A GTPase Controls Cell-Substrate Adhesion in *Xenopus* XTC Fibroblasts

Marc H. Symons and Tim J. Mitchison

Department of Pharmacology, University of California, San Francisco, California 94143-0450

Abstract. Cell-substrate adhesion is crucial at various stages of development and for the maintenance of normal tissues. Little is known about the regulation of these adhesive interactions. To investigate the role of GTPases in the control of cell morphology and cell-substrate adhesion we have injected guanine nucleotide analogs into *Xenopus* XTC fibroblasts. Injection of GTPγS inhibited ruffling and increased spreading, suggesting an increase in adhesion. To further investigate this, we made use of GRGDSP, a peptide which inhibits binding of integrins to vitronectin and fibronectin. XTC fibroblasts injected with non-hydrolyzable analogs of GTP took much more time to round up than mock-injected cells in response to treatment with GRGDSP, while GDPβS-injected cells rounded up in less time than controls. Injection with GTPγS did not inhibit cell rounding induced by trypsin however, showing that cell contractility is not significantly affected by the activation of GTPases. These data provide evidence for the existence of a GTPase which can control cell-substrate adhesion from the cytoplasm. Treatment of XTC fibroblasts with the phorbol ester 12-*o*-tetradecanoylphorbol-13-acetate reduced cell spreading and accelerated cell rounding in response to GRGDSP, which is essentially opposite to the effect exerted by non-hydrolyzable GTP analogs. These results suggest the existence of at least two distinct pathways controlling cell-substrate adhesion in XTC fibroblasts, one depending on a GTPase and another one involving protein kinase C.

INTEGRIN-mediated adhesion of cells to the extracellular matrix (ECM)[1] plays a key role in development, cell motility, and metastasis (Dedhar, 1990; DiMilla et al., 1991; Dodd and Jessell, 1988; Hynes and Lander, 1992; Zetter and Brightman, 1990). Evidence has accumulated that integrin function can be controlled from the cytoplasm, and that this regulation occurs by modulation of the affinity of the receptor for its ligand. Examples come from lymphocyte, platelet, and macrophage activation (O'Toole et al., 1990; Phillips et al., 1991; Shaw et al., 1990; Springer, 1990), terminal differentiation of keratinocytes (Adams and Watt, 1990) and development of retinal neurons (Neugebauer and Reichardt, 1991). It was recently shown that the cytoplasmic tails of the integrin subunits are important for such regulation (Hibbs et al., 1991; O'Toole et al., 1991). Various second messenger systems have been implicated in the regulation of integrin affinity, including calcium, protein kinase C (PKC) and G-proteins involved in lipid metabolism and cAMP formation (Brass et al., 1991; Dustin and Springer, 1989; Shattil and Brass, 1987; Shaw et al., 1990; van Kooyk et al., 1989). The detailed mechanism by which these second messengers regulate integrin affinity has not been determined however.

Over the past few years, evidence has accumulated for the involvement of GTPases in the control of the actin cytoskeleton, cell motility, and intracellular adhesion (Aznavoorian et al., 1990; Bar-Sagi and Feramisco, 1986; Doherty et al., 1991; Lester et al., 1989; Paterson et al., 1990; Sha'afi and Molski, 1988). GTPases act as switches, being "on" in the GTP-bound state and "off" in the GDP-bound state (Bourne et al., 1990; Hall, 1990). The precise signalling pathways in which these GTPases participate still remain to be elucidated. The superfamily of GTPases can be subdivided in essentially three different categories (Bourne et al., 1990): the receptor-coupled trimeric GTPases (Gilman, 1987), called G proteins, the ras family of small GTPases (Hall, 1990), and the GTPases controlling protein synthesis. Activation of G proteins in neutrophils has been shown to strongly increase the amount of polymerized actin (Bengtsson et al., 1990; Downey et al., 1989; Therrien and Naccache, 1989). Two small GTPases of the ras-family, ras itself and rho, also appear to be involved in the regulation of actin dynamics, as injection of oncogenic H-ras and rho^{val14} (a mutated, constitutively active form of rho) have a pronounced effect on cell morphology and the actin cytoskeleton (Bar-Sagi and Feramisco, 1986; Paterson et al., 1990). In addition, two other members of the ras-family, CDC42 and RSR1/BUD1, have been shown to be involved in the control of budding and actin organization in the yeast *Saccharomyces cerevisiae* (Bender and Pringle, 1989; Chant and Herskowitz, 1991; Johnson and Pringle, 1990).

ECM receptors are known to interact with the actin cytoskeleton (Burridge et al., 1988). In this work therefore,

Marc Symons' present address is ONYX Pharmaceuticals, 3031 Research Dr., Richmond, CA 94806.

1. *Abbreviations used in this paper*: DNP, 2,4-dinitrophenol; DOG, 2-deoxy-D-glucose; ECM, extracellular matrix; Fn, fibronectin; NDP, nucleoside-diphosphate; PKC, protein kinase C; TPA, 12-*o*-tetradecanoylphorbol-13-acetate; Vn, vitronectin.

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we have investigated the involvement of GTPases in cell morphology and cell-substrate adhesion by injection of guanine nucleotide analogs into fibroblasts. We observed that injection of non-hydrolyzable GTP analogs stimulated spreading, inhibited ruffling and inhibited cell rounding caused by treatment with GRGDSP. These results suggest the existence of a GTPase activity which can control cell-substrate adhesion.

Materials and Methods

Cell Culture and Microinjection

Xenopus XTC cells were grown in 75% L-15 medium, supplemented with 10% FBS and penicillin and streptomycin, at room temperature (24°C). Cells for microinjection were grown on 25-mm-diam glass coverslips. All experiments were carried out 14–24 h after plating.

For the microinjection experiments, the coverslips were mounted in their growth medium in a homemade thermostatted chamber, kept at 24°C. The chamber was fixed to the stage of an inverted microscope (IM35; Carl Zeiss, Oberkochen, Germany). Injections were performed using continuous flow and were done by inserting the needle in the perinuclear area. Nucleotide analogs were dissolved in injection buffer (K glutamate, 2.5 mM, KCl, 100 mM). All nucleotide analogs were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), except GMPCPP which was a gift of A. Hyman (University of California, San Francisco, CA). Cells were followed during injection and permeabilization using video microscopy. Time-lapse video recording was performed on an inverted microscope (IM35, Carl Zeiss) as previously described (Sawin and Mitchison, 1991).

