Retinoids Suppress Epidermal Growth Factor-associated Cell Proliferation by Inhibiting Epidermal Growth Factor Receptor-dependent ERK1/2 Activation*  

Received for publication, November 13, 2001, and in revised form, January 7, 2002  
Published, JBC Papers in Press, January 11, 2002, DOI 10.1074/jbc.M110897200  

Jerome F. Sah, Richard L. Eckert, Roshantha A. S. Chandraratna, and Ellen A. Rorke  

From the Departments of Environmental Health Sciences, Physiology and Biophysics, and Reproductive Biology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106 and Retinoid Research, Allergan Pharmaceuticals, Inc., Irvine, California 92612  

Human papillomavirus (HPV) is an important etiological agent in the genesis of cervical cancer. HPV-positive cervical tumors and human papillomavirus-positive cell lines display increased epidermal growth factor receptor (EGFR) expression, which is associated with increased cell proliferation. ECE16-1 cells are an HPV-immortalized human ectocervical epithelial cell line that is a model of HPV-associated cervical neoplasia and displays elevated EGFR levels. In the present study, we evaluated the effects of receptor-selective retinoid ligands on EGFR-associated signal transduction. We show that retinoic acid receptor (RAR)-selective ligands reduce EGFR level and the magnitude and duration of EGFR activation in EGF-stimulated cells. These effects are reversed by cotreatment with an RAR antagonist. To identify the mechanism, we examined the effects of retinoid treatments on EGFR-dependent signaling. Stimulation with EGF causes a biphasic activation of the ERK1/2 MAPK. The first peak of activation is present at 20 min, and the second is present at 36 h. This activation subsequently leads to an increase in the cyclin D1 level and increased cell proliferation. Simultaneous treatment with EGF and a RAR-selective retinoid inhibits both phases of ERK1/2 activation, completely eliminates the cyclin D1 induction, and suppresses EGF-dependent cell proliferation. This effect is specific as retinoid treatment does not alter the level or activity of other EGFR-regulated kinases, including AKT and the MAPKs p38 and JNK. Retinoid X receptor-selective ligands, in contrast, did not regulate these responses. These results suggest that RAR ligand-associated down-regulation of EGFR activity reduces cell proliferation by reducing the magnitude and duration of EGF-dependent ERK1/2 activation.

Retinoids are analogs of vitamin A that control cell growth and differentiation of a variety of epithelial tissues, including the lining of the uterine cervix (1, 2). Under conditions of retinoid deprivation, the normal mucus-producing cervical epithelium is converted to squamous-like metastatic epithelium, a process that is reversed by readministration of vitamin A (3, 4). Both preneoplastic and dysplastic cervical lesions have been reported to revert to normal following retinoid treatment (5), suggesting that retinoids may be efficacious for the treatment of cervical disease (6).

Human cervical cancer cells are characterized by the presence of the high risk forms of the human papillomavirus (7, 8). HPV16 is the predominant subtype (9). HPV encodes two oncoproteins, E6 and E7, which immortalize cervical cells in culture, and are believed to play a key causal role, along with chemical mutagens, in the genesis of cervical cancer (10). Retinoid regulation of cervical cell function has been studied in both normal and HPV16-immortalized human cervical epithelial cells. These studies show that, at physiological levels, retinoids do not affect the growth of normal human ectocervical cells but markedly suppress the growth of HPV16-immortalized cells. Moreover, cell proliferation can be inhibited under conditions where E6 and E7 expression is not reduced (2, 11, 12).

Trans-retinoic acid metabolism in vivo generates many metabolites with varying retinoid activity (13, 14). These products interact with and activate retinoid nuclear receptors that act to regulate transcription (15). There are two retinoid receptor families, RAR and RXR, and each family includes α, β, and γ members (16). The RARs bind preferentially to all-trans-retinoic acid, while the RXRs bind to a stereoisomer, 9-cis-retinoic acid (17). These receptors frequently exist as RAR/RXR heterodimers, although RAR homodimers may also form (18–20). Although retinoic acid has been shown to be a potent antineoplastic agent, side effects have limited its clinical usefulness. For this reason there is substantial interest in designing retinoids that have a better therapeutic index and in understanding how receptor-selective ligands influence cancer cell function.

