The Ras/Erk Pathway Induces Primitive Endoderm but Prevents Parietal Endoderm Differentiation of F9 Embryonal Carcinoma Cells*

Mark H. G. Verheijen‡, Rob M. F. Woltthus§, Johannes L. Bos§, and Libert H. K. Defize‡

From the ‡Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands and the §Laboratory for Physiological Chemistry, Utrecht University, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands

The formation of parietal endoderm (PE) is one of the first differentiation processes during mouse development and can be studied in vitro using F9 embryonal carcinoma (EC) cells. Treatment of F9 EC cells with retinoic acid (RA) induces differentiation toward primitive endoderm (PrE), while differentiation toward PE is induced by subsequent addition of parathyroid hormone (PTH) or PTH-like peptide (PTHrP). The signal transduction mechanisms involved in this two-step process are largely unclear.

We show that the RA-induced differentiation toward PrE is accompanied by a sustained increase in Ras activity and that ectopic expression of oncogenic Ha-Ras is sufficient to induce PrE differentiation. Ras activity subsequently decreases upon PTH-induced differentiation toward PE. This is a necessary event, since expression of oncogenic Ha-Ras in PrE-like cells prevents PTH-induced PE differentiation. Expression of active PKA in PrE-like F9 cells mimics PTH-induced PE differentiation and is again prevented by oncogenic Ha-Ras. The effect of oncogenic Ras on both differentiation steps is abolished by the MEK inhibitor PD98059 and can be mimicked by constitutively active forms of Raf and MEK.

In conclusion, our data suggest that activation of the Ras/Erk pathway is sufficient to induce differentiation to PrE and to prevent subsequent differentiation toward PE. Activation of PKA down-regulates Ras activity, resulting in disappearance of this blockade and transmission of signals(s) triggering PE differentiation.

Murine F9 embryonal carcinoma (EC) cells are a suitable in vitro model system to study the formation of extraembryonic endoderm. Treatment with retinoic acid (RA) induces differentiation toward a primitive endoderm (PrE)-like phenotype (1). Subsequently, the cells can be differentiated toward a parietal endoderm (PE)-like phenotype by addition of cAMP-elevating agents (2). The first step of differentiation is accompanied by morphological changes and disappearance of the stage-specific embryonic antigen (SSEA-1) (3) and appearance of differentiation markers like tissue plasminogen activator (tPA) (1), laminin (2), and TROMA-1 (4, 5). The second step of differentiation is accompanied by morphological changes as well and is characterized by the appearance of thrombomodulin (6, 7), while expression of proteins like tPA and laminin is strongly elevated (2).

We and others have shown that PTH or PTHrP can substitute for Bt2cAMP in the differentiation of PrE to PE (8, 9). Furthermore, expression patterns of PTHrP and the PTH/PTHrP receptor suggest that this signaling system functions in PE formation in vivo (9–11). PTH and PTHrP bind to a common receptor, which couples to at least two G proteins, Gs and Gq, resulting in activation of both the adenylate cyclase and the phospholipase Cβ-mediated signal transduction pathways (12, 13). We recently demonstrated that PTH, by elevation of intracellular cAMP levels, can affect the Ras/Erk pathway in a cell-type-specific fashion (14, 15).

The mammalian ras family encodes membrane-bound GTPases, which are essential in transduction of extracellular signals involved in cell proliferation and differentiation. Activation of receptors catalyzes the exchange of GDP for GTP on Ras. In the active GTP-bound state, Ras will bind to its effectors and influence their biological activity. Ras is inactivated by GTPase-activating proteins (GAPs), which increase the rate of GTP hydrolysis, resulting in an inactive GDP-bound state (16, 17). The best characterized Ras-dependent signal transduction pathway involves the activation of the Raf/Erk cascade. Activated Ras interacts with Raf-1 and translocates it to the plasma membrane where it is exposed to activating kinases and/or lipids (18). Activated Raf-1 phosphorylates and activates MEK, leading to the phosphorylation and activation of Erk (16).

