Activation of the MAP kinase pathway induces chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) expression in human breast cancer cell lines

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Abstract

Growth factors are essential for cellular growth and differentiation in both normal and malignant human breast epithelial cells. In the present study we investigated the effect of epidermal growth factor (EGF), transforming growth factor alpha (TGFα) and phorbol myristate acetate (PMA) on chicken ovalbumin upstream promoter-transcription factor (COUP-TF) expression in human breast cancer cells. The orphan receptors COUP-TFI and COUP-TFII are members of the nuclear receptor superfamily. The high degree of evolutionary conservation of these proteins strongly argues for an important biological function.

COUP-TF expression was highest in SK-BR3 cells (approximately 130 amol/µg total RNA), while the lowest COUP-TF expression was observed in MCF-7 cells (3·5 amol/µg total RNA). While treatment of EGF, TGFα and PMA induced expression of COUP-TFII, COUP-TFI did not respond to these agents. Oncostatin M (OSM) is known to exert an antiproliferative effect in breast cancer cells. Treatment of MCF-7 cells with OSM resulted in an approximately 90% reduction of COUP-TFII mRNA expression.

In SK-BR3 cells, treatment with the MEK inhibitor UO126 resulted in a profound suppression of endogenous COUP-TFII expression. Furthermore, cotreatment with UO126 prevented induction of COUP-TFII expression by EGF in MCF-7 cells. In conclusion, our data provide evidence, for the first time, that mitogenic substances which activate the MAP kinase pathway, can induce COUP-TFII expression. Our results strongly suggest that an active MAP kinase pathway is essential for COUP-TFII expression in human breast cancer cells.

Integrating Previous Knowledge

Chicken ovalbumin upstream promoter–transcription factors (COUP-TFs) are members of the nuclear receptor superfamily with a DNA binding domain consisting of two zinc fingers separated from a ligand binding domain by a flexible hinge region (Wang et al. 1989). Since no ligand has been identified for these proteins, COUP-TFs are considered as ‘orphan receptors’. It has been demonstrated that COUP-TFs bind to a wide spectrum of response elements (Cooney et al. 1992), which can be recognized by several other members of the superfamily, including the vitamin D receptor (VDR), the thyroid hormone receptor (TR), the retinoic acid receptor (RAR), the retinoid X receptor (RXR), the peroxisome proliferator activated receptor (PPAR) and the hepatocyte nuclear factor-4 (HNF-4) (Sladek et al. 1990, Umesono et al. 1991, Miyata et al. 1993). Competing with these receptors for binding to their cognate response elements, COUP-TFs can function as potent repressors of gene transcription (passive repression) (Cooney et al. 1992, Kliewer et al. 1992, Ladias et al. 1992, Mietus-Snyder et al. 1992, Tran et al. 1992). Several investigators could demonstrate that COUP-TFs can also repress transcription by other mechanisms, such as active repression and trans-repression (Cooney et al. 1993, Leng et al. 1996, Achatz et al. 1997).

