The EMBO Journal vol.14 no.6 pp.1145–1155, 1995

Thyroid hormone regulates stromelysin expression, protease secretion and the morphogenetic potential of normal polarized mammary epithelial cells

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Communicated by H.Beug

Stromelysins are a group of proteases which degrade the extracellular matrix and activate other secreted proteases. Stromelysin (ST)-1 and ST-2 genes are induced by tumor promoters, oncogenes and growth factors, and have been involved in acquisition of the malignant phenotype. We show here that the thyroid hormone (T_3) increases ST-1 and ST-2 expression in a non-transformed mouse mammary epithelial cell line (EpH4) in a way that is dependent on the level of thyroid receptor/c-*erb*A (TRα-1) expression. In agreement with this, T_3 increases the secreted stromelysin activity and enhances the gelatinolytic activity of type IV collagenase. We have also demonstrated that T_3 affects the epithelial polarity of EpH4 cells, diminishing the transepithelial electrical resistance of monolayers cultured on permeable filters, causing an abnormal distribution of polarization markers and the disruption of the organized 3-D structures formed by these cells in type I collagen gels. These results indicate that the ligand-activated TRα-1 plays an important role in regulating the morphogenetic and invasive capacities of mammary epithelial cells. Because the c-*erb*A locus is altered in several types of carcinoma, an altered or deregulated TRα-1 expression may also be important for breast cancer development and metastasis.

Key words: *erb*A/mammary epithelial cells/proteases/stromelysin/thyroid hormone

Introduction

Thyroid hormone (T_3; 3,5,3′-L-triiodothyronine) plays multiple and crucial roles in vertebrates regulating developmental processes in numerous cell types. T_3 receptors (TR) are encoded by two genes (TR/c-*erb*Aα and TR/c-*erb*Aβ), which are cellular counterparts of the retroviral v-*erb*A oncogene involved in avian erythroleukemia (Sap *et al.*, 1986; Weinberger *et al.*, 1986). A number of clinical and experimental studies suggest the involvement of T_3 and its receptors in processes related to carcinogenesis. T_3 increases the transforming potential of chemical carcinogens (Borek *et al.*, 1983), radiations (Guernsey *et al.*, 1980, 1981) and viruses (Fisher *et al.*, 1983; Babiss *et al.*, 1985), and is able to enhance the activity of the *ras* oncogene in cultured cells (López *et al.*, 1989). Also, an ill-defined interaction exists between TR/c-*erb*A and the activity of the Fos and Jun proteins forming the AP-1 transcription factor. TR/c-*erb*A and v-*erb*A have been reported to increase c-*jun* transcriptional activity (Sharif and Privalsky, 1991), whereas other studies describe mutual antagonism between TR/c-*erb*A and AP-1 (for a recent review see Pfahl, 1993). Recently, alterations in the *erb*A loci have been found in human colon (Markowitz *et al.*, 1989), lung (Dobrovic *et al.*, 1988; Drabkin *et al.*, 1989; Leduc *et al.*, 1989), stomach (Yokota *et al.*, 1988) and hepatocellular carcinomas (Arbuthnot *et al.*, 1989), and in leukemia (Dayton *et al.*, 1988). Thyroid hormone receptors are expressed in the normal mammary gland (Bhattacharya *et al.*, 1977; Hayden and Forsyth, 1977; Wilson and Gorewit, 1980; Selliti *et al.*, 1983) as well as in breast cancer cells (Burke and McGuire, 1978; Smallridge and Latham, 1980; Cerbon *et al.*, 1981; Eil *et al.*, 1981; Ruzicka and Rose, 1983). Both amplifications and deletions of the *erb*A genes (van de Vijver *et al.*, 1987; Ali *et al.*, 1989; Futreal *et al.*, 1992) have been described in breast cancer, further suggesting an involvement of aberrant, T_3-dependent gene regulation in these tumors. However, despite extensive studies on the relationship between thyroid status on the one hand and human breast cancer and other diseases on the other, data on the beneficial or adverse effects of T_3 are contradictory (for recent reviews see Vorherr, 1987; Goldman, 1990; Goldman *et al.*, 1992).

Breast cancer is the most common cancer among women in western countries. One in eight women are expected to develop breast cancer during their lifetime, with a tendency for rates to increase (Marshall, 1993). It is obvious that the study of agents acting on mammary epithelial cells, whose transformation *in vivo* leads to breast carcinoma, is of prime interest. To elucidate putative roles of T_3, we have analyzed its effects on a clonal non-transformed and non-tumorigenic cell line (EpH4) derived from spontaneously immortalized mouse mammary cells described previously (Reichmann *et al.*, 1989). For the following reasons, EpH4 cells appear to be an ideal system to address the question of whether T_3 has an effect during the initial steps of mammary epithelial transformation, clearly superior to the heavily altered cells usually established from late phases of tumor growth. EpH4 cells exhibit a stable fully polarized epithelial phenotype, resembling in many aspects that of luminal epithelial cells present in the normal mammary gland. They form regular compact monolayers on culture dishes and exhibit the typical characteristics of a polarized epithelial cell, such as a high

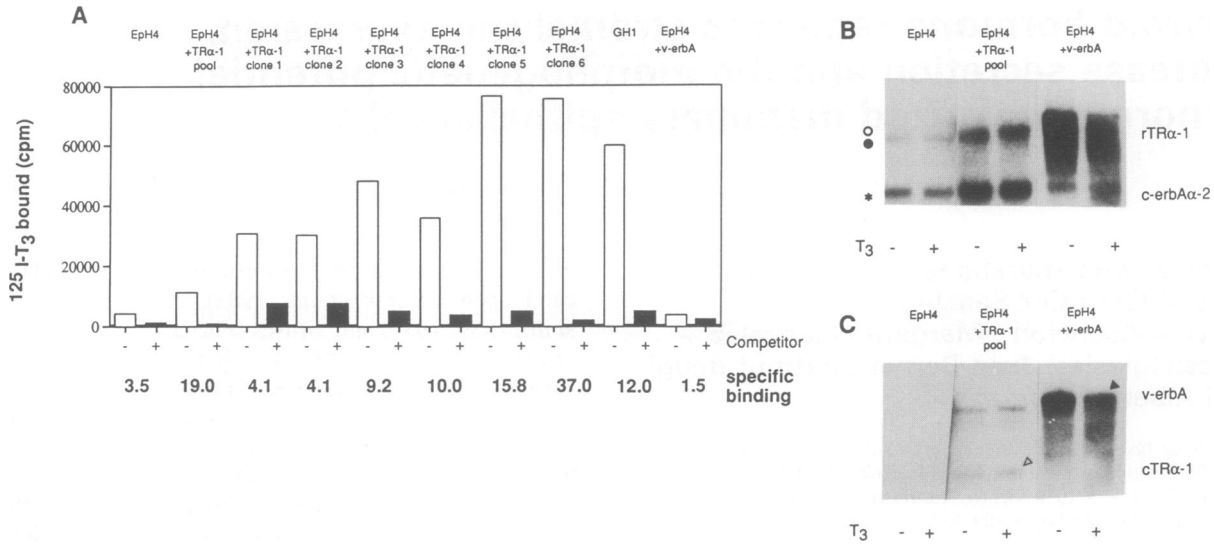

Fig. 1. *erb*A expression in EpH4 cells. (**A**) Parental EpH4, EpH4+TRα-1 and EpH4+v-*erb*A cells were analyzed for T_3-specific binding as described (Sap *et al.*, 1986; Muñoz *et al.*, 1990) in the presence (+, open bars) or absence (−, solid bars) of a 1000-fold molar excess (0.3 mM) of cold T_3 as competitor. Rat pituitary GH1 cells expressing high levels of endogenous T_3 receptors were used as the control. (**B**) Analysis by Northern blots of endogenous TRα mRNA expression in parental EpH4, EpH4+TRα-1 and EpH4+v-*erb*A cells. 10 μg poly(A)$^+$ RNA were analyzed per lane. The exposure time was 6 days. TRβ was not detected. Mature endogenous TRα-1 mRNA (5 kb, solid circle) and its precursor (6.5 kb, open circle) are indicated. The band corresponding to endogenous TR/c-*erb*Aα-2 mRNA (2.6 kb, asterisk) is also marked. It coincides with that of the viral subgenomic RNA encoded by the internal promoter. This RNA, as well as the large unspliced viral genomic RNA (indicated by an arrow), cross-hybridize with the rat TRα probe. (**C**) Analysis of exogenous retrovirally expressed chicken TRα (cTRα-1) and v-*erb*A mRNA by Northern blots. 10 μg poly(A)$^+$ RNA were analyzed per lane. We used as probe a 700 bp *Bst*EII−*Sac*I fragment corresponding to the hormone binding domain of the chicken TRα cDNA which does not cross-hybridize with the endogenous mouse TRα RNA. Large viral genomic (7.5 kb, solid arrowhead) and the smaller subgenomic (2.6 kb, open arrowhead) RNAs were detected in the corresponding infected cells. The exposure time was 2 days.

