Biochemical Effects of Retinoic Acid on GTP-binding Protein/Transglutaminases in HeLa Cells

STIMULATION OF GTP-BINDING AND TRANSGLUTAMINASE ACTIVITY, MEMBRANE ASSOCIATION, AND PHOSPHATIDYLINOSITOL LIPID TURNOVER

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Treatment of HeLa cells with retinoic acid (RA) gives rise to a marked stimulation in the incorporation of \([\alpha-^32P]GTP\) into an \(87-kDa\) cytosolic protein that cross-reacts with a monoclonal antibody raised against tissue transglutaminases. In the absence of RA treatment, the transglutaminase immunoreactivity elutes from a gel filtration column with an apparent size of \(600\) kDa (designated TGa), whereas following RA treatment, a second peak of transglutaminase immunoreactivity (designated TGb) is detected with an apparent size of \(150\) kDa. The TGa fractions show little or no GTP-binding or GTP hydrolytic activity and very little transglutaminase activity. However, the TGb fractions show all three activities. Retinoic acid treatment also promotes the association of the GTP-binding protein/transglutaminase with membrane fractions, as detected by Western blotting and photoaffinity cross-linking with \([\alpha-^32P]GTP\. In\. In\. In addition, the TGb fraction shows a markedly enhanced ability (relative to TGa) to associate with membranes from control (non-RA-treated) cells. The ability of the GTP-binding protein/transglutaminase to bind to membranes is correlated with the stimulation of a membrane-associated phospholipase C activity. Thus, these findings indicate that RA treatment results in a number of changes in the biochemical properties of a GTP-binding protein/transglutaminase which strongly enhance its ability to bind GTP, associate with plasma membranes, and stimulate phosphoinositide lipid turnover.

Transglutaminases (TGases) are \(Ca^{2+}\)-dependent enzymes that catalyze the formation of covalent bonds between peptide-bound glutamyl residues and the primary amino groups in a variety of compounds, including the \(\epsilon\)-amino group of lysine in certain proteins (1). These enzymes have been found in a variety of species from sea urchins to mammals as well as in a number of different cell types (2). In addition, transglutaminase activity has been detected in both extracellular and intracellular locations. A number of different members of the TGase family have been identified and characterized. These include TGase1, a membrane-associated activity that appears to be involved in the formation of the cornified cell envelope of the epidermis (3–5), TGase2 which is ubiquitously distributed, soluble enzyme implicated in apoptosis and cell adhesion (6–9), TGase3 which is soluble and like TGase1 may be involved in terminal differentiation events in the epidermis (6, 10), and the catalytic \(\alpha\) subunit of the blood-clotting Factor XIII protein which has been shown to catalyze the intermolecular cross-linking of fibrin to other fibrin molecules and to plasma proteins and to be important for wound healing (11–14).

Chues to the mechanisms that underlie the biological functions of the TGase2 enzyme have come from two lines of research. The first was the realization that TGase2 is in essence a dual function GTP-binding protein/transglutaminase. Achuthan and Greenberg (15) first reported that TGase2 activity was regulated by guanine nucleotides, and then subsequently it was demonstrated that purified TGase2 preparations were capable of hydrolyzing GTP (16). More recently, it has been shown that a GTP-binding protein/transglutaminase that shares sequence similarity with TGase2 is present in the nucleus (17) and that the TGase activity of this protein is specifically inhibited by GTP but not by GDP. It also appears that a GTP-binding protein/transglutaminase with similar characteristics is present in plasma membranes and is capable of stimulating phosphoinositide lipid turnover (18). This protein, originally named Gh (19), shares strong sequence similarity with TGase2 and appears to be responsible for mediating the coupling of the \(\alpha_1\)-adrenergic receptor to a potentially new isoform of phospholipase C (20).

A second important clue to the biochemical mechanisms that regulate TGase2 function has come from the findings that retinoic acid (RA) and other retinoids induce TGase activity in different cells (21–24). Retinoids have been shown to play a potentially important role in the regulation of cell proliferation and differentiation (23, 25, 27). The cellular effects of RA are felt to be elicited by changes in gene expression as an outcome of the interactions of RA with its cellular binding proteins and nuclear receptors (28, 29). However, the RA-induced stimulation of TGase2 activity in HeLa cells appeared to occur independently of any change in the levels of TGase2 mRNA (30), suggesting that the RA-induced stimulation of enzyme activity represented a direct effect on the TGase2 protein, rather than simply an increase in TGase2 expression. Nonetheless, it was proposed that the stimulation of TGase2 activity and the ensuing cross-linking of cellular proteins was directly related to the apoptotic activity of RA.

