossof-function clones (Fig. 1), mimicking the loss of G1α₂ in F9 stem cells induced to PE by RA (9). The gain-of-function clones express the Q205L mutant of G1α₂, which is constitutively activated (FQ). Owing to expression of the Q205L mutant form, immunoreactive G1α₂ levels are increased in immunoblots of FQ
clones, whereas immunoreactive Gi\textsubscript{a2} is essentially absent in the F33 clones. The levels of Gq\textsubscript{a}, Gi\textsubscript{a3}, and Gb\textsubscript{1}, in contrast, were quite similar in F33 and F9 clones (Fig. 1).

In order to determine to what extent PLC activity was altered, IP\textsubscript{3} mass accumulation was measured. IP\textsubscript{3} levels were found to increase severalfold as F9 stem cells (STEM) progressed to PE in response to RA (Fig. 2A). Both F9 stem cells and PE displayed elevated IP\textsubscript{3} levels in response to stimulation of PLC by 5-hydroxytryramine (5HT; Fig. 2B), as well as to bradykinin and \alpha-adrenergic agonists (not shown).

We tested the linkage further using loss-of-function mutants, stable transfectants expressing RNA antisense to Gi\textsubscript{a2} and lacking Gi\textsubscript{a2} (F33). In the absence of morphogen, loss-of-function mutants displayed elevated IP\textsubscript{3} levels and progression to PE. Gain-of-function mutants, stably expressing the Q205L activating mutant Gi\textsubscript{a2} (FQ), displayed no activation of PLC, no tPA production, and a stem-like phenotype in the absence (Fig. 2) or presence (not shown) of RA. Analysis by HPLC of the water-soluble inositol phosphates from cells metabolically labeled with [3H]inositol confirmed the mass assays of IP\textsubscript{3} levels for the different clones (Fig. 2C). Levels of IP\textsubscript{3} (arrows) were elevated markedly in cells induced to PE by RA and in the F33 Gi\textsubscript{a2}-deficient clones that progress to PE in the absence of RA. Quantification of the label recovered as IP\textsubscript{3} from HPLC of

**Fig. 2.** RA-induced decline in Gi\textsubscript{a2} activates PLC and promotes F9 stem cell progression to PE. A, basal PLC activity, measured by accumulation of IP\textsubscript{3}, is increased in RA-induced PE cells and mimicked by F9 stem cells deficient in G\textsubscript{i3} (F33). B, activation of PLC, measured by IP\textsubscript{3} accumulation, in response to 5HT (10^{-5} M) is potentiated in RA-induced PE cells, mimicked by F9 stem cells deficient in Gi\textsubscript{a3} (F33), and abolished in stem cells expressing Q205L Gi\textsubscript{a2} (FQ). Data are means \pm S.E. (n = 4). By Student’s test, significance of difference is p < 0.05 (*) or < 0.01 (**) with respect to wild type. C and D, progression of F9 stem cells to PE either by RA or by deficiency of Gi\textsubscript{a3} (F33) activates PLC and increases IP\textsubscript{3} levels, as measured by metabolic labeling (C) and quantified by liquid scintillation (D). The elution of IP\textsubscript{3} in indicated by the arrows. Metabolic labeling of the different clones was equivalent quantitatively on a “per cell” basis, and equivalent amounts of label were subjected to analysis by HPLC. E and F, deficiency in Gi\textsubscript{a2} (F33), like the action of RA on F9 stem cells, induced progression of stem cell to PE phenotype, as analyzed by the PE-marker, tPA (E) or by phase-contrast microscopy (F). One unit of tPA is arbitrarily defined as that amount of tPA that results in a reaction rate of 10^{-7} ΔA\textsubscript{405} min^{-2}. For phase-contrast microscopy, the cells were fixed with 3% (w/v) paraformaldehyde and photographed with a Zeiss Axiphot. The micrographs shown are representative of five independent experiments for each clone or condition. Stem cells stably transfected with vectors alone, showed activities similar to that F9 stem cells throughout (data not shown).
samples loaded with equivalent total counts is shown (Fig. 2D). In stem or FQ cells, in contrast, the IP$_3$ levels were lower (Fig. 2C). Excepting the changes in IP$_3$ levels, overall profiles of inositol metabolites (Fig. 2C) and labeling of inositol-containing lipids (not shown) were similar. Expression of the PE-specific marker, tPA, and phase-contrast microscopy define morphogen-induced progression to PE (Fig. 2, E and F, respectively). Stem cells stably transfected with vectors alone showed activities similar to that F9 stem cells (data not shown). The data are mean values ± S.E. from three separate experiments. By Student's test significance of difference is $p < 0.05$ (*) or $p < 0.01$ (**) with respect to wild type.