Besides its transforming potential, Ras can affect the differentiation of several cell types. Oncogenic Ras inhibits myoblast differentiation (19) and induces dedifferentiation of thyroid cells (20). On the other hand, oncogenic Ras is sufficient to induce differentiation of fibroblasts to adipocytes (21) and to induce neuronal differentiation of PC12 cells (22).

Here we examined the role of Ras and Erk in endoderm differentiation of F9 EC cells. We show that the Ras/Erk pathway plays an important role in this two-step differentiation process, since sustained Ras activity is observed during PrE differentiation.


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differentiation and is sufficient to induce it, while Ras activity needs to be down-regulated by PTH prior to differentiation toward PE. Finally, we show that the effect of Ras on both differentiation processes is mediated by Erk.

EXPERIMENTAL PROCEDURES

Materials—Rat PTH(1–34) was purchased from Peninsula Laboratories Europe (St. Helens, United Kingdom), Bt2cAMP from Aldrich (Zwijndrecht, The Netherlands), all-trans-RA from Sigma; PD98059 was from Calbiochem. γ-[32P]ATP and ECL were obtained from Amersham Pharmacia Biotech (Klosters, Switzerland). Monoclonal antibodies against SSEA-1 were a gift from Dr. D. Solter (Wistar Institute, Philadelphia, PA), monoclonal antibodies against TROMA-1 were a gift from Dr. R. Kemler (Max-Planck-Institute for Immunobiology, Freiburg, Germany), and monoclonal antibodies 273-34A against thrombomodulin were a gift from Dr. S. J. Kennel (Oak Ridge National Laboratory, Oak Ridge, TN). Raf-CAAX was a gift from Drs. S. Leevers and C. Marshall (24), and MEK-D218/222 was a gift from Drs. Brunet and J. Pouyssegur (25). The expression plasmids pSV2lacZ (26), pMT-PKAcα (27), RSV-Rasα61 (32) and RSV-Rasα17 (28), have been described previously, while pSGS-HA-p42 MAP kinase was provided by Arjan Buist and constructed by subcloning the HA-tagged MAP kinase from a cytomegalovirus-driven promoter (29) into pSGS.

Cat. 2:40,000—Induction of Differentiation, and Transfections—F9 EC cells were obtained from the ATCC and cultured on gelatinized surface in medium consisting of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 containing 7.5% fetal calf serum. Unless denoted otherwise, differentiation to PrE-like cells was performed by culture in monolayer in the presence of 1 μM RA for 3 days. To obtain PE-like cells, this treatment was followed by 1 mM Bt2cAMP or 100 nM PTH(1–34) for 2 days. Transient transfections were performed using LipofectAMINE (Life Technologies, Inc.) according to manufacturer's instructions.

In the case of transfections with β-gal to identify transfected cells, undifferentiated F9 cells and F9 cells treated with RA for 3 days, were plated in concentrations of, respectively, 3 × 10^4 and 2 × 10^5 cells/cm^2 on 13-mm tissue coverslips (Sarstedt Inc., Newton, NC) in 25-compartment clusters (Greiner). The following day cells were transfected for 6 h, using LipofectAMINE, with pSV2lacZ (0.3 μg) and Raα61 (0.3 μg), Raα17 (0.3 μg), Raf-CAAX (0.3 μg), MEK-D218/222 (0.3 μg), or pMT-PKAcα (0.3 μg), unless denoted otherwise. PUC-RSV plasmid was added to bring the total amount of DNA to 1 μg. Where appropriate, RA, PTH(1–34), and Bt2cAMP were added the day after transfection.

In the case of transfected for transient HA-Erk assays, undifferentiated F9 cells, and F9 cells treated with RA for 3 days, were plated in concentrations of, respectively, 3 × 10^4 and 2 × 10^5 cells/cm^2 in six-well tissue culture clusters. The following day cells were transfected for 6 h, using LipofectAMINE, with PGS5-HA-Erk (0.6 μg) and Raα61 (0.6 μg), Raf-CAAX (0.6 μg) or MEK-D218/222 (0.6 μg), unless denoted otherwise. PUC-RSV plasmid was added to bring the total amount of DNA to 2 μg.