Our laboratory has for some time been interested in the mechanisms by which GTP-binding proteins are regulated. The tissue TGases represent a potentially new class of GTP-binding proteins where high affinity GTP-binding and GTPase activity is coupled to a possible effector (TGase) activity within the same molecule. The reported effects of RA on cellular TGase activity suggested that this differentiation/apoptotic agent...
might exert regulatory effects on the biochemical and GTP-binding activities of TGase2 enzymes. In the present study, we show that RA treatment of HeLa cells results in a variety of effects on the biochemical properties of GTP-binding protein/transglutaminases. These include a change in the elution properties of the GTP-binding protein/transglutaminase from a gel filtration column, suggestive of a change in the oligomeric state of the protein and/or in its ability to associate with other cellular proteins. This change is accompanied by stimulated GTP-binding, GTAPase activity, and TGase activity. Treatment with RA also results in the appearance of a GTP-binding protein/transglutaminase in membranes, which is accompanied by a marked stimulation of GTP-binding activity and the stimulation of phosphoinositide lipid turnover.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM t-glutamine, 100 IU/ml penicillin and streptomycin, 2.0 g/liter sodium bicarbonate, and 10 mM HEPES in humidified atmosphere with 5% (v/v) CO2 at 37 °C. 5 × 10⁵ cells/cm² were plated in fresh growth medium. The growth medium containing 5 μM RA (from a stock solution of 5 mM RA dissolved in 70% ethanol) was replaced daily. Control cell cultures were supplemented with media containing 0.07% ethanol.

**Storage**—Thawed cells were resuspended in 25 mM HEPES, pH 7.4, 25 mM NaCl, 1 mM EGTA, 100 mM Na2HPO4, 500 mM AMP-PNP, 20% (v/v) glycerol, 2 μg of protein (5%, w/v) were added. This was followed by the addition of 100 μl of 40% trichloroacetic acid. The suspension was centrifuged at 12,000 × g for 30 min and then the supernatant was collected and spun down at 100,000 × g for 30 min. The supernatant was collected and spun down 100,000 × g for 45 min. The supernatant was used as the cytosolic fraction, and the membrane pellet was resuspended in 25 mM HEPES, pH 7.4, 1% Triton X-100, 150 mM NaCl, 0.5 mM EGTA, 0.2% benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride. After incubating at 4 °C for 1 h, the extract was centrifuged at 12,000 × g for 1 h, and the supernatant was used for the studies outlined under “Results.”

**Immunoprecipitations**—The clear supernatant (200 μl) representing the cytosolic fraction was transferred to fresh microcentrifuge tubes and incubated with anti-TGase2 monoclonal antibody (CUB7402, Neomarkers, Fremont, CA) (100 μg/ml) for 2 h at 4 °C. Protein G-Sepharose (4 mg/mg) was added to the sample and incubated for 2 h at 4 °C. A parallel set of control samples containing the same amount of mouse IgG were also performed. The immunoprecipitates were pelleted, washed three times with buffer 1 (137 mM NaCl, 15.7 mM NaH2PO4, 1.5 mM KH2PO4, 2.7 mM KCl, pH 7.4, containing 1% Nonidet P-40), and twice with TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5) and finally suspended in buffer 1 (200 μl) for further experiments. For Western blots, GTAPase assays, and GTAP affinity-labeling experiments, 20-μl aliquots of the resuspended immunoprecipitates were used.