transepithelial electrical resistance and the expression of apical and basolateral marker genes at the correct plasma membrane domains when seeded on permeable supports. They also exhibit dome formation on plastic and form organized 3-D tubular structures in collagen gels which are typical of normal luminal epithelial cells (I.Fialka, E.Reichmann, M.Busslinger and H.Beug, manuscript in preparation).

Epithelial morphogenesis as well as abnormal events occurring during carcinogenesis are thought to involve metalloproteases. Stromelysin-1 [ST-1, transin or matrix metalloproteinase-3 (MMP-3)] and stromelysin-2 (ST-2, transin-2 or MMP-10) are two such metalloproteases which actively degrade the extracellular matrix and are also involved in the activation of other secreted cellular proteases (Matrisian and Bowden, 1990). ST-1 and ST-2 are induced by tumor promoters [12-*O*-tetradecanoyl-13-phorbolacetate (TPA), phorbol 12-myristate 13-acetate (PMA)], oncogenes and growth factors (Kerr *et al.*, 1988; Kreig *et al.*, 1988; Machida *et al.*, 1988; Chan *et al.*, 1992; De Vouge and Mukherjee, 1992; Sreenath *et al.*, 1992). Regulation of the ST-1 gene is complex. Growth factors such as platelet-derived growth factor (PDGF; Kerr *et al.*, 1988) and epidermal growth factor (EGF; Machida *et al.*, 1988) are positive regulators of ST-1 expression. Further supporting the important role of ST-1 in tumor progression, its expression has been found to be inhibited by transforming growth factor (TGF) β (Machida *et al.*, 1988; Kerr *et al.*, 1990) and retinoic acid (Nicholson *et al.*, 1990). In both cases, inhibition is mediated by AP-1 sites. Since TGFβ is induced by antiestrogens (Knabbe *et al.*, 1987), a role for ST-1 in mammary cells seems plausible. Interestingly enough, ST-1 and ST-2 expression has been shown to correlate with an increased malignancy of cancer cells during tumor progression (Matrisian *et al.*, 1986a; Ostrowski *et al.*, 1988; Matrisian and Bowden, 1990; Sreenath *et al.*, 1992). ST-1 expression is higher following the oncogenic transformation of rat fibroblast cell lines (Matrisian *et al.*, 1985, 1986b; Breathnach *et al.*, 1987; Engel *et al.*, 1992; Sreenath *et al.*, 1992) and in the progression of benign mouse skin papillomas to malignant squamous cell carcinomas (Matrisian *et al.*, 1986a; Ostrowski *et al.*, 1988). ST-1 also correlates with the metastatic potential of oncogenic-transformed rat embryo cells (Matrisian and Bowden, 1990).

In this study we show that T_3 increases ST-1 and ST-2 mRNA levels and secreted activity in a way that is dependent on and correlates with the level of TR/c-*erb*A receptors in the EpH4 mammary epithelial cells. Moreover, T_3 also enhances the collagenolytic activity of the cells. Finally, T_3 affects epithelial polarity. T_3 markedly reduces the transepithelial electrical resistance in monolayers of EpH4 cells expressing TRα-1 grown on permeable filter supports. It also causes the partial redistribution of both apically and basolaterally sorted proteins. Probably as a consequence of this, T_3 treatment abolishes the ability of the cells to form ordered 3-D structures in collagen gels, suggesting that TR/c-*erb*A receptors are regulating morphogenetic events in the formation of epithelia. These results indicate that T_3 exerts profound effects on aspects of mammary epithelial cell function which may be crucial during breast cancer development.

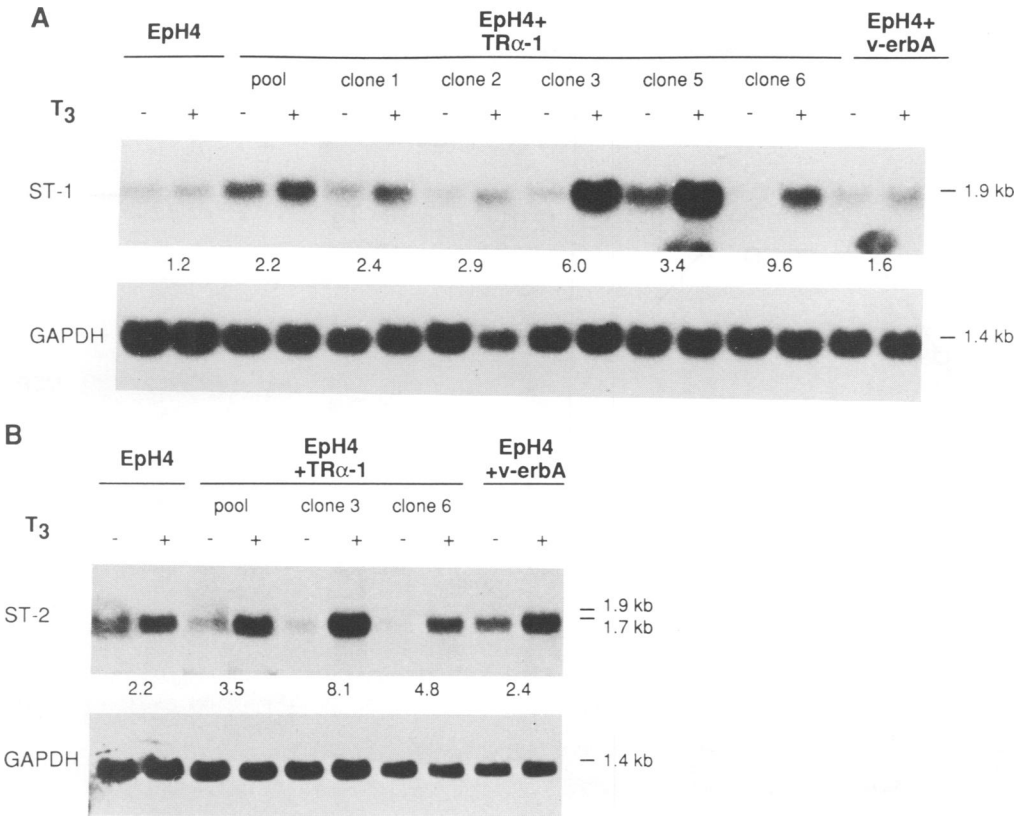

Fig. 2. Increase of ST-1 and ST-2 mRNA levels by T_3 in EpH4 cells. (**A** and **B**) Northern analysis of ST-1 and ST-2 mRNA levels, respectively. 10 μg poly $(A)^+$ RNA were analyzed per lane. Parental EpH4, EpH4+TRα-1 (pool and individual clones) and EpH4+v-*erb*A cells were treated (+) or not (−) with T_3 (150 nM) for 2 days in serum-free medium. Specific probes for each ST gene were used: a *Bgl*II−*Eco*RI fragment containing nucleotides 1128–1771 of rat ST-1 and a 703 bp *Eco*RI fragment of rat ST-2 (Breathnach *et al.*, 1987). Numbers represent the fold increase in ST-1 or ST-2 mRNA levels caused by T_3 treatment after normalization versus GAPDH mRNA levels. Exposure times: ST-1 and ST-2, 3 days; GAPDH, 6 h.