EGFR is an important regulator of cell proliferation that is expressed in many cell types (21–24). Moreover, altered EGFR expression and function have been noted during initiation and progression in several cancer models (25–27). For example, EGFR levels are increased in the majority of lung, vulval, and cervical carcinomas (28). Based on these observations, inhibition of the activity of the EGFR and the downstream targets of EGFR is a major goal of anticancer therapy. Several anticancer drugs have been designed based on this principle (29). Our previous studies show that EGFR levels are increased in cervical cells following human papillomavirus-dependent immor...
talization (12). This increase is associated with enhanced proliferation. Importantly EGFR levels can be returned to normal levels by retinoid treatment, and this reduction is correlated with reduced cell proliferation.

Two questions are particularly important. First, which retinoid receptor subtype mediates the retinoid-dependent reduction in EGFR level. This information would be helpful for the design of the optimal therapeutic retinoid. In the present manuscript, we describe experiments that identify the RAR-selective retinoids as the most efficient regulators of EGFR level. Second, the mechanism responsible for the retinoid-dependent suppression of EGFR function is fundamentally important. We show here that RAR-specific retinoids selectively influence EGFR-dependent ERK1/2 activation but do not influence AKT, p38, or JNK activity.

**MATERIALS AND METHODS**

_cells—ECE16-1 cells are an immortalized line of human ectocervical epithelial cells derived by stable transfection with a plasmid encoding the complete HPV16 genome (12).

_reagents—Dubellco’s modified Eagle’s medium, Ham’s F-12 medium, and fetal bovine serum were purchased from Invitrogen. The retinoids, including TTNPB, AGN193109, AGN191183, and AGN190168, were synthesized in the Department of Chemistry at Allergan Pharmaceuticals, Inc. The receptor binding specificity of each ligand is summarized in Table I. Human recombinant EGF was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-activated EGFR antibody (E12120) was obtained from Translab, anti-phospho-ERK (K-23), anti-ERK (E-4), and anti-EGFR (sc-05) were from Santa Cruz Biotechnology. Anti-AKT (9272), anti-p38 (9212), anti-SAPK/JNK (9252), anti-phospho-AKT (9271), anti-phospho-SAPK/JNK (9251), anti-phospho-p38 (9211), the ERK1/2 MAP kinase assay kit (catalog no. 9800), and the MEK1/2 inhibitor U0126 were purchased from New England Biolabs (Beverly, MA). The EGFR kinase inhibitor AG1478 was procured from Calbiochem. The anti-phosphotyrosine antibody, PY-20, was from ICN Biomedicals (Cost Mesa, CA). [35S]Cysteine and carrier-free Na 125I were purchased from Amersham Biosciences, Inc. Anti-β-actin and trans-retinoic acid was from Sigma.

_cell proliferation—ECE16-1 cells were maintained in Dulbecco’s modified Eagle’s medium: Ham’s F-12 medium: Ham’s F-12 (3:1) supplemented as described previously (2). Briefly, the medium was supplemented with 4% fetal calf serum, 5 μg/ml insulin, 5 μg/ml transferrin, 1 nM T3, 10 ng/ml EGF, 0.18 nM adenine, nonessential amino acids, l-glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Retinoids were dissolved in Me 2SO as 1000-fold concentrates and stored at −20 °C. For cell proliferation experiments, ECE16-1 cells were plated in 24-well plates at 10,000 cells/well in growth medium. Two days after plating, fresh medium was added in which the fetal calf serum was replaced with 0.4% delipidized fetal calf serum and the appropriate retinoid. EGF was always present unless stated otherwise. Fresh retinoid-containing medium was added on alternate days. Cells were harvested in triplicate with trypsin at the indicated times for counting using a Coulter counter.

_EGF Binding Assay—The ECE16-1 cells were treated with retinoids as described above. In the presence of the EGF ligand, the EGF receptor

**TABLE I**

Relative binding affinities of retinoids for various RAR and RXR receptor subtypes

| Ligand           | Structure | RAR   | RXR   |
|------------------|-----------|-------|-------|
|                  |           | α     | β     | γ     | α     | β     | γ     |
| All-trans-retinoic acid | ![Structure](image) | 15    | 13    | 18    | **   | **   | **   |
| TTNPB            | ![Structure](image) | 30    | 3     | 2     | *    | *    | *    |
| AGN190168***     | ![Structure](image) | **    | 67    | 57    | **   | **   | **   |
| SR11217          | ![Structure](image) | *     | *     | 388   | 121   | 915   |
| AGN193109        | ![Structure](image) | 2     | 2     | 3     | *    | *    | *    |