**Identification of Proteins by Western Blot Assays**—20 μl of the immunoprecipitates were diluted with an equal volume of 2 × Laemmli sample buffer and boiled for 5 min. The supernatants were subjected to electrophoresis on a 10% polyacrylamide gel with a 4% stacking gel. Proteins were transferred to 0.45 μm polyvinylidene difluoride filters (Millipore), and the filters were blocked using TBS (20 mM Tris, 137 mM NaCl, pH 7.4)/2% bovine serum albumin for 1 h. The filters were washed in TBS/0.2% Tween 20 (TTBS) three times at 15-min intervals. To identify the protein bands, the blots were exposed to primary antibody against TGase (100 μg/ml) in TTBS at 1:500 dilution and exposed to secondary antibody (anti-mouse horseradish peroxidase, Amersham Corp.) at 1:5000 dilution in TTBS/1% bovine serum albumin for 1 h. Filters were washed three times with TTBS and twice with TBS and visualized using a chemiluminescence system (ECL, Amersham Corp.).

**Gel Filtration**—The cytosolic fractions were subjected to gel filtration column chromatography on a Superdex 200 XK 16 (Pharmacia Biotech Inc.) column connected to a fast protein liquid chromatography system at a flow rate of 0.5 ml/h using TXED (50 mM Tris-LiCl, pH 7.4, containing 0.1% Triton X-100, 1 mM DTT, 1 mM EDTA, and 100 mM NaCl). Fractions of 1.5 ml each were collected. 20-μl aliquots of each fraction were subjected to Western blotting and GTAP affinity labeling.

**Transglutaminase Activity Assays**—Transglutaminase activity was measured as the Ca²⁺-dependent incorporation of [14C]putrescine (Amersham Corp.) into N,N-dimethyleasine as outlined by Nara et al. (31). Samples were added to the reaction mixtures consisting of 25 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM MgO, 1 mM KCl, 5 mM glucose, 1 mM CaCl₂, 1 mM dimethylsulfoxide, 20 mM DTT, 0.8% (v/v) glycerol, and 250 μM [¹⁴C]putrescine (0.5 μCi) in a final volume of 300 μl. The reaction mixtures were incubated for 1 h at 30 °C and then stopped by the addition of 100 μl of 40% trichloroacetic acid. The suspension was centrifuged at 12,000 × g for 30 min and then the precipitates were washed with 12% trichloroacetic acid (twice) and dissolved in 100 μl of H2O and 10 μl of 1 N NaOH. Radioactivity was measured by liquid scintillation. Transglutaminase activity was expressed as nanomoles of putrescine incorporated into casein in 1 h at 30 °C per mg of protein.

**Photoaffinity Labeling of GTP-binding Proteins**—Photoaffinity labeling of GTP-binding proteins with [α-³²P]GTP was carried out as described by Singh et al. (17). Given that TGase2 appears to be a high affinity GTP-binding protein/transglutaminase from a gel filtration column, suggestive of a change in the oligomeric state of the protein and/or in its ability to associate with other cellular proteins. This change is accompanied by stimulated GTP-binding activity and the stimulation of phosphoinositide lipid turnover.

**RESULTS**

**Retinoic Acid (RA) Stimulates the GTP-binding Activity of a Cytosolic GTP-binding Protein**—Previously, studies have shown that RA treatment of HeLa cells results in a stimulation of cellular transglutaminase (TGase) activity (30). Given that TGase2 appears to be a high affinity GTP-binding protein (15, 17–19), as well as a potential activator of phospholipase C activity (18), we set out to examine whether RA treatment has any effect on the GTP-binding activity of TGase2. The data presented in Fig. 1 show the results obtained when HeLa cells were treated with 5 μM RA (i.e. which corresponds to the time period that is necessary for RA to induce cellular changes that have been previously correlated with changes in TGase2 activity (30)). Cytosolic fractions from cells that were untreated or RA-treated were then prepared and assayed for photoconversion of [α-³²P]GTP. The results show that RA causes a strong stimulation of the incorporation of [α-³²P]GTP into two proteins with apparent molecular masses of ~87 kDa and ~66 kDa. The apparent sizes of these proteins were similar to the sizes for two nuclear GTP-binding proteins from rabbit liver that we have recently characterized.
and shown to share a high degree of sequence identity with members of the TGase2 family (17). In that study, we showed that the 36-kDa protein was likely a proteolytic fragment of the larger (~80 kDa) GTP-binding protein, and that the larger protein could be immunoprecipitated and Western-blotted with a specific anti-TGase2 monoclonal antibody. Thus, we examined whether the RA-stimulated GTP-binding activity in HeLa cells could be immunoprecipitated with this antibody.