In humans, the COUP-TF family comprises at least three proteins, namely COUP-TFI (Wang et al. 1989, Mietus-Snyder et al. 1992), COUP-TFII (Ladias et al. 1992) and the ErbA-related protein-2 (EAR2) (Miyajima et al. 1988). COUP-TFI and -II are highly homologous
proteins, with almost identical DNA binding and ligand binding domains, while the sequence of EAR2 is more divergent. Members of the COUP-TF family are potent repressors of transcriptional activities of multiple genes including several apolipoprotein-encoding genes, the erythropoietin, and the alpha-fetoprotein gene (Paulweber & Levy-Wilson 1991, Paulweber et al. 1991a,b, Ladis et al. 1992, Liu & Teng 1992, Liu & Chiu 1994, Galson et al. 1995). Expression of a considerable number of genes, such as the arrestin gene (Lu et al. 1994), the variant HNF-1 (vHNF-1) gene (Power & Cereghini 1996) and the RARβ gene is, however, stimulated by COUP-TFs (Lin et al. 2000). COUP-TFs have been highly conserved throughout evolution and homologous proteins have been identified in numerous species, such as sea urchin (Chan et al. 1992), Drosophila (Mlodzik et al. 1990), mouse (Qiu et al. 1994) and zebrafish (Fjose et al. 1993). This high degree of evolutionary conservation of the COUP-TF proteins strongly argues for an important biological function. In zebrafish the COUP-TF homologs, svp44 and sup46, are expressed in the developing nervous system, suggesting their involvement in neurogenesis (Fjose et al. 1993). In developing mouse embryos, in situ hybridization experiments also have shown that mouse COUP-TFs are highly expressed in the central nervous system (Pereira et al. 1995). Moreover, COUP-TFs are highly expressed in numerous other developing organs, including lung, testis, prostate, skin, intestine, pancreas and several others (Jonk et al. 1994, Lu et al. 1994, Pereira et al. 1995).

Kerber et al. (1998) recently reported that seven-up (svp), the COUP-TF homolog in Drosophila, is a key regulator in morphogenesis of the insect kidney that is induced in response to stimulation of the epidermal growth factor receptor (EGFR) signal transduction cascade.

Therefore, in the present study we asked whether in human cells COUP-TF expression is regulated by factors controlling cell growth and differentiation, such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα), 12-phorbol-13-myristate-acetate (PMA) and the cytosolic agent oncogen M (OSM). Furthermore, we investigated whether ERK activity correlates with COUP-TF expression.

Materials and Methods

Human breast cancer cell lines

Ten human breast cancer cell lines were examined. Five of them were estrogen receptor alpha positive (T-47D, ZR 751, MDA-MB 361, MCF-7, ALAB), and five were estrogen receptor alpha negative (MDA-MB 231, HS 578T, SK-BR3, MT-SV1–7, HBL-100). The estrogen receptor alpha status in cells investigated in this study was described by ATCC (American Type Culture Collection, Manassas, VA, USA) and recently re-evaluated by Dandachi and coworkers (2001) who used the same cell lines in their work. These cell lines were a generous gift from P Obrist (Institute of Pathology, Innsbruck, Austria). HBL-100 is a transformed cell line, originating from normal lactating breast (Caron et al. 1985). Cells were grown in monolayer in RPMI-1640 medium (Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies Inc.) at 37 °C in a fully humidified atmosphere, containing 10% CO₂ in air. Cells were passaged twice a week using trypsin/EDTA (Life Technologies Inc.).

Quantitative competitive RT-PCR for measurement of COUP-TFI, COUP-TFII, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels

For exact quantification of COUP-TFI, COUP-TFII, and GAPDH mRNA levels in various human breast cancer cell lines, a highly sensitive competitive RT-PCR technique was developed. The method involves co-amplification of cDNA transcribed from sample RNA together with cDNA transcribed from RNA of an internal standard to reduce tube to tube differences in efficiency of reverse transcription or amplification.

Synthesis of internal standards

Internal standards contained the same primer binding sites as the target cDNAs of COUP-TFI, -II and GAPDH. The standard sense primer (5’-GCT GCC TCA AAG CCA TCG TGG ACA AGC AGC AGC AGC AAG AGA AGG CTG GGG CTC ATT GCA G-3’) was inserted into a pBluescript SK(−) vector, which had been digested with XbaI and BamHI. The standard antisense primer (5’-AAG GTC TAG GAG CAC TGG ATG GA CGA ACT GCC CGT GGG TCG GCC ATC CAC AGT CTT CTG GGT GGC AG-3’) was cloned into the pBluescript SK(−) vector after EcoRI and PstI (Roche Diagnostics GmbH, Germany) digestion. The length of the standard PCR fragment was modified by placing a piece of spacer DNA between sense and antisense primer. Downstream of the standard antisense primer a poly-A stretch was inserted into the vector to allow for reverse transcription of the internal standard RNA using oligo-dT as a primer.

