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Inhibition of mink lung epithelial cell proliferation by transforming growth factor-β is coupled through a pertussis-toxin-sensitive substrate

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Transforming growth factor β1 (TGFβ1) inhibits the proliferative response of mink lung epithelial cells (CCL64) to serum and to epidermal growth factor (EGF). This response to TGFβ1 can be inhibited by prior exposure of the cells to nanogram concentrations of pertussis toxin (PT), suggesting the involvement of a guanine-nucleotide-binding regulatory protein (G-protein) in mediating TGFβ1-induced growth inhibition. To characterize further this G-protein dependence, we have isolated, by chemical mutagenesis, a CCL64 variant (CCL64-D1) that is resistant to TGFβ1. Whereas in the parental CCL64 cells TGFβ1 stimulates both GTP[^{35}S] (guanosine 5′-[γ-[^{35}S]thio]triphosphate) binding and GTPase activity, in the CCL64-D1 variants TGFβ1 is without effect. Quantitative immunoblotting with antisera for G-protein α- and β-subunits, as well as PT-catalysed ADP-ribosylation analyses, revealed no appreciable changes in the level of G-protein expression in the CCL64-D1 variants compared with parental cells. In contrast with another TGFβ-resistant clone, MLE-M, which we show lacks detectable type I receptor protein, the CCL64-D1 cells retain all three TGFβ cell-surface binding proteins. On the basis of these studies, we propose that a necessary component of TGFβ1-mediated growth inhibition in CCL64 epithelial cells is the coupling of TGFβ1 receptor binding to G-protein activation.

INTRODUCTION

Transforming growth factor β1 (TGFβ1) is a 25 kDa homodimer prototypic of a family of structurally related polypeptides which regulate cell growth and differentiation [1]. Although mitogenic for mesenchymal-derived cells, TGFβ1 is an inhibitor of the proliferation of cells derived from epithelial, endothelial, haematopoietic, myogenic and neural tissues [2]. The means by which TGFβ1 elicits these cellular responses is at present unclear, although it is fairly well established that the effects of TGFβ1 involve changes in the expression of growth regulatory genes [3,4] and cytoskeletal/matrix-associated genes [5,6]. The intracellular pathways mediating these changes in gene expression remain to be elucidated.

Two polypeptides, termed TGFβ1 and TGFβ2, which exhibit approx. 31% and 71% sequence similarity in their *N*- and *C*-termini respectively have thus far been characterized [7]. Cell-surface binding species for both TGFβ1 and TGFβ2 have been identified on almost all cell types examined [7,8]. These have been termed TGFβ receptor types I, II and III. Types I and II are glycoproteins of 53 kDa and 73–95 kDa respectively, which have a higher affinity for TGFβ1 than for TGFβ2. The type III receptor is a 300 kDa glycoprotein which displays similar affinity for TGFβ1 and TGFβ2. At present it is not known whether one receptor type or all three receptor forms are involved in the mediation of the TGFβ signal, although recent data with epithelial cell variants defective in their growth inhibitory response to TGFβ implicate the type I receptor as the mediator of TGFβ action [8].

Guanine nucleotide-binding regulatory proteins (G-proteins) are a family of heterotrimers composed of α-, β- and γ-subunits that transduce signals between cell-surface receptors and intracellular enzyme effector systems (for review see [9]). Distinct G-proteins, termed G_s (stimulatory) and G_i (inhibitory), modulate the activity of adenylate cyclase. The activity of light-activated cyclic GMP phosphodiesterase, involved in visual excitation in the retina, is controlled by a specific G-protein termed transducin (G_t). In addition, the activity of phospholipase C [10], phospholipase A_2 [11], the neuronal Ca^{2+} channel [12] and the atrial K^+ channel [13] are also postulated to be regulated by G-proteins. The ability to inhibit these effector systems with various bacterial exotoxins is not only evidence for G-protein involvement, but also facilitates identification of these proteins. Pertussis toxin (PT) ADP-ribosylates G_i, the protein which inhibits adenylate cyclase, whereas cholera toxin catalyses the ADP-ribosylation of G_s, the protein which activates adenylate cyclase. G_t possesses both PT and cholera toxin ADP-ribosylation sites [9].

Recently, the mitogenic signals of polypeptide growth factors such as bombesin [14], insulin [15], tumour necrosis factor [16] and colony-stimulating factor 1 [17] have been postulated to be coupled through G-proteins. Similarly, recent studies from this laboratory and others have demonstrated that the stimulatory effects of TGFβ1 in AKR-2B fibroblasts are coupled through a PT-

Abbreviations used: TGFβ1, transforming growth factor β1; G-protein, guanine-nucleotide-binding regulatory protein; PT, pertussis toxin; FBS, fetal bovine serum; EMS, methanesulphonic acid ethyl ester; GTP[^{35}S], guanosine 5′-[γ-[^{35}S]thio]triphosphate.