* Indicates Kd values >1000 nM.
** Indicates Kd values >10,000 nM.
*** AGN190168 is an ethyl ester that is converted by cells to the free acid that has affinity for the receptor as follows: RARα 1250 nM, RARβ >1000 nM.
is activated, endocytosed, and finally degraded. Therefore, prior to measurement of EGFR level, cells were transferred to EGF-free medium (retinoids or vehicle were included) for 12–18 h to facilitate receptor measurement. To measure EGF binding, [125I]-EGF labeled by the chloramine-T method was added to 0.5 ml of treatment medium. After 3 h at 4°C, the cells were washed with Hank's balanced salt solution and solubilized in 0.2 N NaOH. The cell-associated radioactivity was counted in a γ counter. Nonspecific binding was determined from incubations in which a 1000-fold excess of unlabeled EGF was used. Specific binding was measured as described under “Materials and Methods.” C and D, AGN193109 reverses the TTNBP-dependent suppression of ECE16-1 cell proliferation and EGF binding. ECE16-1 cells were plated as above and treated in the presence or absence of 1000 nM TTNBP with increasing concentrations of the RAR antagonist AGN193109. After a 4-day treatment, the cells were harvested, counted, and assayed for [125I]-EGF binding as described under “Materials and Methods.” E, RAR-selective ligands do not reverse the TTNBP-dependent suppression of ECE16-1 cell proliferation. ECE16-1 cells were grown in the presence or absence of 1000 nM SR11217 and increasing levels of TTNBP. After 4 days, the cells were harvested, and [125I]-EGF binding was measured. All experiments are representative of at least three separate determinations. Where not visible, error bars are smaller than the symbol size.

**Results**

All-trans-retinoic Acid (t-RA) and RAR-selective Ligands Suppress ECE16-1 Cell Proliferation and 125I-EGF Binding—We began by examining the effect of various receptor-selective retinoids on ECE16-1 cell proliferation. As shown in Fig. 1A, the natural pan-agonist t-RA and the RAR-selective ligands AGN190168 and TTNBP suppress ECE16-1 proliferation in a concentration-dependent fashion. In contrast, the RAR antagonist AGN193109 and the RXR-selective ligand SR11217 do not regulate cell proliferation. Further, as apparent from Fig. 1B, the RAR-selective ligand-associated reduction in cell proliferation is associated with a concomitant reduction in 125I-EGF binding. SR11217, the RXR-selective ligand, which
does not affect cell proliferation, had no effect on 125I-EGF binding.

**Antagonism of the RAR-dependent Regulation by AGN193109** — AGN193109, a retinoid inverse agonist, is known to reverse retinoid action in some cell types, including ECE16-1 (30, 31). To further assess retinoid specificity, we compared the effect of treatment with increasing concentrations of the RAR-selective antagonist AGN193109 and the RXR agonist SR11217 on TTNPB-dependent responses. Fig. 1C shows that treatment with 100 nM TTNPB suppresses cell proliferation and that AGN193109 causes a dose-dependent reversal of the TTNPB-mediated effect. Further, the AGN193109-dependent reversal of the TTNPB-dependent growth suppression is accompanied by restoration of 125I-EGF binding (Fig. 1D). As shown in Fig. 1E, ECE16-1 cells treated with varying concentrations of TTNPB show a dose-dependent inhibition of 125I-EGF binding, and this response is not reversed by treatment with 100 nM SR11217.

**t-RA and TTNPB Down-regulate Cellular EGFR Levels** — The reduction in 125I-EGF binding subsequent to treatment with RAR-specific ligands may be due either to a reduction in EGFR expression or a modification of the affinity of the receptor for ligand. The analysis presented in Fig. 2A shows that 125I-EGF binding is directly related to the level of EGFR detected by immunoblot. The EGFR level is reduced by t-RA and the RAR-selective ligand TTNPB. In contrast, neither the RXR-selective ligand SR11217 nor the RAR antagonist AGN193109 influence EGFR levels. However, co-administration of the RAR antagonist with TTNPB at a concentration approaching the ED50 partially restores EGFR expression. SR11217, the RXR-selective agent, does not inhibit the TTNPB-dependent reduction in EGFR level.

