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Transforming growth factor β1 treatment of AKR-2B cells is coupled through a pertussis-toxin-sensitive G-protein(s)

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Transforming growth factor β (TGFβ1) is a potent regulator of DNA synthesis and cellular proliferation. In this study, we investigated whether the growth stimulatory signal of TGFβ1 is transduced intracellularly by guanine nucleotide regulatory proteins (G-proteins). In plasma membranes from AKR-2B cells, TGFβ1 increased binding of the radiolabelled, non-hydrolysable GTP analogue, guanosine 5'-[γ-[^{35}S]thio]triphosphate (GTP[^{35}S]), in a dose-dependent manner. Maximal effects occurred between 0.4 and 1.0 nM-TGFβ1. Specific binding of GTP[^{35}S] occurred with a K_d of 3.2×10^{-8} M which was not affected by addition of TGFβ1. Instead, TGFβ1 increased the number of available binding sites for GTP[^{35}S] from 16.2 ± 1.2 to 21.6 ± 2.1 pmol/mg of protein. GTP[^{35}S] binding was both nucleotide- and growth-factor-specific. Only guanine nucleotides were able to compete for binding, and of the growth factors tested (epidermal growth factor, platelet-derived growth factor, insulin, TGFβ1 and TGFβ2) only TGFβ1 affected GTP[^{35}S] binding. TGFβ1 increased GTPase activity, as determined by the release of $^{32}PO_4^{3-}$ from GTPγ[^{32}P], from 116 ± 5.5 to 175 ± 4.3 pmol/mg of protein following a 15 min incubation. Pretreatment of the membranes with pertussis toxin inhibited both TGFβ1-stimulated binding of GTP[^{35}S] as well as TGFβ1-stimulated GTPase activity. These inhibitory actions of pertussis toxin were associated with toxin-induced ADP-ribosylation of a 41 kDa protein. Furthermore, the stimulatory effects of TGFβ1 on c-*sis* mRNA expression were shown to be pertussis-toxin sensitive and could be mimicked by direct activation of G-proteins with AlF_4^-. These results demonstrate that in AKR-2B cells a pertussis-toxin-sensitive guanine nucleotide regulatory protein(s) is coupled to TGFβ1 receptor binding.

INTRODUCTION

Transforming growth factor β (TGFβ) is a 25 kDa homodimeric polypeptide produced by a variety of normal and transformed cells *in vitro* and *in vivo* [1]. While growth-inhibitory for most cell types (i.e. non-mesenchymal), it is capable of stimulating the monolayer growth of mesenchymal-derived cells as well as the anchorage-independent growth of cells that are normally anchorage-dependent [2]. The means by which TGFβ1 exerts these cellular responses is at present unclear, although it is fairly well established that one of the earliest effects of TGFβ1 involves the regulation of gene expression. TGFβ1 stimulation of target cells results in the increased expression of cytoskeletal/matrix-associated genes such as collagen [3,4], fibronectin [3], integrin [4] and actin [5], as well as various proto-oncogenes such as c-*sis*, c-*myc*, and c-*fos* [6,7]. There are three known forms of TGFβ, termed TGFβ1, β2 and β3. These are structurally and biologically similar and exhibit approx. 31 and 71% identity in their *N*- and *C*-termini, respectively [8,9]. Both TGFβ1 and β2 bind to three distinct receptor types with varying affinities [8]; however, the transducing pathways to which these receptors are coupled remain unknown.

The coupling between some membrane receptors and activation of intracellular effector enzymes has been shown to involve guanine nucleotide regulatory G-proteins [10]. Distinct G-proteins, termed G_s (stimulatory) and G_i (inhibitory), modulate the activity of adenylyl cyclase. The activity of light-activated cGMP phosphodiesterase, involved in visual excitation in the retina, is controlled by a specific G-protein, termed transducin or G_t [10]. In addition, the activity of phospholipase C [11], phospholipase A_2 [12], the neuronal Ca^{2+} channel [13] and the atrial K^+ channel [14] are also thought to be regulated by G-proteins. The ability to inhibit these effector systems with specific bacterial toxins is not only evidence of G-protein involvement but also facilitates identification of these proteins.

Pertussis toxin has been shown to disrupt receptor–G-protein interactions by catalysing the ADP-ribosylation of the α-subunit of several G-proteins. It is therefore a unique probe to study mechanisms involved in receptor-mediated signal transduction [15]. Recently, it has been demonstrated that pertussis-toxin-sensitive G-proteins might be involved in growth-factor-mediated cell proliferation in several fibroblastic cell lines [16,17]. In addition, TGFβ1 signal transduction has recently been postulated to be coupled through a G-protein [18,19]. For instance, both decreased membrane binding of ^{125}I-TGFβ in the presence of guanosine 5'-[βγ-imido]-triphosphate (Gpp[NH]p) and pertussis-toxin-sensitive GTPase activity stimulated by TGFβ have been reported [19]. Furthermore, previous studies from this laboratory have shown that stimulation of fibroblast cells in culture

Abbreviations used: TGFβ, transforming growth factor (types β1, β2, and β3); G-protein, guanine nucleotide regulatory protein; GTP[^{35}S], guanosine 5'-[γ-[^{35}S]thio]triphosphate; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; *1B15*, gene encoding cyclophilin (cyclosporin A binding protein).