Immunofluorescence Procedures

For the immunofluorescence experiments cells were plated 14–18 h before microinjection on marked glass coverslips, which greatly facilitated the relocation of injected cells after staining. The marks consist of tiny capital letters, inscribed with a diamond pen. After injection, cells were simultaneously permeabilized and fixed for 10 min on the stage in cytoskeleton buffer (Symons and Mitchison, 1991): 10 mM MES, pH 6.1, KCl 138 mM, $MgCl_2$ 3 mM, and EGTA 2 mM, containing 0.5% Triton X-100 and 0.5% glutaraldehyde. Cells were subsequently rinsed in TBS-TX (0.15 M NaCl, 0.02 M Tris-Cl, pH 7.4, containing 0.1% Triton X-100) and reduced with 1 mg/ml $NaBH_4$ in TBS, for 5 min, three times. The coverslips were rinsed in TBS-TX and blocked with 10 mg/ml BSA dissolved in TBS-TX for 45 min, incubated with DM1β, a anti-β-tubulin mAb (Sigma Chemical Co., St. Louis, MO), for 1 h, rinsed with TBS-TX, and incubated with FITC goat anti-mouse together with Rhodamine phalloidin (Molecular Probes, Eugene, OR) for 30 min, rinsed with TBS-TX, and mounted as previously described (Symons and Mitchison, 1991).

Fluorescence image collection was performed on an Olympus inverted microscope using a 16×, 0.5 NA Plan-neofluar Zeiss objective, and a Peltier-cooled CCD camera equipped with a Texas Instruments 512 × 512 pixel chip, as previously described (Symons and Mitchison, 1991). Pictures were made using a Dunn digital camera and Technical Pan film.

Data Analysis

Imaging for the cell morphology experiments was performed using a Zeiss 40× Neofluar 0.75 NA objective. For the quantification of cell morphometry we used an Image I (Interactive Video Systems, Media, PA) analysis system.

Surface area measurements were determined by outlining the cell contour with a mouse. Cell ruffling was determined as the total length of ruffles visible for each time point analyzed, i.e., about every one or two minutes.

For the large scale adhesion experiments the cells were plated on marked coverslips, see above. In each experiment, typically two series of about 25 single cells were injected with nucleotides or buffer. Spindle-shaped cells were avoided. All the injection events were recorded with the video camera. Imaging was performed using a 16× objective. The average time between injection and application of the adhesion peptide was 25 min. At regular intervals after peptide application, the injected cells, as well as a similar amount of uninjected control cells, were recorded. Subsequently, for each cell, the time from peptide application to complete rounding was determined (see Fig. 5). For each series of cells, the average cell rounding time was determined as the time needed to round up 63% of the cells (see Fig. 6). The cut-off level of 63% was chosen, because this level yielded slightly larger differences in rounding time between the guanine nucleotide analog-injected cells and the controls. We observed a small but significant increase in the rounding time of buffer-injected cells, 25 ± 2 (SEM) min (n = 4) compared with that of uninjected cells, 19 ± 2 (SEM) min (n = 4). We therefore corrected the rounding time of the uninjected controls cells accordingly. The cause of this difference is not clear, but it might be due to the fact that not all cells are readily injectable, and that this population of cells adheres less tightly to the substrate.

For the contractility assays, one 16× field of single cells was injected. After ~20 min, the cells were rinsed with serum-free 75% L-15 medium, immediately followed by application of 75% Saline A containing 75 nM Trypsin and 0.4 mM EDTA. All these events were recorded with the video camera at 5-s intervals. For the trypsin experiments, cell rounding was quantified by determining the spread area for each frame using Image I. The spread area of each cell was normalized to its area before trypsin application. After a lag time, which probably reflects the time necessary for the proteolysis of extracellular matrix components (Zetter et al., 1976), very fast rounding of the cells is observed. The rate of cell rounding was calculated as follows: the spread area was calculated for each recorded video frame, with a time lapse of 5 s, and the maximum decrease in area during any 5-s time interval was determined. From this decrease in area, the time necessary for the cell to decrease its area by 50% was calculated. The rate of cell rounding was then calculated as the inverse of this time.

Results

Effects of Guanine Nucleotide Analogs on Cell Morphology

To study the effect of non-hydrolyzable analogs of GTP on fibroblast morphology, these analogs were introduced into the cytoplasm of Xenopus XTC cells by pressure microinjection. The cells were observed using phase contrast video microscopy. Injection of 1 mM GTPγS rapidly caused a strong inhibition of ruffling and an increase in cell spreading (compare Fig. 1 *a* and *b*). Quantification of these phenomena showed that the inhibition of ruffling was complete within 10 min and resumed about 1 h after injection (Fig. 2). Maximal spreading occurred after ~30 min and slowly reversed with kinetics similar to that of ruffling (Fig. 2). These phenomena were very reproducible: inhibition of ruffling was observed in all injected cells, while the average increase in cell area was 36 ± 7 (SEM) % (n = 20). The lowest concentration of GTPγS in the injection needle to produce observable effects was 250 μM. Thus, assuming that the injected volume amounted to 5–10% of the cell volume, the threshold concentration of cytoplasmic GTPγS which inhibits ruffling and stimulates spreading was <25 μM, which is lower than the estimated 100 μM of unbound GTP in the cytoplasm (Otero, 1990). In NIH-3T3 and human foreskin fibroblasts, injection of GTPγS also inhibited ruffling and induced spreading, but the reversal occurred after a much shorter time (15 min). Similar phenomena were observed after injection of other non-hydrolyzable GTP analogs, GMPCPP (Sandoval et al., 1977) and GMPPNP, but not with AMPPNP, a non-hydrolyzable analog of ATP. Injection of GTP does not have a significant effect on cell morphology either. The latter is in line with the concept that the activation of GTPases consists in the stimulation of the release of bound GDP, which then rapidly gets exchanged with GTP, which is in excess over GDP in the cytoplasm (Bourne et al., 1990). The injection of the non-hydrolyzable GTP analogs therefore strongly suggests that the activation of a GTPase is responsible for the observed changes in cell morphology. Injection of 2.5 mM of