Calcium mobilization in response to elevated IP$_3$ levels was probed by in vitro assays of calcium/calmodulin-dependent protein kinase II (CaM kinase II, Fig. 3). Progression to PE by RA elevated CaM kinase II activity in the absence of activator calcium and calmodulin (+CaM). The in situ assay of CaM kinase II (26, 31) revealed a sharp increase in the $\beta$-form (mass of 60 kDa) compared to $\alpha$-form (50 kDa) of kinase activity (inset, Fig. 3). G$_{i2}$ deficiency alone (F33), like RA, increased CaM kinase II activity, well above that of PE.
with the β-isofrom activity predominant. Expression of a constitutively active \( \text{Gi}_{a2} \) (FQ), in contrast, not only blocks progression, but also reduces CaM kinase II activity to levels below those of stem cells. Ionophore (A23187 or ionomycin)-induced activation of CaM kinase II activity revealed that less than half of the CaM kinase II was activated in stem cells, whereas >70% was activated by RA treatment (PE) or loss of \( \text{Gi}_{a2} \) (F33, data not shown).

PKC plays a prominent role in cell signaling, growth and proliferation (32, 33). The levels of DAG, a metabolite of the PLC pathway and intracellular activator of PKC, were explored in cells progressing to PE by RA or by loss of \( \text{Gi}_{a2} \) (F33). DAG content of stem cells nearly doubled when induced to PE by RA (Fig. 4). Likewise, \( \text{Gi}_{a2} \) deficiency alone, in the absence of RA, elevated DAG content nearly 2.5-fold. Expression of Q205L mutant \( \text{Gi}_{a2} \) displayed DAG levels similar to that of stem cells. 5HT elevated DAG levels in stem, PE and \( \text{Gi}_{a2} \)-deficient cells, whereas in cells expressing the Q205L mutant \( \text{Gi}_{a2} \) the response to 5HT was essentially abolished.

A critical linkage between PKC and both \( \text{Gi}_{a2} \) and stem cell progression is illustrated in Fig. 7. RA-induced progression to PE and deficiency in \( \text{Gi}_{a2} \) (F33) activate MAPK. A, immunoblotting of immunoprecipitated whole-cell extracts with antibodies to MAPK p42. MAPK activity of F9 stem cells and PE (B), and FQ and F33 clones (C), as measured after separation on Mono Q FPLC. Stem cells stably transfected with vectors alone, showed activities similar to that F9 stem cells (data not shown). D, serum-starved cells (18 h) were treated with mouse EGF (50 ng/ml) for 5 min. Cell lysates were then prepared, resolved by Mono Q FPLC, and assayed for MAPK activity. E, Western blot analysis of Mono Q FPLC fractions with a monoclonal anti-MAP2 kinase antibody reveals increased p42 protein in fractions from PE and F33 cells. Data are representative of three independent experiments for each condition.
progression was revealed by measurement of PKC activity. In the absence of an exogenous activator (PMA), PKC activity in PE was more than 10-fold greater than in F9 stem cells not induced by RA (Fig. 5). Remarkably, total PKC activity measured in the presence of PMA showed the same striking difference following progression, i.e. PE display a 14-fold greater amount of PKC activity than stem cells. The G_{in2}-deficient clones (F33) that progress to PE in the absence of RA mimicked the cells induced to PE by RA, displaying elevated basal and total PKC activity. Expression of the constitutively active Q205L mutant G_{in2} (FQ), in stark contrast, resulted in an stem cell-like phenotype refractory to RA with levels of PKC similar to those of F9 stem cells prior to progression. Immunoblots of DEAE-cellulose-purified whole-cell extracts stained with antibodies to PKCa are consistent with the activity measurements (Fig. 6A). Northern analysis of total cellular RNA identified PKCα transcripts of 3.5 and 8.1 kilobases in PE and F33 clones. PKCα transcripts were not detected in Northern blots of RNA from either F9 stem cells or FQ clones (Fig. 6B, top), with relative equivalent sample loading (Fig. 6C, bottom). PKC isoforms other than PKCα do not appear to be major contributors to the changes in activity, as PKCβ protein is undetected (data not shown), and the level of PKCβ and PKCγ transcripts is reduced, rather than increased, in progression to PE (34). Thus, stimulation by the morphogen RA leads not only to an increase in DAG generation and thereby PKC activation, but also to a sharp increase in expression of the PKCα mRNA.