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with TGFβ1 or transfection of the same with activated *ras* genes induces both anchorage-independent growth and morphological transformation [20]. Because the *ras* gene product, p21, has been shown to exhibit properties similar to other G-proteins [21], and because TGFβ1 produces similar phenotypic effects as do transfected *ras* genes [20], in the present study we further examined the role of G-proteins in transducing the TGFβ1 signal. To determine whether TGFβ receptors are coupled to G-protein(s), we investigated the effect of TGFβ1 on guanosine 5′-[γ-[^{35}S]thio]triphosphate (GTP[^{35}S]) binding and GTPase activity in AKR-2B fibroblast plasma membranes. In addition, the effects of pertussis toxin and AlF_4^- were studied to determine whether these substances inhibit, stimulate or mimic TGFβ1-induced responses.

MATERIALS AND METHODS

Chemicals

TGFβ1 and TGFβ2 were purchased from R & D Systems, Inc. (Minneapolis, MN, U.S.A.). Purified platelet-derived growth factor (PDGF, B chain) was a gift from W. J. Pledger. Mouse receptor grade epidermal growth factor (EGF) was obtained from Collaborative Research, Inc. (Bedford, MA, U.S.A.). GTP[^{35}S] (1400 Ci/mmol) and [adenylate-^{32}P]NAD (800 Ci/mmol) were from Dupont–New England Nuclear. GTPγ[^{32}P] (10–30 Ci/mmol) was obtained from Amersham Corporation. Other nucleotides were obtained from Boehringer-Mannheim Biochemicals. Pertussis toxin was from List Biological Laboratories (Campbell, CA, U.S.A.). The sources of the other reagents are those described in previous papers [5,7].

Cell culture and toxin treatment

Mouse AKR-2B cells are a fibroblastic cell line of embryonic mesenchymal origin. Cells were initially plated at a density of 5×10^3 cells/cm^2 in McCoys 5A medium supplemented with 5% fetal bovine serum and allowed to grow at 37 °C in a humidified atmosphere of 5% CO_2 for 7 days. The quiescent density-arrested cultures were then used as a source for the preparation of plasma membranes or stimulated with the indicated reagents in serum-free MCDB 402 medium containing 10 μg of bovine serum albumin/ml for the times indicated in the legend for each experiment. For toxin treatment in intact cells, cultures were incubated with pertussis toxin (100 ng/ml) for 3 h in serum-free MCDB 402 medium prior to the addition of the indicated reagents or the preparation of plasma membranes.

Preparation of plasma membranes

Quiescent cultures were scraped with a rubber policeman and cells collected by centrifugation (235 *g* for 5 min). The cell pellet was lysed by Dounce homogenization in a hypo-osmotic buffer containing 10 mM-Tris (pH 7.4), 10 mM-NaCl, 1.0 mM-EGTA and 2.0 mM-$MgCl_2$. Sucrose was added to the homogenate to 0.125 M and the nuclei were removed by centrifugation (1400 *g*). The supernatant was layered over a pad of 20% sucrose and spun for 60 min at 100000 *g*. Pelleted membranes were resuspended in 50 mM-Tris (pH 7.4)/1.0 mM-EGTA/100 μg of leupeptin/ml (membrane resuspension buffer), and protein concentration was determined by 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.

ADP-ribosylation in plasma membrane preparations

Pertussis toxin was activated by incubation at 37 °C for 20 min in 20 mM-dithiothreitol [22]. The activated toxin (1 μg) was incubated with 50 μg of plasma membranes at 37 °C for 30 min in a final reaction volume of 200 μl containing 2.5 μM-NAD/1.0 mM-ATP/1.0 mM-GTP/10 mM-thymidine, and where indicated 25 μCi of [α-^{32}P]NAD/ml. The reaction was stopped by the addition of ice-cold 25 mM-Tris/HCl (pH 7.4)/5 mM-$MgCl_2$ and centrifuged at 10000 *g* for 10 min. The pellet was resuspended in membrane resuspension buffer to a final concentration of 1 mg/ml and stored as described above. When the reaction was to be analysed by autoradiography (see Fig. 4), the incubation was stopped by the addition of 100% trichloroacetic acid to a final concentration of 10% and the ADP-ribosylated products were separated on 7–15% linear gradient SDS/polyacrylamide-gels as described by Laemmli [23].

Assay of GTP[^{35}S] binding to isolated plasma membranes

Binding reactions were carried out at 37 °C in a final volume of 100 μl. The binding buffer consisted of 50 mM-Tris (pH 7.4)/2 mM-$MgCl_2$/1 mM-EGTA/100 μg of bovine serum albumin/ml/5 mM-dithiothreitol, and the indicated concentrations of GTP[^{35}S] and TGFβ1. Reactions were initiated by the addition of AKR-2B membranes (1–10 μg) 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 rapidly washed three times with ice-cold binding buffer by repeated centrifugation and then resuspended in 150 μl of the same buffer. All assays were carried out in triplicate, along with triplicate control samples containing 1000-fold unlabelled GTP for determination of non-specific binding. Specific binding in the presence or absence of TGFβ1 was obtained by subtracting non-specific binding from total binding. Non-specific binding was consistently < 10% of total binding. Bound radioactivity was measured by liquid scintillation spectrometry. In preliminary experiments (results not shown), binding of GTP[^{35}S] to AKR-2B membranes was shown to be rapid, reaching a steady state level after 15 min at 37 °C. Therefore this incubation time was chosen for subsequent experiments.