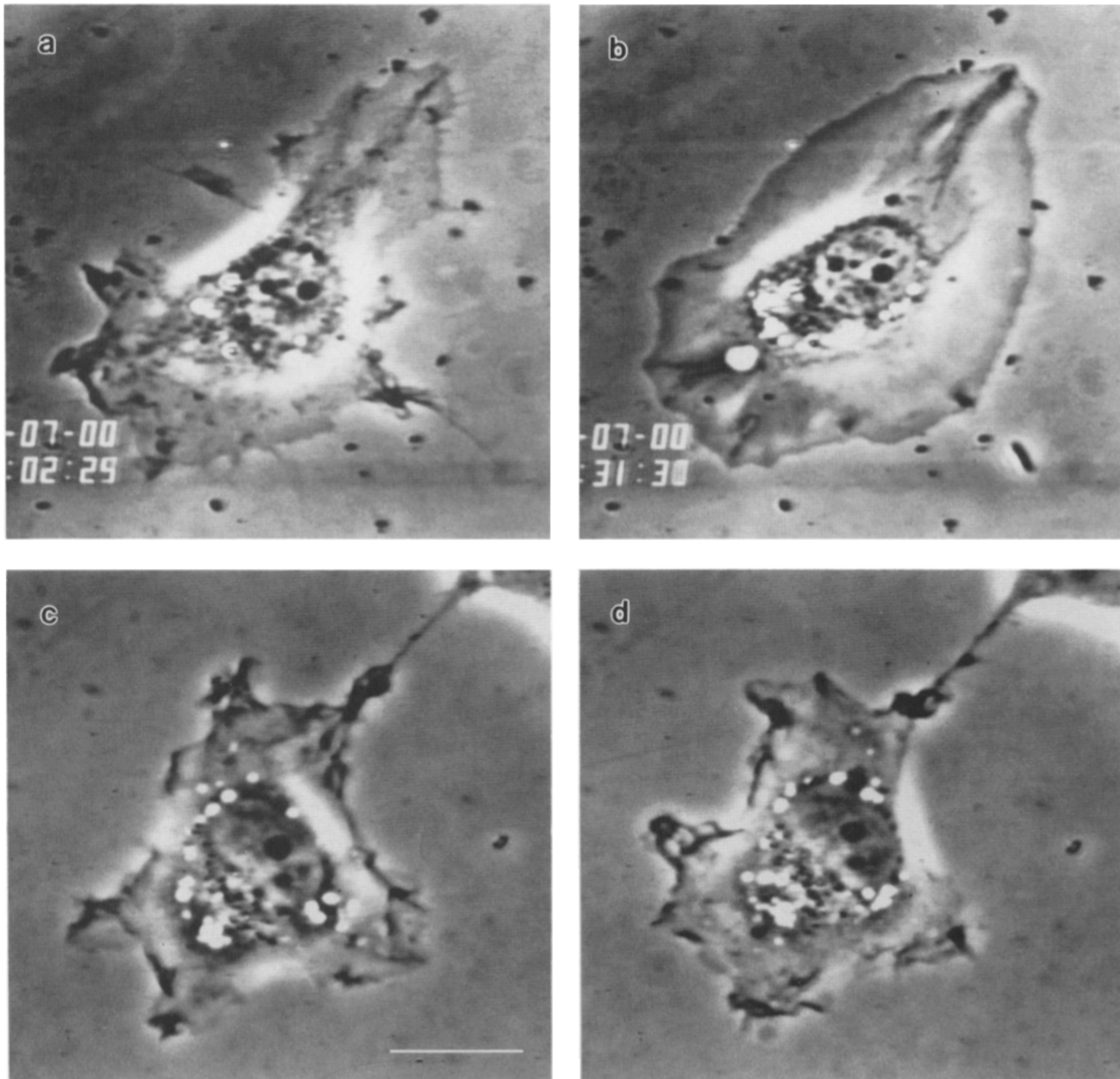

Figure 1. GTPγS-induced spreading and inhibition of ruffling. (*a*) Phase micrograph of a XTC fibroblast 1 min before and (*b*) 28 min after injection with 1 mM GTPγS. (*c*) XTC fibroblast 1 min before and (*d*) 28 min after buffer injection. Bar, 20 μm.

GDPβS, which locks GTPases in the inactive state (Eckstein et al., 1979), had no significant effect on cell morphology. Injection of higher concentrations of GDPβS in XTC fibroblasts caused cell death. In NIH-3T3 fibroblasts however, injection of 10 mM GDPβS produced an increase in ruffling and a decrease in cell spreading, which is opposite to the effects caused by GTPγS injections.

The changes in cell morphology induced by non-hydrolyzable GTP analogs prompted us to inspect the actin cytoskeleton of the injected cells. XTC fibroblasts were injected with 1 mM GTPγS, fixed 20–30 min after injection, and stained with phalloidin. The most prominent changes in the actin cytoskeleton of GTPγS-injected cells consisted of the reorganization of the peripheral actin cytoskeleton. Whereas in the controls only part of the periphery of the fibroblasts showed well-defined lamellipodia, in the GTPγS-treated cells, lamellipodia covered almost the entire circumference (compare Fig. 3, *a* and *b*). We define lamellipodia here as regions of the cell periphery in which the actin cytoskeleton is much more dense than in the remainder of the cell (Small, 1988; Symons and Mitchison, 1991). Lamellipodia of uninjected or buffer-injected cells were irregular and frequently interspersed with microspikes extending into filopodia. Lamellipodia of GTPγS-injected cells appeared very homogenous, with smooth edges and were devoid of filopodia. No significant redistribution or change in thickness of stress fibers could be observed in the GTPγS-injected cells. Furthermore, quantitative immunofluorescence did not reveal any significant difference between GTPγS- and buffer-injected cells in either filamentous actin (ratio 1.16 $\pm$ 0.34 [SEM], $n = 11$) ($P < 0.2$, t test) or microtubule content (ratio 0.77 $\pm$ 0.29 [SEM], $n = 11$) ($P < 0.05$, t test). A detailed account

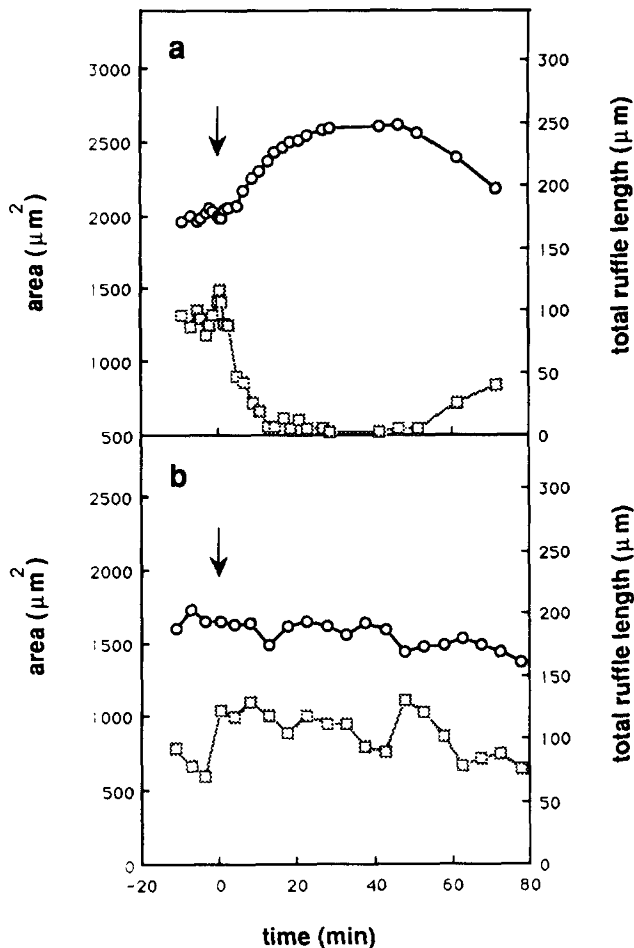

Figure 2. Kinetics of the increase in spread area (○) and inhibition of ruffling (□) induced by injection of (*a*) 1 mM GTPγS and (*b*) buffer. Quantification of the area and total length of ruffles for the cells shown in Fig. 1.

of the effect of guanine nucleotide analogs on the actin cytoskeleton and lamellipodial and filopodial dynamics will be presented elsewhere.