Activation and Phosphorylation of Erk—To culture F9 EC cells in such a concentration that cell densities were comparable after 5 days of culture, cells to be treated with or without RA for 1, 2, 3, 4, or 5 days were plated at concentrations of, respectively, 150, 200, 300, 500, 800, or 1000 cells/cm^2 in six-well tissue culture clusters. Erk phosphorylation was measured by Western blotting with anti-p42 MAP kinase antibodies as described previously (30). Phosphorylated p42 MAP kinase is detected as a band with reduced mobility compared with unphosphorylated p42 MAP kinase (31). Experiments were repeated at least three times and representative results are shown.

For determination of Erk activity, epitope-tagged p42 HA-MAP kinase was immunoprecipitated with protein A-Sepharose beads coupled to monoclonal antibody 12CA5, as described previously (15). After the kinase reaction with myelin basic protein as a substrate, the reaction mix was subjected to SDS-polyacrylamide gel electrophoresis. Phosphorylation of myelin basic protein was measured using a PhosphorImager and ImageQuant software (Molecular Dynamics).

Activation of Ras—F9 cells were plated in 9-cm tissue culture dishes at similar concentrations as described above in “activation and phosphorylation of Erk.” In vivo Ras activity was determined with the minimal Ras-binding domain (RBD) of Raf-1 as an activation-specific probe for Ras, as described previously (32, 33). In short, cell lysates were brought to equal protein concentrations, and active Ras was precipitated with glutathione-agarose beads coupled to GST-RBD. Precipitated Ras was detected by Western blotting using the rat monoclonal antibody Y19-209.

β-Galactosidase Staining and Immunfluorescence—Cells were washed twice with PBS, fixed with 2% paraformaldehyde, incubated with an 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside staining solution (PBS with 0.8 mg/ml X-gal (Life Technologies, Inc.), 1 mg/ml K2Fe(CN)6, 1.3 mg/ml K3Fe(CN)6, and subsequently incubated with 20 mM NH4Cl for 30 min. For detection of SSEA-1, cells were incubated in PBS, 0.2% bovine serum albumin and subsequent antibody incubations were done in PBS, 0.2% bovine serum albumin as well. For detection of TROMA-1 and thrombomodulin, cells were incubated for 1 h in NETGEL (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% (v/v) Nonidet P-40, 0.25% (w/v) gelatin, 0.02% (w/v) sodium azide), and subsequent antibody incubations were done in NETGEL as well. Incubations with first antibodies were for 1 h at room temperature, the cells were rinsed extensively in PBS and incubated with CY3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature, again extensively rinsed, and mounted in Moviol.

RESULTS

Up-regulation of Ras and Erk Activity during RA-induced PrE Differentiation of F9 EC Cells—Previous work has suggested an involvement of the Ras/Erk pathway in PrE differentiation of F9 EC cells. It was shown that expression of oncogenic Ras in F9 EC cells is sufficient to induce differentiation to endoderm-like cells (34) and furthermore that RA-generated PrE-like F9 cells have a higher level of p42 MAP kinase activity than undifferentiated F9 cells (35). To get more insight in the role of Ras and Erk in PrE differentiation, we determined the activity of Ras and Erk during RA-induced differentiation. This was performed by using the RBD of Raf-1 as a specific probe for activated Ras, as described previously (32, 33, 36). Ras activity was measured as the amount of Ras precipitated with GST-Raf-RBD. Treatment of F9 EC cells with RA strongly induced Ras activity, which was not due to increased Ras expression (Fig. 1A). RA activated Ras already after 24 h, while longer treatments resulted in a further up-regulation of Ras activity. Comparable kinetics were observed for the RA-induced Erk phosphorylation (Fig. 1B), indicating that Erk activation is mediated by Ras activation.

