K-ras Transformation Greatly Increases the Toxin-dependent ADP-ribosylation of GTP Binding Proteins in Thyroid Cells

IN VOL VEMENT OF AN INHIBITOR OF THE ADP-RIBOSYLATION REACTION*

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The GTP binding (G) proteins of normal (FRTL5) and ras-transformed thyroid cells (KiKi) were characterized by cholera and pertussis toxin-induced ADP-ribosylation and immunoblot analysis. Two pertussis toxin substrates with molecular masses of 40 and 41 kDa were identified in normal cells as the α2 and α3 subunits. The molecular masses of the cholera toxin substrates were 42 and 45 kDa. The same cholera and pertussis toxin substrates were present in the K-ras-transformed cell line. However, the toxin-dependent ADP-ribosylation was markedly higher in KiKi than in normal cell membranes (more than 50-fold). The reason for this difference was investigated; it could not be explained by the relative amounts of G proteins in the two cell systems, since the levels of α2 subunit as measured by quantitative immunoblot in K-ras-transformed cells were only slightly (65%) higher than in normal cells. The difference in ADP-ribosylation was not due to poly-ADP-ribosylation nor to a different degree of subunit dissociation of G proteins in the two cell lines. Rather, the enhanced ADP-ribosylation in K-ras-transformed cells appears to be due to the loss of an inhibitory factor present in the normal cells. Partial characterization indicates that such a factor is a peripheral membrane protein of <25 kDa capable of directly interfering with the ADP-ribosylation reaction.

A large number of receptors for neurotransmitters, hormones, and growth factors are coupled to GTP binding (G) proteins, which modulate membrane-associated effector systems responsible for the formation of second messengers. G proteins are heterotrimers which are believed to dissociate upon receptor activation into α and βγ subunits (1-4). There are several classes of G proteins (Gs, Gi, Go, Td) which are derived from a large gene family (1-4). The G protein α subunits bind GTP with high affinity, have an intrinsic GTPase activity, and some α subunits are substrate for ADP-ribosylation by cholera and/or pertussis toxin (1-4). The β and γ subunits are tightly associated with each other and may serve to anchor the heterotrimer to the plasma membrane (5-8).

In a thyroid cell line (FRTL5) which maintains the differentiated function of the original tissue, G protein-coupled receptors (such as thyrotropin, adrenergic, purinergic, and muscarinic receptors) modulate the cellular activity by interacting with effector enzymes such as phospholipase C, phospholipase A2, and adenylyl cyclase (9-14). When these cells are transformed by the k-ras oncogene (KiKi cells) they lose the thyrotropin dependence of growth and other markers of differentiation (15, 16). We have previously analyzed the signal transduction pathways in these cells and shown that although basal adenylyl cyclase and phospholipase C activities are not affected by ras-transformation, there is a significant increase in the activity of a phosphoinositide-specific phospholipase A2 (17, 18). Moreover, the hormones regulating normal cells become unable to stimulate effector enzyme activity in transformed cells. In the latter cells, thyrotropin, norepinephrine, and carbachol do not stimulate phospholipase A2, phospholipase C, and adenylyl cyclase.

Since the above observations suggest the possibility of a G protein defect, we have characterized the G proteins present in normal and ras-transformed cells and evaluated their sensitivity to pertussis and cholera toxin. We show that in ras-transformed cells the G proteins are more abundant and their toxin-induced ADP-ribosylation is greatly increased. This increase appears to be due to the loss of an inhibitory factor active in normal cells.

MATERIALS AND METHODS

Hormones used in the tissue culture media, Coon's modified Ham's F-12 medium, and pertussis toxin were obtained from Sigma. Tissue culture materials were from GIBCO. Cholera toxin was from Calbiochem. GTP and ATP from Boehringer Mannheim (Federal Republic of Germany). Superose 12 HR 10/30 size exclusion column was from Pharmacia. [3H]NAD was purchased from Du Pont-New England Nuclear (Federal Republic of Germany). All chemicals were obtained from commercial sources as the highest purity material available.