Fig. 2A shows the results obtained when HeLa cell cytosol was incubated with the anti-TGase2 antibody, followed by precipitation with protein G-Sepharose and photoaffinity labeling with [\(\alpha^{32}\)P]GTP. Fig. 2B shows the results from a similar experiment where the pellet was resuspended and subjected to Western blot analysis using the anti-TGase2 antibody. In this experiment, we found that both the [\(\alpha^{32}\)P]GTP-labeled 87-kDa and 36-kDa proteins were immunoprecipitated with the anti-

TGase2 antibody. However, we were only able to detect the 87-kDa GTP-binding protein and not the 36-kDa protein by Western blotting with the anti-TGase2 antibody, just as was the case for the rabbit liver 36-kDa GTP-binding protein/TGase. This probably reflects an association of the 36-kDa protein with the 87-kDa protein, as was previously suggested for the nuclear 80-kDa and 36-kDa TGases (17). The RA-stimulated GTP-binding activity cannot be directly attributed to a change in the levels of expression of the 87-kDa GTP-binding protein/TGase, because Western blot analysis suggests that there are essentially identical levels of this protein in the cytosol of both untreated and RA-treated HeLa cells (Fig. 2B). This is consistent with our finding that Western blot analysis of whole cell lysates also showed no significant differences in the levels of TGase2 protein in the presence and absence of RA treatment (data not shown).

**Gel Filtration Chromatography of the GTP-binding Protein/TGase in the Presence and Absence of RA Treatment**—As a first step toward determining whether RA stimulated the association of the GTP-binding protein/TGase with (or dissociation from) some kind of regulatory protein that was responsible for catalyzing the striking changes in GTP-binding activity, we examined the behavior of the cytosolic GTP-binding protein/TGase on a gel filtration column, before and after RA treatment. In the absence of RA treatment, the cytosolic TGase2 immunoreactivity eluted at an apparent size of ~600 kDa on a Superdex gel filtration column. The peak fractions containing immunoreactivity (fractions 21–24) are shown in the lower left panel of Fig. 3. At present, we do not know whether the presence of the TGase2 molecules in these fractions is due to some degree of higher oligomerization (the keratinocyte TGase has been reported to exist as dimeric complexes (34); also, see below) and/or an association between TGase2 and other cellular proteins. However, when the cells were first treated with RA and then the cytosolic fractions were subjected to gel filtration, two peaks of TGase2 immunoreactivity were detected (Fig. 3, upper panels). One corresponded to the larger molecular mass (~600-kDa) fractions, while the second peak of immunoreactivity (fractions 60–64) corresponded to an apparent size of ~150 kDa. From here on, these two TGase2 species will be referred to as TGa (fractions 20–24) and TGb (fractions 60–64). As shown in Fig. 4 (upper right panel), TGb contained the majority of the GTP-binding activity, as measured by photoaffinity incorporation of [\(\alpha^{32}\)P]GTP, whereas the higher molecular mass TGa species showed little or no ability to bind GTP (whether generated in cells treated or not treated with RA). Likewise, the GTP hydrolytic activity was significantly

**Fig. 1.** Photolabeling of HeLa cell cytosol with [\(\alpha^{32}\)P]GTP. HeLa cells were treated with retinoic acid (RA) (+) or were untreated (−) for 3 days and then the cytosolic fractions were prepared and photolabeled as described under “Experimental Procedures.” The arrows mark the positions of proteins with apparent molecular masses of 87 and 36 kDa based on the mobility of known standards.

**Fig. 2.** Immunoprecipitation from HeLa cell cytosol with an anti-transglutaminase antibody. Aliquots (200 \(\mu\)l) from the cytosolic fractions from HeLa cells treated with retinoic acid (RA) (+) and from untreated cells (−) were immunoprecipitated with a monoclonal antibody (CUB 7402) raised against the guinea pig liver transglutaminase as outlined under “Experimental Procedures.” The proteins were first labeled with [\(\alpha^{32}\)P]GTP, electrophoresed on SDS-PAGE, and visualized by autoradiography (A) or electrophoresed, transferred to nitrocellulose, probed with the anti-transglutaminase antibody, and then visualized with ECL (Amersham) (B).
higher for TGb compared with TGa (Fig. 5). In addition, the TGb species contained virtually all of the measurable TGase2 activity, as assayed by the incorporation of [3H]putrescine into N,N-dimethylcasein. Specifically, the activity measured for TGa was 0.6 ± 0.2 nmol of putrescine incorporated per mg of protein after 30 min (room temperature) whereas the TGase activity measured for TGb was 130 ± 10 nmol of putrescine incorporated per mg of protein.

