2492–2498 Nucleic Acids Research, 1995, Vol. 23, No. 13

Activation of transfer RNA-guanine ribosyltransferase by protein kinase C

Rana C. Morris, Bonnie J. Brooks, Panayota Eriotou, Deborah F. Kelly, Sandeep Sagar, K. Lenore Hart and Mark S. Elliott*

Old Dominion University, Department of Chemistry and Biochemistry, Norfolk, VA 23529, USA

Received March 16, 1995; Revised and Accepted May 23, 1995

ABSTRACT

Transfer RNA-guanine ribosyltransferase (TGRase) irreversibly incorporates queuine into the first position in the anticodon of four tRNA isoacceptors. Rat brain protein kinase C (PKC) was shown to stimulate rat liver TGRase activity. TGRase preparations derived from rat liver have been observed to decrease in activity over time in storage at –20 or –70°C. Contamination of the samples by phosphatases was indicated by a *p*-nitrophenylphosphate conversion test. The addition of micromolar concentrations of the phosphatase inhibitors sodium pyrophosphate and sodium fluoride into TGRase isolation buffers resulted in a greater return of TGRase activity than without these inhibitors. Inactive TGRase preparations were reactivated to their original activity with the addition of PKC. In assays combining both TGRase and PKC enzymes, inhibitors of protein kinase C (sphingosine, staurosporine, H-7 and calphostin C) all blocked the reactivation of TGRase, whereas activators of protein kinase C (calcium, diacylglycerol and phosphatidyl serine) increased the activity of TGRase. None of the PKC modulators affected TGRase activity directly. Alkaline phosphatase, when added to assays, decreased the activity of TGRase and also blocked the reactivation of TGRase with PKC. Denaturing PAGE and autoradiography was performed on TGRase isolates that had been labelled with ^{32}P by PKC. The resulting strong 60 kDa band (containing the major site for phosphorylation) and weak 34.5 kDa band (containing the TGRase activity) are suggested to associate to make up a 104 kDa heterodimer that comprises the TGRase enzyme. This was corroberated by native and denaturing size-exclusion chromatography. These results suggest that PKC-dependent phosphorylation of TGRase is tied to efficient enzymatic function and therefore control of the queuine modification of tRNA.

INTRODUCTION

There are a large number of modified nucleosides found in transfer RNA (tRNA) molecules. For the most part, these modified nucleosides have undefined functions in the molecular physiology of the cell. Yet, various individual members of this group of modifications have been suggested to play important roles in the function of tRNA. One specific example is the queuosine modification of tRNA. The pre-formed base, queuine, is incorporated into tRNA by an irreversible post-transcriptional exchange of queuine-for-guanine in the first position of the anticodon of four tRNA isoacceptors (aspartyl, asparaginyl, tyrosyl and histidyl-tRNAs) (1–4). This unique base exchange reaction is catalyzed by the enzyme tRNA-guanine ribosyltransferase (TGRase) (EC 2.4.2.29) (3,4).

A great deal of research regarding the function of queuine in tRNA has been undertaken. The overall picture that emerges positions queuosine-modified tRNA as a controlling agent in the differentiation, development and stress management of several cell types. Alterations in the levels of queuosine-modified tRNA have been observed during differentiation and development for *D.discoideum* (5), plants (6) and *Drosophila* (7–9), and during development and aging in the rat (10). Queuosine-modified tRNA in *E.coli* appear to protect the organism from stress invoked by suboptimal growth conditions (11). Queuosine-modified tRNA also appears to be involved with lactate dehydrogenase (12,13) and cytochrome b_{559} (14) expression in mammalian systems, and therefore may be involved with management of oxidative stress in eukaryotic cells.

Transfer RNA isolated from neoplastic tissues and transformed cell lines is queuosine-hypomodified to various degrees (2,15,16). The degree of hypomodification has been related to the staging toward malignancy in human lymphomas and leukemias (17), and in lung (18) and ovarian tumors (19). Queuosine-deficient murine erythroleukemia cells demonstrated a significant increase in tRNA queuosine levels when forced to differentiate (20). The differentiation of these cells and the corresponding increase in queuosine level were effectively blocked by chronic treatment with the tumor promoter TPA (20). These data suggest that undermodification of tRNA with queuosine is related to growth enhancing or tumor promoting events.