GTP Analogs Inhibit Cell Rounding Induced by GRGDSP

Both the increase in cell spreading and inhibition of ruffling caused by injection of non-hydrolyzable GTP analogs suggested to us that these analogs caused an increase in cell-substrate adhesion. This would imply the existence of a GTPase which in its activated state stimulates adhesion. To test this hypothesis, we examined whether non-hydrolyzable GTP analogs could inhibit cell rounding induced by peptide inhibitors of integrin-ECM interactions (Hayman et al., 1985). As the XTC fibroblasts were plated on glass in the presence of serum, the major ECM components to which they adhere are vitronectin (Vn) and fibronectin (Fn) (Hayman et al., 1985). We therefore used GRGDSP, a peptide which specifically inhibits integrin binding to Vn and Fn (Pierschbacher and Ruoslahti, 1987).

Application of 0.5 mg/ml GRGDSP caused rapid rounding up of buffer-injected cells (Fig. 4, *a–c, left cell*), whereas cells injected with GTPγS were much less affected by the peptide (Fig. 4, *a–c, right cell*). The kinetics of GRGDSP-induced cell rounding for the cells depicted in Fig. 4 is shown in Fig. 5. Whereas complete cell rounding took less than 15 min for the buffer-injected cell, rounding of the GTPγS-injected cell was much slower. Complete rounding in the GTPγS-injected cell was only reached after an incubation time of more than 1 h, similar to the time of reversal of the effect of GTPγS on cell morphology (Fig. 2). GRGESP, a control peptide, did not cause rounding of either buffer-injected cells or GTPγS-injected cells (not shown).

To quantitate the effect of guanine nucleotide analogs for a larger population of cells, we injected 20 to 30 cells at a time on marked coverslips. At regular time intervals after application of GRGDSP, images were taken of the injected cells and stored for subsequent analysis. For each time point, the number of cells which rounded up completely was scored. The results for buffer, GTPγS and GDPβS injections are shown in Fig. 6. Compared to buffer-injected cells, injection of GTPγS slowed down cell rounding after addition of GRGDSP, whereas GDPβS had a small, but opposite effect. As can be seen in Fig. 6, there was a strong cell to cell variation with respect to rounding in response to the GRGDSP peptide. We therefore quantified the effect of nucleotide injection by measuring the time needed to round up 63% of the cells in response to GRGDSP and an average was calculated over several experiments. The results are summarized in Fig. 7. Injection of GTPγS markedly increased the resistance of the cells to the peptide, while GDPβS induced a statistically significant decrease. GMPCPP had an effect similar to that of GTPγS. Thus, the opposite effects of GDPβS and the nonhydrolyzable GTP analogs strongly suggest the existence of a GTPase activity which can modulate cell-substrate adhesion. Surprisingly, injection of ATPγS had effects on cell morphology and inhibition of GRGDSP-induced cell rounding which were similar to those of GTPγS and GMPCPP, which could indicate the additional involvement of protein kinases in the regulation of cell morphology and adhesion. The effects of ATPγS however may be entirely due to transfer of the thiophosphate of ATPγS to GDP via nucleoside-diphosphate (NDP) kinase, as has been shown for the activation of a variety of G-proteins with ATPγS (Otero et al., 1988; Wieland and Jacobs, 1989). However, the facilitation of GRGDSP-induced cell rounding by GDPβS and inhibition by GMPCPP cannot be accounted for by any known transphosphorylation event and therefore strongly suggest the involvement of a GTPase activity in the control of cell-substrate adhesion.

GTP Analogs Do Not Affect Cell Contractility

In principle, the inhibition of cell rounding in response to GRGDSP could be explained by either an increase in cell-substrate adhesion or a decrease in cell contractility. The latter could be brought about via an increase in cAMP levels (Lamb et al., 1988). To investigate this possibility, we followed the kinetics of cell rounding induced by application of trypsin (75 nM) in the presence of 0.5 mM EDTA, a combination which rapidly inactivates the interaction of the cell with ECM components. We expected that under these conditions the rate of cell rounding would be limited by the contractile properties of the cells rather than by cell-substrate

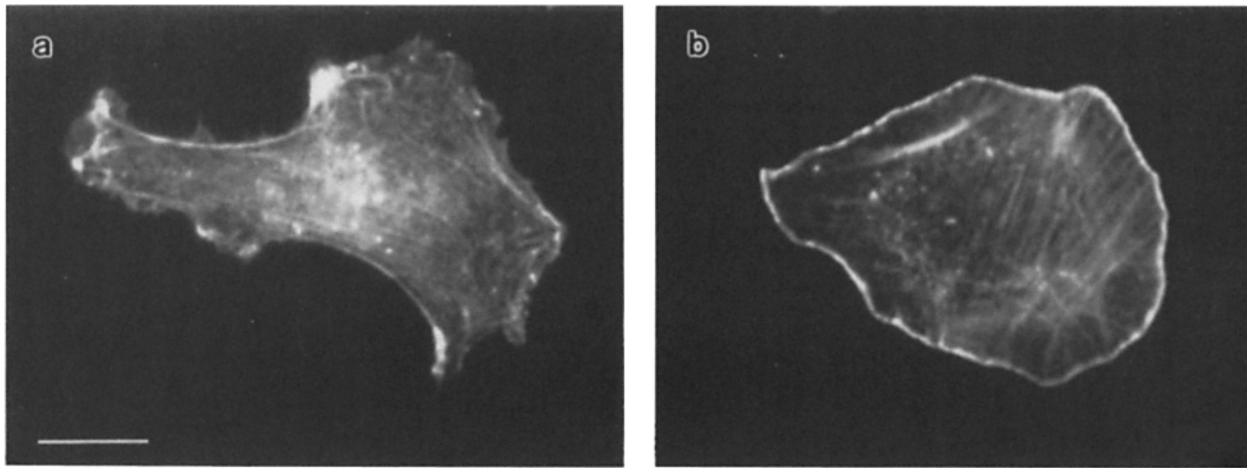

Figure 3. Rhodamine-phalloidin fluorescence micrographs of buffer- and GTPγS-injected XTC fibroblasts. (*a*) Control cell fixed 30 min after buffer injection and (*b*) cell fixed 25 min after injection with 1 mM GTPγS. Bar, 10 μm.