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sensitive G-protein and that addition of TGFβ1 to membrane preparations from these cells stimulates binding of the GTP analogue guanosine 5'-[γ-thio]-triphosphate (GTP[S]) [18] and GTPase activity [18,19]. In this paper, we characterize two mink lung epithelial cell variants which are insensitive to TGFβ1-mediated growth inhibition; one clone has lost detectable expression of a functional type I TGFβ receptor, whereas the other retains all three TGFβ binding species. However, both clones are defective in TGFβ1–G-protein coupling, a property necessary for TGFβ1-mediated growth inhibition.

MATERIALS AND METHODS

Cell culture

Mink lung epithelial cells (American Type Culture Collection CCL64), as well as the variant stocks CCL64-D1 and MLE-M, were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS) and allowed to grow at 37 °C in a humidified atmosphere of air/CO_2 (19:1, v/v).

Selection of TGFβ1-resistant variants

CCL64 cells were plated at 1×10^6 cells/T-75 flask and allowed to attach overnight. Methanesulphonic acid ethyl ester (EMS) was added to a final concentration of 4 mM and the cultures were grown for a further 24 h. This concentration of EMS has been shown to result in a minimum of 50% cell death with CCL64 cells (results not shown). The flasks were washed free of EMS and grown in McCoy's 5A medium with 10% FBS for 24 h to allow recovery from the mutagen and the expression of mutations. TGFβ1-resistant cells were selected by addition of 12 pM-TGFβ1. The cultures were cloned by limiting dilution in 96-well microtitre plates containing 10% FBS-supplemented McCoy's and 40 pM-TGFβ1.

DNA synthesis measurements

Inhibition of DNA synthesis of TGFβ1 in CCL64 cells and variants was evaluated by [^{3}H]thymidine incorporation into trichloroacetic acid-insoluble material. Proliferating cultures were assayed 2 days after initial plating at 2×10^4 cells/cm^2, and quiescent density-arrested cells were used 5–7 days after plating. Cells were pulsed with 1 μCi of [^{3}H]thymidine/ml at the indicated times for 1–2 h at 37 °C and then processed for scintillation counting.

Affinity cross-linking of ^{125}I-labelled TGFβ1

Chemical cross-linking of ^{125}I-labelled TGFβ1 to density-arrested quiescent cultures of the various cell lines was performed as described previously [8]. Briefly, cultures in 100 cm^2 dishes were labelled with 80 pM-^{125}I-TGFβ1 (kindly provided by Dr. Harold Moses, Vanderbilt University) in the presence or absence of a 100-fold concentration of competing TGFβ1 for 4 h at 4 °C. The cells were then washed extensively to remove unbound label and cross-linked using 200 μM-disuccinimidyl suberate (DSS; Pierce Chemical Co.). The cells were solubilized in a Triton X-100-containing buffer (1% Triton X-100, 125 mM-NaCl, 1 mM-EDTA, 10 mM-Tris, pH 7.0, and proteinase inhibitors) and analysed by gel electrophoresis and autoradiography on 6–8% gradient Laemmli gels [20].

Preparation of membranes and ADP-ribosylation

Membranes from quiescent cultures of the various cell lines were prepared and subjected to ADP-ribosylation with [α-^{32}P]NAD$^+$ (800 Ci/mmol; DuPont–NEN) as described previously [18], and protein concentration was determined with a dye-binding assay (Bio-Rad) after solubilization for 15 min with 0.1 M-NaOH. Membranes were used immediately or snap-frozen in liquid N_2 and stored at −70 °C.

Immunoblotting

Membranes from the various cell lines (50 μg) were electrophoresed on 12.5% polyacrylamide gels. Proteins were then transferred on to nitrocellulose and processed for immunoblotting as described previously [21] with antisera against consensus sequences of α (J-881; a serum obtained from a different rabbit but with the same antigen as A-569) and β (U-49) G-protein subunits kindly provided by Drs. S. M. Mumby and A. G. Gilman (University of Texas Health Science Center, Dallas, TX, U.S.A.).

Assay of GTP[^{35}S] binding in membranes

Binding reactions were carried out at 37 °C for 15 min in a final volume of 100 μl as described previously [18]. The binding buffer consisted of 50 mM-Tris (pH 7.4), 2 mM-$MgCl_2$, 1 mM-EGTA, 100 μg of BSA/ml, 5 mM-dithiothreitol, 10 nM-GTP[^{35}S] (1400 Ci/mmol; DuPont–NEN) and 200 pM-TGFβ1. Reactions were initiated by the addition of 1 μg of various cell membrane preparations and terminated by the addition of 10 vol. of ice-cold binding buffer, followed by centrifugation at 10000 *g* for 1 min. The pellets were washed rapidly three times with ice-cold binding buffer by repeated centrifugation and then resuspended in liquid scintillation fluid for determination of bound radioactivity. All assays were carried out in triplicate, along with triplicate control samples containing a 1000-fold concn. of unlabelled GTP for determination of non-specific binding. Non-specific binding was consistently < 10% of total binding.