MAPKs are members of a group of serine/threonine kinases that are activated in response to growth factors, mitogenic stimuli and neurotransmitters, that control cell proliferation and differentiation (35). Tyrosine kinase-encoded growth factor receptors and G_{i}-coupled receptors (e.g. thrombin receptor) are capable of activating MAPKs in response to stimulation (36). Phorbol esters, like PMA, also activate MAPK in many cells. Immunoblotting of immunoprecipitated whole-cell extracts with antibodies to p42 reveals an enhanced expression of MAPK in PE and the F33 clones (Fig. 7A). In an effort to probe the downstream events triggered by G_{in2} decline and PKC activation, MAPK activity was assessed in cell extracts first subjected to anion-exchange chromatography FPLC. MAPK activity resolves as two peaks, the major p42 MAPK eluting in fractions 12–16 and a minor p44 MAPK in fractions 17–19. In F9 stem cells, MAPK activity was relatively low. Cells induced to PE by RA displayed a striking, 5-fold increase in MAPK activity (Fig. 7B). The G_{in2}-deficient F33 clones, like the PE cells, displayed a striking increase in MAPK activity in the absence of RA. The FQ clones expressing Q205L G_{in2}, in contrast, were shown to have profiles of MAPK not unlike the untreated ES cells (Fig. 7C). Treatment of the cells with EGF provides a positive control, demonstrating that RA-induced progression to PE is accompanied by a substantial, though lesser activation of MAPK, approximately 60% of that obtained by stimulation with EGF (Fig. 7D).

For PE and F33 clones, immunoblotting of MAPK shows a modest increase in protein and an elution profile in which the p42 isoform is most prominent (Fig. 7E), which, together with the MAPK protein expression of the whole-cell extracts, suggests that activation of MAPK is the basis for increased activity both in F9 stem cells promoted to PE by RA and in G_{in2}-deficient cells that progress to PE in the absence of RA. Activation of the upstream element regulating MAPK activity, MEK, revealed a similar pattern in which progression to PE induced by RA activated MEK to a significant but lesser level that EGF (Fig. 8).

To probe events downstream of RA-induced G_{in2} loss mediating progression of stem cells to PE, the effects of protein kinase inhibitors on F9 stem cell progression to PE were explored (Table I). F9 stem cells were treated with RA in the absence and presence of selective inhibitors of PKC, protein kinase A, or CaM kinase, after establishing the dose-response curve for inhibition in F9 cells to be equivalent (not shown) to those in the literature performed with other cell lines (37–41). PKC-selective inhibitors calphostin C (37) (0.1 µM), bisindoylmaleimide (38) (0.1 µM), and H7 (39) (25 µM) each effectively blocked the ability of RA to activate MAPK and to promote F9 stem cell progression to PE. In agreement with earlier observations that cyclic AMP analogues and cholera toxin treatment do not influence progression to PE, inhibitors of protein kinase A, such as KT5720 (40) and HA1004 (39) at concentrations 20-fold in excess of their IC_{50} values were shown to be without effect on either parameter. KN62 (41), a potent inhibitor of CaM kinase, blocked CaM kinase activity (data not shown), but neither MAPK activation nor progression to PE. CaM kinase II activity increases dramatically in stem cells progressing to PE and the RA-induced change may well be important to both calcium...
signaling and longer term features of gene expression associated with the PE-phenotype. Clearly, RA triggers a decline in Gi2, thereby activating PLC, PKC, MAPK, and progression of F9 stem cells to PE.

To probe the linkage between MAPK and RA-induced progression to PE, we employed phosphorothioate oligodeoxynucleotides antisense to MAPK p42 (42). Oligodeoxynucleotides antisense, but not sense, to MAPK nearly abolished progression to PE (Fig. 9, bottom). Measurement of MAPK activity by in situ assay confirmed the loss of MAPK (not shown). Elimination of MAPK abolished the ability of RA to induce progression of F9 stem cells to PE (Fig. 9, bottom), establishing an obligate role of MAPK in the cascade from Gi2 to stem cell progression.

RA-induced differentiation of a variety of tumor cell types is accompanied by increased expression of PKC, especially the α isozymes (34, 43). The role of Gi2 in progression of these tumor cells in response to RA is not known. Loss-of-function mutants mimic the loss of Gi2 and promote progression in the absence of RA (present study). Gain-of-function mutants with constitutively active Gi2 show no activation of PLC, PKC, MAPK, and progression in response to RA (present study). Like the recent discovery of a critical role of Gi2 in Caenorhabditis elegans behavior (44), the current study highlights a new dimension of G-protein function in development, one in which a G-protein (Gi2) triggers stem cell progression via activation (derepression) of PLC, and thereby activation of PKC and the MAPK regulatory network.

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