adhesion. Quantification of the spread area of the XTC fibroblasts as a function of time after application of the trypsin/EDTA solution showed that, after a lag time, the cells round up rapidly, at a speed which is very similar for the GTPγS- and mock-injected cells (Fig. 8). Analysis of a large number of cells showed that the rounding rates (see Materials and Methods) for GTPγS- and buffer-injected cells were 0.116 ± 0.008 (SEM) (n = 12) and 0.112 ± 0.015 (SEM) (n = 9) s^{-1}, respectively. The onset of rounding however took significantly longer for GTPγS-injected cells (27 ± 4 [SEM] s) than for controls (15 ± 3 [SEM] s). This longer lag time probably reflects the GTPγS-induced increase in adhesion to the substrate.

Since cell contractility is an energy-dependent process (Cande et al., 1983; Chen 1981), we investigated the effect of DOG and DNP, a combination which drastically lowers the ATP content of cells (Spurck et al., 1986). Application of 1 mM DOG and 0.5 mM DNP to the cells stopped ruffling and induced retraction of filopodia within minutes. This process was rapidly reversible after washing out the drugs, as shown previously by others (Svitkina et al., 1986). As shown in Fig. 8, trypsin-induced cell rounding is strongly inhibited by DOG/DNP; the contractility rate dropped to 0.019 ± 0.003 (SEM) s^{-1} (n = 11). Normal cell contractility was restored after washing out the metabolic drugs (not shown). The similarity of the contractility rates of GTPγS- and mock-injected cells treated with trypsin argues against any significant decrease in cell contractility mediated by activation of GTPases. Thus, the GTPγS-induced inhibition of cell rounding by GRGDSP is most likely due to an increase in adhesion to the substrate.

TPA Inhibits Cell-substrate Adhesion in XTC Fibroblasts

As phorbol esters are known to modulate integrin-mediated adhesion in a number of cell types (Adams and Watt, 1990; Dustin and Springer, 1989; Shaw et al., 1990; van Kooyk et al., 1989), we investigated the effect of 12-*o*-tetradecanoylphorbol-13-acetate (TPA) on cell morphology and cell rounding induced by GRGDSP and trypsin/EDTA. TPA at 200 ng/ml completely inhibited filopodial formation and caused a decrease in the length (tip to base) of lamellipodia, which was apparent within 15 after application (Fig. 9, *a* and *c*). Application of 0.2% DMSO had no effect on cell morphology (Fig. 9, *b* and *d*). TPA also caused a decrease in cell spreading, whereas 0.2% DMSO had no effect (Fig. 10). The average decrease in cell spreading after 1 h of TPA treatment was 30 ± 5 (SEM) % (n = 10) of the original spread area. By this time, about 50% of the cells became elongated and had formed thin processes extending away from the cell body. Cells incubated with 200 ng/ml TPA for 1 h showed much faster cell rounding in response to GRGDSP than control cells (Fig. 11). 63% cell rounding was reached after 8 ± 2 (SEM) minutes (n = 6) vs. 15 ± 2 (SEM) min (n = 4) for controls in the presence of 0.2% DMSO. The rate of cell rounding in response to trypsin/EDTA, 0.12 ± 0.01 (SEM) (n = 11), was identical to that of DMSO-treated control cells, indicating that cell contractility is not altered by TPA. These results clearly show that TPA causes a decrease in cell-substrate adhesion, essentially the opposite effect to that of the non-hydrolyzable GTP analogs.

Discussion

A GTPase Controls Cell-substrate Adhesion

We have examined the effect of nucleotide analog injection on cell-substrate adhesion of XTC fibroblasts. Non-hydrolyzable nucleotide analogs which put GTPases in their active state, such as GTPγS and GMPCPP, promoted adhesion, as indicated by the resistance to treatment with GRGDSP. The GTPγS-induced increase in cell spreading and cessation of ruffling are in line with this. GDPβS, which keeps GTPases in their inactive state, did not induce significant changes in cell morphology, but facilitated the cell rounding induced by GRGDSP. We excluded the possibility that these effects are due to changes in overall cell contractility by showing that injection of GTPγS does not alter the rate of contraction of