Results

T_3 receptors specifically increase ST-1 and ST-2 mRNA levels

EpH4 cells were first analyzed for the presence of active T_3 receptors. According to reports on normal mammary gland (Bhattacharya and Vonderhaar, 1977; Hayden and Forsyth, 1977; Wilson and Gorewit, 1980; Selliti *et al.*, 1983), we detected low but significant levels (Figure 1A). However, as found with most cultured cells, the mRNA concentration for the non-hormone binding inhibitory c-*erb*Aα2 form was higher than that corresponding to the active TRα-1 (Figure 1B). The second TR/c-*erb*A gene (TRβ) is not expressed in these cells. To avoid masking or blocking actions of c-*erb*Aα2 and more clearly reveal T_3 effects, we increased the number of functional hormone receptors in the cells by stably expressing an exogenous (chicken) TRα-1 gene using retroviral vectors containing the neomycin resistance gene as a selectable marker (see Materials and methods). This strategy has been shown to be successful in demonstrating relevant T_3 effects in other systems (Carnac *et al.*, 1993; Muñoz *et al.*, 1993). V-*erb*A was also used in parallel for comparison. Expression of the erbA genes was verified in Northern blots and hormone binding assays (Figure 1A and C).

Since the ability to degrade extracellular matrix and basement membrane components is a prerequisite for epithelial cell plasticity, morphogenesis and metastasis, we studied T_3 effects on the expression of protease genes. First, the ST-1 gene was analyzed. Results from Figure 2A demonstrate that hormone treatment increased the 1.9 kb ST-1 mRNA levels in all EpH4+TRα-1 clones tested. A comparison with Figure 1A shows that this effect correlates with the amount of active thyroid receptor expressed in each clone. Thus, parental EpH4 and EpH4+v-*erb*A cells essentially did not respond to T_3, whilst ST-1 mRNA levels in EpH4+TRα-1 clones expressing relatively low levels of receptor (nos 1 and 2) as well as in EpH4+TRα-1 pool cells were enhanced by a factor of two to three. In clones exhibiting higher levels of specific T_3 binding (nos 3, 5 and 6), ST-1 RNA levels were upregulated 6- to ~10-fold. A similar result was obtained for the highly homologous ST-2 gene. Using a specific ST-2 probe, a clear induction by T_3 of the two ST-2 mRNAs of 1.7 and 1.9 kb was observed (Figure 2B). This induction was again strong in EpH4+TRα-1 clones 3 and 6, moderate in EpH4+TRα-1 pool cells and low in EpH4+v-*erb*A cells and parental EpH4 cells. Another gene regulatory activity of TRα-1, i.e. its ability to inhibit basal transcription in the absence of ligand, is also clearly seen in the EpH4+TRα-1 cells (compare Figure 2B, lanes 1, 3, 5 and 7). A kinetic analysis showed that the up-regulation of ST-1 mRNA levels by T_3 is quite slow (Figure 3A). The induced increase was maximum after 40 h of T_3 treatment, suggesting an indirect or post-transcriptional mechanism. To determine whether T_3

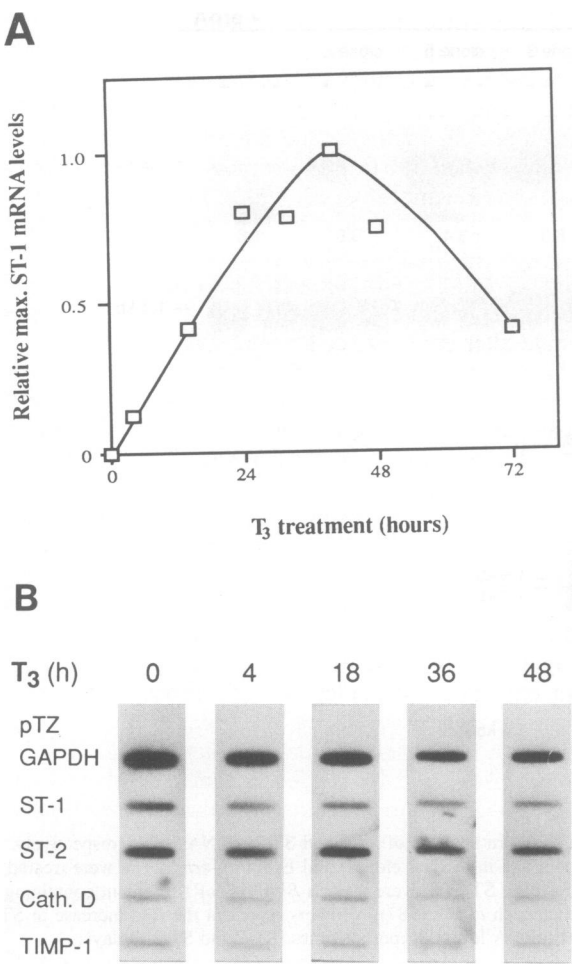

Fig. 3. Effect of T_3 on ST-1 and ST-2 mRNA levels and transcription in EpH4+TRα-1 cells. (**A**) Kinetics of the increase in ST-1 mRNA levels by T_3. EpH4+TRα-1 clone 6 cells were treated in serum-free medium with T_3 (150 nM) for the indicated times. Filters containing 10 μg poly $(A)^+$ mRNA per lane were hybridized consecutively with the ST-1 and GAPDH probes. Densitometric scanning of the ratio between ST-1 and GAPDH RNAs was performed. Relative values with respect to maximum increase in the ST-1:GAPDH ratio observed at 40 h of T_3 treatment are shown. (**B**) Transcription rate of ST-1 and ST-2 genes in T_3-treated EpH4+TRα-1 cells. Nuclei isolated from monolayers of EpH4+TRα-1 clone 6 cells untreated or treated for the indicated times with 150 nM T_3 were assayed for transcription, as described in Materials and methods. Labeled RNAs (10^7 c.p.m.) were hybridized to nylon membranes containing 5 μg of linearized plasmids encoding the cDNAs indicated. GAPDH was used as an internal control and pTZ as a background control. The RNA polymerase II inhibitor α-amanitin (2 μg/ml) was used as a control in the reaction (results not shown). Similar results were obtained in two experiments.

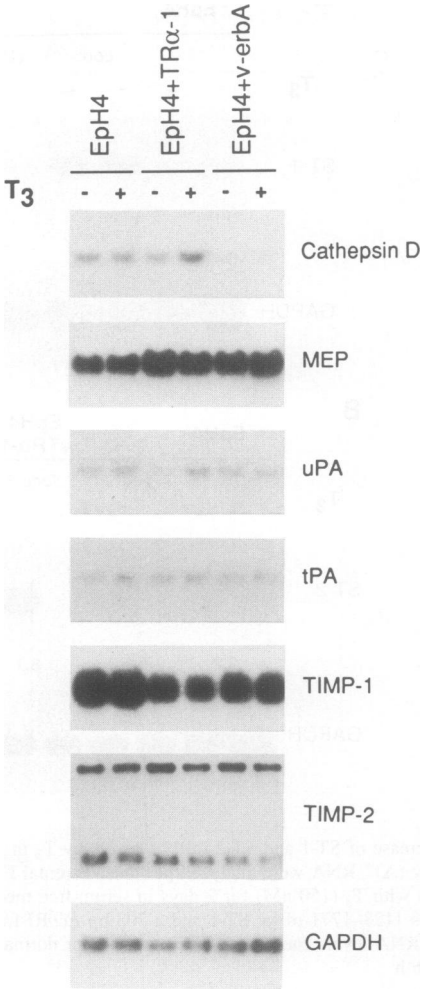

Fig. 4. Effect of T_3 on the steady-state mRNA levels of other proteases and protease inhibitors in EpH4 cells. Parental EpH4, EpH4+TRα-1 (pool) and EpH4+v-*erb*A cells were treated or not with 150 nM T_3 in serum-free medium for 48 h. Filters containing 10 μg poly $(A)^+$ mRNA per lane were hybridized consecutively with the cathepsin D, MEP, uPA, tPA, TIMP-1 and TIMP-2 probes. Exposure times: cathepsin D and TIMP-1, 1 day; MEP and TIMP-2, 6 h; uPA and tPA, 2 weeks; GAPDH, 6 h.

affects the rate of transcription of the ST-1 and ST-2 genes, we performed nuclear run-on experiments. Nuclei from control or T_3-treated EpH4+TRα-1 cells were isolated and nascent transcripts hybridized to filter-bound plasmid probes as described in Materials and methods. As shown in Figure 3B, the transcriptional rate of ST-1 and ST-2 genes was unaffected by T_3 at any time studied.