GTPase activity in membranes

Reactions were performed as described above for GTP[^{35}S] binding except that the incubation mixture contained 2.5 mM-ATP, and 0.02 μM-[γ-^{32}P]GTP (10–30 Ci/mmol; Amersham) replaced GTP[^{35}S]. Incubations were initiated by the addition of 1 μg of the various cell membrane preparations, carried out at 37 °C for 15 min and stopped by the addition of 0.5 ml of activated charcoal (Norit A) in 20 mM-Na_2HPO_4 buffer, pH 7.4. After centrifugation at 10000 *g* for 10 min, the supernatant was counted for $^{32}PO_4^{3-}$ as described previously [22]. GTPase activity was expressed as pmol of GTP hydrolysed per mg of protein.

RESULTS

Effect of TGFβ1 on mink lung epithelial cell growth

The inhibition of the pseudo-diploid mink lung epithelial cell line (CCL64) by picomolar concentrations of TGFβ1 has been reported previously by several laboratories [8,23,24]. Previous studies have described the isolation of TGFβ1-resistant clones from this cell line [8,24], and have categorized these variants into two general classes; those that have lost a functional type I

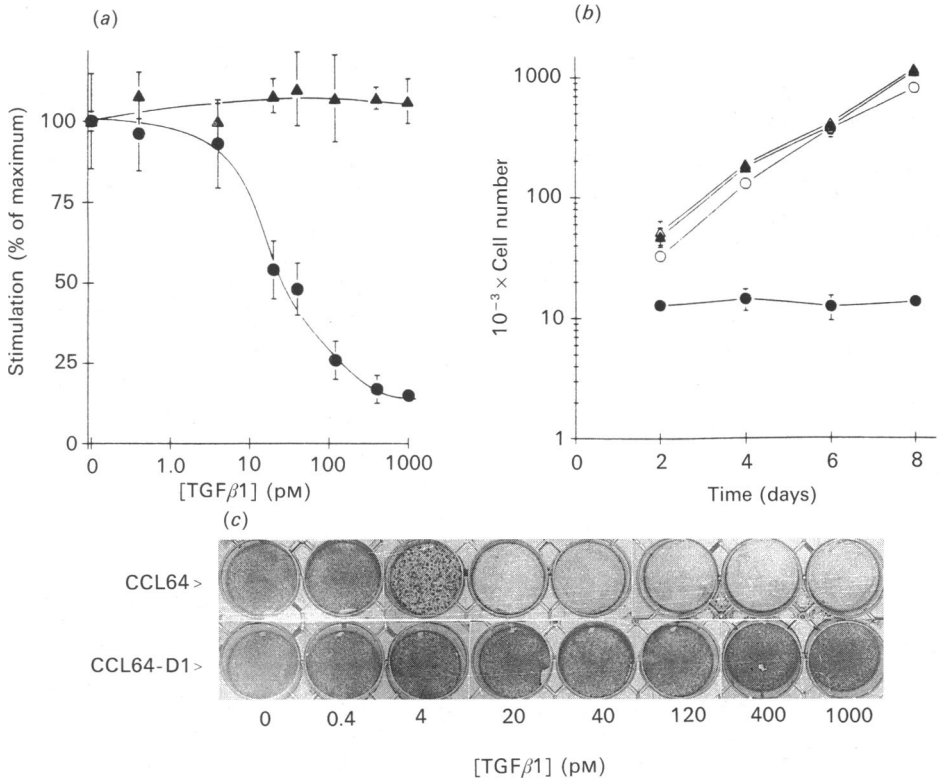

Fig. 1. Effect of TGFβ1 on CCL64 and CCL64-D1 cell growth

(*a*) CCL64 (●) or CCL64-D1 (▲) cells were plated at 2×10^4 cells/cm² in McCoy's 5A medium containing 10% FBS. After 5 days growth at 37 °C, fresh McCoy's containing 10% FBS + 3.3 nM-epidermal growth factor was added in the presence of the indicated concentrations of TGFβ1. The cells were pulsed for 1 h with 1 μCi of [³H]thymidine/ml after 22 h of stimulation. The absence of TGFβ1 resulted in 5.5-fold and 7.0-fold stimulation of CCL64 and CCL64-D1 cells respectively. (*b*) CCL64 (○, ●) or CCL64-D1 (△, ▲) cells were plated at 5×10^3 cells/cm² in McCoy's 5A medium containing 10% FCS in the presence (●, ▲) or absence (○, △) of 80 pM-TGFβ1. Cell counts were performed on the indicated days from duplicate wells and the cultures were replenished with fresh medium containing 10% FBS ± 80 pM-TGFβ1 on day 4. (*c*) CCL64 (1×10^3 cells/cm²) and CCL64-D1 (2.6 ± 10^3 cells/cm²) cells were plated at 37 °C in McCoy's 5A medium supplemented with 10% FBS and the indicated concentrations of TGFβ1. Fresh medium containing FBS and TGFβ1 was added on day 4 and the cells were fixed with 100% methanol and stained with hematoxylin on day 7. Results in (*a*) and (*b*) are the means ± S.E.M. of two experiments conducted in triplicate.