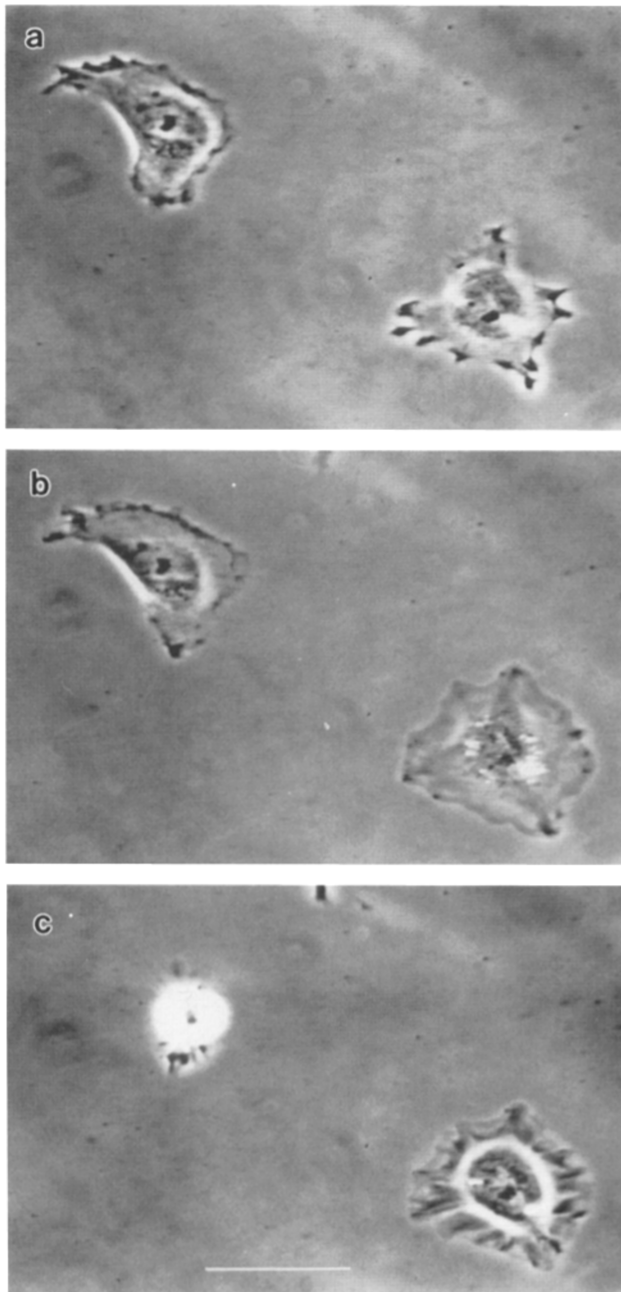

Figure 4. Effect of guanine nucleotide analogs on GRGDSP-induced cell rounding. (*a*) XTC cells before injection. The cell at the left was injected with buffer and the cell at the right was injected with 1 mM GTPγS; (*b*) same cells, 30 min after buffer injection and 24 min after GTPγS injection. This image was taken just before addition of the GRGDSP peptide; (*c*) same field, 25 min after addition of GRGDSP. GRGDSP was dissolved to a final concentration of 0.5 mg/ml in serum-free 75% L-15 medium. Bar, 50 μm.

the fibroblasts induced by trypsin treatment. Together, these observations provide strong evidence for the existence of a GTPase which can control cell-substrate adhesion from the inside of the cell out.

Possible Mechanisms of the GTPase-modulated Adhesion

In principle, the increase in cell-substrate adhesion caused by the non-hydrolyzable guanine nucleotide analogs could be brought about by either a change in the properties of ECM receptors involved themselves or by an increase of ECM receptor expression on the cell surface. The latter mechanism is very unlikely, since GTPγS inhibits most of the steps involved in protein transport to the plasma membrane (Goud and McCaffrey, 1991). We therefore favor a mechanism for the GTPase-dependent modulation of adhesion whereby the GTPase in its activated state increases the adhesive properties of the ECM receptors themselves.

Inside-out modulation of the binding properties of inte-

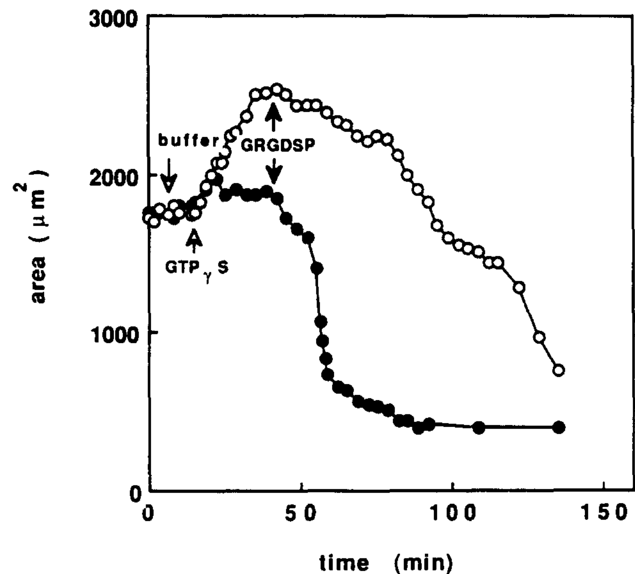

Figure 5. Kinetics of the changes in cell area induced by addition of GRGDSP. (●) Buffer-injected cell; (○) GTPγS-injected cell. The times of the respective injections and of GRGDSP application are indicated.

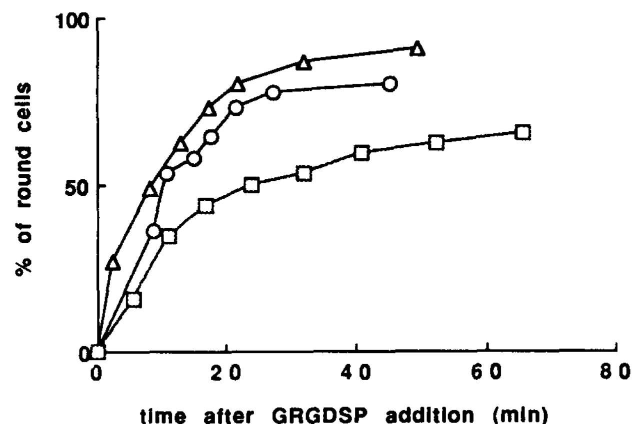

Figure 6. Quantification of GRGDSP-induced cell rounding for various injected guanine nucleotide analogs. (△) Cells injected with 2.5 mM GDPβS; (○) Buffer-injected controls; and (□) cells injected with 1 mM GTPγS. For each experiment, at least 20 cells were injected with the respective nucleotide or buffer. On the average 25 min after injection, the medium was replaced by serum-free L-15 medium to which GRGDSP was added to a final concentration of 0.5 mg/ml.

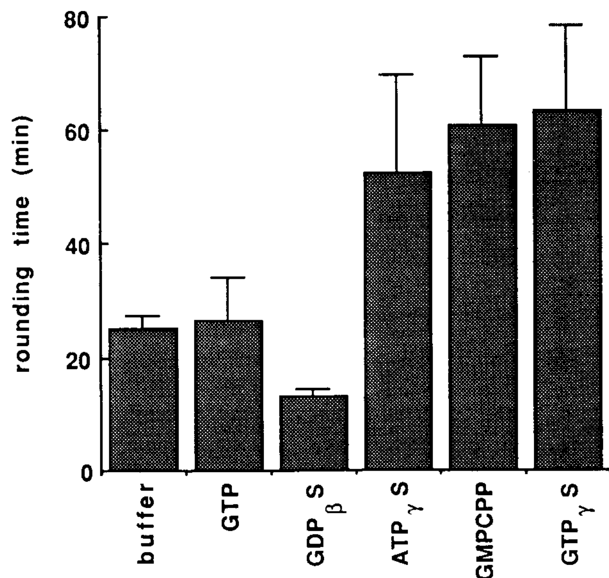

Figure 7. Cell rounding for various injected nucleotide analogs. The time indicated is the time needed for 63% of the injected cells to round up completely. Each bar corresponds to the average (± SEM) of 3 to 5 independent experiments, such as depicted in Fig. 6. Concentration in the needle of GTPγS, GMPCPP, and GTP was 1 mM, GDPβS, 2.5 mM, and ATPγS, 1 to 3 mM. For the latter, there was no significant difference between the two concentrations. The differences between GDPβS, GTPγS, GMPCPP, and buffer-injected control are significant at the $P < 0.001$ level. The result with ATPγS is significantly different from the control at the $P < 0.02$ level.