Oligonucleotides

Sequences of the PCR sense (S) and antisense (AS) primers used were as follows: COUP-TFI-S: 5’-GCT GCC TCA AAG CCA TCG TG-3’; COUP-TFI-AS: 5’-AAG GTC TAG GAG CAC TGG ATG GA CGA ACT GCC CGT GGG TCG GCC ATC CAC AGT CTT CTG GGT GGC AG-3’; COUP-TFII-S: 5’-GAC AAG CAG CAG CAG CAG CAA-3’; COUP-TFII-AS: 5’-CGA ACT GCC CGT
GGG TCG G-3'; GAPDH-S: 5'-GAG AAG GCT GGG GCT CAT TTG CAG-3'; GAPDH-AS: 5'-CCA TCC ACA GTC TTC TGG GTG GCA G-3'.

Reverse transcription
After cultivation for 12 to 72 h at 37 °C in a fully humidified atmosphere containing 10% CO2 in air, cells were trypsinized and RNA was isolated using a Mini-Blood RNA Isolation Kit (Qiagen GmbH, Hilden, Germany) according to the instructions provided by the manufacturer. After DNase I treatment (Life Technologies Inc.), reverse transcription was performed from 1 µg total RNA and various different amounts of internal standard RNA using 'Ready-To-Go, You-Prime First-Strand Beads' (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA).

PCR conditions
Each cycle of PCR consisted of a denaturation step of 1 min at 95 °C, an annealing step of 1 min at 60 °C for COUP-TFI, at 66 °C for COUP-TFII and at 60 °C for GAPDH, and an extension step of 1 min and 30 s at 72 °C for all primer pairs. For each PCR amplification 30 cycles were performed.

The lengths of the PCR products for COUP-TFI, COUP-TFII and GAPDH were 318 bp, 289 bp and 315 bp respectively.

After co-amplification of both the target cDNA fragment (COUP-TF or GAPDH) and the internal standard fragment, PCR products were separated by electrophoresis on 2% agarose gels. Optical density of the bands was analyzed by the Scanalytics Software (Scanalytics, Billerica, MA, USA). Absolute target mRNA concentrations were calculated by linear regression analysis based upon the ratios between the values (after logarithmic transformation) obtained for targets and internal standards.

Treatment of cultured cells with various growth factors and cytokines
To determine the effect of various growth factors and cytokines on COUP-TFI expression, cultured cells were treated with EGF (0.01 µM), TGFα (10 ng/ml), PMA (100 nM), OSM (10 ng/ml) and UO126 (20 µM). These concentrations have been found to generate maximal biological effects in other experimental systems (Rehemtulla et al. 1990, Liu et al. 1997, DeSilva et al. 1998, Favata et al. 1998). EGF, TGFα, PMA and OSM were purchased from Sigma Chemical Co. (St Louis, MO, USA), and UO126 was from Promega Corporation (Madison, WI, USA). Cells were seeded at an initial density of 500 000 cells/ml and cultured in 12 ml RPMI-1649 supplemented with 10% FBS. Growth factors or cytokines were added to the culture medium after cells were fully spread and treated with these substances for 12, 24, 48 h until the end of experiments (72 h) at the concentrations indicated above. Control experiments were performed in parallel using untreated cells. Every 48 h the incubation medium was refreshed (with or without growth factors/cytokines).