TGFβ1 receptor, termed R mutants, and those that retain all three TGFβ receptor types but are not inhibited by TGFβ, presumably due to a defect in the TGFβ signal-transducing mechanism, termed S mutants [8]. In the present studies we have also subjected CCL64 cells to chemical mutagenesis with EMS. From 2×10^7 cells we isolated 12 clones in the presence of TGFβ1. Of these, two regained their sensitivity to TGFβ1 with time in culture and failed to grow, eight demonstrated a partial resistance to TGFβ1 and two remained resistant to TGFβ1 for several months in culture propagated in the absence of TGFβ1. Characterization of one of these, CCL64-D1, is described in the following experiments.

As shown in Fig. 1, clone CCL64-D1 is resistant to TGFβ1-mediated growth inhibition by a variety of criteria. Restimulation of quiescent cultures in the presence of TGFβ1 had little or no effect on [³H]thymidine incorporation into DNA in the CCL64-D1 clone, whereas in the parental CCL64 cells growth inhibition was observed at approx. 5–10 pM-TGFβ1 (Fig. 1*a*). TGFβ1 inhibited significantly the logarithmic growth of sparse parental cells but was without inhibitory effect on the variant TGFβ1-D1 cell line (Fig. 1*b*). Furthermore, the clonagenic growth of the two cell lines revealed that a concentration of 20 pM-TGFβ1 almost completely inhibited the parental cell line, whereas concentrations as high as 1 nM-TGFβ1 had no detectable effect on the CCL64-D1 variant (Fig. 1*c*). Having isolated a stable, non-revertant, variant which is resistant to TGFβ1, we next wished to determine whether this mutation was due to a loss of a TGFβ1 receptor (R mutant) or due to an impairment in the TGFβ1 transducing pathway (S mutant).

Analysis of TGFβ1 binding species by chemical cross-linking

To determine the mechanisms by which CCL64-D1 cells are resistant to TGFβ1, we first investigated whether there was any detectable alteration in the TGFβ1 binding species. Affinity cross-linking of ^{125}I-TGFβ1 via disuccinmidyl suberate was performed with CCL64 cells, CCL64-D1 cells and MLE-M clone 4 cells (MLE-M clone 4 cells are another TGFβ1-resistant CCL64 cell line isolated previously and kindly provided by Mike Chinkers, Vanderbilt University [24]). Affinity cross-linking of ^{125}I-TGFβ1 to CCL64 cells has been demon-

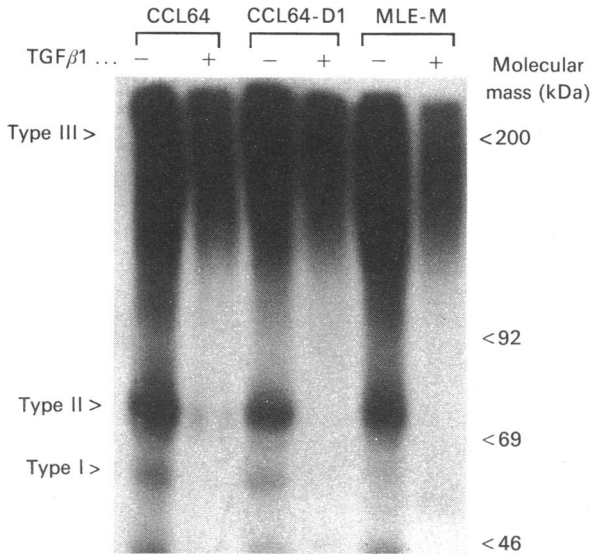

Fig. 2. Affinity labelling of ^{125}I-TGFβ1 to cell-surface receptors

Confluent cultures of CCL64, CCL64-D1 or MLE-M cells were treated with 80 pM-^{125}I-TGFβ1 in the presence (+) or absence (−) of unlabelled TGFβ1 for 4 h at 4 °C, cross-linked with 200 μM-disuccinimidyl suberate and analysed by polyacrylamide-gel electrophoresis as described in the Materials and methods section.