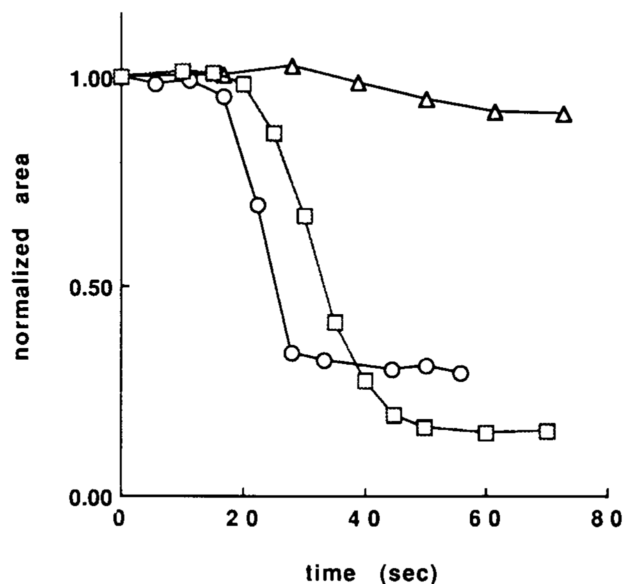

Figure 8. Kinetics of cell rounding induced by trypsin/EDTA. Trypsin (75 nM) and EDTA (0.4 mM) were dissolved in 75% Saline A and applied at time 0. (○) Buffer-injected control cell. (□) Cell injected with 1 mM GTPγS. (△) Quantification of cell spreading of a XTC cell treated with DNP (0.5 mM) and deoxyglucose (1 mM) for 20 min before application of trypsin/EDTA. The energy inhibitors were present during the entire experiment, including rinsing and trypsinization steps.

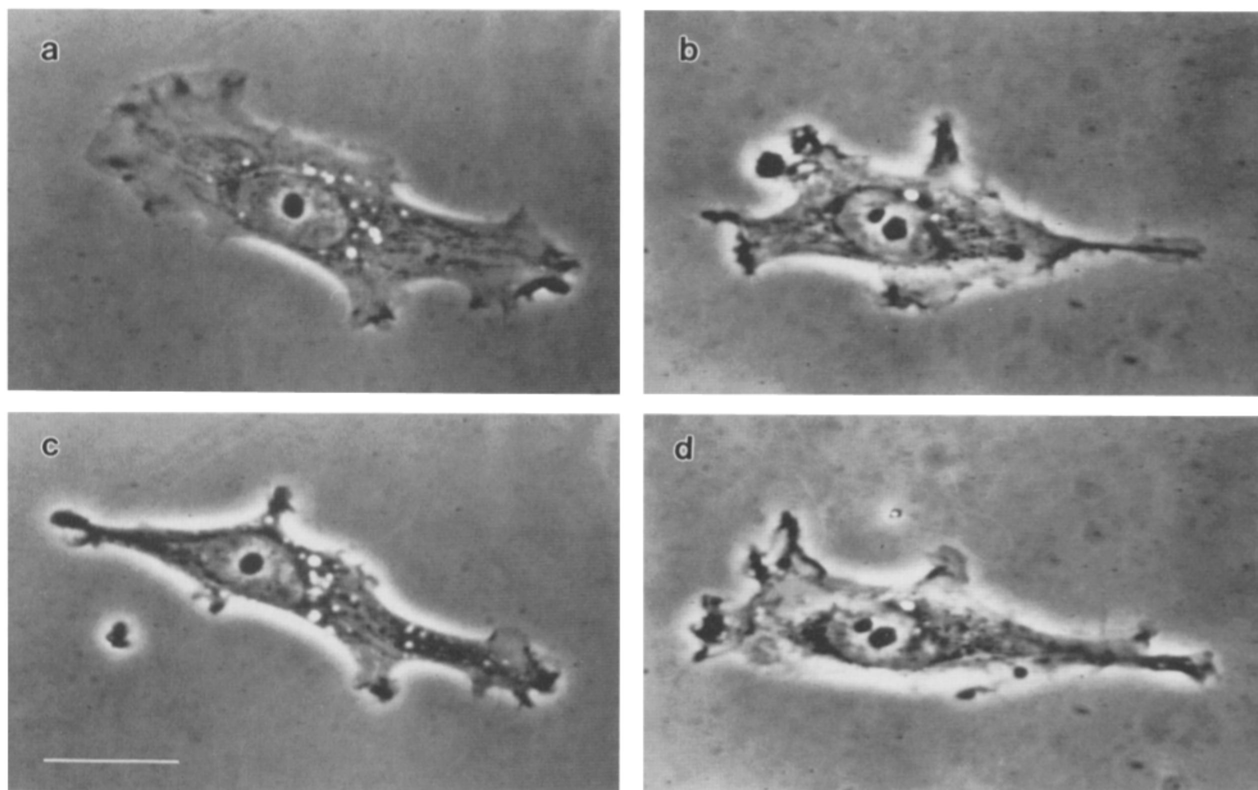

Figure 9. TPA-induced changes in cell morphology. Phase micrographs of a cell (*a*) before and (*c*) 34 min after application of 200 ng/ml TPA and a cell (*b*) before and (*d*) 34 min after addition of 0.2% DMSO. Bar, 25 μm.

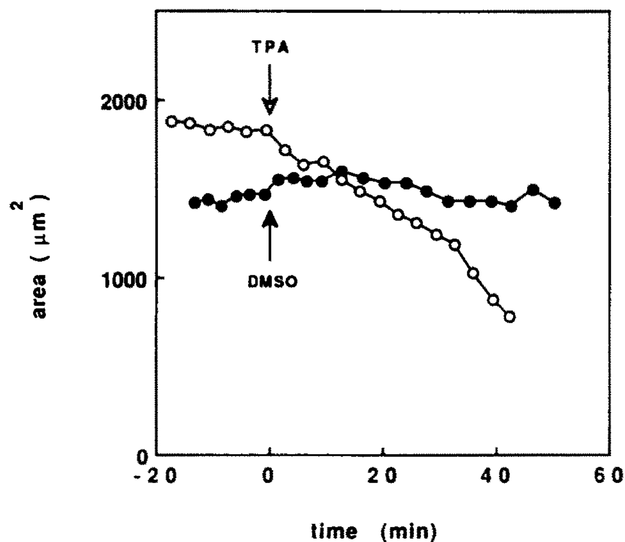

Figure 10. Kinetics of TPA-induced inhibition of cell spreading. The spread areas of the cells shown in Fig. 9 are plotted. (○) Cell treated with 200 ng/ml TPA; (●) Cell treated 0.2% DMSO.