Next, we studied the effect of T_3 on the expression of genes coding for other proteases and protease inhibitors. A slight increase was found in the steady-state mRNA levels of cathepsin D, an estrogen-regulated acidic protease whose expression and secretion are enhanced in breast cancer and correlate with the acquisition of the metastatic potential of cancer cells (Garcia *et al.*, 1990; Rochefort *et al.*, 1990). No major reproducible changes were found in the case of the tissue and urokinase-type plasminogen activator genes (tPA, uPA), major excreted protein (MEP; Figure 4) or the interstitial collagenase I and matrilysin (PUMP-1; results not shown). Genes coding for protease inhibitors were also analyzed. RNA levels of tissue inhibitors of metalloproteinases (TIMP)-1 and TIMP-2 (Figure 4) and of plasminogen activator inhibitor (PAI)-1 were unchanged (results not shown). PAI-2 RNA was undetectable. These results show that the increase of ST-1 and ST-2 mRNA levels by T_3 is specific and does not reflect a generalized effect on protease and protease inhibitor genes.

T_3 enhances extracellular protease activity

To investigate the effect of T_3 on ST-1 activity and on other secreted proteolytic enzymes, we carried out zymography analysis using different substrates. Casein

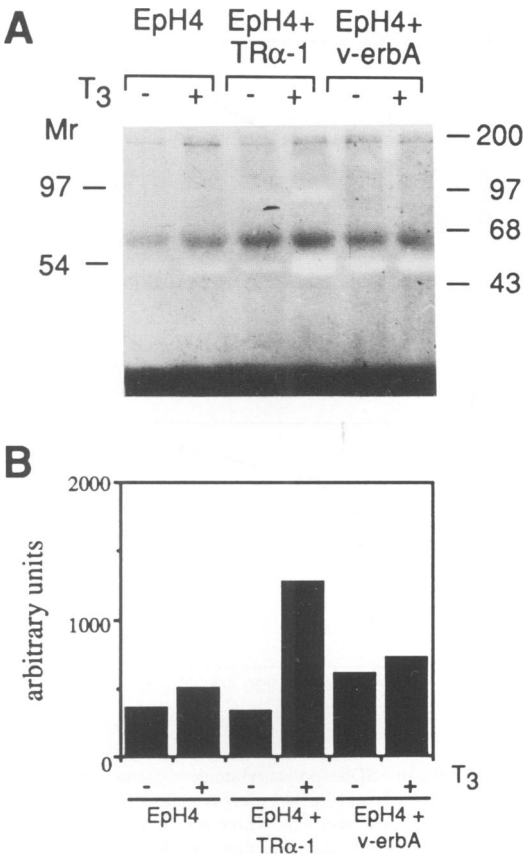

Fig. 5. Casein zymography of parental EpH4, EpH4+TRα-1 and EpH4+v-*erb*A cells. (**A**) Casein–SDS–polyacrylamide substrate gels were used to visualize the caseinolytic activity of conditioned media from parental EpH4, EpH4+TRα-1 (pool) and EpH4+v-*erb*A cells after 2 days incubation in the presence or absence of 150 nM T_3 in serum-free medium, as described in Materials and methods. The sizes of the active proteins found are indicated on the left. Molecular weight markers are shown on the right. (**B**) Densitometric scanning of the caseinolytic activity corresponding to the protein of M_r 54 000.

zymography was used to study T_3 effects on stromelysin activity. In agreement with the mRNA expression data, T_3 caused EpH4+TRα-1 cells to secrete nearly 4-fold levels of a caseinolytic protein with a size (M_r 54 000) corresponding to that of the ST-1 and ST-2 (Sreenath *et al.*, 1992; Figure 5A and B). In addition, the activity of a M_r 97 000 protease was also enhanced by T_3. As expected, only minor effects of T_3 were observed in parental EpH4 and EpH4+v-*erb*A cells. This result shows that the increase in ST-1 and ST-2 mRNAs levels caused by T_3 is accompanied by a higher secreted ST-1 enzymatic activity.

Hormonal effects on the collagenolytic activity of the cells were studied on gelatin zymograms. As shown in Figure 6A and B, T_3 also increased (60–70% after 48 h) the secretion of a M_r 97 000 gelatinase which is probably the mouse homolog to the human M_r 92 000 type IV collagenase/gelatinase B or MMP-9. Again, such an effect was not observed in EpH4+v-*erb*A cells. Secretion of this enzyme was enhanced by T_3 in a time- and dose-dependent manner (Figure 6C and D). These results clearly demonstrate that the T_3-activated c-*erb*A/TRα-1 simultaneously induces different metalloproteinases with distinct substrate specificities.

TRα-1 affects epithelial polarity and disturbs epithelial morphogenesis in collagen gels

EpH4 cells exhibit a typical polarized epithelial phenotype, as revealed by their high transepithelial electrical resistance (TER) and their specific expression of apical and basolateral protein markers at the respective plasma membrane domains when allowed to form monolayers on permeable supports (Reichmann *et al.*, 1992). In addition, these cells are able to form ordered tubular structures when seeded into type I collagen gels (Reichmann *et al.*, 1992; M.Oft, I.Fialka and H.Beug, unpublished results). We therefore analyzed whether the ligand-activated TRα-1 protein would alter epithelial cell polarity and morphogenesis in this system.

In control EpH4 cells seeded on porous supports, T_3 treatment did not affect the progressive formation of monolayers with a high TER (Figure 7A). In contrast, the addition of T_3 did prevent the establishment of such a monolayer in TRα-1-expressing EpH4 cells. In the absence of T_3, the formation of compact monolayers which reached high TER values proceeded essentially as normal (Figure 7B, □). However, the addition of T_3 to TRα-1-expressing EpH4 cells completely blocked the increase in TER (Figure 7B, ●). These results demonstrate that TRα-1 is not only able to regulate specific protease genes but is also able to alter the normal epithelial phenotype of EpH4 cells in a hormone-dependent fashion.

The above results suggested that the ligand-activated TRα-1 was able to inhibit or modulate epithelial polarization. To investigate this further, we analyzed the apical/basolateral distribution of several proteins established as apical or basolateral marker proteins in both parental EpH4 and EpH4+TRα-1 cells. For this determination, cells grown on porous filters were analyzed by labeling with suitable antibodies using confocal immunofluorescence microscopy (see Materials and methods). As seen in Figure 8, T_3 treatment led to increased expression of the apical marker dipeptidyl peptidase (DPP IV; Marguet *et al.*, 1992; Zurzolo *et al.*, 1992) in EpH4+TRα-1 cells. T_3 also caused a redistribution of DPP IV expression towards the lateral plasma membrane. The ligand-activated TRα-1 also caused a partial redistribution of basolateral markers. Three components of the adherens junctions (E-cadherin/uvomorulin, fodrin and β-catenin) that show a distinct lateral expression in control cells and EpH4+TRα-1 in the absence of T_3, became much more diffusely distributed over the whole lateral plasma membrane when the cells were treated with T_3 (Figure 8). However, none of these proteins was detected apically. This is in line with our observation that the distribution of ZO-1, a tight junction protein that may be involved in forming a diffusion barrier between apical and basolateral membrane proteins, exhibited only moderate redistribution (data not shown). As expected, neither of these effects of T_3 was found in the parental EpH4 cells (Figure 8). Our results clearly show that the ligand-activated TRα-1 causes a distinct alteration in the ability of epithelial cells to build up epithelial polarity, leading to a partial redistribution of both apically and basolaterally sorted proteins.

The observed ability of TRα-1 to alter epithelial polarization prompted us to study the ability of a series of EpH4 cell clones expressing different levels of TRα-1 receptors to form 3-D tubular structures in collagen gels.