Oncogenic Ha-Ras Induces Differentiation of F9 EC to PrE—It has been reported that oncogenic Ras induces endoderm differentiation of F9 EC cells (34). However, a lack of proper PrE- and/or PE-specific markers made it difficult to distinguish between both cell types at that time. Recently, thrombomodulin has been shown to be a suitable marker for
PE, both in vitro as well as in vivo (7). We therefore transiently transfected F9 EC cells with constructs encoding β-galactosidase (β-gal) and oncogenic Ras, Ras<sub>Leu-61</sub>, and determined the phenotype of β-gal-expressing cells by morphology and by immunofluorescence using antibodies against phenotype-specific markers. When F9 EC cells were transfected with control plasmids, they remained undifferentiated, as reflected in the expression of the stem cell marker SSEA-1 (3) (Fig. 2, A, B, E, and F). However, when cells were transfected with Ras<sub>Leu-61</sub>, the number of transfected SSEA-1-positive cells decreased with approximately 80% (Fig. 2, C, D, and I). This was comparable with the decrease in SSEA-1 expression induced by RA-treatment (Fig. 2I).

As a positive marker for PrE differentiation we used TROMA-1 (4, 5). Undifferentiated cells did not express TROMA-1 (Fig. 2, E, F, and J), while Ras<sub>Leu-61</sub>-induced expression of TROMA-1 in approximately 70% of the transfected cells (Fig. 2, G, H, and J), which was even higher than in RA-treated cells (35%) (Fig. 2J). Furthermore, Ras<sub>Leu-61</sub> expressing cells showed an enlarged and flattened morphology, which was similar to the morphology of RA-differentiated cells, and is characteristic for PrE cells (1). We could not detect expression of thrombomodulin in the Ras<sub>Leu-61</sub>-differentiated cells (not shown). Taken together, these data indicate that the transient expression of oncogenic Ras induced differentiation toward a PrE-like phenotype, without further differentiation to PE.

Oncogenic Ras-induced PrE Differentiation Is Mediated by Erk—As was shown in Fig. 1B, the RA-induced PrE differentiation is accompanied by a sustained increase in Erk activity. The importance of Erk activity in RA- and/or Ras-induced PrE differentiation was investigated with the specific MEK inhibitor PD98059 (37). A continuous treatment of F9 EC cells with PD98059 completely blocked the activation of a transfected hemagglutinin-tagged p42 MAP kinase by RA or cotransfected Ras<sub>Leu-61</sub> (Fig. 3A). Under these conditions, Ras<sub>Leu-61</sub>-induced PrE differentiation was completely abolished, while RA-induced PrE differentiation was unaffected, as based on the appearance of a PrE-like morphology (not shown) and expression of the differentiation marker TROMA-1 (Fig. 3B). Similar results were found by cotransfecting NΔraf, a dominant-nega-
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Ras-induced PrE differentiation is dependent on Erk, in contrast to RA-induced PrE differentiation. To determine whether activation of the Ras/Erk cascade is sufficient to induce PrE differentiation, we transiently transfected F9 EC cells with constitutive active forms of Raf (Raf-CAAX) and MAP kinase kinase (MEK-D218/222). Both Raf-CAAX as well as MEK-D218/222 strongly induce a PrE-like morphology and TROMA-1 expression (Fig. 3C). Taken together, the data show that activation of the Ras/Erk cascade is sufficient to induce PrE differentiation, but that RA can induce PrE differentiation via Ras/Erk-independent pathway(s) as well.

Down-regulation of Ras Activity during PTH-induced PE Differentiation—When F9 EC cells are differentiated to PrE, they can subsequently be differentiated to PE by addition of Bt2cAMP, PTH, or PTHrP (2, 8, 9). In previous studies we demonstrated that PTH, by elevation of cAMP levels, can either inhibit or activate Erk activity, depending on the cell type studied (14, 15). Here, we determined the effect of PTH and Bt2cAMP on Ras and Erk activity in PrE-like F9 cells. Endogenous Ras activity decreased when cells were differentiated to PE upon addition of PTH(1–34) (Fig. 4A). The PTH-induced and C depict the percentage of β-gal-expressing cells positive for TROMA-1, as similar to Fig. 2 and represent the mean ± S.D. of at least three independent experiments.
Inhibition of Ras activity is likely mediated by elevation of cAMP levels, since treatment with Bt2cAMP rapidly inhibited Ras activity (Fig. 4B). Longer treatment resulted in a further decrease in Ras activity, while Ras activity was almost completely inhibited after treatment with Bt2cAMP for 48 h, when the cells were fully differentiated toward PE. Importantly, expression levels of Ras were not changed. The inhibition of Ras activity was reflected in the down-regulation of Erk activity (Fig. 4C), which occurred with similar kinetics, suggesting a causal relation between the inhibitory effect of PTH on Ras and Erk activity.