Cell Culture—FRTL5 cells are a continuous line of differentiated epithelial cells derived from normal Fisher rat thyroids (obtained from F. S. Ambesi-Impicabat), their growth conditions have been detailed elsewhere (19-20). The KiKi cells, which are derived from FRTL5 cells upon infection and transformation with kiMSV-kMuLV and express high level of ras p21, were cultured as described (15, 16).

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Membrane Preparation—FRTL5 and KiKi cell membranes (21), HL-60 and S49 membrane preparations (22), and human brain membranes (23) were prepared as described. Protein concentration was measured by a modification of the Lowry procedure (24). In all experiments the relative amounts per sample are expressed as micrograms of membrane protein, in good correlation with the data number of FRTL5 and KiKi cells, since comparable numbers of cells yield the same amount of membrane proteins.

**ADP-ribosylation Assay**—ADP-ribosyltransferase activity was measured by following the incorporation of [32P]ADP-ribose into membrane components. 50 µg of cell membrane proteins were incubated at 27 °C for 60 min (unless otherwise specified) in a volume of 50 µl containing 10 mM Tris/HCl (pH 7.5), 25 mM dithiothreitol, 0.5 mM EDTA, 1 mM ATP, 0.1 mM GTP, 3.5-5 x 10^6 cpm adenylic [32P]NAD (Du Pont-New England Nuclear, specific activity 800 Ci/mmole) and, where indicated, 10 µg/ml activated pertussis toxin. The toxin was preactivated immediately before use for 1 h at room temperature in 62.5 mM dithiothreitol. When the experiments were performed in the presence of cholera toxin, membranes were instead incubated in a volume of 50 µl containing 100 mM potassium phosphate buffer (pH 7.5), 2.5 mM MgCl_2, 1 mM ATP, 10 mM thymidine, 10 µg/ml activated cholera toxin, and 3.5-5 x 10^6 cpm adenylic [32P]NAD (Du Pont-New England Nuclear, specific activity 800 Ci/mmole) and, where indicated, 10 µg/ml activated pertussis toxin. The toxin was preactivated immediately before use for 1 h at room temperature in 62.5 mM dithiothreitol.

The assay was stopped by diluting the sample with 50 µl of sample buffer. The samples were immediately boiled for 5 min and analyzed on a 8% SDS-polyacrylamide, 4.5 % gels (26). All gels were run in phosphate buffer, pH 6.8, 0.125% SDS, 5% glycerol, and 0.001% bromophenol blue. The gels were dried and exposed to X-ray film. The density of the radiolabeled bands was measured by an LKB ultra scan-XL densitometer equipped with an integrated densitometer. The gels were stained with Coomassie blue R-250 to identify the pertussis toxin substrates after separation by SDS-PAGE.

**Immunoblotting Analysis**—Proteins were transferred from gel to nitrocellulose with constant current (125 mA) for 30 min in TBS with 2% gelatin and for 12-24 h in the same buffer containing 1% gelatin and 1:200 dilution of rabbit antiserum. Immunoblotting was performed using Kodak X-AR films. The density of the radiolabeled bands was measured using a radiodensity measurement system equipped with an integrated densitometer.

**Size-exclusion Chromatography**—FRTL5 and KiKi membranes were resuspended at a concentration of 1 mg of protein/ml in 20 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 2 mM KCl, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 0.1 mM phenethylmethylsulfonyl fluoride. The suspension was stirred at 4 °C for 60 min. 0.2 ml of the KCl extracts obtained after centrifugation at 20,000 x g for 30 min were applied at a flow rate of 0.4 ml/min to a Superose 12 HR 10/30 size exclusion column (Pharmacia) equilibrated with 20 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 0.15 M KCl. Fractions of 0.4 ml were collected. The protein elution pattern was evaluated by the absorbance at 254 nm. The column was calibrated with the following molecular mass standards (Pharmacia): aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), ribonuclease (13.7 kDa), and apropin (6.5 kDa). The void volume of the column was determined with blue dextran.