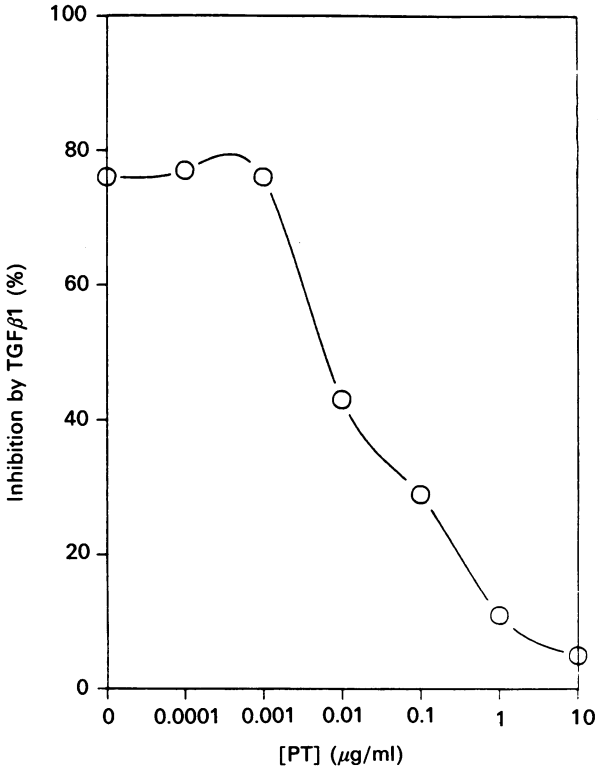

Fig. 3. Effect of PT on TGBβ1-induced growth inhibition

Quiescent CCL64 cells were prepared as described in the legend to Fig. 1(*a*). The spent serum-containing medium was removed after 5 days growth and replaced with McCoy's 5A medium containing 10 μg of BSA/ml and the indicated concentrations of PT for 3 h at 37 °C. Concentrated stocks of growth factors were added to obtain final concentrations of 10% FBS, 3.3 nM-epidermal growth factor and 40 pM-TGFβ1. After incubation for a further 22 h at 37 °C, the cells were pulsed for 2 h with 1 μCi of [^{3}H]thymidine/ml, and the trichloroacetic acid-precipitable material was determined as described in the Materials and methods section.

strated previously to result primarily in the labelling of three TGFβ binding species of approx. 53, 73 and 300 kDa (Fig. 2 and [7,8]). The CCL64-D1 clone revealed a similar TGFβ1 labelling pattern as that of the parental cells, displaying all three TGFβ1 binding species. However, the MLE-M variant yielded a labelling pattern in which the 53 kDa type I receptor was absent or diminished as compared with the parental cell line, although both the 73 and 300 kDa TGFβ receptors were present. Although it has previously been shown by saturation binding studies that ^{125}I-TGFβ1 binding in the MLE-M cells was not significantly different from that in parental CCL64 cells, analysis of the individual binding species in these cells was not determined [8].

Effects of PT on TGFβ1-induced growth inhibition

We next determined whether aspects of TGFβ1 signal transduction might be altered in the CCL64-D1 variants. Whereas the effector system(s) coupled to the TGFβ1 receptor(s) is not known, earlier work from this laboratory and others [18,19] has implicated the involvement of a PT-sensitive G-protein in stimulation of AKR-2B fibroblasts by TGFβ1. If TGFβ1-mediated growth inhibition were similarly coupled, alteration in this coupling by PT should then abolish growth inhibition caused by TGFβ1. Fig. 3 shows that a 3 h pretreatment of quiescent CCL64 cells with PT before restimulation of the cultures in the presence of TGFβ1 abrogates TGFβ1-induced growth inhibition. This inhibition of TGFβ1 action was half-maximal at a PT concentration of 0.01 μg/ml and essentially complete at 1 μg/ml. Although this experimental design cannot confirm directly the association of a PT-sensitive G-protein with TGFβ1-mediated growth inhibition, the results are consistent with the primary mechanism of PT action, namely the prevention of G-protein–receptor coupling [25,26].

Analysis of TGFβ1-stimulated GTP[^{35}S] binding and GTPase activity

The results shown in Fig. 3 demonstrate that the activity of a PT-sensitive substrate is required for TGFβ1-mediated growth inhibition. These data suggest that one mechanism by which epithelial cultures might become resistant to growth inhibition (Fig. 1) is through an interruption in TGFβ1 receptor–G-protein coupling. To this end, we next investigated whether there were any differences in TGFβ1-stimulated GTP[^{35}S] binding in membrane preparations from CCL64, CCL64-D1 and MLE-M cells. An increased binding of GTP following receptor activation is an established test for demonstrating G-protein involvement in receptor function which we have used previously with TGFβ1 in AKR-2B fibroblast membranes [18]. As shown in Table 1, both the CCL64-D1 and MLE-M variants were unable to increase GTP[^{35}S] binding on addition of TGFβ1. However, in the parental CCL64 cells, addition of TGFβ1 resulted in an approx. 41% stimulation of GTP[^{35}S] binding.