**Oncogenic Ha-Ras Inhibits Differentiation to PE**—The down-regulation of Ras and Erk activity might play a crucial role in the PTH-induced PE differentiation. We therefore investigated the effects of ectopic expression of either oncogenic Ras, thereby overruling the PTH-induced inhibition of Ras activity, or a dominant negative mutant of Ras, RasAsn-17, thus mimicking the inhibition of endogenous Ras activity. RA-treated F9 cells were cotransfected with a construct encoding β-gal, thereby identifying transfected cells. PE differentiation of β-gal-expressing cells was assessed using β-gal staining and immunofluorescent staining of thrombomodulin.

**Fig. 5. Oncogenic Ras inhibits PE differentiation of RA-treated F9 cells.** Shown is β-gal staining (A, C, E, G, I) or immunofluorescent staining of thrombomodulin (B, D, F, H, J). RA-treated F9 cells were transiently transfected with β-gal alone (A, B, C, D) or cotransfected with RasLeu-61 (E, F), with cPKA (G, H), or with both cPKA and RasLeu-61 (I, J). Cells were subsequently left untreated for 3 days, or treated with 100 nM PTH (C, D, E, F) or 1 μM Bt2cAMP during the last 2 days. K, L, and M diagrams depict the percentage of β-gal-expressing cells staining positive for thrombomodulin. Plasmids used are indicated in each diagram. Data in diagrams represent the mean ± S.D. of at least three independent experiments. Bar, 50 μm.
cells was measured by morphology and thrombomodulin expression. The RA-treated control cells had a PrE-like morphology and did not express thrombomodulin (Fig. 5, A and B). Transfections with either RasLeu-61 or RasAsn-17 did not change the PrE-like morphology nor did they induce expression of thrombomodulin (Fig. 5K, no ligand).

Treatment with PTH-(1–34) induced differentiation toward PE, resulting in the typical change in morphology and expression of thrombomodulin in approximately 20% of the cells (Fig. 5, C, D, and K). It should be noted that this low efficiency of PTH-induced PE differentiation is probably due to the transfection procedure, since normally PTH induces PE differentiation in approximately 50% of the cells. Transfection with oncogenic Ras had a dramatic effect on PTH action, the cells maintained their PrE-like morphology and did not express thrombomodulin, resulting in the occurrence of a PE-like phenotype in only 5% of the transfected cells (Fig. 5, E, F, and K). Expression of RasAsn-17 to interfere with Ras activation slightly enhanced the PTH-induced PE differentiation (Fig. 5K). These results show that high levels of Ras activity in PTH-treated cells prevent differentiation toward PE, and that inhibition of Ras activity by itself is necessary but not sufficient for PE differentiation to occur.

The inhibition of PTH-induced PE differentiation by Ras can be at different levels. Ras could interfere at the level of the PTH/PTHrP receptor or downstream of the receptor within the phospholipase C or the adenylate cyclase pathway. We therefore determined the effect of oncogenic Ras on Bt2cAMP-induced PE differentiation. Addition of Bt2cAMP to RA-generated PrE-like F9 cells induced a PE-like phenotype in 45–50% of the cells (Fig. 5L). This was strongly inhibited by expression of oncogenic Ras, to approximately 10%, suggesting that Ras interferes with PTH-induced PE differentiation downstream of cAMP.

The major effector of cAMP elevation is PKA. However, cAMP has been shown to induce PKA-independent effects, like direct regulation of ion channels and transporter proteins (39–41). Furthermore, Bt2cAMP can also mediate cAMP-independent effects via butyrate (42). To determine whether activation of PKA is sufficient to induce PE differentiation, we transfected RA-treated F9 cells with the catalytic subunit of PKA. This induced a PE-like phenotype in approximately 45% of the transfected cells (Fig. 5, G, H, and M). Also the PKA-induced PE differentiation could be inhibited by coexpression of oncogenic Ras, which reduced the number of PKA-induced thrombomodulin expressing cells to 5–10% (Fig. 5, I, J, and M). This suggests that Ras interferes with PTH-induced PE differentiation at the level of or downstream of PKA.