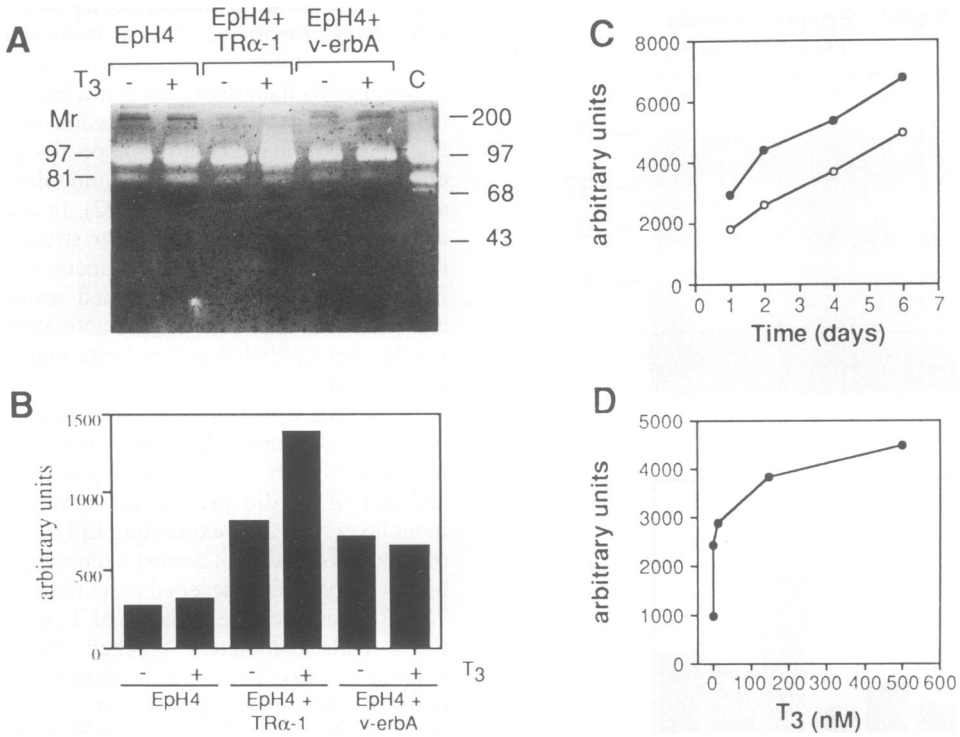

Fig. 6. Gelatin zymography of parental EpH4, EpH4+TRα-1 and EpH4+v-*erb*A cells. (**A**) Gelatin−SDS−polyacrylamide substrate gels were used to visualize the gelatinolytic activity of conditioned media from parental EpH4, EpH4+TRα-1 (pool) and EpH4+v-*erb*A cells after 2 days incubation in the presence or absence of 150 nM T_3 in serum-free media, as described in Materials and methods. Three times larger volumes of conditioned medium from parental EpH4 cells than from EpH4+TRα-1 and EpH4+v-*erb*A cells were loaded. Purified type XI collagenase (1 μg/ml) was added to lane C as a positive control. The sizes of the active proteins found are indicated on the left. Molecular weight markers are shown on the right. (**B**) Densitometric scanning of the gelatinolytic activity corresponding to the protein of M_r 97 000. Values have been normalized to the same volume of conditioned medium. (**C**) Kinetic study of the gelatinolytic activity corresponding to the protein of M_r 97 000 secreted by EpH4+TRα-1 clone 6 cells following T_3 treatment. Densitometric scanning: ○, untreated cells; ●, cells treated with 150 nM T_3 for the indicated times. (**D**) Dose curve of T_3 induction of the gelatinolytic activity corresponding to the protein of M_r 97 000 secreted by EpH4+TRα-1 clone 6 cells after 2 days of treatment.

As expected from their highly polarized non-transformed phenotype, both parental EpH4 and EpH4+TRα-1 cells developed tubular branched structures which are reminiscent of those present in the normal mammary gland (Figure 9). These structures sometimes showed a visible lumen, and were surrounded by deposits of basement membrane components such as type IV collagen and laminin, as assessed by immunofluorescence analysis (see below and data not shown). Most significantly, the addition of T_3 to such structures formed by EpH4+TRα-1 cells after 7 days caused a progressive disruption of this tubular architecture (Figure 9). T_3 promoted a progressive thinning and loss of lumen in the structures, followed by cell disaggregation, eventually leading to the complete separation of the structures into individual cells (Figure 9). These effects tended to be stronger in clones showing high hormone binding capacity (Figure 1). In contrast, T_3 essentially did not affect tubular morphogenesis in control EpH4 cells containing only endogenous TRα-1.

To analyze if and how the distribution of polarization markers would be altered during the T_3-induced breakdown of tubular structures and if the ligand-activated TRα-1 would cause alterations in the deposition of basement membrane components, the expression of basolateral markers on the one hand and of type IV collagen and laminin on the other was analyzed by staining frozen sections prepared from the structures obtained. As in the cells grown on porous filter supports (see Figure 8), the lateral distribution of uvomorulin, β-catenin and fodrin was partially disrupted by T_3 (Figure 10B and data not shown). In addition, the ordered deposition of basement membrane components was affected. While in structures formed by EpH4+TRα-1 cells in the absence of T_3 laminin was deposited on the basement membrane only, T_3 disrupted this ordered deposition, leading to the deposition of laminin all around or even within the cells (Figure 10A). These results are in line with our assumption that the enhanced secretion of stromelysins and collagenase IV induced by the ligand-activated TRα-1 may be responsible for the aberrant deposition of basement membrane components such as laminin (Figure 10B) and collagen IV (data not shown), both of which are substrates of the TRα-1-induced proteases.

Discussion

Breast cancer results from stepwise alterations of epithelial cells in the mammary gland, first forming *in situ* carcinomas which later can increase in malignancy and give rise to metastases. Despite the enormous interest in breast cancer due to its clinical significance, the molecular basis of mammary epithelial cell transformation and the events and factors involved in the progressive malignization of breast cancer epithelial cells are mostly unknown.

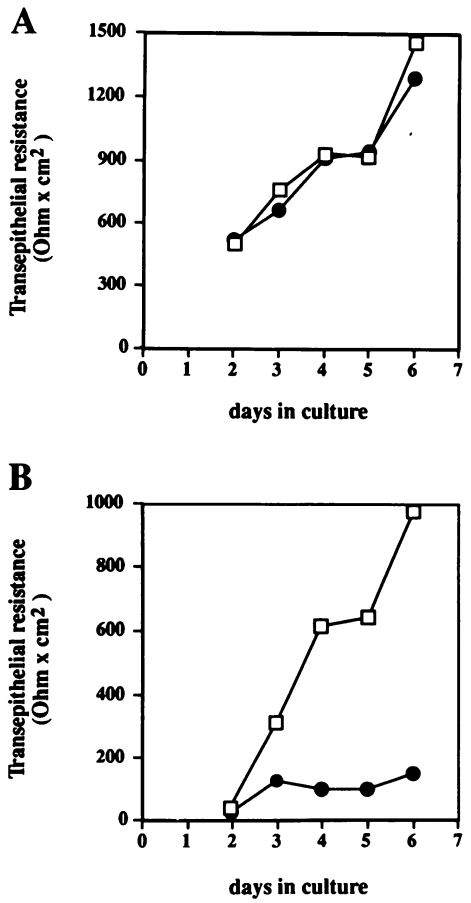

Fig. 7. Transepithelial electrical resistance of parental and TRα-1-expressing EpH4 cells. Parental EpH4 (**A**) and EpH4+TRα-1 clone 6 cells (**B**) were seeded onto porous supports in the absence (□) or presence (●) of 150 nM T_3 and transepithelial electrical resistance measured at the times indicated. Partial medium changes plus re-addition of fresh hormone were performed daily.