**Suppression of Activated EGFR** — Upon activation, EGFR is autophosphorylated at specific tyrosine residues. To determine whether the change in receptor level is correlated with reduced receptor activation, lysates prepared from cells exposed to EGF for 5 min were probed with antibodies specific for phosphorylated EGFR (P-EGFR) and phosphorytosine (P-tyrosine). In each case, loading was normalized based on 10,000 cell equivalents/lane. β-Actin levels were monitored to assure equal loading in all lanes. C, proliferating ECE16-1 cells were treated with 100 nM of each retinoid in EGF-containing DM as described under Materials and Methods. After 48 h, the treatment was continued in EGF-free DM for 16 h. The cells were subsequently harvested and counted. D, absence of retinoid regulation of MAPK level. Proliferating ECE16-1 cells were treated with 100 nM of each of the indicated retinoids as described in A. Cell extracts were prepared, and 10,000 cell equivalents were electrophoresed in each lane. ERK1/2, AKT, p38, and JNK1/2 levels were monitored by immunoblot. This experiment is representative of four separate determinations.

**Retinoids Do Not Alter ERK, AKT, p38, or SAPK/JNK Levels** — The MAPK and AKT signaling cascades are downstream targets of EGFR. Prior to evaluating the effects of EGF and
Retinoids Suppress EGFR-mediated ERK Activation

Fig. 3. Retinoid suppression of EGFR-dependent ERK1/2 activation. A, proliferating ECE16-1 cells were treated with 100 nM of each retinoid in EGF-containing DM as described under “Materials and Methods.” After 48 h, the treatment was continued in EGF-free DM for 16 h to prevent EGFR internalization and endocytosis prior to assay. Total protein lysates were electrophoresed at 10,000 cell equivalents/lane and blotted for detection of activated (phosphorylated) ERK1/2, AKT, p38, and JNK1/2 (P-ERK1/2, P-AKT, P-p38, and P-JNK1/2). B, ECE16-1 cells were treated exactly as in A with the exception that prior to preparing the cell lysates the cells were stimulated with 5 ng/ml EGF for 5 min. Extracts were electrophoresed at 10,000 cell equivalents/lane and blotted for detection of activated (phosphorylated) ERK1/2, AKT, p38, and JNK1/2 (P-ERK1/2, P-AKT, P-p38, and P-JNK1/2). Similar results were observed in each of three separate experiments. The histogram depicts the output of a scan of the ERK1/2 immunoblot presented in arbitrary density units (a.u.).

Retinoid treatment on these downstream effectors, we determined whether retinoid treatment influences their endogenous expression. As shown in Fig. 2D, retinoids do not influence total ERK, AKT, p38, or SAPK/JNK levels.

t-RA and RAR-selective Ligands Reduce EGF-dependent ERK Activation—We next examined the effect of retinoid treatment on the EGFR-associated activation of ERK1/2, JNK, AKT, and p38 in ECE16-1 cells. Fig. 3 shows the effects of RAR treatment of ECE16-1 cells in the absence (Fig. 3A) or presence (Fig. 3B) of EGF. An examination of all the blots that make up Fig. 3A reveals no effect of retinoid treatment on MAPK activity in the absence of EGF. That is, baseline MAPK cascade activity is not influenced by retinoid treatment.

As shown in Fig. 3B, stimulation with EGF causes an increase in the level of phosphorylated (activated) ERK, p38, JNK, and AKT (compare control lane in Fig. 3B with control lane in Fig. 3A). The RAR-selective ligands (t-RA and TTNPB) inhibit the activation of ERK1/2 by 50%, but SR11217 and AGN193109 do not reduce the EGF-dependent activation. However, AGN193109 does block the inhibitory effect of TTNPB on EGF-dependent ERK activation. Thus, retinoids produce parallel changes in EGFR and ERK. None of the retinoids influenced the EGF-dependent AKT, p38, or SAPK/JNK activation.

Further examination of the effect of retinoids on EGF-dependent ERK1/2 activity was performed using a kinase assay. Fig. 4A shows that although total ERK1/2 levels are not affected, ERK1/2 activity, as measured by its ability to phosphorylate ELK-1, is reduced by treatment with t-RA or TTNPB. The RAR-specific ligand is without effect, and the antagonist AGN193109, when administered with TTNPB, restores ERK1/2 kinase activity. As shown in Fig. 4B, the reduced ERK1/2 activity is directly reflected in reduced cell proliferation.

t-RA and TTNPB Regulate the Time Course of EGF-dependent EGFR and ERK Activation—The duration of the ERK activation is thought to play a pivotal role in regulating cell proliferation. Sustained ERK activation is part of the required response for G1 phase progression (32). We therefore monitored the level of activated EGFR and ERK with time. In these experiments, EGF was removed from the medium for 12 h prior to assay to permit receptor levels to stabilize. Fig. 5A shows that EGFR, as measured by formation of P-EGFR, remains activated for 8 h after EGF treatment in the absence of retinoid treatment (Fig. 5A, Control). However, the magnitude and duration of the EGF-dependent activity is reduced by t-RA or TTNPB treatment. The level of activity is reduced by 50%, and the response terminates 4 h earlier. This change is reflected at the level of ERK activation. In the presence of t-RA or TTNPB, maximal ERK1/2 activation is one-half that observed in control cells (Fig. 5A, P-ERK1/2). Moreover, the second peak of ERK activity, observed at 36 h, is absent in the retinoid-treated cells.