GTPase activity in plasma membranes

Reactions were performed similarly to those described above for GTP[^{35}S] binding except that the incubation mixture contained 2.5 mM-ATP, and 0.02 μM-GTPγ[^{32}P] replaced GTP[^{35}S]. Incubations were initiated by the addition of untreated or pertussis-toxin-treated membranes (1–10 μg), carried out at 37 °C for the indicated times, and stopped by the addition of 0.5 ml of activated charcoal (Norit A). After centrifugation at 10000 *g* for 10 min, a 50 μl aliquot of the supernatant was counted for $^{32}PO_4^{3-}$ as previously described [24]. GTPase activity was expressed as pmol of GTP hydrolysed/mg of protein.

Preparation of RNA and Northern hybridization analysis

Total poly(A)$^+$ containing RNA and Northern hybridization were done as described previously [7]. At the end of the incubation period, cultures were directly lysed by

the addition of lysis buffer (100 mM-NaCl/2 mM-EDTA/0.8% SDS/10 mM-Tris/HCl, pH 7.4). The poly(A)$^+$-containing RNA was purified by one cycle of oligo(dT) cellulose chromatography, and equivalent quantities were electrophoresed in 1.2% agarose gels containing 2.2 M-formaldehyde. RNA was transferred to nitrocellulose in 20 × SSC (3 M-NaCl/0.3 M-sodium citrate, pH 7.0) for 18 h and fixed by baking at 80 °C for 4 h. Conditions for prehybridization, hybridization and posthybridization washes were performed as previously described [7]. The human c-*sis* riboprobe containing the 507 bp *Aval* fragment was provided by T. O. Daniel, Vanderbilt University [25]. The constituitively expressed *1B15* (gene encoding cyclophilin, the cyclosporin binding protein) riboprobe used as an internal marker to normalize RNA loading was obtained from J. Douglass, Oregon Health Sciences University [26]. Quantification was performed using an LKB Ultra Scan Laser Densitometer by designating the control cell treatment (media alone) as 1.0 (i.e. *sis* expression with media alone/*1B15* expression in medium alone, as equivalent to 1.0).

RESULTS

Effect of TGFβ1 on GTP[^{35}S] binding to AKR-2B plasma membranes

An increased binding of GTP following receptor stimulation is a common characteristic of G-protein activation. This activation results not only in an increased binding of GTP, but in a displacement of bound GDP and stimulation of a specific GTPase activity [10]. To determine whether the TGFβ1 signal is coupled to a G-protein, we used the non-hydrolysable, radiolabelled GTP analogue GTP[^{35}S] to establish if TGFβ1 addition results in increased GTP binding in plasma membranes prepared from AKR-2B cells. Fig. 1 shows that specific binding of 10 nM-GTP[^{35}S] was stimulated in a dose-dependent manner by the addition of TGFβ1. Maximal GTP[^{35}S] binding occurred between 0.4 and 1.0 nM-TGFβ1. These concentrations of TGFβ1 are similar to those previously reported to elicit maximal biological effects in various cell types [5,7,27,28]. Basal GTP[^{35}S] binding to AKR membranes was 5.7 ± 0.5 pmol/mg of protein and was increased to 10.0 ± 1.1 pmol/mg of protein in the presence of 2.0 nM-TGFβ1, representing an approx. 76% stimulation. Time-course experiments showed that stimulated GTP[^{35}S] binding was rapid and reached steady state levels within 15 min at 37 °C (results not shown).

To determine if the TGFβ1-stimulated binding of GTP[^{35}S] was due to an increased affinity of the G-protein for GTP[^{35}S] or to changes in the number of GTP[^{35}S] binding sites, saturation binding experiments were performed (Fig. 2). The binding data resulted in linear Scatchard plots [29] as calculated using the 'ligand' binding program developed by Munson & Rodbard [30]. Specific binding of GTP[^{35}S] occurred with an apparent dissociation constant (K_d) of 3.2×10^{-8} M, and this was not appreciably affected by TGFβ1 addition (K_d 3.45×10^{-8}). In the presence of 0.4 nM-TGFβ1, however, the number of available binding sites for GTP[^{35}S] was increased from 16.2 ± 1.2 to 21.6 ± 2.0 pmol/mg of protein. Thus, the TGFβ1-stimulated binding of GTP[^{35}S] appears to be due not to an increased affinity of the G-protein for GTP[^{35}S] but to an increased exchange of bound GDP, resulting in enhanced GTP[^{35}S] binding.