RA-stimulated Membrane Association of a Cytosolic GTP-binding Protein/TGase—Because it had been previously reported that a membrane-associated GTP-binding protein/TGase was involved in mediating hormonal stimulation of phosphoinositide lipid turnover (18), we examined whether RA treatment had any influence on the activities of a membrane-associated TGase2. The results presented in Fig. 6 show that similar to the case for cytosolic TGase2, the treatment of HeLa cells with RA resulted in a striking stimulation of the GTP-binding activities of a membrane-associated 87-kDa protein and a 36-kDa protein. However, in contrast to our findings with the cytosolic TGase2, where the levels of protein (i.e. TGase2 antibody reactivity) were essentially the same in the presence and absence of RA treatment (Fig. 2B), there was a marked difference in the amount of TGase2 immunoreactivity at 87 kDa that was membrane-associated in cells pretreated with RA (Fig. 6D).

Thus far, it has not been possible for us to definitively determine whether the RA-induced appearance of TGase2 activity in membrane fractions is the outcome of a loss of TGase2 from the...
cytosol or from other cellular compartments (e.g. the nucleus also, see below), in part, because the membrane-associated TGa2 appears to be a small percentage of the total cellular TGase2 protein. However, we set out to determine whether RA treatment of cells might cause changes in the properties of the cytosolic GTP-binding protein/TGase such that it is able to associate with membranes. To address this possibility, we used the experimental protocol outlined in the scheme shown in Fig. 7. First, HeLa cells were either treated with RA or with control buffer, and then gel filtration chromatography on a Superdex 2000 XK 16 column was performed (see “Experimental Procedures”) to isolate the TGa fraction (i.e. fractions 21–24 in Fig. 3) from the untreated cytosol and the TGb fraction (fractions 60–64 in Fig. 3) from the RA-treated cytosol. Each set of fractions was passed through a 1-ml column of DEAE-Sephalose to remove Triton X-100 (from the original gel filtration-elution buffer). The pooled TGa and TGb fractions were eluted from DEAE-Sephalose with 0.5 mM NaCl in TKM buffer (25 mM Tris-HCl, pH 7.4, containing 5 mM MgCl2, 25 mM KCl, and 0.25 mM sucrose). Fractions (0.5 ml) containing TGase2 immunoreactivity as determined by Western blot analysis were further diluted 5× by TKM sucrose solution. Membrane pellets obtained from the untreated HeLa cells were suspended in TKM sucrose buffer, and equal aliquots of the membrane suspensions (containing 100 μg of protein, each) were incubated with TGa and TGb (100 μg of total protein, each) for 30 min at room temperature. The membranes were solubilized in 1% Triton X-100 (in 25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT), and then the TGa and TGb species were precipitated using the anti-TGase2 antibody or using GTP-agarose. In each case, the resuspended precipitates were assayed for [α-32P]GTP binding and Western-blotted with the anti-TGase antibody using the anti-TGase antibody.

Fig. 8A shows the results obtained when the solubilized membrane proteins were precipitated with the specific anti-TGase2 antibody (left panel) or with GTP-agarose (right panel) and visualized by Western blotting with the specific anti-TGase2 antibody. The TGb fractions show a significantly stronger association with the membranes compared to the TGa fractions. Fig. 8B shows that similar results were obtained when visualizing by photoaffinity labeling with [α-32P]GTP. Taken together, the results indicate that RA treatment causes some kind of change in the TGase molecule such that it elutes differently (i.e. at an apparently smaller size) from gel filtration columns and is able to more effectively associate with the membrane fraction.