Chronic exposure of the phorbol ester tumor promoter PDD to cultured normal human fibroblasts induced a large and transient inhibition of queuosine modification levels in tRNA (21). The decrease in queuosine content of tRNA always occured immediately before an increase in the population density of the cultures

* To whom correspondence should be addressed

(21). Concurrent addition of exogenous queuine effectively blocked the PDD-induced increase in population density, and maintained the queuosine modification at near normal levels. In addition, it was demonstrated that PDD was unable to directly inhibit TGRase preparations *in vitro* (22). These data imply an indirect mechanism of action for phorbol esters on TGRase, queuosine modification of tRNA, and their relationship to cell growth.

Mammals are incapable of synthesizing their own queuine, and must obtain it from their diet or gut flora (23,24). Mammalian cells grown in culture obtain queuine from animal serum used to supplement the growth media (3,25). This suggests that efficient cellular uptake of dietary queuine across the cell membrane is a prerequisite for the insertion of the base into tRNA by TGRase. It was demonstrated that cellular uptake of queuine is sensitive to phorbol esters (22,26), and more recently that queuine uptake is modulated by protein kinase C (PKC) activity (27). Activators of PKC-stimulated queuine uptake in cultured human fibroblasts, whereas inhibitors of PKC reduced queuine uptake to a measurable base-line level (27).

Chronic exposure of cultured cells to phorbol esters has been shown to induce a down-regulation of PKC (28). We have observed a decrease in the queuosine-modification of tRNA with chronic exposure of PDD to human fibroblasts, and a reversal of this phenomenon by concurrent addition of exogenous queuine (21). If PKC levels were diminished in cells chronically exposed to PDD, we would expect a decrease in the queuine uptake rate that would reduce substrate availability for TGRase. However, since there is a base-line level of uptake for queuine, it is unlikely that the profound level of queuosine-hypomodification seen in cultured cells is due to partial substrate depletion alone. Therefore, this study was undertaken to address the possibility that PKC may also be exerting a modulatory effect directly on TGRase activity.

MATERIALS AND METHODS

Materials

Yeast tRNA and PKC purified from rat brain was purchased from Boehringer Mannheim. ATP (biotech grade) was purchased from Fisher Scientific. Sphingosine was purchased from Biomol Research Laboratories. ^{3}H-guanine (1 mCi, 168 Ci/mmol) and [γ-^{32}P]ATP (250 μCi, 3000 Ci/mmol) were purchased from Amersham Corporation. H-7 was purchased from Seikagaku America, Inc. All other chemicals or reagents were purchased from Sigma Chemical Company.

Buffers

Homogenization buffer. Twenty mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$, 1.0 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.05% Triton X-100 and 10 μg/ml of the following protease inhibitors: aprotinin, calpain inhibitor I, chymostatin, leupeptin, pepstatin, phenylmethylsulfonylfluoride.

Phosphate buffer. Twenty mM sodium phosphate, pH 7.2, 0.5 mM dithiothreitol, 1 mM $MgCl_2$ and 10% glycerol.

Storage buffer. Twenty mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM EDTA and 50% glycerol.

Mono-Q buffer. Ten mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$ and 0.5 mM dithiothreitol.

Reaction buffer. Ten mM Tris-Cl, pH 7.5, 35 mM KCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol.

Denaturing sample buffer. Ten mM Tris-Cl, pH 7.4, 0.5% SDS, 20% glycerol and 0.01% bromophenol blue.