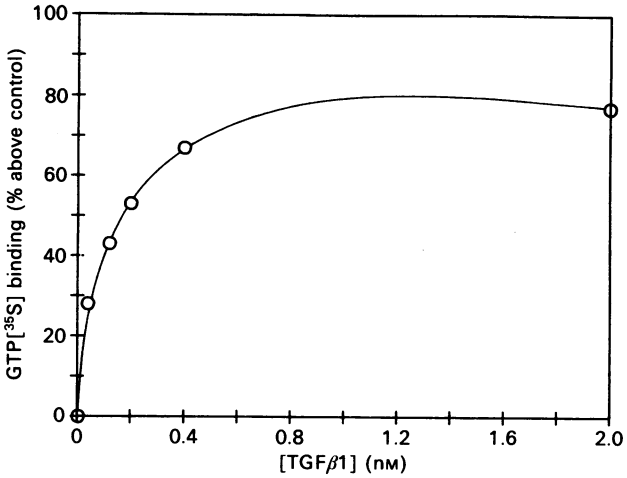

Fig. 1. Dose-response effect of TGFβ1 on GTP[^{35}S] binding to AKR-2B membranes

Plasma membranes isolated from quiescent density-arrested AKR-2B cells were incubated with 10 nM-GTP[^{35}S] for 15 min at 37 °C in the presence of the indicated concentrations of TGFβ1. The amount of bound GTP[^{35}S] was determined by liquid scintillation spectrometry as described in the Materials and methods section. Results are the mean of three experiments conducted in triplicate.

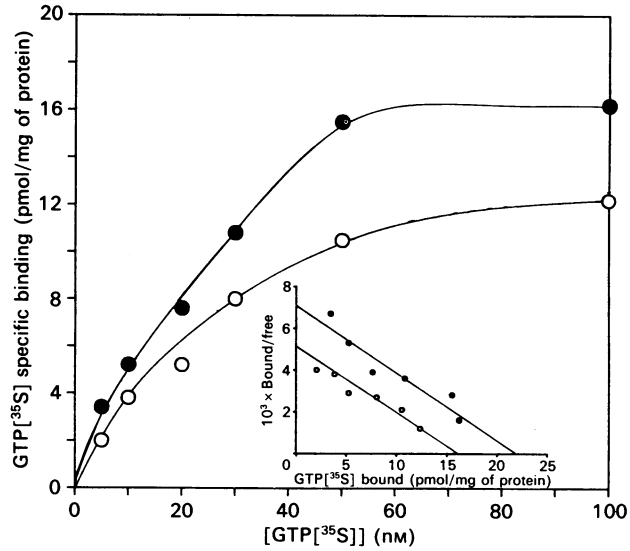

Fig. 2. Effect of TGFβ1 on equilibrium binding of GTP[^{35}S] to AKR-2B membranes

Plasma membranes from untreated AKR-2B cells were incubated in the absence (○) or presence (●) of 0.4 nM-TGFβ1 for 15 min at 37 °C with the indicated concentrations of GTP[^{35}S], and binding was determined as described in the Materials and methods section. Results are the means of two experiments conducted in triplicate. The insert depicts the Scatchard analysis of the binding data.

Nucleotide specificity to inhibit GTP[^{35}S] binding

The specificity with which excess unlabelled nucleotides were able to compete with GTP[^{35}S] binding is shown in Fig. 3. The basal binding of GTP[^{35}S] was inhibited by increasing concentrations of GTP with half-maximal

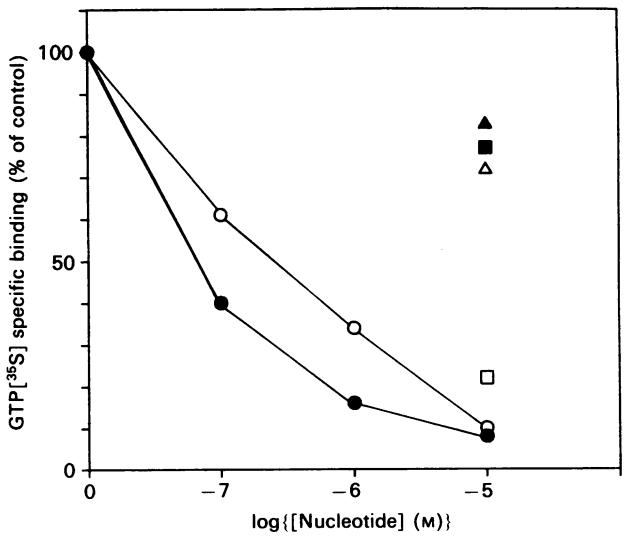

Fig. 3. Effect of nucleotides on GTP[^{35}S] binding on AKR-2B membranes

Plasma membranes from untreated AKR-2B cells were incubated with 10 nM-GTP-[^{35}S] for 15 min at 37 °C in the presence (●) or absence (○) of 0.4 nM-TGFβ1, and the indicated concentration of unlabelled GTP. Inhibition of basal GTP[^{35}S] seen with 10^{-5} M-ATP (△), -CTP (■), -TTP (▲), or -GDP (□) is indicated. Basal GTP[^{35}S] binding in the absence of unlabelled nucleotide (100% value) was 5.4±0.75 pmol/mg of protein, and TGFβ1-stimulated GTP[^{35}S] control (100% value) was 8.03±1.05 pmol/mg of protein. Values represent the means of two experiments conducted in triplicate.