Activation of G-proteins results not only in increased binding of GTP, but also in increased GTP hydrolysis by

Table 1. GTP[^{35}S]binding and GTPase activity in CCL64, CCL64-D1 and MLE-M cell membranes

Membranes from CCL64, CCL64-D1 and MLE-M cells were incubated in the presence or absence of 200 pM-TGFβ1, and GTP[^{35}S] binding and GTPase activity were measured as described in the Materials and methods section. Values represent the means ± S.E.M. of two separate experiments conducted in triplicate. The control value for each membrane type is listed as 100 %.

Cell type	Addition of 200 pM-TGFβ1	GTP[^{35}S] binding (pmol/mg of protein)	GTP[^{35}S] binding (% of control)	GTPase activity (pmol of P_i hydrolysed/min per mg of protein)	GTPase activity (% of control)
CCL64	–	6.64±0.42	100	8.97±1.3	100
	+	9.38±0.77	141	13.80±1.4	155
CCL64-D1	–	5.31±0.41	100	8.44±1.0	100
	+	5.15±0.86	97	8.06±0.8	96
MLE-M	–	4.23±0.34	100	5.65±0.4	100
	+	4.38±0.72	103	6.19±0.5	109

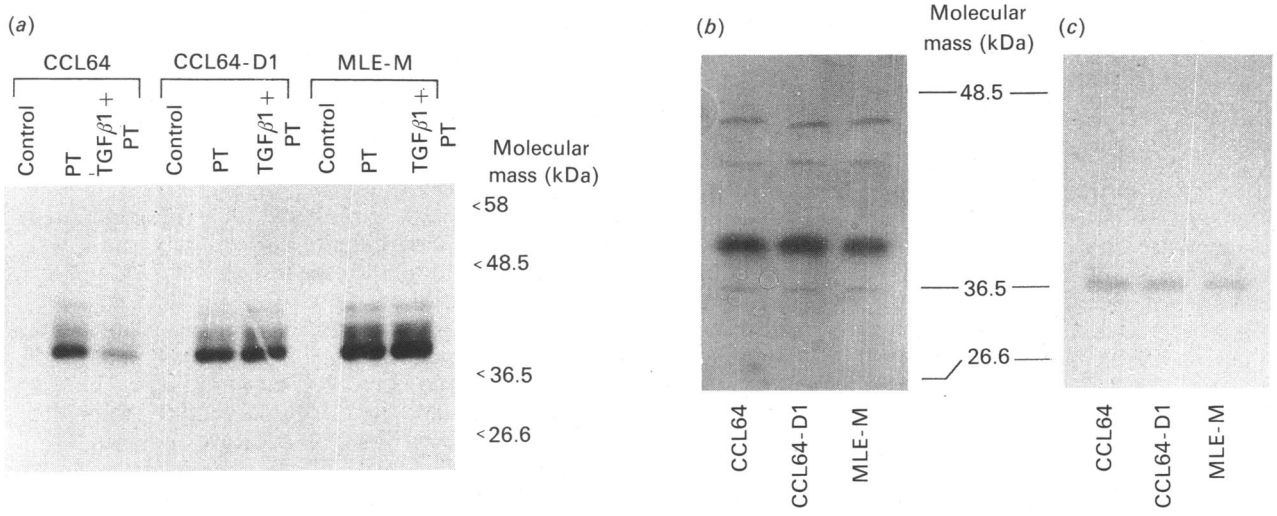

Fig. 4. Analysis of G-proteins

(*a*) Autoradiographic analysis of PT-treated membranes. Membranes (50 μg) from CCL64, CCL64-D1 and MLE-M cells were ADP-ribosylated *in vitro* by activated PT for 30 min at 37 °C before processing for 10–15 % gradient polyacrylamide-gel electrophoresis. TGFβ1-treated membranes from all three cell types were incubated with 200 pM-TGFβ1 at 37 °C for 15 min before ADP-ribosylation *in vitro* with [α-^{32}P]NAD$^+$. (*b*), (*c*) Immunoblotting analysis of α-subunit (*b*) and β-subunit (*c*) of G-proteins. Membranes (50 μg) from the various cell lines were subjected to SDS/polyacrylamide-gel electrophoresis and immunoblotting using antisera to consensus α- or β-subunits as described in the Materials and methods section.

a specific GTPase activity [25]. GTP hydrolysis was also studied in the various cell types by monitoring the release of $^{32}PO_4^{3-}$ from [γ-^{32}P]GTP. Table 1 demonstrates that basal GTPase activity in the CCL64 cells was stimulated by approx. 55 % after addition of TGFβ1, but that the variants failed to show any significant TGFβ1-induced GTPase activity. Thus resistance to TGFβ1-induced growth inhibition is coupled to an inability of TGFβ1 to stimulate either GTP[^{35}S] binding or GTPase activity.