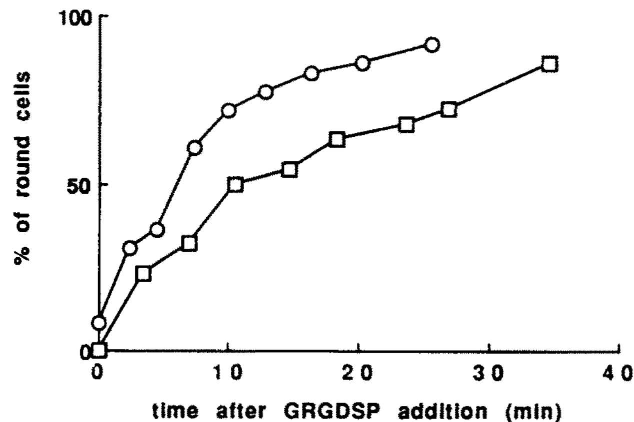

Figure 11. Quantification of GRGDSP-induced cell rounding for TPA- and DMSO-treated cells. (○) Cells treated with 200 ng/ml TPA; (□) Cells treated with 0.2% DMSO. Other conditions as in Fig. 6.

grins has been demonstrated in a variety of cell types, $\alpha_{IIb}\beta_3$ in platelets and β_2 in leukocytes constituting the best-characterized examples (see Hynes, 1992 for a recent review). Activation of these integrins has been shown to be accompanied by a conformational change. It has recently been shown that the cytoplasmic tails of the integrins play an important role in this regulation (Hibbs et al., 1991; O'Toole et al., 1991), suggesting that the actin cytoskeleton could be involved in inside-out signalling to integrins (Pardi et al., 1992). Thus, the GTPase inferred from our studies could exert its effect either by binding to the cytoplasmic portion of ECM receptors themselves or by modulating ECM receptor binding to other entities, such as actin binding proteins. In this respect, it is interesting to note that GTPγS has been found to increase the association of the FMLP receptor to actin filaments in neutrophils (Särndahl et al., 1989). It is also possible however that the GTPase regulates cell-substrate adhesion by reorganizing the ECM receptors in the plane of the plasma membrane, via a redistribution of cytoskeletal components. Such a reorganization could consist of the modulation of the degree of ECM receptor clustering for example (Edelman, 1992; Gingell and Owens, 1992).

At this moment, we do not know the identity of the ECM receptor involved in GTPase-modulated adhesion. We have observed GTPγS-induced spreading of XTC fibroblasts plated plated in the absence of serum on purified vitronectin, fibronectin, laminin and collagen I (unpublished observations), indicating that a large variety of integrins or other ECM receptors could be involved, although we were not able to significantly decrease the spreading of XTC cells, GTPγS- or buffer injected, plated on Vn and Fn with GRGDSP and GRGDNP (Pierschbacher and Ruoslahti, 1987) peptides, respectively. Other adhesion assays will therefore be needed to resolve the question of receptor specificity in detail.

The identity of the GTPase involved in the control of adhesion is also unknown. Two outstanding characteristics of G-proteins are that unlike the small GTPases, (*a*) most, but not all, members of the G-protein family are ADP-ribosylated in a specific manner by either pertussis or cholera toxin (Simon et al., 1991) and (*b*) they are all activated by fluoride (Kahn, 1991). In contrast to the activation of adhesion in platelets (Shattil and Brass, 1987), we could not detect any effect of aluminium fluoride and cholera and pertussis toxins on cell spreading and ruffling (data not shown), suggesting therefore that a GTPase belonging to the ras family is involved in the control of cell-substrate adhesion. The changes in cell morphology induced by GTPγS however, are clearly distinct from those observed after injection of H-ras or rho^{val14} (Bar-Sagi and Feramisco, 1986; Paterson et al., 1990). This is to some extent surprising, as cellular ras and rho also should be activated by non-hydrolyzable GTP analogs and suggests that the GTPase inferred from our injection studies overrides the function of ras and rho either because they act downstream of these GTPases or because of quantitative differences in guanine nucleotide binding properties. Two other members of the ras family involved in the control of actin dynamics and cell morphology in *Saccharomyces cerevisiae* have been described, CDC42 and RSR1/BUD1 (Bender and Pringle, 1989; Chant and Herskowitz, 1991; Johnson and Pringle, 1990). The mammalian homologues of these GTPases have been identified as CDC42Hs and smg-p21A/rap1A/Krev-1 (Kawata et al., 1988; Kitayama et al., 1989; Munemitsu et al., 1990; Pizon et al., 1988; Shinjo et al., 1990) and provide possible candidates for the GTPase activity inferred from our results.

Phorbol esters are known to stimulate integrin-mediated adhesion in a variety of cell types, including T-lymphocytes, platelets, and macrophages (Dustin and Springer, 1989; Shaw et al., 1990; van Kooyk et al., 1989). We observed that in XTC fibroblasts TPA strongly facilitates cell rounding caused by application of GRGDSP without an effect on cell contractility, indicating a decrease in cell substrate adhesion. This is reminiscent of the situation in human keratinocytes where TPA induces differentiation, which is accompanied by a loss of cell adhesion to ECM components (Adams and Watt, 1990). As phorbol esters are thought to act via activation of PKC (Nishizuka, 1986), the opposite effects of non-hydrolyzable GTP analogs and TPA on adhesion indicate that

the GTPase-mediated pathway does not involve activation of PKC and therefore suggests the existence of at least two different pathways controlling cell-substrate adhesion in XTC fibroblasts.

Implications for the Regulation of Cell Adhesion and Motility

The results reported here demonstrate the existence of a signalling pathway involving one or more GTPases, by which the cell can regulate its adhesive properties and thus control its interaction with the environment from the inside out. Such a mechanism may be used by the cell to allow directional migration by setting up a decreasing gradient of adhesion from tip to tail (DiMilla et al., 1991; Springer, 1990). Regulation of adhesion to the ECM is thought to be involved in oncogenic transformation (Giancotti and Ruoslahti, 1990) and metastasis (Dedhar, 1990; Liotta, 1986). The central importance of a GTPase-mediated pathway in metastasis is also suggested by the observation that overexpression of 23 nm, whose gene product has NDP kinase activity, significantly reduced the metastatic potential of melanoma cells (Leone et al., 1991). Overexpression of NDP kinase could activate GTPases by increasing the cellular GTP content or by exchange of GDP on the GTPase itself (Kahn, 1991), raising the possibility that the GTPase involved in the control of cell adhesion could be a primary target of NDP kinase. A GTPase-dependent pathway may also be utilized by the cell to release ECM components at the onset of mitosis. In line with this, we have observed that GTPγS causes premature spreading of mitotic fibroblasts (unpublished observations). Investigation of this novel signaling device thus promises to yield important insights into the control of cell shape and motility.

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