inhibition occurring at approx. 100 nM. At 10 μM concentration, GTP and GDP inhibited basal GTP[^{35}S] binding by 90 and 78% respectively. The other nucleotides, ATP, CTP and TTP, were relatively inefficient in their abilities to inhibit basal GTP[^{35}S] binding. The TGFβ1-stimulated binding of GTP[^{35}S] was also inhibited by GTP with approximately the same kinetics as that observed with basal GTP[^{35}S] binding, albeit slightly more sensitive to unlabelled GTP. At 1 μM-GTP, TGFβ1-stimulated GTP[^{35}S] was inhibited by approx. 82%. The high potency and specificity of the guanine nucleotides to inhibit GTP[^{35}S] binding, as compared with the other nucleotides, further suggest the involvement of a G-protein.

Growth factor specificity for stimulation of GTP[^{35}S] binding

To test whether this enhanced GTP[^{35}S] binding in AKR-2B membranes was specific to TGFβ1, we investigated the effects of other known growth factors. Despite the fact that all the growth factors tested have previously been shown to elicit biological activity in AKR-2B cells, the results presented in Table 1 demonstrate that only TGFβ1 stimulated GTP[^{35}S] binding. EGF in combination with insulin resulted in a minor increase, but even TGFβ2, contrary to our expectation, was without effect on GTP[^{35}S] binding. The results obtained with EGF, insulin and PDGF are not totally unexpected since a G-protein involvement in their signalling pathway has not been conclusively demonstrated. The fact that TGFβ2 did not stimulate GTP[^{35}S] binding is of interest and could contribute further insight into the mechanism by which the different TGFβ receptor types transduce their signal.

Table 1. Growth factor specificity of stimulated GTP[^{35}S] binding to AKR-2B membranes

Plasma membranes isolated from AKR-2B cells were incubated with 10 nM-GTP[^{35}S] for 15 min at 37 °C in the presence of the indicated growth factors. Bound GTP[^{35}S] was determined as described in the Materials and methods section. Values represent the means±S.E.M. of two experiments conducted in triplicate.

Addition	GTP[^{35}S] binding (pmol/mg of protein)	% of control
None	5.8±0.64	100
TGFβ1 (0.4 nM)	8.8±0.75	153
TGFβ2 (0.4 nM)	5.7±0.21	99
EGF (1.7 nM)	5.6±0.80	97
Insulin (50 nM)	5.7±0.84	98
EGF+insulin	6.2±0.43	107
PDGF (B chain) (1.5 nM)	5.6±0.21	96

Table 2. Inhibition of TGFβ1-stimulated GTP[^{35}S] binding to AKR-2B membranes by pertussis toxin

Plasma membranes isolated from AKR-2B cells were incubated in the presence or absence of 5 μg of activated pertussis toxin/ml for 30 min at 37 °C. The reaction was stopped by the addition of ice-cold 25 mM/HCl (pH 7.4)/5 mM-$MgCl_2$ and centrifuged at 10000 *g* for 10 min. The pelleted protein was resuspended in membrane resuspension buffer and GTP[^{35}S] binding was performed as described in the Materials and methods section. Values represent the means±S.E.M. of two experiments conducted in triplicate. The control value for toxin-untreated and treated membranes is listed as 100%.

Pertussis toxin treatment	Addition of 0.4 nM-TGFβ1	GTP[^{35}S] binding (pmol/mg of protein)	% of control
−	−	4.5±0.61	100
−	+	7.1±0.93	157
+	−	5.5±0.95	100
+	+	5.6±0.61	102

Effect of pertussis toxin on TGFβ1-stimulated GTP[^{35}S] binding and GTPase activity

Covalent modification of G-proteins by pertussis toxin has been shown to prevent their normal coupling with receptors [15]. To determine whether the G-protein associated with the TGFβ1 receptor could be modified by ADP-ribosylation, we investigated the effect of pertussis toxin on GTP[^{35}S] binding. As can be seen in Table 2, ribosylation of AKR-2B membranes *in vitro* by 5 μg of activated pertussis toxin/ml caused a minor increase in basal binding but, more importantly, resulted in a

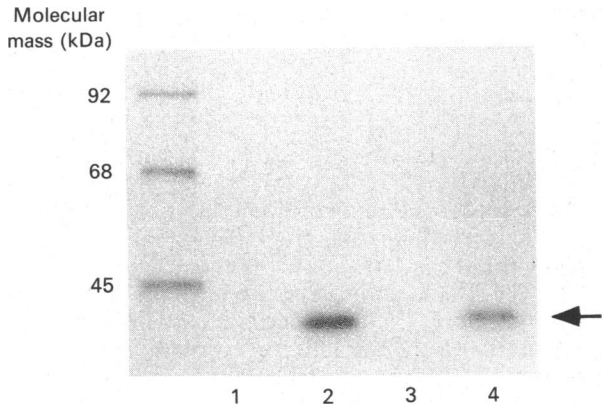

Fig. 4. Autoadiographic analysis of pertussis toxin-treated AKR-2B membranes

Plasma membranes were ADP-ribosylated *in vitro* by pertussis toxin with [α-^{32}P]NAD in the presence or absence of 0.4 nM-TGFβ1 and separated on a 7–15% gradient polyacrylamide gel as outlined in the Materials and methods section. Lane 1, control (buffer alone); lane 2, pertussis toxin; lane 3, control + TGFβ1; lane 4, pertussis toxin + TGFβ1. Quantification of the 41 kDa labelled protein was calculated using an LKB Ultra Scan Laser Densitometer.