Isolation of TGRase from rat liver

The purification of TGRase was performed by adaptation of previously published methods (29). Five adult Sprague–Dawley rats were sacrificed by cervical dislocation and their livers were rapidly excised and rinsed in ice-cold homogenization buffer. The tissue was homogenized in 100 ml buffer, filtered through two layers of sterile gauze and centrifuged at 10 000 *g* for 30 min at 4°C. The resulting supernatant was centrifuged at 100 000 *g* for 1 h at 4°C. The supernatant was decanted through sterile gauze, brought to a final volume of 300 ml in homogenization buffer and loaded by gravity feed onto a 13 × 5 cm DE-52 ion exchange pre-equilibrated with homogenization buffer. A 2 l wash of homogenization buffer lacking the protease inhibitors and Triton X-100 was drawn through the column by low pressure vacuum using a water-aspirator. TGRase was eluted from the column with 1 l of homogenization buffer containing 0.2 M KCl. Protein in the 0.2 M KCl fraction was precipitated by adding ammonium sulfate to a final saturation of 55%. The solution was stirred on ice for 30 min and then allowed to stand for an additional 30 min. The protein precipitate was collected by centrifugation for 20 min at 10 000 *g*.

The precipitate was redissolved in 25 ml phosphate buffer, and dialyzed against 4 l of the buffer overnight at 4°C with at least two changes of buffer. Denatured precipitate in the dialysate was removed by centrifugation at 10 000 *g* for 20 min, and the supernatant was collected and applied to a P-11 phosphocellulose column (2 × 10 cm) which had been pre-equilibrated at 4°C with phosphate buffer. The phosphocellulose column was washed with 100 ml of phosphate buffer at a flow rate of 1.0 ml/min. The sample was eluted with a 200 ml 0–1.0 M KCl gradient in phosphate buffer. Fractions were collected and tested for the capability of ^{3}H-guanine exchange with yeast tRNA. Those fractions that resulted in ^{3}H-guanine incorporation were judged to contain TGRase activity. The pooled TGRase-active fractions from the 0.25 to 0.30 M KCl region of the gradient were either stored at sub-zero temperatures (–20 or –70°C), or further purified using a 1 × 10 cm Pharmacia Mono-Q ion exchange column.

Samples to be stored were dialyzed against storage buffer for ≥ 3 h and then stored in 1 ml aliquots at either –20 or –70°C. Samples to be purified further by Mono-Q FPLC ion exchange chromatography were concentrated to 5 ml in an 50 ml Amicon pressure cell using a YM 10 membrane (10 000 MWCO) under 40 p.s.i. N_2 gas. The sample was then diluted to 50 ml with Mono-Q buffer and reconcentrated to 5 ml.

Five to ten milligrams of protein (as determined by Bradford assay) in 1.0 ml aliquots of the TGRase preparation were injected onto a pre-equilibrated 1 × 10 cm Mono-Q column. The column was eluted at a flow rate of 1.0 ml/min with a 100 ml 0–0.5 M KCl linear gradient in Mono-Q buffer. The fractions collected were assayed for TGRase activity. The protein preparation exhibiting TGRase activity eluted in the 0.20–0.25 M region of the gradient, and corresponded to the major peak of the A_{280} profile of the

elution gradient. The Mono-Q fractions were concentrated to 3.0 ml in an Amicon pressure cell as before. The 3.0 ml samples were concentrated further using a Centricon 10 concentrator to volumes of 0.5–1.0 ml. These preparations were then used as samples in enzyme assays, electrophoresis and autoradiography, and Superose 6 size-exclusion chromatography.

Reaction conditions for TGRase assays

The assay for TGRase activity parallels one described previously (29), and is based on the enzyme's ability to reversibly exchange ^{3}H-guanine for guanine in the first position of the anticodon of yeast tRNA. Yeast tRNA is unique in that it does not contain queuosine (30), therefore it is an excellent substrate for TGRase. TGRase assays were conducted in a total volume of 0.3 ml reaction buffer with 20 μM ^{3}H-guanine (1 μCi), 0.1 A_{260} unit yeast tRNA (5 μg) and TGRase. Assays were incubated for 1 h at 37°C, terminated by the addition of 100 μl of ice-cold 30% trichloroacetic acid (TCA) and chilled on ice for 10 min. The resulting precipitate was collected by suction filtration through glass fiber filters, washed with 45 ml of ice-cold 5% TCA and analyzed by liquid scintillation.