RA-stimulated Phosphoinositide Turnover in the Plasma Membrane Fraction—Given the suggestion that RA treatment resulted in an increased association of GTP-binding protein/TGase activity with the plasma membrane, and the earlier findings that a TGase was involved in the stimulation of phosphoinositide (PI) lipid turnover in the plasma membranes from rat liver (18), we set out to examine whether RA treatment may result in a stimulated PI turnover. The results shown in Table I indicate that this is in fact the case. Plasma membrane preparations from HeLa cells treated with RA, or from control (non-RA-treated) cells, were assayed for PI turnover by measuring the generation of [3H]inositol trisphosphate (IP3). We consistently found that the membranes prepared from RA-treated cells were capable of 2-fold higher levels of IP3 production compared with control cells. Cytosolic fractions, on the other hand, showed no appreciable difference in IP3 production, whether they were obtained from RA-treated or control cells. However, under conditions where the TGb fractions were isolated by gel filtration from the cytosol of RA-treated cells and then added to plasma membrane fractions from control (non-RA-treated) cells, an -2-fold stimulation of PI turnover was again measured. These findings suggest that there is a corre-
TABLE I

| Treatment | Source | IP₃ produced |
|-----------|--------|--------------|
| Control   | Membrane fraction | 2.1 ± 0.2 |
| RA-treated cells | Membrane fraction | 4.0 ± 0.3 |
| Control   | Cytosol | 1.5 ± 0.1 |
| RA-treated cells | Cytosol | 1.4 ± 0.15 |
| Control   | Membranes plus TGa | 2.2 ± 0.1 |
| Control   | Membranes plus TGb | 3.9 ± 0.2 |

Effects of Retinoic Acid on Transglutaminases

In the studies described above, we have demonstrated that RA treatment of HeLa cells elicits a variety of changes in the biochemical properties of a GTP-binding protein/TGase that appears to be highly related if not identical to TGase2. Among the most notable changes is a (RA-induced) stimulation of the GTP-binding activity of two proteins of apparent size 87 kDa and 36 kDa. The properties of these two GTP-binding proteins from HeLa cell cytosol are very similar to a pair of GTP-binding proteins (~80 kDa and 36 kDa) that we initially identified in rabbit liver nuclei (17) and showed to be highly similar in sequence to the tissue TGase2 protein. We proposed that the rabbit liver 36-kDa nuclear GTP-binding protein was a proteolytic fragment of the 80-kDa nuclear protein (based on microsequence analysis), such that the 36-kDa protein represented the amino-terminal half of a GTP-binding protein/TGase and included the entire GTP-binding domain. We suspect that the HeLa cell 87-kDa and 36-kDa GTP-binding proteins represent a similar case.

The RA-stimulated increase in GTP-binding activity of the cytosolic 87-kDa protein was accompanied by changes in its elution properties during gel filtration chromatography. In the absence of RA treatment, the protein eluted as part of a larger protein complex with an apparent mass of ~600 kDa (designated TGa). However, upon RA treatment, the cytosolic GTP-binding protein eluted with an apparent size of ~150 kDa (TGb). This is close to the predicted size for a dimeric version of the 87-kDa protein and may be related to the situation for the keratinocyte TGase (TGase1) which typically exists as a dimer of apparent molecular mass 190 kDa (34). Interestingly, we find that the TGb species contains virtually all of the GTP-binding activity as well as a markedly enhanced GTP hydrolytic activity and transglutaminase activity. Thus, taken together, our results would suggest that RA treatment causes a kind of change in the oligomeric state and/or state of association of the 87-kDa GTP-binding protein/TGase with other cellular proteins, such that when the GTP-binding protein/TGase dissociates from this larger molecular mass complex, it is able to bind GTP. The RA-induced change that elicits this effect could either be due to a change in the post-translational modification of the 87-kDa GTP-binding protein/TGase or of another protein that binds to the 87-kDa GTP-binding protein/TGase within the larger molecular mass complex. Such a change could be caused by an increased expression of a protein kinase or an enzyme responsible for fatty acid modification. This would be consistent with our findings that inhibitors of protein synthesis block the ability of RA to stimulate the GTP-binding activity of the 87-kDa GTP-binding protein/TGase2 and that 1–3 days are necessary for the RA-induced effects reported here. Both protein kinase C-catalyzed phosphorylation (35) and palmitoylation (and myristoylation) (36) of keratinocyte TGase have been reported. However, the RA-induced changes shown here do not appear to be simply due to alterations in the total protein levels of the GTP-binding protein/TGase because such a change is not indicated by Western blot analysis nor would it easily account for the changes in the behavior of the protein during gel filtration chromatography or for the increased ability of the TGb fractions to bind more effectively to membranes (see below).