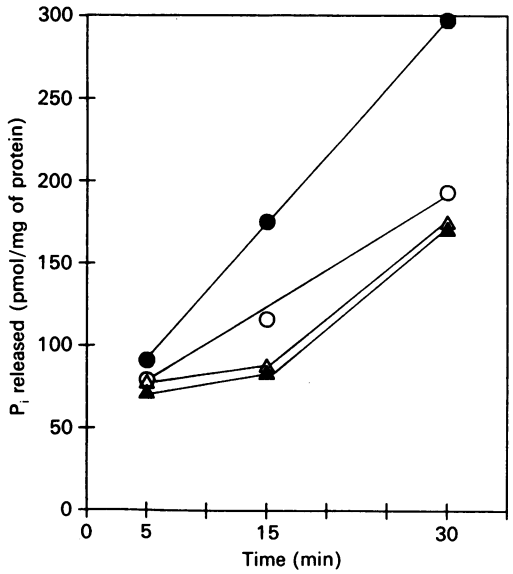

Fig. 5. TGFβ1-induced GTPase activity and its inhibition by pertussis toxin in AKR-2B membranes

Plasma membranes from untreated and pertussis-toxin-treated AKR-2B cells were incubated in a reaction mixture containing GTPγ[^{32}P] in the presence or absence of 0.4 nM-TGFβ1. GTP hydrolysis was determined by measuring the release of $^{32}PO_4^{3-}$ as described in the Materials and methods section. ○, untreated membranes; ● untreated membranes + TGFβ1; △, pertussis-toxin-treated membranes; ▲, pertussis-toxin-treated membranes + TGFβ1. Values represent the means of two separate experiments each conducted in triplicate.

complete inhibition of TGFβ1-stimulated GTP[^{35}S] binding.

Treatment of AKR-2B cell membranes with activated pertussis toxin in the presence of [α-^{32}P]NAD resulted in the ADP-ribosylation of a protein of approx. 41 kDa which is similar to that reported for the α-subunit of G_i or G_o (Fig. 4, lane 2; [15]). ADP-ribosylation of the α subunit is thought to occur primarily when the G-protein is in its 'inactive' (GDP-bound) heterotrimer complex with βγ and not when the monomeric α-subunit is liberated as following receptor activation [15]. Consistent with these findings are the data presented in Fig. 4 (lane 4) in which membranes were ADP-ribosylated in the presence of activated pertussis toxin and TGFβ1. Under these conditions there was an approx. 35% decrease in the labelling of the 41 kDa protein as compared with the pertussis toxin alone (lane 2). This 35% decrease in labelling could represent the amount of α-subunit released, following TGFβ1 binding, which is no longer available as a substrate for ADP-ribosylation.

As previously stated, ligand-stimulated activation of G-proteins results in an enhanced GTPase activity. Fig. 5 demonstrates that in AKR-2B membranes basal GTPase activity was stimulated by approx. 51% during a 15 min incubation with 0.4 nM-TGFβ1 (116 ± 5.5 versus 175 ± 4.3 pmol/mg of protein). This increase in GTPase activity was observed within 5 min and continued during a 30 min incubation with TGFβ1. In contrast, membranes prepared from pertussis-toxin-treated cells exhibited not only a lower basal GTPase activity following a 15 min incubation (85.4 ± 7.6 pmol/mg of protein), but a GTPase activity which could not be stimulated by TGFβ1 (84 ± 3.0 pmol/mg of protein). These results are similar to those of Murthy *et al.* [19] and provide not only further evidence for the role of a G-protein(s) in the transduction of the TGFβ1 signal, but also confirm the postulated uncoupling actions of pertussis toxin [15]; namely, that ADP-ribosylation of the 41 kDa α-subunit (Fig. 4) leads to a decrease in TGFβ1-stimulated GTP[^{35}S] binding (Table 2), and to an inhibition of TGFβ1-stimulated GTPase activity (Fig. 5).

AlF_4^--induced accumulation of c-*sis* mRNA

One of the earliest effects of TGFβ1 is its stimulation of the proto-oncogenes c-*fos*, c-*myc* and c-*sis* [6,7]. We chose therefore to investigate the effects of fluoride ions and pertussis toxin on c-*sis* mRNA expression. Fluoride, and in particular AlF_4^-, acts as a high-affinity analogue of the γ-phosphate group of GTP, and is capable of maintaining G-proteins in their active conformation [31,32]. As can be readily observed in Fig. 6(*a*), stimulation of AKR-2B cells with 0.4 nM-TGFβ1 for 2 h resulted in an increased expression of c-*sis* mRNA. Under these same conditions, NaF and NaF + $AlCl_3$ (AlF_4^-) similarly stimulated c-*sis* expression, while $AlCl_3$ alone had no significant effect. *1B15* mRNA was used as a reference in the quantification of c-*sis* levels since it is constituitively expressed and it remains constant upon TGFβ1 stimulation (Fig. 6*b*). A 3 h pretreatment of the cells with 100 ng of pertussis toxin/ml was found to selectively inhibit TGFβ1-induced effects, but was relatively ineffective in inhibiting AlF_4^--stimulated c-*sis* expression. Similar results were obtained when c-*myc* mRNA levels were analysed under identical experimental conditions (results not shown). These results demonstrate that the effects of TGFβ1 on proto-oncogene expression can be inhibited by pertussis toxin and mimicked by fluoride ions (direct activators of G-proteins), further