Analysis of G-proteins

Treatment of cell membranes with activated PT in the presence of [α-^{32}P]NAD$^+$ has been shown to result in the ADP-ribosylation of proteins of approx. molecular mass 41 kDa and 39 kDa which are postulated to be the α-subunits of G_i and G_t or G_o respectively [9]. Similarly, ADP-ribosylation of membranes prepared from CCL64, CCL64-D1 and MLE-M cells also resulted in the ADP-ribosylation of proteins migrating with similar molecular masses (Fig. 4*a*). Whereas the identity of the bands in Fig. 4(*a*) is presently unknown, the major ribosylated species has an approximate molecular mass of 39 kDa. ADP-ribosylation of the α-subunit is thought to occur primarily when the G-protein is in its 'inactive' (GDP-bound) heterotrimer complex with $\beta\gamma$, and not when the monomeric α-subunit is liberated as following receptor activation [26]. Consistent with these findings are the data presented in Figure 4(*a*). In this experiment, parental CCL64 cell membranes were incubated in the presence of 200 pM-TGFβ1 for 15 min before PT-induced ADP-ribosylation. Under these conditions there was an approx. 42 % decrease in the labelling of the 39 kDa protein as compared with the PT-treated membranes alone (Fig. 4). This decrease in labelling could represent the amount of

α-subunit released following TGFβ1 binding, which is then no longer available as a substrate for PT-induced ADP-ribosylation. In addition, ribosylation of the two minor bands at 41–43 kDa was also decreased by prior TGFβ1 treatment. Analysis of the ADP-ribosylation patterns in membrane preparations from the two TGFβ1-resistant variants also revealed the presence of both the 39 kDa and the 41–43 kDa substrates, as in the parental cells. The amount of [^{32}P]ADP-ribose incorporated per 50 μg of each membrane type was equivalent to that in the parental cells; however, the labelling of each substrate was not diminished by prior treatment of the membranes with TGFβ1. These data suggest that, unlike the parental cells, the two variants are unable to couple TGFβ1 binding to G-protein activation.

PT-catalysed radiolabelling suggested that the abundance of G-protein α-subunits present in the two variant cell types was not significantly different from that in the parental cells. This prediction was verified by quantitative immunoblotting using antisera specific for either the consensus α-subunit or the consensus β-subunit (Figs. 4*b* and *c*; [21]). The consensus α-subunit antiserum recognized several peptides with molecular masses ranging from approx. 45 kDa to 37 kDa, consistent with the molecular mass of known G-protein α-subunits; however, there were no detectable differences in the labelling of these bands in the different cell lines. Consistent with the ribosylation pattern observed in Fig. 4(*a*), the major band recognized by this antiserum had an approximate molecular mass of 39 kDa. The consensus β-subunit antiserum was specific for a 36 kDa peptide, consistent with the molecular mass of G-protein β-subunits; however, as with the α-subunit antiserum, no significant differences were observed in the labelling patterns between the parental and variant cell types.

DISCUSSION

Two general classes of stable TGFβ1-resistant CCL64 variants have been reported previously [8]: those that have lost the type I TGFβ receptor (R mutants), and those that still possess these receptors but are resistant to TGFβ, presumably due to an impairment in the TGFβ transducing pathway, termed S mutants. We describe here the characterization of two such CCL64 epithelial cell variants; one is an R type mutant (MLE-M) lacking detectable type I TGFβ1 receptors (53 kDa), and the other is an S type mutant (CCL64-D1) which we show is associated with the loss of functional TGFβ1 receptor–G-protein coupling.

The MLE-M variant has been described previously as a TGFβ-resistant cell line [24]. It was concluded from saturation binding studies with ^{125}I-TGFβ1 that these cells still retained a functional TGFβ receptor [24]. However, analysis of individual TGFβ receptor types by affinity cross-linking of ^{125}I-TGFβ1 via disuccinimidyl suberate shows that the MLE-M cells, unlike both the parental CCL64 and the mutant CCL64-D1 cells, appear to be lacking or have greatly reduced levels of the 53 kDa type I receptors (Fig. 2). The frequent and selective loss of this receptor type in a variety of mutagenized cells has been interpreted as evidence that this receptor type is in fact the mediator of TGFβ action [8]. Our data support this general hypothesis. It is possible that the mutation in the MLE-M cells results not in the loss of the receptor, but in an increase in the K_d of the receptor. The affinity-labelling experiments shown in Fig. 2 cannot distinguish between these two possibilities, since they were performed with a fixed concentration of ^{125}I-TGFβ1 (80 pM). However, both the CCL64-D1 cells and the MLE-M cells were resistant to concentrations of TGFβ1 as high as 1 nM (Fig. 1 and results not shown), suggesting that an alteration in the K_d of the receptor is not responsible for the loss of TGFβ1-mediated growth inhibition.