An important mechanistic question that is raised by these findings concerns the relationship between the RA-stimulated GTP-binding activity of the GTP-binding protein/transglutaminases and the accompanying increases in GTP hydrolytic activity and transglutaminase activity. It seems most likely that the apparent increase in GTP hydrolytic activity is a consequence of the stimulated GTP-binding, rather than due to a direct effect on GTPase activity. That is, in the presence of RA, more of the 87-kDa protein is able to bind GTP and so more total GTP hydrolysis is measured. This would be exactly analogous to the situation for heterotrimeric G proteins, where it has been well documented in reconstitution studies that hormone receptors that activate a particular G protein by stimulating the exchange of tightly bound GDP for GTP also give rise to an apparent stimulation of GTP hydrolytic activity (37). However, the relationship between RA-stimulated GTP-binding activity and RA-stimulated TGase activity reported here and observed by other investigators (15, 18) is more difficult to understand, in light of previous studies (17, 18) that indicated that GTP binding significantly inhibited TGase activity. Taken together, these different findings point to a more complicated mechanism for the regulation of TGase activity in cells. GTP binding may induce a conformational change in the GTP-binding protein/TGase that enables it to undergo a GTP hydrolytic event and releases some kind of inhibitory constraint on TGase activity perhaps through the actions of a cellular regulatory factor (also, see below).

Another important outcome of RA treatment of HeLa cells is the appearance of the 87-kDa GTP-binding protein/TGase in membrane fractions. Although we have not been able to definitively determine that the membrane-associated pool of the 87-kDa protein represents a translocation from the cytosol, we have found that the cytosolic 87-kDa protein from RA-treated cells, which elutes as an apparent dimer from gel filtration columns (i.e. the TGb species), shows a markedly enhanced capability to associate with membranes (relative to the 87-kDa protein from control, non-RA-treated cells). Thus, perhaps the simplest interpretation is that whatever (RA-stimulated) change in the 87-kDa protein that enables it to dissociate from a larger molecular mass complex and to bind GTP more effectively, also enables it to bind to membranes. This may simply reflect the dissociation of the 87-kDa protein from other proteins that tend to maintain it in the cytosol.

We also have found that under conditions where the 87-kDa protein is able to associate with membranes, there is a stimulation of phospholipase C activity as measured by the production of [³²P]inositol trisphosphate. Previously, it had been reported that a GTP-binding protein with sequence similarity to TGase2 was able to mediate hormonal stimulation of phospholipase C activity in rat liver plasma membranes (18). Thus, we would suggest that the RA-promoted increase in the membrane association of the 87-kDa protein provides this GTP-binding protein with proximity to one of its target/effector molecules.

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8 U. S. Singh, unpublished data.
e.g. a member of the phospholipase C family. The identity of the specific isoform of this family of enzymes which is responsible for mediating the RA-stimulated phosphoinositide lipid turnover reported here has not yet been determined. However, recent studies suggest that the GTP-binding protein/TGase activity originally designated G6 is capable of specifically stimulating phospholipase C enzymes and phosphoinositide lipid turnover has been reported on a number of pathways as well as overall membrane structure (1), is responsible for RA-promoted differentiation and/or apoptosis (23, 25, 27, 30). However, among a number of important future questions are whether the biochemical changes in the GTP-binding protein/TGase stimulated by RA are entirely directed toward changes in phosphoinositide lipid turnover, and, if so, what consequences does this have in the nucleus with regard to the 80-kDa GTP-binding protein/TGase that appears to be present in the nuclear envelope and pore/lamina fractions (17). Although the presence of phospholipase C enzymes and phosphoinositide lipid turnover has been reported in the nucleus (38), thus far, we have not found any significant stimulation of this nuclear activity by RA. This then raises the possibility that there may be other important target-effector molecules for GTP-binding protein/TGases (i.e. in addition to phospholipase C enzymes) that may play a role in mediating the various biological responses associated with RA treatment of cells. Related to these issues is the question of the identities of the regulatory proteins that mediate the effects of RA on the different activities of GTP-binding protein/TGases and whether it will be possible to purify one or more of these regulators from the higher molecular mass complexes eluted from gel filtration columns.

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