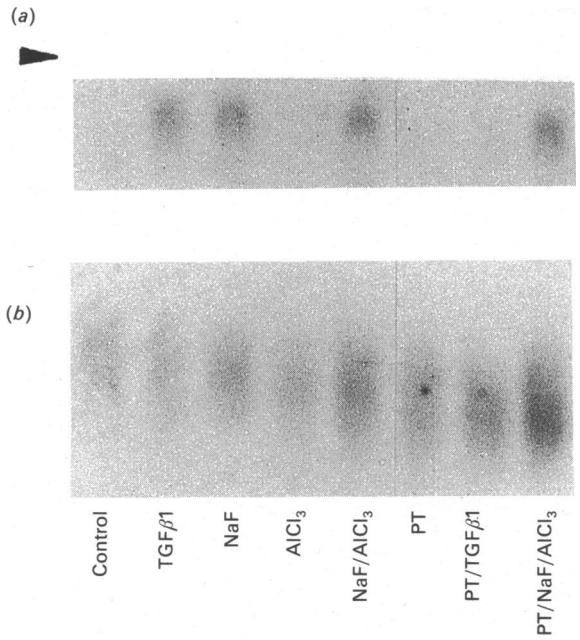

Fig. 6. Stimulation by TGFβ1 and AlF_4^- of proto-oncogene expression

Quiescent density-arrested cultures of AKR-2B cells were pretreated for 3 h at 37 °C with media alone or containing 100 ng of pertussis toxin (PT)/ml. TGFβ1 (0.2 nM), NaF (3 mM), $AlCl_3$ (10 μM) or NaF in combination with $AlCl_3$ (AlF_4^-) were directly added to the indicated plates for an additional 2 h incubation at 37 °C. Total poly(A)$^+$-containing mRNA was purified by oligo(dT) cellulose chromatography and processed for Northern hybridization with a ^{32}P-labelled probe specific for c-*sis* (*a*) as described in the Materials and methods section. RNA loading in (*a*) was normalized to the signal obtained with the constitutively expressed *1B15* gene for each condition (*b*). Arrow indicates position of 28S ribosomal RNA marker. This experiment is representative of two separate experiments.

supporting the involvement of a G-protein(s) in TGFβ1 signal transduction.

DISCUSSION

Activation of G-proteins, following receptor–ligand interaction, is associated with an increased displacement of bound GDP by GTP. The use of radiolabelled GTP analogues, such as GTP[^{35}S], which bind with high affinity to G-proteins but are not hydrolysed [10], allows for the quantification of agonist-stimulated G-protein activation. Agonist-stimulated binding of radiolabelled GTP analogues to membranes is an established test of G-protein involvement in receptor function and has been used in a variety of membrane preparations [10,33–36]. The ability of TGFβ1 to stimulate the GTP[^{35}S] binding in AKR-2B membranes, as presented here, strongly suggests that the TGFβ receptor(s) is coupled to a subsequent effector system(s) by a guanine nucleotide regulatory G-protein(s).

As analysed by equilibrium saturation experiments (Fig. 2), AKR-2B membranes bind GTP[^{35}S] with high affinity, displaying a dissociation constant of 3.2×10^{-8} M which is consistent with the affinity of other characterized G-proteins [33,35]. Addition of TGFβ1 did not affect the affinity of GTP[^{35}S] binding as revealed by the parallel Scatchard plots. Instead, TGFβ1 addition was shown to increase the number of available binding sites for GTP[^{35}S] by approx. 34% from 16.2 to 21.6 pmol/mg of protein, presumably by causing an increased displacement of bound GDP. These findings are in agreement with those observed in the adrenergic and cholinergic receptor systems [34], but are inconsistent with those described for the interleukin-2 receptor where agonist-induced GTP[^{35}S] binding was reportedly due to an increase in affinity [37]. Furthermore, a G-protein concentration of 16–22 pmol/mg of protein in AKR-2B membranes is similar to that seen for G-proteins in platelets, liver and cerebral cortex membranes [34,38], as well as that observed in human monocyte and leukaemic (HL60) cell membranes [39,40].