**RESULTS AND DISCUSSION**

Fig. 1A shows that two proteins of 40 and 41 kDa, respectively, are substrates of the ADP-ribosyltransferase activity of pertussis toxin in FRTL5 cell membranes. The ADP-ribosyltransferase activity of cholera toxin reveals two substrates of 42 and 45 kDa, as reported previously (Fig. 2A) (21).

Antibodies specific for different α subunits were employed to identify the pertussis toxin substrates after separation by urea/SDS-polyacrylamide gel electrophoresis (Fig. 1B) (26). The antisera AS 58, raised against the carboxy-terminal sequence of transducin α, which recognizes transducin α, α2, and α3, revealed a single band comigrating with the 40 kDa pertussis toxin substrate. The same band was also recognized by the antibody R 230, which is specific for α3 (Fig. 1B). Thus, the 40-kDa pertussis toxin substrate appears to be α3. The second substrate did not react with the antibody AS 58 which is strongly reactive with α1, indicating that it could not correspond to α1 (Fig. 1B). Moreover, it did not comigrate with the brain α3 and α2 (Fig. 1A, for identification of the α subunits see Refs. 4 and 33), nor was it recognized by an antibody specific for the α3 subunit (RV3, data not shown). The data indicate that the 40- and 41-kDa pertussis toxin substrates of FRTL5 cells correspond to α3 and α2. The α3 subunit of FRTL5 comigrates with the α3 subunit of S49

![Fig. 1. A.](image-url)

**Fig. 1.** A. [32P]ADP-ribosylation by pertussis toxin of membrane proteins from FRTL5 cells compared with those from S49 cells, HL60 cells, bovine brain, and human brain. Samples were analyzed by SDS-PAGE and autoradiography. Only the region of ~40 kDa is shown. The apparent molecular masses are indicated. Membranes were prepared as described under "Materials and Methods." B, immunoblots of G proteins from HL-60, FRTL5 cells, and rat brain membranes. Membrane proteins were subjected to SDS-PAGE and then blotted onto nitrocellulose and immunostained as described under "Materials and Methods." Similar results were obtained in at least ten separate experiments. See text for details.
cells; both subunits, however, seem to migrate slightly slower than the HL 60 cell $\alpha_\alpha$ subunit (Fig. 1A; Ref. 26). This small discrepancy in the apparent molecular mass of the $\alpha_\alpha$ subunits of the three cell systems could be due to the differences in amino acid sequence between rat and human $\alpha_\alpha$ (34).

Multiple isoforms of $\alpha_\beta$ and $\beta_\gamma$ proteins have been demonstrated in membrane preparations of bovine, canine, and porcine thyroid (35, 36). As in other cellular systems, these may represent functionally different $G$ proteins involved in transducing receptor signals into the activation of effector enzymes. We have previously identified two effector enzymes whose activity is controlled by $G$ proteins highly sensitive to pertussis toxin in FRTL5 cells, namely phospholipase A$_2$ (coupled to $\alpha_\beta$-adrenergic, muscarinic, and thyrotropin receptors) and phospholipase C (coupled to an inhibitory muscarinic receptor) (10–14). It could thus be proposed that the $\alpha_2$ and $\alpha_3$ subunits activate these signal pathways in FRTL5 cells. A more precise assignment of each a subunit to either phospholipase A$_2$ or phospholipase C, however, requires further work.