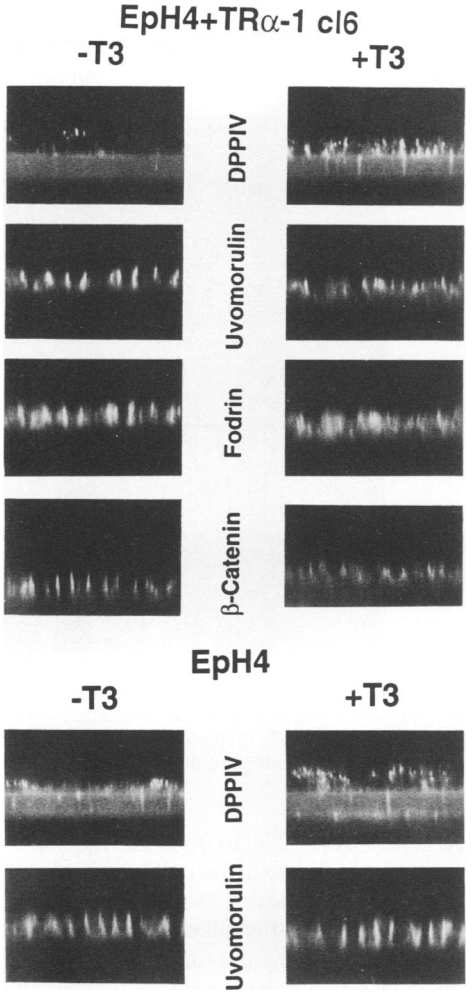

Fig. 8. Partial disruption of epithelial cell polarity by the T_3-activated TRα-1. EpH4+TRα-1 cells (clone 6) and control EpH4 cells were grown on permeable supports in the absence or presence of T_3 for 6 days, stained with the apical marker DPP IV and the basolaterally expressed adherens junction proteins uvomorulin, fodrin and β-catenin, and visualized by confocal microscopy.

The estrogen dependence of certain mammary tumors and the importance of estrogen and also progesterone receptors for normal mammary cell development and tumor growth is well established. Other agents, however, should contribute to their loss of epithelial polarity, increased proteolytic activity and tissue architecture destruction, as well as to the acquisition of full metastatic potential. When considering (i) the expression of T_3 receptors in normal and cancer breast cells and (ii) the homology between steroid and thyroid receptors in structure, activity as ligand-dependent transcription factors and even DNA recognition sequences in target genes (Evans, 1988; O'Malley, 1990), it is surprising that no studies on T_3 effects in mammary epithelial cells have been reported. We show here that T_3 clearly alters distinct biological and biochemical features of a polarized mammary epithelial cell line, increasing protease gene expression and secreted proteolytic activity in these cells as well as affecting the formation of compact monolayers and blocking their ability to form ordered tubular structures characteristic of normal epithelial cells.

Mammary epithelial development (Singh and Bern, 1969; Vonderhaar, 1975; Vonderhaar and Greco, 1979; Rosato *et al.*, 1992) and milk protein synthesis (Vonderhaar, 1977; Houdebine *et al.*, 1978) are sensitive to T_3, most probably due to the presence of thyroid receptors. However, to our knowledge this is the first study dealing with T_3 effects on the polarized epithelial phenotype of mammary epithelial cells. We have demonstrated that T_3 is able to enhance the protease activity of non-transformed mammary epithelial cells, a necessary step for basement membrane degradation in the process of tumor formation. At least two protease genes, ST-1 and ST-2, are induced by T_3 in EpH4 cells. Evidence exists linking ST-1 (see Matrisian and Bowden, 1990, for a review) and ST-2 (Chan *et al.*, 1992; De Vouge and Mukherjee, 1992; Sreenath *et al.*, 1992) to cancer. Stromelysin activity is blocked by specific inhibitors like TIMP, which are also under growth factor control. Actual extracellular stromelysin activity is the balanced result of the respective ST and TIMP expression. TIMP-1 and TIMP-2 expression are not altered by T_3 in EpH4 cells, supporting the correlation between the increase in ST-1 expression and secreted activity. In line with our data, ST-1 mRNA has been detected recently in the periphery of some human breast carcinomas but not in benign lesions (Polette *et al.*, 1993). Also, we have reported previously

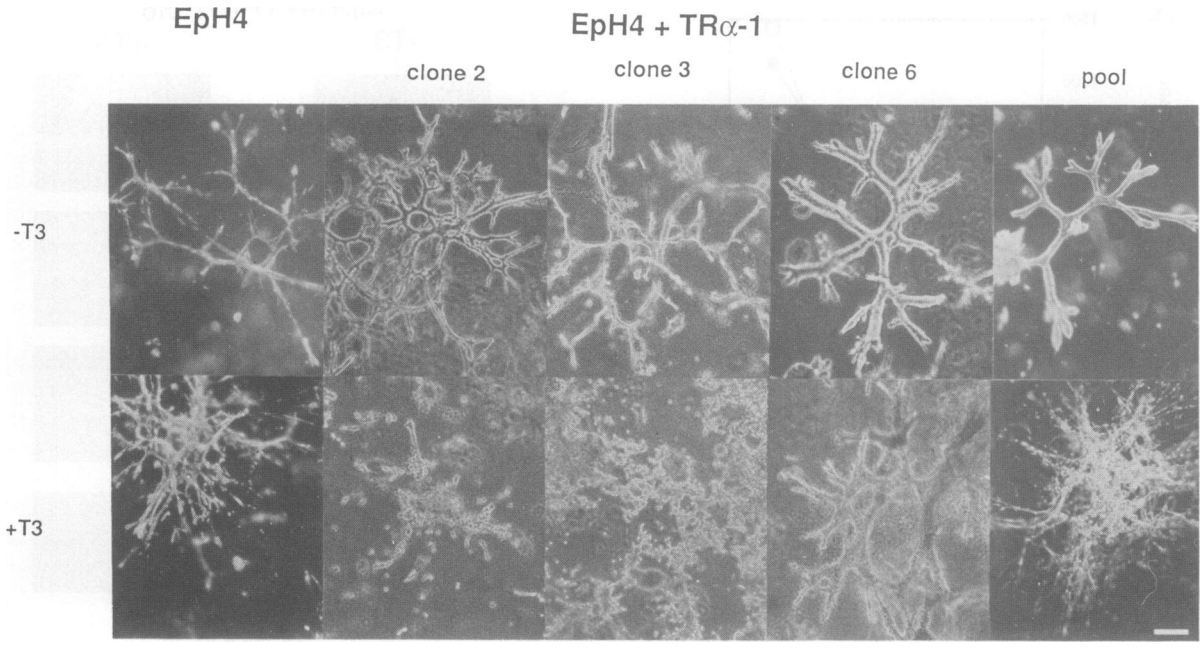

Fig. 9. T_3 effects on the epithelial morphogenetic potential of EpH4 cells. Parental EpH4 cells, three individual clones (nos 2, 3 and 6) and a pool of EpH4+TRα-1 cells were plated in duplicate in collagen gels, allowing them to form tubular structures as described in Materials and methods. Once the structures were developed, T_3 (150 nM) was added daily to one set of plates for an additional 6 days, and phase-contrast micrographs were taken. Three separate experiments gave the same results. Bar, 625 μM.

that *erb*A genes were able to regulate the induction of ST-1 by nerve growth factor in PC12 cells. In this case, the addition of T_3 relieves the suppression of ST-1 expression caused by unliganded TRα-1 (Muñoz *et al.*, 1993). Further supporting our data, T_3 has been found recently to induce two genes respectively homologous to human ST-3 and rat collagenase during amphibian tail resorption (Wang and Brown, 1993). Taken together, available data suggest that ST-1 expression is a relatively late event during tumor progression accompanying malignant conversion, and may therefore correlate with the invasive behavior of the tumor. The increase in ST-1 and ST-2 mRNA levels by T_3, reported here in five independent clones of mammary epithelial cells expressing different levels of TRα-1, raises questions concerning the role of T_3 and its receptors in tumor progression.

From the gelatin zymography experiments it is clear that T_3 also enhances the activity of an enzyme which probably is the mouse homolog to the human M_r 92 000 type IV collagenase. An elevated expression of the M_r 92 000 type IV collagenase correlates with the metastatic phenotype of cultured rodent cells (Ballin *et al.*, 1988; Bernhard *et al.*, 1990). Type IV collagenase activity is also secreted by cultured cells with invasive capacity and correlates with the metastatic potential of clonal breast cancer cell lines (Nakajima *et al.*, 1987). Furthermore, activated type IV collagenases are also found in a significant proportion of human breast carcinoma biopsies (Brown *et al.*, 1993) and, interestingly, the 92 000 type IV collagenase and ST-1 are co-expressed in some human tumor epithelial cell lines (Sato *et al.*, 1992). Since type IV collagen, which is also a substrate of ST-1 and ST-2, is a major structural component of the basement membrane, its degradation has been linked to the apparition of the malignant phenotype.