Suppression of Cyclin D1 Expression by t-RA and TTNPB—Sustained ERK activation results in increased cyclin D1 expression, which contributes to G1 phase progression and cell proliferation. To determine whether retinoids influence the EGF-dependent increase in cyclin D1, we measured cyclin D1 levels in EGF-treated cells in the presence or absence of retinoid treatment. In the absence of retinoid treatment, cyclin D1 levels were markedly increased at 24 and 36 h after EGF treatment (Fig. 5B). However, the presence of t-RA or TTNPB markedly attenuated this response. Further, neither t-RA- nor TTNPB-treated cells show a change in cell number during the first 24 h after EGF stimulation. During the same time period, control cell number increased by 22% (Fig. 5C). This represents a substantial increase considering the brief time period.

Suppression of EGFR-mediated ERK Activation Causes Growth Inhibition—The importance of the EGFR-dependent ERK activation was further examined by comparing the effects of retinoids, inhibitors of the EGFR receptor kinase, and inhibitors of MEK1/2 on cell proliferation and ERK1/2 activity. AG1478, a specific inhibitor of the EGFR tyrosine kinase, and U0126, a specific inhibitor of MEK1/2 kinase, were used. As is evident in Fig. 6A, both AG1478 and U0126 suppress cell proliferation within 24 h (the earliest time point examined), suggesting that ECE16-1 cells require EGFR-dependent ERK activation to proliferate. Consistent with this observation is the fact that ECE16-1 cells do not proliferate when EGF is withdrawn from the medium (DM). t-RA and the RAR-selective ligand TTNPB also inhibit cell proliferation. However, the onset of growth inhibition is delayed. This time difference is most likely due to mechanistic differences; unlike the kinase inhibitors that immediately bind to and inactivate either EGFR or MEK1/2, retinoids act through the reduction of EGFR protein levels.

We also cultured ECE16-1 cells in the absence of EGF to provide a measure of ERK1/2 activity in resting, growth-arrested cells (Fig. 6B, DM). ECE16-1 cells grown under these conditions display detectable ERK1/2 activity. This activity is increased 2.5-fold in the presence of EGF (Fig. 6B). ERK activation in cells treated in the presence of EGF plus either retinoids or the MEK1/2 inhibitor U0126 was significantly less that that found in EGF treated cells but higher than in growth arrested cells. Only AG1478 inhibited ERK1/2 activation below the level observed in nonproliferating cells. These alterations represent real changes in activity as total ERK1/2 levels were not altered (Fig. 6B, ERK1/2).

As a final experiment to show that the retinoid regulation of ERK1/2 activity requires EGF, we grew cells in the absence of EGF and monitored cell number, EGFR level, EGFR activity, and ERK1/2 activity. The results shown in Fig. 6C indicate that the cells do not proliferate in the absence of EGF nor is cell proliferation.
number suppressed by retinoid treatment of EGF-free cultures. Fig. 6D shows that retinoid treatment of EGF-free cultures lowers EGFR level as observed in EGF-treated cultures. However, because no EGF is present, no active EGFR (Fig. 6D, P-EGFR) is detected. This absence of activated EGFR is associated with an absence of retinoid regulation of ERK1/2 (Fig. 6D, P-ERK1/2) by retinoids.

**DISCUSSION**

EGFR overexpression and activation is observed in many epithelial cancers, including cervical cancer (25–27). This increased EGFR expression is not due to gene amplification (33). In disease, EGFR levels are regulated by a number of factors, including viral oncogenes. For example, expression of the HPV16 E6/E7 oncoproteins in ECE16-1 cells results in a 3–5-fold increase in EGFR level (12). This increased EGFR is associated with enhanced cell proliferation. Conversely treatment of E6/E7-positive cell lines from established cervical tumors with E6/E7 antisense leads to diminished EGFR expression and reduced cell proliferation (for a review, see Ref. 34). In the present study, we used ECE16-1, an established HPV16-immortalized cell line, to study the role of retinoids as regulators of EGFR-dependent signaling and cell proliferation. These cells...
are known to overexpress EGFR (12).