Ras Inhibits PE Differentiation via Activation of Erk—The decrease in endogenous Ras activity, which was observed when PrE-like F9 cells were differentiated to PE by addition of PTH or Bt2cAMP, strongly correlated with the decrease in Erk activity (Fig. 4). To determine whether the inhibitory effect of Ras on PE differentiation was dependent on Erk activity, we used PD98059. Like in F9 EC cells (Fig. 3A), a continuous treatment with PD98059 blocked the RA- as well as the RasLeu-61-induced Erk activity in PrE-like F9 cells (not shown). PD98059 did not induce a PE-like morphology by itself (not shown) nor thrombomodulin expression (Fig. 6A), suggesting that inhibition of Erk activity is not sufficient to induce differentiation of PrE-like F9 cells to PE. Interestingly, PD98059 did however significantly enhance PKA-induced PE differentiation (29.6 versus 46.9%, Fig. 6A), indicating that the basal Erk activity interferes with differentiation to PE. Furthermore, the inhibitory effect of RasLeu-61 on PKA-induced PE differentiation was completely abolished by PD98059 (Fig. 6A) and could be mimicked by Raf-CAAX and MEK-D218/222 (Fig. 6B). Together these data strongly suggest that the inhibitory effect of Ras on PE differentiation is mediated by Erk and that down-regulation of the Erk activity in PrE-like F9 cells is necessary but not sufficient for PE differentiation to occur.

DISCUSSION

One of the first differentiation processes during mouse embryogenesis takes place in the blastocyst around day 4.5 post-coitus. Cells of the inner cell mass differentiate into PrE, which subsequently differentiates into PE. The signals involved in this two-step process are poorly understood. F9 EC cells are a suitable in vitro model system to study the formation of PrE and PE. However, lack of proper PrE- and/or PE-specific markers made a good distinction between PrE or PE difficult in the past. In this study we made use of antibodies against thrombomodulin, a suitable marker for PE (6, 7), to distinguish between PrE and PE. We show that both differentiation processes are regulated by the Ras/Erk pathway, since expression of constitutively active forms of Ras, Raf, and MAP kinase kinase in F9 EC cells induced differentiation toward a PrE-like phenotype, while they inhibited the subsequent differentiation.
Involvement of Ras in PrE Differentiation—Expression of oncogenic Ha-Ras in F9 EC cells has been reported to induce differentiation toward an endoderm-like phenotype (34). Our data suggest that this Ras-induced phenotype resembles PrE and not PE. Importantly, also when expressed in embryonic stem (E14) cells, which can be differentiated in vitro to form derivatives of all three germ layers, oncogenic Ras induces a PrE-like morphology and expression of Troma-1. This indicates that Ras/Erk-mediated PrE differentiation is not only restricted to the F9 EC system, but a more general feature for embryonic stem and embryonic carcinoma cells (Fig. 7).

Ras activity is up-regulated during RA-induced PrE differentiation, again suggesting an important role for Ras in mediating PrE differentiation. The mechanism of Ras activation by RA is unclear. RA-induced PrE differentiation is accompanied by expression of growth factors and/or their receptors, such as platelet-derived growth factor, epidermal growth factor, fibroblast growth factor, and transforming growth factor (43, 44), and thus RA might elevate Ras activity by establishing autocrine stimulation of these receptors. Expression of Ras<sup>Thr<sub>17</sub></sup>, to interfere with RA-induced Ras activation, did not affect RA-induced PrE differentiation. However, the inhibition of RA-induced Ras activation might have been incomplete, as indicated by residual RA-induced Erk activation in the presence of Ras<sup>Thr<sub>17</sub></sup> (data not shown). Nevertheless, while PD98059 completely inhibited Ras<sup>Leu<sub>61</sub></sup>, and RA-induced Erk activation in EC cells, only the Ras-induced and not the RA-induced PrE differentiation was blocked. This is in apparent contrast with the findings of Gao et al. (35), who, using antisense p42 MAP kinase oligonucleotides, found an inhibition of RA-induced expression of the differentiation marker TPA. We observed, however, that complete inhibition of p42 MAP kinase by PD98059 had no effect on RA-induced PrE-like morphology, expression of Troma-1, or on subsequent PTH-mediated PE differentiation (data not shown). Thus, RA induces PrE differentiation via a Ras/Erk-independent pathway as well. A possible target of RA and Ras involved in PrE differentiation is AP-1, a transcription factor family consisting of homo- or heterodimers of Jun, Fos, or ATF proteins, which can be activated via Ras-dependent and Ras-independent pathways (45). Both RA as well as oncogenic Ras induce c-Jun expression in EC cells (34, 46, 47), while forced expression of c-Fos or c-Jun in EC cells is sufficient to induce differentiation (34, 47, 48, 49). The role of AP-1 activation in RA- and Ras-induced PrE differentiation is currently being investigated.