Western blot analysis
To determine changes in COUP-TFI protein expression, logarithmically growing cells were lysed in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM phenyl-methylsulfonylfluoride and 10 µg/ml aprotinin) for 15 min. After this incubation period cells were harvested, cell lysates were collected, transferred to Eppendorf tubes and cleared by centrifugation. Supernatants were assayed for protein using the Bio-Rad DC-Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Eighty micrograms total protein extract were subjected to electrophoresis on 7-5% SDS-PAGE gels. After electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, USA). After transfer, membranes were blocked in 5% dry milk in LS-Buffer (2 M Tris–base, 20% Tween-20, 150 mM NaCl) for 1 h at room temperature. Immunostaining was performed using a polyclonal COUP-TFII antibody (dilution of 1:5000) at 4 °C overnight. The antibody was purchased from Research Genetics Inc. (Huntsville, AL, USA). It had been raised in rabbits by injection of an amino-terminal COUP-TFII peptide and purified by affinity chromatography. The specificity of this antibody has been tested in several experiments in our laboratory and shows no crossreaction with the COUP-TFI protein. COUP-TFI protein was observed at the expected size of 46 kDa. Anti-total ERK1/2 pAb and anti-phosphorylated ERK1/2 pAb were purchased from Promega and diluted as recommended. Subsequently, membranes were incubated with an appropriate peroxidase-conjugated immunoglobulin G secondary antibody (Amersham Pharmacia Biotech UK Limited, Bucks, UK) for 1 h at room temperature at a 1:1000 dilution. The peroxidase-catalyzed reaction was visualized using the ECL Western blotting detection reagent (Amersham Pharmacia Biotech UK Limited).

Results
Expression of COUP-TFII in human breast cancer cell lines
In the present study COUP-TFI and -II mRNA levels were determined in a series of human breast cancer cell lines. Levels of COUP-TFII and -II mRNA varied over a wide range in these cell lines (Fig. 1). We focused our
Figure 1 COUP-TFII expression in various human breast cancer cell lines. (A) Human breast cancer cell lines T-47D, ZR 751, MDA-MB 361, MCF-7 and ALAB cells are estrogen receptor positive, MDA-MB-231, HS 578T, HBL-100, MT-SV1-7, and SK-BR3 cells are estrogen receptor negative. Cells were harvested when they had reached a level of confluency of about 70%. Levels of COUP-TFI and -II mRNA were determined by quantitative competitive RT-PCR. All values are expressed as amol target mRNA per µg total RNA. The data were normalized for differences in GAPDH mRNA levels. (B) COUP-TFII protein levels were determined by Western blotting. Eighty micrograms total protein were analyzed in each lane.
interest on two breast cancer cell lines in which the highest and the lowest COUP-TF expression could be detected. As illustrated in Fig. 1 the expression of COUP-TFI and -II is 3.5 and 3.0 amol/µg total RNA respectively in MCF-7 cells, an estrogen receptor positive cell line. In SK-BR3 (an estrogen receptor negative cell line) expression reached a maximum of 27.5 and 84.8 amol/µg total RNA for COUP-TFI and -II mRNA. In these experiments RNA was isolated from exponentially growing cells at 48 h after splitting. Interestingly, in serum-free medium COUP-TFI and -II expression did not change significantly (data not shown), we therefore decided to perform all our experiments in medium containing serum.

Next, we determined the time course of COUP-TF expression in MCF-7 cells. In Fig. 2 COUP-TFI and -II mRNA levels in MCF-7 cells cultured under normal culture conditions at 12, 24, 48 and 72 h after seeding are presented. In this experiment investigating the time course of COUP-TF expression every 48 h from the timepoint of seeding, the medium was removed and replaced by fresh medium. The data demonstrate that expression of both COUP-TFI and -II decreases profoundly during the culture period, independently from refreshing the medium. Twelve hours after splitting, expression of COUP-TFI and -II was 4.2 amol/µg total RNA and 3.0 amol/µg total RNA respectively. After 72 h, when cells nearly reached confluency, expression of COUP-TFI and -II had dropped to 0.7 amol/µg total RNA for COUP-TFI and 1.0 amol/µg total RNA for COUP-TFI.