The extracellular matrix regulates the growth and differentiation of many cell types, including normal mammary epithelial cells, *in vivo* and in culture. Normal mammary cells are able to form organized tubular structures when cultured within collagen gels or natural extracellular matrix. In contrast, breast cancer cells and oncogene-transformed mammary epithelial lines cannot express this phenotype (Petersen *et al.*, 1992; D'Souza *et al.*, 1993). Furthermore, the capacity to form complex multilobular structures in culture has been reported recently to be one of the best criteria to discriminate between normal and malignant primary human mammary epithelial cells (Bergstraesser and Weitzman, 1993). We have shown here that T_3 addition causes the disruption of the 3-D tubular structures formed by up to six independent EpH4 clones, the severity of the phenotype being directly related to the expression levels of thyroid hormone receptors. This result demonstrates that T_3 may be involved in morphogenetic events carried out by these mammary epithelial cells. There are several explanations for this effect. One is the induction of secreted proteolytic activity which could be responsible for the degradation or improper formation of the thick basement membrane forming the walls of the tubules. The recent description of ST-1 expression in transgenic mice supports this possibility. Results from Sympson *et al.* (1994) demonstrate a role of ST-1 in morphogenesis. They have found that whereas low ST-1 expression increases morphogenesis and differentiation in virgin animals, high ST-1 expression during lactation and pregnancy causes a decrease in basement membrane components and a loss of its integrity, together with dramatic alterations of alveolar morphology. Still, other mechanisms cannot be ruled out at present, such as the action of TRα-1 on the synthesis or post-translational modification of basement membrane

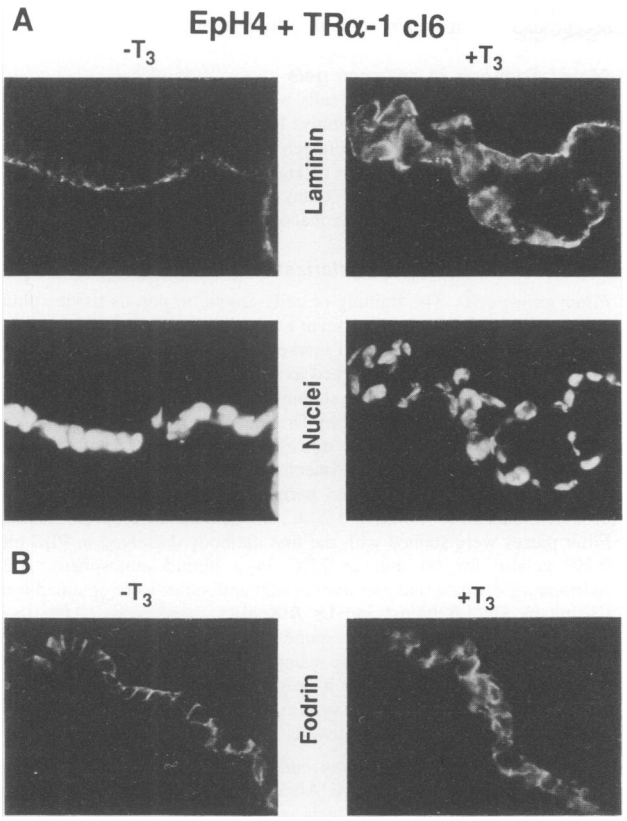

Fig. 10. Effects of T_3 on epithelial marker and basement membrane protein distribution in EpH4+TRα-1 cells grown in collagen gels. EpH4+TRα-1 cells (clone 6) were pregrown in collagen gels for 10 days, further cultivated in the absence or presence of T_3 for 6 days and processed for immunofluorescence analysis using antibodies against laminin (**A**, top) or fodrin (**B**), as described in Materials and methods. The bottom panels of (A) show the 4′6-diamidino-2-phenylindole-stained nuclei of the same fields shown for laminin fluorescence in the top panels.

components, on changes in cell−cell or cell−substrate adhesion, on signal transduction pathways triggered by such adhesion events or on the production of and response to cell motility factors like the hepatocyte growth factor/scatter factor.

Our data also show that the ligand-activated TRα-1 results in distinct alterations of the polarized epithelial phenotype of EpH4 cells expressing exogenous TRα-1. In contrast to other oncogenes inducing a complete loss of epithelial polarity (e.g. c-FosER; Reichmann *et al.*, 1992), TRα-1 caused only a partial redistribution of apically and basolaterally sorted proteins, leading to more diffuse localization but not allowing basolateral proteins to become apically located. It is tempting to speculate that the primary cause for these changes is the inability of the cells to form a proper basement membrane. As discussed in Schoenenberger *et al.* (1991) and Schoenenberger and Matlin (1991), the basal deposition of basement membrane proteins may well be the prerequisite for the epithelial cell to build up epithelial polarity. If this process is affected by an imbalance in the production and/or activity of proteases and their inhibitors, the observed partial inability of the epithelial cell to polarize may well be the result. Recent results suggest that the disruption of epithelial polarity may be one of the first steps leading to more dramatic changes in the epithelial cell phenotype, e.g. epithelial−mesenchymal transition, eventually leading to the loss of expression of epithelial markers and the up-regulation of proteins typical for mesenchymal cells (Reichmann *et al.*, 1992; I.Fialka, E.Reichmann, M.Busslinger and H.Beug, manuscript in preparation). Therefore, the observed effects of the ligand-activated TRα-1 on epithelial polarity may well represent a type of change that could contribute to tumor development.

Since the correlation between proteolytic activity and malignant progression is well established (for a review see Liotta *et al.*, 1991), we can speculate that T_3 and/or its receptors play a role in the process of cellular transformation of mammary epithelia. Similar to the current opinion about estrogen involvement in breast cancer, T_3 could in some circumstances (e.g. when its receptor is overexpressed or mutated) lead to the uncontrolled activation of proteases, and so have a deleterious effect contributing to the process of tumor formation. Strikingly, the number of thyroid receptors in the mammary gland is not only conserved in the transition to neoplasm but is even increased in spontaneous rat mammary tumors (Selliti *et al.*, 1983). Thus, it is conceivable that carcinogenesis may be accompanied by an increased sensitivity to T_3. In view of our results, a re-evaluation of the relationship between thyroid hormone and breast cancer clearly seems to be merited. Most of the studies existing in the literature have been carried out by trying to correlate the circulating hormone levels with the apparition or development of the neoplasia. Instead, we suggest that as is common in the actual clinical practice for the receptors of estrogen, progesterone and several growth factors, the number and also the integrity/functionality of thyroid receptors in breast cancer patients at different stages of tumor progression may be of great significance.

Materials and methods

Cell culture and infection with erbA retroviruses

EpH4 cells were derived by subcloning of the mammary epithelial cell line Ep-1 described previously (Reichmann *et al.*, 1992), a derivative of the parental line IM-2 (Reichmann *et al.*, 1989), and selecting for purely epithelial single-cell colonies. EpH4 cells and cell clones were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1 mM glutamine (all from Gibco BRL). When indicated, serum was omitted and replaced by 100 µg/ml human transferrin and 0.1 mg/ml bovine serum albumin (BSA), fraction V (both from Sigma).

The recombinant retroviruses encoding the chicken TRα-1 and v-*erb*A genes used to infect EpH4 cells have been described already (Muñoz *et al.*, 1990). Both are derived from the Moloney murine leukemia virus and encode the neo^r gene providing resistance to the antibiotic G418. For each infection, 5×10^5 EpH4 cells were seeded in 60 mm dishes on top of a monolayer of mitomycin-C-treated virus-producer Psi-2 fibroblasts. Mitomycin-C (10 µg/ml; Sigma) was added for 2 h and then the cells washed three times with PBS, incubated at 37°C for 30 min, washed again twice with PBS and used for the coculture. Polybrene (8 µg/ml; Sigma) was present in the medium. Selection with 800 µg/ml G418 (Gibco BRL) started 48 h later. Six clones of resistant TRα-1-infected cells could be expanded separately (clones 1–6). In contrast, no individual v-*erb*A-infected clones could be expanded. Therefore, a pool of G418-resistant v-*erb*A-expressing clones was used.