Reaction conditions for PKC assays

PKC reactions were performed in a total volume of 0.3 ml reaction buffer containing 1 mM $CaCl_2$, 5.0 μg diolein, 5.0 μg phosphatidyl serine and 100 μM [γ-^{32}P]ATP (1 μCi). Various concentrations of either histone fraction IV (positive control) or the TGRase preparation and PKC were added, incubated at 30°C for 30 min, and terminated by the addition of 100 μl of ice-cold 30% TCA. The precipitate was allowed to stand on ice for 10 min, collected by suction filtration through glass fiber filters, washed with 40 ml of cold 5% TCA and analyzed by liquid scintillation.

Reaction conditions for the combined TGRase, PKC and alkaline phosphatase assays

TGRase, PKC and alkaline phosphatase (AP) assays were combined using the aforementioned assay conditions. Reactions were performed in a total volume of 0.3 ml reaction buffer containing 1 mM $CaCl_2$, 5.0 μg diolein, 5.0 μg phosphatidyl serine, 20 μM ^{3}H-guanine (1 μCi), 100 μM ATP, 0.1 A_{260} yeast tRNA (5 μg) and various concentrations of TGRase, PKC and/or AP as indicated. The reaction mixtures were incubated at 30°C for 45 min. The reaction was terminated and prepared for liquid scintillation analysis as previously described.

SDS–PAGE and ^{32}P-autoradiography

Combination reactions using the TGRase and PKC preparations were performed to assess the ability of PKC to incorporate ^{32}P from [γ-^{32}P]ATP into TGRase. Reaction conditions were as described with the PKC assays with the differing reaction mixture samples containing 10 μg histone fraction IV (positive control), P-11 concentrated sample or Mono-Q purified samples. Reaction mixtures were incubated at 30°C for 45 min and terminated by the addition of denaturing sample buffer. The samples were boiled for 5 min and allowed to cool to room temperature. The samples were loaded onto 16 × 16 cm SDS–PAGE gels prepared by the method of Laemmli (31). The gel was run at 50 mA constant current for ~5 h. The gel was fixed, stained (40% methanol, 10% acetic acid, 0.05% Coomassie Blue stain) and destained to reveal banding patterns. Pictures were taken of the gel, then the gel was wrapped in cellophane and exposed to X-ray film at –70°C for 48 h. The X-ray film was developed by an automated film developer.

Denaturing and native size-exclusion chromatography

Samples of two sequential Mono-Q fractions exhibiting TGRase activity and the major site for PKC catalyzed ^{32}P-incorporation were analyzed with Pharmacia's Superose-6 high resolution size exclusion column. Protein (~800 μg) from each sample was loaded onto a pre-equilibrated 1 × 30 cm Superose-6 column and eluted with 10 mM Tris-Cl, pH 7.5 at a flow rate of 0.5 ml/min. Molecular weights of major peaks were compared with standards on the basis of elution time as detected by a UV monitor at 280 nm. When a discrepancy arose between the SDS–PAGE and the profile of the Mono-Q fraction exhibiting ^{32}P-incorporation, a denaturing chromatography experiment was undertaken.

The Mono-Q fraction (~800 μg) exhibiting ^{32}P-incorporation was brought to a concentration of 4 M guanidinium chloride and incubated at room temperature for 30 min. Then the sample was applied to the Superose-6 column and eluted with 10 mM Tris-Cl, pH 7.5 at a flow rate of 0.5 ml/min. The results of the denaturing and non-denaturing Superose-6 chromatography were then compared with the SDS–PAGE results for the consecutive Mono-Q fractions containing TGRase activity and the major site for ^{32}P-incorporation.

RESULTS

TGRase was isolated from rat liver as described. Data in Table 1 represents a typical isolation protocol and the resulting enzymatic characterization of the TGRase activity after each major step. A phosphocellulose preparation of TGRase was stored in Tris-buffered 50% glycerol at –20 or –70°C. It was observed that the activity of the enzyme preparation was labile regardless of storage conditions. Measurable TGRase activity in cold stored preparations would decrease rapidly to a low background level within 1–4 weeks. In one instance a TGRase preparation lost activity within only 4 days (Fig. 1).