Since the coupling between receptors and effector enzymes is altered in transformed cells, we have examined whether the expression or the function(s) of $G$ proteins might be in some way modified as a consequence of K-ras-induced transformation. ADP-ribosylation experiments similar to those described above were performed using membranes prepared from the K-ras-transformed cell line (KiKi cells). The $G$ proteins of these cells comigrated with those of the normal FRTL5 cells in SDS gels, indicating that the same $G$ proteins are expressed in normal and transformed cells (Fig. 2, A–C). A major difference, however, was observed in the degree of ADP-ribosylation of the $\alpha_\alpha$ and $\alpha_\beta$ subunits between KiKi and normal cell membranes (Fig. 2, A and B): the labeling of the $\alpha_\alpha$ subunit by pertussis toxin in membranes from KiKi cells was enormously enhanced (>50-fold increase; 1 $\mu$g of membrane proteins from transformed cells was labeled to a higher degree than 50 $\mu$g of membrane proteins from FRTL5 cells) (Fig. 2, A and C, lanes 4 and 10). The origin of this difference was analyzed in a series of experiments.

The $\alpha$ subunits in the two cell systems were quantitated by Western blot analysis using antibody AS 58. In a series of eight experiments employing different concentrations of membrane proteins (50, 100, 150, and 200 $\mu$g) the $\alpha_2$ subunit was found to be higher by 65 ± 12% (mean ± S.E.) in KiKi cells than in FRTL5 cells. This difference, however, could not explain the ~50-fold increase in the pertussis toxin-dependent ADP-ribosylation (Fig. 2, lanes 4 and 10). Another possible reason for the increased ADP-ribosylation could be a different degree of dissociation of the $G$ proteins into $\alpha$ and $\beta_\gamma$ subunits in normal and in ras-transformed cells; this would alter toxin-induced ADP-ribosylation, since the heterotrimeric $\alpha_\beta_\gamma$ form of the $G$ protein is the preferred substrate for ADP-ribosylation (37, 38). To test this possibility, an excess of purified $\beta_\gamma$ subunit (0.5 $\mu$g, which represented 1% of the total proteins in the assay) was added to the ADP-ribosylating mixture. Under these conditions the labeling of $\alpha_\alpha$ subunits of normal and transformed cell membranes was slightly increased, but the difference between the two types of membranes was not altered (Fig. 3A).

The possibility of a poly-ADP-ribosylation reaction taking place in ras-transformed cells was also examined by carrying out experiments in the presence of the inhibitor thymidine (10 mM) (39). This agent, however, did not affect the reaction (data not shown). A further possible explanation was that the amount of NAD available for the reaction might be decreased by an NAD-glycohydrolase acting in normal but not in transformed cells. The availability of NAD in the reaction mixture was evaluated by thin layer chromatography after 5 min of incubation, a time at which a clear difference in $G$ protein ADP-ribosylation by pertussis toxin was already very marked between normal and ras-transformed cells (Fig. 3B). A similarly low extent of NAD degradation was observed in the two systems (Fig. 3C). Moreover, inhibitors of NAD-glycohydrolase, such as imidazole, isoniazide, and nicotinamide at concentrations reported to inhibit the enzyme (0.4–20 mM; Refs. 40 and 41) did not affect the labeling of $\alpha_\alpha$ and $\alpha_\beta$ proteins by pertussis and cholera toxin both in normal and transformed cell membranes (data not shown).

De-ADP-ribosylating enzymes capable of hydrolyzing ADP-ribose from the $G$ protein as well as endogenous inhibitors of the ADP-ribosylation reaction have been described and partially purified (42, 43). We excluded that a de-ADP-ribosylating enzyme active only in normal cells could cause the difference in pertussis toxin-dependent ADP-ribosylation of $G$ proteins when the FRTL5 cell membranes were added to ADP-ribosylated KiKi cell membranes, only a slight decrease (~20%) in the $G$ protein labeling was observed (data not shown). Such a decrease is not sufficient to explain the 50-fold difference in the ADP-ribosylation of $G$ proteins between the two cell systems.
ADP-ribosylation of G Proteins in Transformed Cells