**COUP-TFI is regulated by the MAP kinase (MAPK) pathway**

In a recent study, Kerber and coworkers (1998) demonstrated that the *Drosophila* homolog of COUP-TF, Seven-up, is a key regulator of insect kidney development that is induced in response to activation of the EGF receptor signal transduction cascade by mitogenic stimuli emanating from the tip cell. Because the EGFR pathway plays an important role in proliferation, we investigated the effect of EGF and TGFα (both are ligands of the EGFR) on COUP-TFII mRNA expression in breast cancer cells (Fig. 3A). In MCF-7 cells EGF and TGFα, which were added to the culture medium at the beginning of the experiments, resulted in a twofold induction of COUP-TFII expression 24 h after treatment. COUP-TFII induction (4.6 amol/µg total RNA) was strongest at 48 h after TGFα treatment. It is important to note that in control cells COUP-TFII expression showed a marked decline, while in cells treated with these growth factors COUP-TFII expression was only slightly reduced (approximately 3.7 amol/µg total RNA by treatment with EGF, 3.2 amol/µg total RNA by treatment with TGFα) 72 h after treatment. To confirm these findings we repeated these experiments in HS 578T cells. As illustrated in Fig. 3B induction of COUP-TFII was already seen 12 h after treatment. HS 578T cells expressed COUP-TFI mRNA at a moderate level and the addition of EGF to the culture medium resulted in a profound increase from 14 amol/µg total RNA to approximately 44 amol/µg total RNA. In both cell lines these growth factors stimulated COUP-TFII mRNA expression and this effect was still detectable at 72 h, while COUP-TFII expression in untreated cells steadily declined during this period. However, addition of EGF or TGFα in serum-free medium revealed no different expression of COUP-TFI or -II (data not shown).

In SK-BR3 cells, which revealed high levels of COUP-TFII expression without external stimuli, no further induction by addition of EGF or TGFα could be detected (data not shown). In contrast to the findings for COUP-TFI, COUP-TFII expression was not influenced by EGF or TGFα in any of the cell lines tested (data not shown). EGF and TGFα stimulate the MAP kinase pathway via activation of Ras, Raf, MEK, and ERK1 and ERK2. To determine whether induction of COUP-TFII expression is mediated by these enzymes, we studied the effect of UO126 on COUP-TFII expression. UO126 specifically blocks the MAP kinase cascade by inhibiting the ability of MEK to phosphorylate ERK without affecting the JNK and p38 pathway. Treatment of MCF-7 cells with UO126 both alone or in combination with EGF, resulted in a marked decrease in COUP-TFII mRNA expression, and...
no induction of COUP-TFII mRNA by co-treatment with EGF could be observed under these conditions. The inhibitory effect of UO126 was detected as early as 12 h after treatment. A more pronounced suppression of COUP-TFII expression was observed at 24 h, which persisted during the subsequent culture period (Fig. 3A).

To determine whether Raf activation by protein kinase C can stimulate COUP-TFII expression, we examined how treatment with PMA, a strong activator of protein kinase C, would affect expression of COUP-TFII in MCF-7 cells. In our experiments addition of PMA to the culture medium resulted in a twofold increase in COUP-TFII expression at 12 h. This induction was still apparent 72 h after treatment, when COUP-TFII expression was downregulated in control cells. In co-treatment experiments with PMA and UO126 the stimulatory effect of PMA on COUP-TFII expression was completely abolished (Fig. 3C). The effect of UO126 could be observed as early as 12 h after treatment and reached its maximum after 24 h.

These data strongly suggest that the MAP kinase pathway is crucially involved in regulation of COUP-TFII expression and that induction of proliferation by addition of growth stimulating agents is accompanied by an increase
in COUP-TFII expression. It is well established that OSM antagonizes cell growth and mitogen-induced proliferation in human breast cancer cells. We therefore tested the effect of OSM treatment on COUP-TFII expression. Addition of OSM to the culture medium resulted in a pronounced reduction (approximately 90%) of COUP-TFII expression at 12 h (Fig. 3D). Twenty-four hours after treatment, only a slight (approximately 40%) difference in COUP-TFII expression between cells treated with OSM and control cells could be detected and this difference was lost by 48 h. Loss of the inhibitory effect of OSM during the culture period may be explained through instability of this cytostatic agent during the culture period.