Recently, it has been proposed that the mitogenic effects of serum [16] and growth factors such as insulin-like growth factor II [41], interleukin-2 [37] and bombesin [17] are mediated via G-proteins. The data herein demonstrate that, of the growth factors tested, only TGFβ1 was able to induce stimulated GTP[^{35}S] binding. Although mitogenic in AKR-2B, the growth factors assayed have receptors which differ from the TGFβ receptor(s) in that they possess tyrosine kinase activity [42]. EGF-receptor-associated tyrosine kinase activity has recently been shown to increase the phosphorylation of phospholipase C in A-431 cells [43]. Thus, a G-protein may not be required in transducing the signal of receptors possessing tyrosine kinase activity, explaining the lack of stimulation of GTP[^{35}S] binding reported here. The inability of TGFβ2 to induce GTP[^{35}S] binding was unexpected but could be attributed to the existence of multiple TGFβ receptor types [8], each possibly with different affinity for G-proteins. We are presently investigating whether TGFβ1 and β2 have other biochemical and/or molecular effects distinct from stimulating GTP[^{35}S] binding. The ability of G-proteins to preferentially couple to one receptor type could also explain the observed different affinities with which TGFβ1 and β2 bind to the type 1 and type 2 TGFβ receptors [8], and their recently reported distinct biological functions [27,28].

Pertussis-toxin-catalysed ADP-ribosylation of the α-subunit of certain G-proteins is thought to induce a conformational change in these proteins preventing their normal coupling to receptors [15]. Treatment of AKR-2B membranes with activated pertussis toxin inhibited both TGFβ1-stimulated GTP[^{35}S] binding and TGFβ1-stimulated GTPase activity. Interestingly, we observed that pertussis toxin lowered basal GTPase activity by 27%. This reduction in basal GTPase activity of AKR-2B membranes treated with pertussis toxin is similar to that recently reported by Murthy *et al.* [19]. While pertussis toxin inhibition of basal GTPase activity has also previously been reported in other systems [37,44], the slight stimulation of basal GTP[^{35}S] binding following toxin treatment is unclear. These inhibitory effects were associated with the ADP-ribosylation of a 41 kDa protein, consistent with the M_r of the α subunit of G_i (41 kDa) and G_o (39 kDa). Furthermore, TGFβ1 addition to AKR-2B membranes in combination with pertussis toxin and [α-^{32}P]NAD was shown to inhibit the labelling of this 41 kDa protein by approx. 35%. It is postulated that TGFβ1 receptor occupation induced the

dissociation of the G-protein heterotrimer into its respective subunits, thereby blocking subsequent ADP-ribosylation [15]. This experimental approach is a common test of receptor–G-protein interaction and has been successfully used to demonstrate G-protein involvement with receptor function in platelets [45,46]. The observations that TGFβ1-regulated c-*sis* expression could be inhibited by pertussis toxin and that the stimulatory effects of TGFβ1 could be mimicked by fluoride ions provide further evidence for the involvement of a G-protein in TGFβ1-mediated proto-oncogene expression. Northern and nuclear run-on analyses demonstrated that pertussis toxin inhibited the TGFβ1 stimulation of c-*sis* mRNA by 50–85% (Fig. 6, and results not shown). The inability to completely abolish c-*sis* expression does not appear to be a result of incomplete substrate ribosylation since treatment of intact cells with 1.0 ng of pertussis toxin/ml for 3 h prior to membrane preparation completely abolished subsequent pertussis toxin ribosylation *in vitro* ([18,19], results not shown). These results indicated that both toxin-sensitive and -insensitive pathways are coupled to TGFβ1-stimulated gene expression. The fact that NaF alone was nearly as effective as AlF_4^- in stimulating c-*sis* mRNA is not inconsistent with earlier studies using fluoride ions [47]. It was subsequently shown that micromolar concentrations of aluminium are required to form the active AlF_4^- species but that these can be etched from glassware with high fluoride concentrations [32]. The fact that pertussis toxin was not able to inhibit AlF_4^--induced c-*sis* expression is contrary to the results obtained in hamster fibroblasts (CCL39) [48], whereas AlF_4^--induced c-*myc* expression was shown to be pertussis-toxin-sensitive. If, as is postulated, the mechanism of G-protein activation by AlF_4^- is to bypass receptor-mediated events and substitute for a γ-phosphate group of GTP [32], then pertussis-toxin-catalysed ADP-ribosylation should not interfere with this response since it has been demonstrated that ADP-ribosylated α-subunits still exhibit functional GTPase activity and GTP[^{35}S] binding [15]. The only effect of pertussis-toxin-catalysed ADP-ribosylation occurs in the presence of receptors [15].

The mechanism(s) by which the TGFβ1 mitogenic signal is directly transduced to the cell nucleus has been difficult to determine. Results presented in this study indicate that the TGFβ receptor is coupled to an undetermined effector system(s) by a guanine nucleotide regulatory G-protein(s). This G-protein contains a 41 kDa substrate for pertussis toxin which is similar to the G_i protein responsible for transducing adenylate cyclase inhibition, and to the G_o protein isolated from brain for which the effector system(s) remains unclear. While there is presently no evidence for cAMP positively transducing the TGFβ1 signal, a recent report by Daniel & Fen [49] demonstrated that increased c-*sis* transcription by TGFβ1 is negatively modulated by cAMP. Although changes in cAMP levels are not detected following TGFβ1 addition (results not shown), this does not eliminate the possibility that a G-protein (i.e. G_i) can interact with multiple effectors [50,51].

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