Other possible mechanisms of modulation of the ADP-ribosylation reaction could result from factors directly affecting the ADP-ribosylating enzyme (42–45). For instance, the cholera toxin-induced ADP-ribosylation of Gs is enhanced by the small GTP-binding protein ARF (ADP-ribosylating factor) (42, 46). To address this possibility, we performed the ADP-ribosylation assay on mixtures of normal and transformed cell membranes. Fig. 4 shows that the ADP-ribosylation of the αs subunits in the ras-transformed cell membranes was greatly decreased by the addition of normal cell membranes (compare lanes 4 and 5). The inhibitory effect was dependent on the amount of normal membranes added to the reaction mixture; a dose-response is shown in Fig. 5. In order to compare the inhibitory activity present in normal and ras-transformed cells, we used transducin, a G protein purified from bovine retinas, as substrate of the pertussis toxin-dependent ADP-ribosylation reaction. Fig. 6 shows that this reaction was inhibited by the normal cell membranes, whereas it was not affected by addition of the ras-transformed cell membranes. These experiments indicate that the normal cell membranes contain a factor able to inhibit the ADP-ribosyl-
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Fig. 6. [³²P]ADP-ribosylation by pertussis toxin of membrane proteins from FRTL5 (lane 1), KiKi cells (lane 2), and of transducin (lane 3). The other lanes show mixed experiments where 50 μg of membrane proteins from FRTL5 (lanes 4 and 6) and from KiKi (lanes 5 and 7) were added to the reaction mixture containing 0.5 μg of purified transducin. Membrane proteins of the two cell types used in the lanes 6 and 7 were pretreated at 60 °C for 5 min. Samples were analyzed by SDS-PAGE and autoradiography. The apparent molecular masses are indicated. Similar results were obtained in at least three separate experiments.

Fig. 7. [³²P]ADP-ribosylation by pertussis toxin of 50 μg of membrane proteins from KiKi cells in the presence of 30 μl of fractions obtained from size-exclusion chromatography of an extract of FRTL5 (A) and KiKi (B) cell membranes. The ADP-ribosylation reaction was carried on for 20 min. The gel filtration column was equilibrated and calibrated as described under "Materials and Methods." 0.2 ml of a KC1 extract (obtained from a 4 mg/ml membrane suspension) were analyzed. The absorbance trace obtained in parallel showed similar pattern of protein distribution. Samples were analyzed by SDS-PAGE and autoradiography. The control sample (i.e. 50 μg of membrane proteins from KiKi cells ADP-ribosylated in the presence of 30 μl of elution buffer) is also shown (dash). The apparent molecular masses are indicated. Similar results were obtained in at least four separate experiments.

Addition of G proteins which is either absent or inactive in ras-transformed cells.

Pretreatment of membranes from normal cells at 60 °C for 5 min abolished the inhibitory activity, suggesting that this factor might be a protein (Fig. 6). Crude membrane preparations (see "Materials and Methods") or membrane that were purified over a Ficoll cushion (47) had a similar ability of inhibiting the pertussis toxin-dependent ADP-ribosylation of G proteins in ras-transformed cells (data not shown). Since it is unlikely that cytosolic components contaminate the membrane preparation under these conditions, we suggest that the inhibitory factor is membrane-associated. To investigate this point further, we analyzed the inhibitory activity by extracting the FRTL5 and KiKi cell membranes with 2 M KCl and applying the extract to a gel filtration column (see "Materials and Methods"). The effects of the different fractions obtained from the FRTL5 and KiKi cell extracts on G proteins ADP-ribosylation are summarized in Fig. 7. Inhibitory activity was recovered in three separate fractions of the FRTL5 cell extract eluate (number 24, 38, and 45) which correspond to >120, ~40, and <25 kDa, respectively (Fig. 7A). The extract of KiKi cell membranes produced results in part different from those obtained with normal cells (Fig. 7B). Fraction 45, which yielded identical absorbance values in the two systems, was either devoid of any inhibitory activity or was partially active (in one case it produced a 10–20% inhibition of G proteins ADP-ribosylation) (an example is shown in Fig. 7B). Inhibitory activity similar to that of the FRTL5 cell extract was recovered from fraction 38 (20–50% inhibition of the G protein ADP-ribosylation; a 30% inhibition is shown in the example of Fig. 7B), and fraction 24 was partially active (0–40% inhibition of the G protein ADP-ribosylation). No additional active fraction was recovered from the KiKi cell membrane extract (Fig. 7B). We therefore conclude that the inhibitory factor present in fraction 45 of the FRTL5 cell extract is greatly reduced (most probably in specific activity) in the KiKi cell membranes.