In conclusion these results demonstrate that activation of the MAP kinase pathway induces COUP-TFII expression, while inhibition of this pathway results in a decrease in COUP-TFII expression.

Levels of phosphorylated ERK1/2 correlate with COUP-TFII expression in MCF-7 and SK-BR3 cells

In many human cancers, Ras mutations can either result in a constitutive activation of ERK1 and/or ERK2 (Iida et al. 1999), or up-regulation of the MAP kinase activity appears to result from enhancement of growth factor pathway activation (Amundadottir et al. 1998). The observation that activation of the MAP kinase pathway by EGF, TGFα or PMA induces COUP-TFII expression in breast cancer cells suggests that protein expression of the phosphorylated ERK1 and ERK2 might correlate with COUP-TFII expression. To test this hypothesis we proceeded to investigate whether activity of these MAP kinases, as determined by measurement of their phosphorylated state, might influence COUP-TFII expression. Protein extracts from exponentially growing cells were harvested 48 h after splitting. As illustrated in Fig. 4 this experiment showed that in SK-BR3 cells, an extremely high COUP-TFII expression was accompanied by a very high active ERK1/2 protein expression. On the other hand in MCF-7 cells a low COUP-TFII expression level was associated with a low active ERK1/2 protein expression. In addition we examined a third breast cancer cell line, HS 578T, in which a correlation between COUP-TFII expression and phosphorylated ERK1/2 protein expression was also observed. A control Western blot was performed to confirm the similar levels of total ERK1/2 protein expression in the three cell lines (Fig. 4 lower panel). These results strongly suggest that phosphorylated ERK1/2 may indeed be involved in the control of COUP-TFII expression. These data, however, do not answer the question whether COUP-TFII expression is directly induced by transcription factors activated by ERK1 and ERK2 or whether induction of COUP-TF is an indirect phenomenon.

Figure 4 Protein levels of phosphorylated (active) ERK1/2 (upper panel) versus total ERK1/2 (lower panel) in MCF-7, HS 578T and SK-BR3 cell lines. Cells were grown in RPMI medium for 48 h (with serum). Protein samples (80 µg) were analyzed by Western blot. Detection was performed with anti-total ERK1/2 pAb and anti-phosphorylated ERK1/2 pAb.

Inhibition of ERK1/2 activity abolishes the high level of COUP-TFII expression observed in SK-BR3 cells

As mentioned above, in SK-BR3 cells a strong active ERK1/2 protein expression activity was accompanied by a high COUP-TFII expression. To study the potential mechanism of an active MAPK pathway on COUP-TFII expression, we measured COUP-TFII expression after inhibition of this pathway. In this cell line COUP-TFII expression reached its maximum of 130 mol/µg total RNA after 48 h, when the cells were logarithmically growing. To answer the question whether suppression of phosphorylated ERK1/2 expression may lead to decreased COUP-TFII expression, SK-BR3 cells were treated with
UO126. As expected, inhibition of ERK activity resulted in a dramatic suppression of COUP-TFII expression. As shown in Fig. 5A and B, after 48 h incubation COUP-TFII mRNA expression in UO126-treated cells was reduced by approximately 80–90% (Fig. 5A and B). The increased COUP-TFII expression in SK-BR3 cells was completely abolished by treatment with this MAPK inhibitor. However, addition of UO126 did not change
total ERK1/2 protein expression (Fig. 5B lower panel). These data suggest that a fully functional MAPK pathway is required for high COUP-TFII expression in SK-BR3 cells.

**Discussion**

Growth factors and cytokines are crucially involved in the complex regulation of growth and differentiation in breast cancer cells (Liu *et al.* 1997). In the present study, we could demonstrate that modulation of the MAP kinase pathway results in significant changes in COUP-TFII expression in human breast cancer cells.