Table 1. Summary of rat liver TGRase isolation after major procedural steps

Active fractions	Unit activity[a]	Specific activity[b]	Purification factor
Homogenate	0.35	0.28	1
Centrifuged supernatant	0.50	2.10	7.5
DE-52 cellulose LC	3.27	81.8	292
$(NH_4)_2SO_4$ precipitant	n/a	n/a	n/a
P-11 phosphocellulose LC	6.61	264.4	944
Mono Q FPLC	15.32	306.4	1094

[a]Unit activity is measured in units of pmol ^{3}H-guanine incorporated per hour per A_{260} unit of tRNA per assay tube (0.3 ml).
[b]Specific activity is measured in units of pmol ^{3}H-guanine incorporated per hour per A_{260} unit of tRNA per milligram of protein.

Phosphatase activity was detected in the TGRase preparations. This was indicated by the ability of the TGRase samples to convert the colorless phosphatase substrate, *p*-nitrophenylphos-

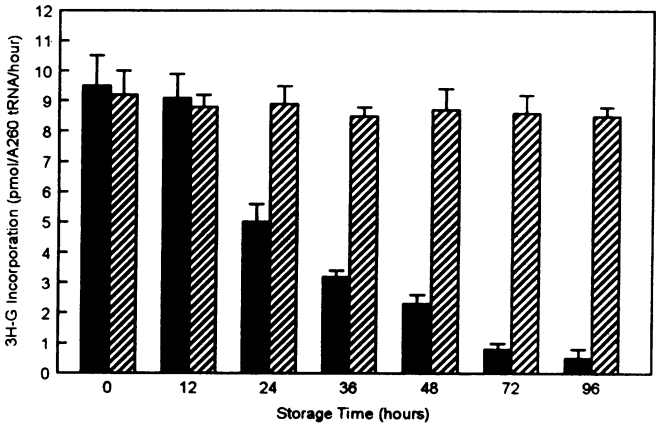

Figure 1. The decay of purified TGRase activity over 4 days. The solid bars indicate the decay of residual TGRase activity. The hashed bars indicate the activity of TGRase after reactivation by the addition of 10 μU protein kinase C to the reaction mixture. Five micrograms of purified protein isolate from the Mono Q-FPLC step were used in each assay.

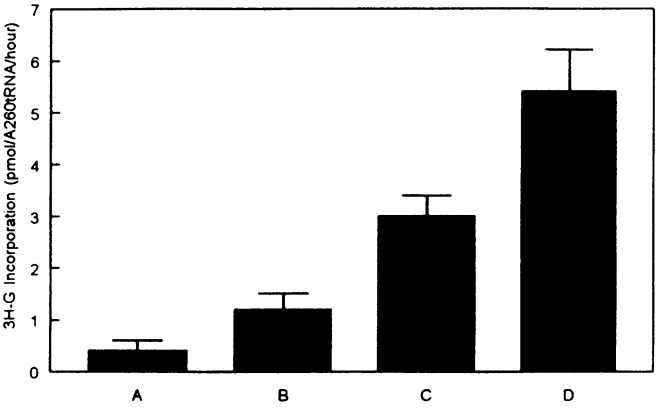

Figure 2. The recovery of crude TGRase activity with the addition of phosphatase inhibitors to the homogenization buffers. Bar A indicates the non-specific binding of ^{3}H-guanine to protein within the sample (no tRNA substrate added). Bar B indicates the residual TGRase activity recovered from homogenization in buffer with no phosphatase inhibitors. Bars C and D indicate the TGRase activity recovered from homogenization in buffer with 10 μM sodium fluoride and 10 μM sodium pyrophosphate, respectively. Five micrograms of protein isolate from the phosphocellulose column step were used in each assay.

phate, to the yellow colored *p*-nitrophenoxide anion (data not shown). The addition of 10 μM phosphatase inhibitors (sodium fluoride and sodium pyrophosphate) to the homogenization buffer enhanced the recovery of TGRase activity from rat liver isolates (Fig. 2). This implies that phosphorylation levels are important to verall TGRase activity.