The mechanisms involved in the inhibition of the ADP-ribosylation of G proteins by the fractions 24, 38, and 45 of the cell membrane extracts were investigated. As shown in Fig. 8, fraction number 24 (>120 kDa) was the only one able to hydrolyze NAD. A NAD-glycohydrolase activity has been previously identified in thyroid cells (40). We can therefore conclude that a NAD-glycohydrolase is associated with the FRTL5 cell membranes; however, other proteins with different mechanism of action participate in modulating the ADP-ribosylation reaction (Figs. 7 and 8). We examined whether a de-ADP-ribosylating activity (which would rapidly hydrolyze
transformed cells, we suggest that the activity present in fraction 45 is the one involved in the different regulation of the two systems.

We investigated further the possible mechanism of action of this inhibitory molecule. One possibility is that this factor could act on the S1 subunit of pertussis toxin or on the A1 subunit of cholera toxin. These subunits share significant homologies, particularly in domains believed to be involved in NAD binding and transfer (49, 50). It is conceivable that an inhibitory component could exist in normal membranes that would interact with these homologous domains, thus interfering with its activity. According to our experimental evidence, this interaction is unlikely since preincubation of pertussis toxin for 1 h with the FRTL5 normal membranes did not affect the toxin enzymatic activity on the transformed cell membranes (in the absence of the normal membranes) (data not shown). It cannot be excluded, however, that there is a transient interaction between the inhibitory factor and the homologous domains of the two toxins. In this case, the two components would be separated under our experimental conditions, which would lead to a loss of toxin inhibition in the second incubation. Another possibility is that the inhibitory factor could interfere with the toxin-dependent ADP-ribosylation by modifying the G protein-lipid interaction, thereby reducing the availability of G proteins as substrates of the reaction (42, 45). In thyroid cells it has been reported that thyrotropin decreases the pertussis toxin-dependent ADP-ribosylation of Gi, probably by reducing the accessibility of the α subunit (21, 49). The interaction between G proteins and lipids in the plasma membranes may play a role in the modulation of the ADP-ribosylation reaction; this is also suggested by the fact that the addition of detergents in different cell systems increases the ADP-ribosylation of G proteins by pertussis and cholera toxin (51–53). However, the inhibitory factor present in FRTL5 cells is active on the ADP-ribosylation of purified transducin which is a protein soluble in aqueous buffers and should be freely accessible to the toxin even in the absence of detergent (data not shown). The extent of inhibition of the pertussis toxin-dependent ADP-ribosylation of purified transducin was comparable with that exerted on the KiKi cell membranes under these conditions (80% inhibition upon 1-h incubation with 50 μg of FRTL5 cell membrane proteins). This indicates that the inhibitory factor could prevent the ADP-ribosylation by directly acting on the G protein. Experiments are in progress to evaluate the possible physical interaction between G proteins and the inhibitory factor.

In summary, we find that G proteins of FRTL5 thyroid cells become a better substrate of toxin-dependent ADP-ribosylation upon transformation by the ras oncogene. This change appears to be related to inactivation or loss of a factor able to inhibit the ADP-ribosylation reaction. Whether this inhibitor directly interacts with G proteins or it alters toxin activity remains to be clarified. Experiments are in progress to further characterize this inhibitor. The information obtained will be useful in addressing the question of the causal relationship between ras-induced transformation and the altered ADP-ribosylation of heterotrimeric G proteins.

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