In the cell lines under investigation COUP-TF expression varies over a wide range. This observation is consistent with results recently reported by Nakshatri *et al.* (2000). Our results, however, are in disagreement with the findings of Nakshatri *et al.* in several other respects. In our experiments, COUP-TFI and -II mRNA levels were extremely low in MCF-7 cells, while Nakshatri *et al.* observed very high COUP-TF mRNA levels in this cell line. Nakshatri *et al.* were unable to detect expression of COUP-TFII in SK-BR3 cells, while in our experiments the highest level of COUP-TFII mRNA (above 130 amol/µg total RNA) was found in SK-BR3 cells. It is difficult to explain the discrepancies between our study and the study reported by Nakshatri *et al.* (2000). One possible explanation for the observed discrepancies is that although cell lines with the same designation have been used, they may, nevertheless, differ in several characteristics.

First, we demonstrated the time course of COUP-TF expression in MCF-7 cells. Since we were not able to observe any changes in COUP-TF expression culturing the cells in medium containing serum or in serum-free medium, we performed all the experiments in RPMI medium containing 10% FBS. Interestingly, COUP-TFI and -II mRNA expression declined during the culture period, independently from refreshing the medium, and no re-activation of either COUP-TFI or -II could be observed after replacing with fresh medium. It might be suggested that downregulation of COUP-TFI and -II during the culture period is a result of contact inhibition which causes antiproliferative mechanisms. Studies concerning this observation have not been done so far.

In studies investigating morphogenesis of the insect kidney it could be demonstrated that EGFR signaling activity emanating from the tip cell induces expression of *svp* in the signal-receiving cells of the developing Malpighian tubules (Kerber *et al.* 1998). This finding suggests that the EGFR pathway provides the mitogenic tip cell signal that regulates cell division and activates *svp* expression in neighboring cells. EGFR receptors possess multiple ligands, including EGF, TGFα, amphiregulin and CRYPTO (Beerli & Hynes 1996). They function as upstream mediators of Ras activation, which in turn phosphorylates and activates a cascade of MAP kinases. To determine whether treatment with EGF and TGFα, which operate as strong mitogenic factors in tumor cells (Cobb *et al.* 1994, Cobb & Goldsmith 1995, Douglas *et al.* 1997), results in changes in COUP-TF expression in human breast cancer cells, MCF-7 cells were treated with these substances and variations in mRNA levels were determined using a very sensitive quantitative RT-PCR assay. Interestingly, in these experiments only COUP-TFI, but not COUP-TFII expression was induced by treatment with these proteins. In control experiments COUP-TFII mRNA levels declined during the observation period of 72 h, while treatment with both EGF and TGFα resulted in a twofold increase in COUP-TFII expression 24 h after treatment. An estrogen-receptor negative cell line, HS 578T, was used to confirm the results observed in MCF-7 cells. Treatment with EGF resulted in a pronounced (approximately threefold) induction of COUP-TFII expression reaching its maximum 12 h after treatment. These results indicate that EGF and TGFα stimulate COUP-TFII mRNA expression most likely through activation of the Ras pathway. Treatment of SK-BR3 cells with these growth factors did not cause any change in COUP-TFII expression, indicating that in these cells expression of COUP-TFII is close to its maximum and no further induction can be obtained. EGF signaling results in activation of Raf via stimulation of Ras. Thus we proceeded to ask whether stimulation of Raf via a different pathway induces COUP-TF expression. To this end, MCF-7 cells were treated with PMA, a strong activator of protein kinase C, which in turn phosphorylates and activates Raf (Schulte *et al.* 1996). PMA induces differentiation processes in a number of breast cancer cells. However, treatment of MCF-7 cells with PMA did not cause differentiation or growth repression; it even had a mitogenic effect in this cell line. Under these conditions a sustained induction of COUP-TFII expression was observed. These results are in good agreement with published data, showing that during proliferation COUP-TF expression is induced, while during differentiation COUP-TF expression declines (Eubank *et al.* 2001). These data strongly suggest that COUP-TFII expression is stimulated by activated MAPK either through activation of Ras by EGFR signaling or through activation of Raf by stimulation of protein kinase C. COUP-TFII expression, however, was not affected by EGF and PMA treatment.