It has been proposed that, since the class R mutants and the class S mutants are remarkably similar in their proliferative, biochemical and morphological responses to TGFβ, the defect in the S class might be an early event in the type I TGFβ receptor signalling pathway [8]. We have demonstrated previously that stimulation of AKR-2B fibroblasts by TGFβ1 is coupled through a PT-sensitive G-protein; both nucleotide and growth factor specificity were shown [18]. If TGFβ1-mediated growth inhibition was similarly regulated, treatment of cultures with PT to prevent TGFβ1 receptor–G-protein coupling should abolish the growth inhibitory response initiated by TGFβ1. In accordance with this hypothesis are the data of Fig 3, which indicate that a PT-sensitive substrate(s) is necessary for inhibition of growth by TGFβ1. Pretreatment of quiescent CCL64 cells with PT before restimulation with FBS plus epidermal growth factor in the presence of TGFβ1 essentially abolished the TGFβ1-mediated growth inhibition. These results suggest that a lack of G-protein expression, or expression of a non-functional TGFβ1-coupling G-protein, would be a logical target to account for the resistance to TGFβ1-induced growth inhibition in the CCL64-D1 culture. To explore further this possibility, the experiments reported in Table 1 were performed. Both the MLE-M cells and the CCL64-D1 cells showed decreased basal GTP[^{35}S] binding and basal GTPase activity as compared with the parental cell type. The reason for this decrease in basal G-protein activity in the two variant cell lines is unclear, but could simply reflect clonal variations. More importantly, however, the data demonstrate that TGFβ1 stimulated both an increased binding of GTP[^{35}S] and an increased GTPase activity in the parental CCL64 cells, but was without effect in the variants. These results are not totally unexpected for the MLE-M cells, since these lack detectable type I receptors and would therefore be unlikely to couple to the transducing protein. These data do suggest, however, that TGFβ1–G-protein coupling is via the 53 kDa receptor, and further support the role of the type I receptor as the mediator of the TGFβ signal [8,27]. Although many possibilities might be envisioned to account for the resistance of the CCL64-D1 cells to TGFβ1, the data in Table 1 and Fig. 4(*a*) suggest that an initial lesion in the response to TGFβ1 might be an inability to activate the appropriate G-protein(s) after TGFβ1 binding. Alternatively, the lesion might be within the TGFβ1 receptor itself, which may be capable of ligand binding but be unable to couple to its appropriate transducer; however, more distal mutations in TGFβ1 signalling cannot be excluded.

PT catalyses the ADP-ribosylation of the α-subunits of G_i, the inhibitory regulator of adenylate cyclase, G_o, an abundant protein of unknown function in the brain, and transducin (G_t), the G-protein that mediates photo-transduction in retinal rod cells through cyclic GMP phosphodiesterase [9]. At present, neither the specific PT-sensitive G-protein nor the specific effector enzyme system(s) to which TGFβ1 is coupled is known. We

show in Fig. 4(*a*) that in epithelial cell membranes PT catalyses the ADP-ribosylation of proteins of approx. 39 kDa and 41–43 kDa, and that prior treatment of parental CCL64 cell membranes with TGFβ1 results in decreased labelling of each of these proteins. Although it is at present unclear whether each of these PT-sensitive substrates is coupled directly to TGFβ1 binding, these results are consistent with the hypothesis that, in the parental cells, TGFβ1 receptor occupation results in the activation and disassociation of the G-protein(s) heterotrimer into its respective subunits, thereby blocking subsequent ADP-ribosylation. However, in the variant cell types, TGFβ1-induced G-protein activation is impaired and the decrease in ADP-ribosylation is not observed (Fig. 4*a*).

We have also used PT-catalysed ADP-ribosylation to quantify the relative abundance of the α-subunit substrates in the various cell types. The results (Fig. 4*a*) demonstrate that there are no detectable differences in the amount of PT substrates found in the parental CCL64 cells as compared with the two variant cell lines. Also, by quantitative immunoblotting using antisera specific to consensus sequences found in α- or β-subunits, it was shown that the relative amounts of these subunits did not vary among the cell types (Figs. 4*b* and *c*). The major species detected by the consensus α-subunit antiserum had a similar molecular mass (approx. 39 kDa) to that of the primary PT-ribosylated protein seen in Fig. 4(*a*). Whether these proteins are identical or represent previously described G-protein α-subunits is unknown at present. These data suggest that the molecular lesion responsible for the CCL64-D1 phenotype might be due to one of at least two possibilities: a deletion of a particular G-protein linked to the TGFβ receptor undetectable by the immunoblotting analysis in Fig. 4, or an inability of this G-protein to functionally transduce the TGFβ1 signal. Similar defects are perhaps best exemplified by the S49 lymphoma mutant cell lines cyc- and unc respectively [28]. The isolation of TGFβ1-resistant epithelial cell clones as reported here should provide useful tools for studying the mechanism of TGFβ1 action. Use of these variants as recipients of genomic DNA transfection to complement such lesions will allow reconstitution of a TGFβ1-sensitive cell line, and aid in the isolation and identification of genes whose products are important in TGFβ-induced signal transduction and growth inhibition.

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