RARs Mediate the Retinoid-dependent Effects on Cell Proliferation—Retinoids are a family of ligands that produce changes in cell function via interaction with ligand-activated nuclear receptors (35). These receptors belong to two classes, the RARs and RXRs. Each receptor class includes an α, β, and γ isofrom (16). An important goal is the identification of receptor-selective ligands that interact with specific members of these families for use in cancer treatment. Thus, it is important to identify the retinoid receptor subtype that is important for clinical response (36). Clinically retinoids promote cervical tumor regression when administered in combination with interferon α (37). However, it is not known whether ligands that interact selectively with the RAR or RXR or with specific subtypes within these families are more efficacious. Our previous studies show that naturally occurring retinoids, including all-trans-retinoic acid, suppress the growth of immortalized and transformed cervical cells, including ECE16-1 cells, while having no effect on normal cervical cell growth (2, 12, 38, 39). Moreover, in some HPV-immortalized cells, t-RA inhibition of cell growth is associated with reduced HPV E6/E7 levels (40). However, E6/E7 oncoprotein expression is not altered by retinoid treatment of ECE16-1 cells (11). Instead retinoid-dependent ECE16-1 growth inhibition is associated with a reduction in EGFR level (12), suggesting the involvement of EGFR in this process.

ECE16-1 cells express the RARα and RXRα receptors and, to a lesser extent, RARγ receptors (41), and RARβ expression is increased by retinoid treatment (38). However, although our previous studies demonstrate that retinoid receptor pan-agonists, agents that interact with both RAR and RXR subclasses, suppress EGFR function, we do not know which receptor class is responsible for this regulation. Thus, our present studies have compared the effects of RAR- and RXR-selective ligands on EGFR function, EGFR-dependent signal transduction, and cell proliferation.

Our present studies show that the reduction in cell proliferation by EGF and retinoids. Cells were plated and then grown in the presence of the indicated treatments. At the indicated times, cells were harvested and counted. A, regulation of cell proliferation by EGF and retinoids. Cells were plated and then grown in the presence of the indicated treatments. At the indicated times, cells were harvested and counted. B, regulation of ERK1/2 function in EGF-treated cells. Cell extracts were prepared at 48 h after initiation of treatment, and P-ERK1/2 and total ERK1/2 levels were monitored by immunoblot. The β-actin level was assayed as a loading control. C, retinoids do not cause growth suppression in the absence of EGF. ECE16-1 cells were plated in 12-well clusters at 20,000 cells/well. After 24 h, the cells were treated in DM (no EGF) or DM containing 100 nM t-RA or 100 nM TTNPB. At the indicated time, cells were harvested for cell counting. D, absence of retinoid regulation of ERK1/2 in the absence of EGF. Cell extracts were prepared at 48 h after initiation of retinoid treatment, and total EGFR, P-EGFR, total ERK1/2, and P-ERK1/2 levels were monitored by immunoblot. The β-actin level was monitored as a loading control. a.u., arbitrary density units.
of the RAR activity is observed for EGF-mediated events. This appears to rule out an independent role for RXRs in the regulation of ERGFR function.

Retinoids and Regulation of EGFR-associated Signal Transduction—Previous characterization of the ECE16-1 cell response to retinoids reveals a loss of EGF cell surface-localized binding in the absence of changes in receptor affinity or internalization (12). This suggests that the growth inhibition induced by retinoids is not due to changes in EGFR affinity, altered endocytosis, or altered subcellular localization. This leaves altered downstream signaling as a possible mechanism to explain the response. The MAPK and AKT cascades are major routes for EGFR-dependent intracellular signal transfer (43, 44). The MAPK cascades include ERK1/2, JNK/SAPK, and p38. We therefore examined how the retinoid-dependent reduction in EGFR level influences these downstream events. Our results indicate that the retinoid-dependent reduction in EGFR level and activity is associated with selective changes in cell signaling pathways. For example, p38, JNK, and AKT activity is not altered by retinoid treatment. EGF produces a significant increase in the activity of these kinases, but this activation is not altered by retinoid treatment. EGF produces a significant decrease in p38 activity. This, in turn, results in a reduced activation of ERK1/2 kinase and reduced cyclin D1 expression.

In summary, we hypothesize that the RAR ligand-selective activation of RAR inhibits EGF-dependent ECE16-1 proliferation via a mechanism that involves reduced EGFR expression/activity. This, in turn, results in a reduced activation of ERK1/2 kinase and reduced cyclin D1 expression.