Stancato and coworkers (1997) demonstrated that OSM leads to activation of Jak1, a signaling protein which is not required for EGF-regulated responses. They found that transfection of Jak1-deficient HeLa cells with wild-type, but not with kinase-inactive Jak1, results in inhibition of basal MAPK activity. By this mechanism Jak1 could function as a negative regulator of the MAPK pathway in a Raf-1 independent manner. This is in good agreement with our results, in that OSM led to a strong repression of COUP-TFII expression. It has been suggested that OSM induces differentiation and reduces cell growth (Liu et al., 1997). In our experiments OSM treatment resulted in strong repression of COUP-TFII expression. This observation reflects the important role of COUP-TF in these processes.

The molecular mechanism by which COUP-TFII is up-regulated following activation of the MAP kinase pathway remains to be elucidated. MAP kinases can phosphorylate a variety of substrates, including several transcription factors that control cell growth, such as Elk-1 (Marais et al. 1993). Whether induction of COUP-TFII expression by the MAP kinase cascade is mediated by activation of such transcription factors or whether it is an indirect consequence of their activation leading to increased cell proliferation cannot be distinguished based on our results obtained in breast cancer cells, in which stimulation of the MAP kinase pathway uniformly leads to increased cell proliferation.

Favata and coworkers (1998) demonstrated that ERK activity is diminished in cells treated with UO126 by inhibition of MEK activity, while Raf and ERK activities are not directly affected by this compound. To determine whether inhibition of MEK activity prevents induction of COUP-TFII expression by EGF and PMA, MCF-7 cells were treated with UO126 and one of these two MAPK activators. Expression of COUP-TFII was dramatically repressed by treatment with UO126 both in the presence and absence of the MAPK activators in these experiments.

Since SK-BR3 revealed extremely high levels of COUP-TFII expression in the absence of external stimuli, we tested the hypothesis that phosphorylated ERK1/2 expression is responsible for differences in COUP-TF expression. In SK-BR3 cells the MAPK pathway may be constitutively active, resulting in high ERK activity, which may be responsible for the high COUP-TFII expression observed in this cell line. The strong ERK1/2 protein phosphorylation found in SK-BR3 cells and the low expression of these enzymes in MCF-7 cells are compatible with this hypothesis. HS 578T cells, in which a moderate COUP-TFII expression correlated with moderate active ERK1/2 expression provided additional support for this hypothesis. No different levels of total ERK1/2 protein expression could be detected in these cell lines. As expected, treatment of SK-BR3 cells with UO126 resulted in a dramatic decrease in phosphorylated ERK1/2, but not in total ERK1/2, protein expression which was accompanied by a strong repression in COUP-TFII expression.

To our knowledge these results represent the first reported evidence that induction of the MAPK cascade by treatment with EGF, TGFα and PMA induces COUP-TFII expression in human breast cancer cells and that effective blockade of this pathway leads to a decrease in COUP-TFII expression. While COUP-TFII mRNA expression was up-regulated by treatment with EGF, TGFα and PMA in our experiments, COUP-TFII expression was unaffected by these agents. These results suggest that transcriptional regulation of the genes coding for COUP-TFII and -II differs substantially. While stimulation of the MAPK pathway is both essential and sufficient for induction of COUP-TFII mRNA expression, activation of COUP-TFII gene expression appears to involve other mechanisms.

This implies that COUP-TFs may be crucially involved in proliferation, differentiation and cell cycle control, which might explain why they have been so highly conserved during evolution.

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