Hormone binding assays

T_3-specific binding assays *in vivo* were performed as described previously (Sap *et al.*, 1986; Muñoz *et al.*, 1990). Specific binding is defined as the ratio of iodinated T_3 bound in the absence and presence of a 1000-fold molar excess of unlabeled T_3.

RNA preparation and Northern analysis

Purification of poly(A)$^+$ RNA was performed as described (Vennström and Bishop, 1982). Northern blots were performed on nylon membranes (Nytran; Schleicher and Schuell) according to standard protocols (Maniatis *et al.*, 1982). All probes were labeled by the random priming method (Feinberg and Vogelstein, 1983). Hybridizations were carried out overnight at 65°C in 7% SDS, 500 mM sodium phosphate buffer, pH 7.2, and 1 mM EDTA according to Church and Gilbert (1984). Filters were washed twice for 30 min each in 1% SDS and 40 mM sodium phosphate buffer, pH 7.2, at 65°C. Before rehybridizing the nylon membranes with probes for other genes, the radioactive probe was stripped off the membrane by placing it in a 75°C water bath for 5 min. The sizes of the respective mRNAs were calculated using RNA ladders as markers (BRL). Membranes were exposed to HyperfilmTM MP films (Amersham).

Rat TRα was obtained from Dr R.M.Evans, rat TRβ from Dr H.C.Towle, rat ST-1/transin and PUMP-1 from Dr R.Breathnach, rat ST-2 from Dr L.M.Matrisian, mouse uPA, MEP and PAI-I from Dr M.Busslinger, human TIMP-1 and tPA from Dr E.Wagner, mouse TIMP-2 from Dr D.R.Edwards, rat glyceraldehyde phosphate dehydrogenase from Dr E.Reichmann and mouse cathepsin D from the American Type Culture Collection.

Run-on assays

Nuclear run-on reactions were performed basically according to Linial *et al.* (1985) using [α-^{32}P]UTP and 2×10^7 nuclei. α-Amanitin (2 μg/ml) was included in one control reaction. Labeled RNA was purified by phenol:chloroform extraction after the addition of three volumes of guanidine isothiocyanate solution (Chomczynski and Sacchi, 1987) and isopropanol precipitation. Unincorporated label was removed by centrifugation through Sephadex G-50 spin columns. The probes were 5 μg of linearized plasmid DNAs immobilized on nylon filters after denaturation in NaOH. Linearized pTZ was also present on the filters to control for background hybridization. Hybridizations were performed in 0.2 M sodium phosphate buffer, pH 7.2, 1 mM EDTA, 7% SDS and 45% formamide containing 250 μg/ml *Escherichia coli* tRNA as a carrier, at 42°C for 3 days. Following hybridization, the filters were washed in 40 mM sodium phosphate buffer, pH 7.2, containing 1% SDS at 37°C.

Preparation of conditioned media

Cells were plated in 150 mm dishes at confluency in 20 ml of growth medium and 24 h later were washed twice with PBS and switched to serum-free medium (15 ml) in either the absence or presence of the indicated T_3 concentration. Cells were allowed to condition the medium for the times indicated in each experiment. Conditioned media were then spun down for 5 min in a refrigerated Sorvall centrifuge at 2500 r.p.m. to remove cell debris and were concentrated 10 times using Centriprep-3 (Amicon).

Proteolytic activity assays

To identify extracellular matrix degradation enzymes secreted by EpH4 cells, gelatin and casein zymograms were performed. Volumes of serum-free conditioned media normalized to protein content (basically the same as if normalized to cell number) were electrophoresed on 10% SDS–polyacrylamide gels containing gelatin (1 mg/ml) from bovine skin (Sigma) or β-casein (0.5 mg/ml) from bovine milk (Sigma). Purified type XI collagenase from *Clostridium histolyticum* (1 μg/ml; Sigma) was loaded as a positive control in the gelatin gels. After electrophoresis, the gels were rinsed twice in 2.5% Triton X-100 for 30 min to remove the SDS and were incubated for 22 h at 37°C in substrate buffer (50 mM Tris–HCl buffer, pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 0.1% Triton X-100, 0.02% NaN_3). The gels were stained in 30% methanol/10% glacial acetic acid containing 0.5% Coomassie brilliant blue and destained in the same solution in the absence of dye. Photographic negatives were used for densitometric scanning using a La Cie scanner connected to a Macintosh IIci computer using Adobe Photoshop^{TM} 2.0 and NIH Image programs.

Transepithelial electrical resistance

The functional integrity of tight junctions was assayed by measuring the electrical resistance towards the ion flux of epithelial cell layers cultured on porous tissue culture inserts (Falcon 3090). Resistance measurements were performed using the Millicell Electrical Resistance System (Millipore Corp.) connected to the electrode system Endohm-24 (World Precision Instruments) according to the manufacturer's specifications. Transepithelial electrical resistances were calculated after subtracting the background contributed by a blank culture insert.

Morphogenesis in collagen gels

In 400 ml of medium, 24 000 cells were mixed with an equal volume of cold type I collagen (Collaborative Research) and poured onto 35 mm tissue culture dishes allowing collagen gel formation for 30 min at 37°C. Medium was then added to the collagen gel and cells were allowed to develop tubular structures for 4–6 days. Subsequently, T_3 (150 nM) was added every 24 h during an additional 6 days and photographs were taken.

Expression of epithelial polarization markers

Filter-grown cells. The staining of cells grown on porous tissue culture inserts (Falcon 3090) by fluorescent antibodies to apical and basolateral markers and the visualization of marker distribution by confocal fluorescence microscopy were performed as described previously (Reichmann *et al.*, 1992). Briefly, DPP IV staining was carried out using a mouse monoclonal antibody (provided by Dr Michel Pierres) on non-fixed cells in serum-free medium for 30 min. Secondary Texas Red-conjugated antibody was obtained from Amersham. For staining with the other antibodies, cells were fixed and permeabilized with a 1:1 mixture of acetone:methanol for 3 min at −20°C, and then air dried before staining. Filter pieces were stained with the first antibody dissolved in PBS plus 0.2% gelatin for 60 min at 23°C in a humid atmosphere. Anti-fodrin anti-β-catenin and anti-uvomorulin antibodies were obtained from Chemicon, Dr J.Behrens, and Dr R.Kemler, respectively. After three washes in PBS–gelatin, the corresponding second antibodies were applied at the appropriate dilutions. The stained cells were mounted in Mowiol (Hoechst) and visualized using a Zeiss IM-35 microscope fitted with a Bio-Rad 600 confocal imaging system. Files were processed and printed using the Adobe Photoshop software.

Staining of frozen sections from collagen gels. Cells were grown in collagen gels as described above. After full development of the expected structures, the whole collagen gels were carefully removed, incubated in 10% dimethyl sulfoxide in growth medium for 5 min and frozen in liquid nitrogen. Frozen sections (7 μm) were obtained using a Zeiss HM 500 OM Cryostat. Fixing, staining and mounting of the stained section was performed as described for the cells on filters, using an anti-laminin antibody (Sigma Immunochemicals) and the anti-fodrin antibody described above. The sections were examined and photographed using a Zeiss Axiophot microscope equipped with epi-illumination.

Acknowledgements

We wish to thank those acknowledged in Materials and methods for generously supplying us with plasmids and probes. We also acknowledge Dr Emilia Mira for her help with the zymography analyses and Gloria Chacón for her excellent technical assistance. M.L.-B. and J.M.G. were supported by fellowships from the Fundación Científica de la Asociación Española contra el Cáncer, M.A. from the Ministerio de Industria, and T.I. from the Gobierno Vasco. This work has been supported with grants from the Fundación Científica de la Asociación Española contra el Cáncer and the European Union (HCM, Laboratory Networks CHRX-CT93-0185).

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Received on May 20, 1994; revised on December 1, 1994