It should be stressed here that the identity of the factor(s) establishing primitive endoderm differentiation in vivo is unclear. Although RA efficiently induces differentiation of F9 EC cells in vitro, its involvement in this differentiation process in vivo is rather unlikely (50–53). Our observation that the Ras/Erk pathway has the capacity to regulate PrE and PE differentiation in vitro suggests that it could play a major role in the regulatory mechanisms governing extraembryonic endoderm differentiation in vivo.

**Differentiation to PE Is Regulated by Ras and PKA**—Treatment of PrE-like F9 cells with PTHrP, besides inducing activation of PKA, also induces an elevation of intracellular calcium levels (data not shown). Here we show for the first time that a strong increase in PKA activity is sufficient to induce PE differentiation, suggesting that PTHrP-induced PE differentiation can be mediated solely through PKA activation.

We recently reported that the PTH-induced PKA activation is sufficient to inhibit both basal as well as growth factor-induced activation of Erk (14). Here we show that PTH and cAMP also inhibit Erk activity in RA-treated F9 cells. It was demonstrated in other cells that activation of PKA interfered with Erk activation at the level of Raf-1, leaving Ras activity unaffected (30). Our data however demonstrate that in PrE-like F9 cells cAMP elevation inhibits Ras activity. We observed a strong correlation between the reduction in Ras and Erk activity, suggesting that the cAMP-induced Erk inhibition is a consequence of the inhibition of Ras activity. The mechanism involved in the inhibition of Ras activity by cAMP remains to be determined. This could be mediated via interference with growth factor-induced Ras activation or via inhibition of Ras-GAP (Ras GTPase activation protein), for instance by PKA-induced phosphorylation (54).

In agreement with an inhibitory role for Ras in PE differentiation, it has been reported that Ras is expressed at very low levels in PE during early rat embryogenesis (55). Inhibition of Ras or Erk activation with Ras<sup>A26</sup> or PD98059, respectively, was by itself not sufficient to induce PE differentiation, suggesting that other events apart from inhibition of Ras activity are involved in cAMP-mediated PE differentiation as well. Inhibition of Erk activity did however enhance PKA-induced PE differentiation, suggesting that the basal Erk activity in PrE-like cells interferes with differentiation of these cells to PE. This is in agreement with the observation that sustained elevation of Erk activity by expression of constitutive active Ras, Raf, or MAP kinase kinase prevents PE differentiation, even when induced by cPKA.

In conclusion, our data suggest the following model for endoderm differentiation of F9 cells (Fig. 7). Activation of the Ras/Erk pathway is sufficient to trigger differentiation to PrE. RA induces activation of the Ras/Erk pathway and accordingly differentiation to PrE. However, RA induces PrE differentiation via an Erk-independent pathway as well. The sustained Ras/Erk activity in PrE-like cells blocks signal(s) inducing the subsequent differentiation toward PE. This Ras activity is down-regulated by PTHrP via PKA, resulting in disappearance of the blockade and transmission of the signal(s) triggering PE differentiation. According to this model, one can speculate that differentiation of PrE and PE in the early mouse embryo is tightly regulated by a subtle cross-talk between (extracellular) signals leading to Ras/Erk activation and signals such as PTHrP, leading to PKA activation.

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