Since PKC has been shown to modulate the rate of queuine uptake in cells, we addressed the possibility that PKC was able to modulate queuine incorporation activity by regulation of TGRase activity as well. In combined TGRase and PKC enzyme assays we observed an increase in the activity of TGRase over that of TGRase controls without PKC (Fig. 1). TGRase was restored to its original activity by exposure to PKC. The PKC preparation was able to incorporate ^{32}P from [γ-^{32}P]ATP into the acid

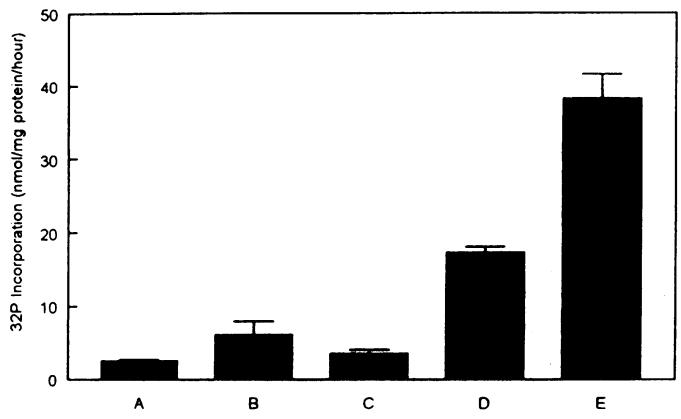

Figure 3. The phosphorylation of histones and purified tRNA-guanine ribosyltransferase (TGRase) by protein kinase C (PKC). Bars A and B indicate non-specific binding of [γ-^{32}P]ATP and any contaminating kinase activity in 5.0 μg histones and TGRase samples, respectively. Bar C indicates the background levels of [γ-^{32}P]ATP binding to 10 μU PKC (no histone or TGRase substrate added). Bars D and E indicate the levels of PKC-catalyzed phosphorylation of 5.0 μg of histone fraction IV and TGRase, respectively.

precipitable component of the purified TGRase preparation derived from the Mono-Q column, and at levels greater than that for histones inthe PKC control assay (Fig. 3). Thus, we believe that PKC is directly phosphorylating TGRase and is likely to be modulating TGRase activity.

The direct effect of PKC modulators on TGRase and PKC activities were tested (Fig. 4a and b) TGRase preparations were assayed for ^{3}H-guanine incorporation into yeast tRNA in the presence of PKC activators (diolein, phosphatidyl serine and/or calcium) and inhibitors (50 μM H-7, 25 μM sphingosine and/or 100 nM staurosporine). None of these agents affected the TGRase activity (Fig. 4a). The methylated purine, 7-methylguanine (10 μM), a known competitive inhibitor of TGRase demonstrated strong inhibitory effects ans served as the inhibited control for this assay. However, PKC's activity was reliably and dramatically inhibited by H-7, sphingosine and staurosporine (Fig. 4b). This inhibition was observed even in the presence of the activators diolein, phosphatidyl serine and calcium. The PKC preparations demonstrated no evidence of latent TGRase activity. In TGRase assays combined with activated PKC, ^{3}H-guanine incorporation was increased as seen before (Fig. 4c). However, inhibitors of PKC appeared to dramatically limit TGRase activity. Thus, the inhibitory effect of the PKC antagonists on ^{3}H-guanine incorporation into tRNA is suggested to be due to an inhibition of a PKC activation of the TGRase enzyme.

Combined assays were run with TGRase, PKC and alkaline phosphatase (Fig. 5). TGRase activity was greatly enhanced by the presence of PKC and it's activators. This induction of TGRase activity was also decreased by the presence of alkaline phosphatase. Baseline TGRase activity was also decreased by the presence of alkaline phosphatase. This suggests that during the isolation procedures there is a basal level of phosphorylation maintained in the isolated TGRase-active fractions. The results of TGRase activity assays in the presence of kinase and antagonist phosphatase verify the phosphorylation-modulation of TGRase.

^{32}P-labelled and unlabelled P-11 and Mono-Q TGRase fractions were analyzed by SDS–PAGE and autoradiography (Fig. 6).

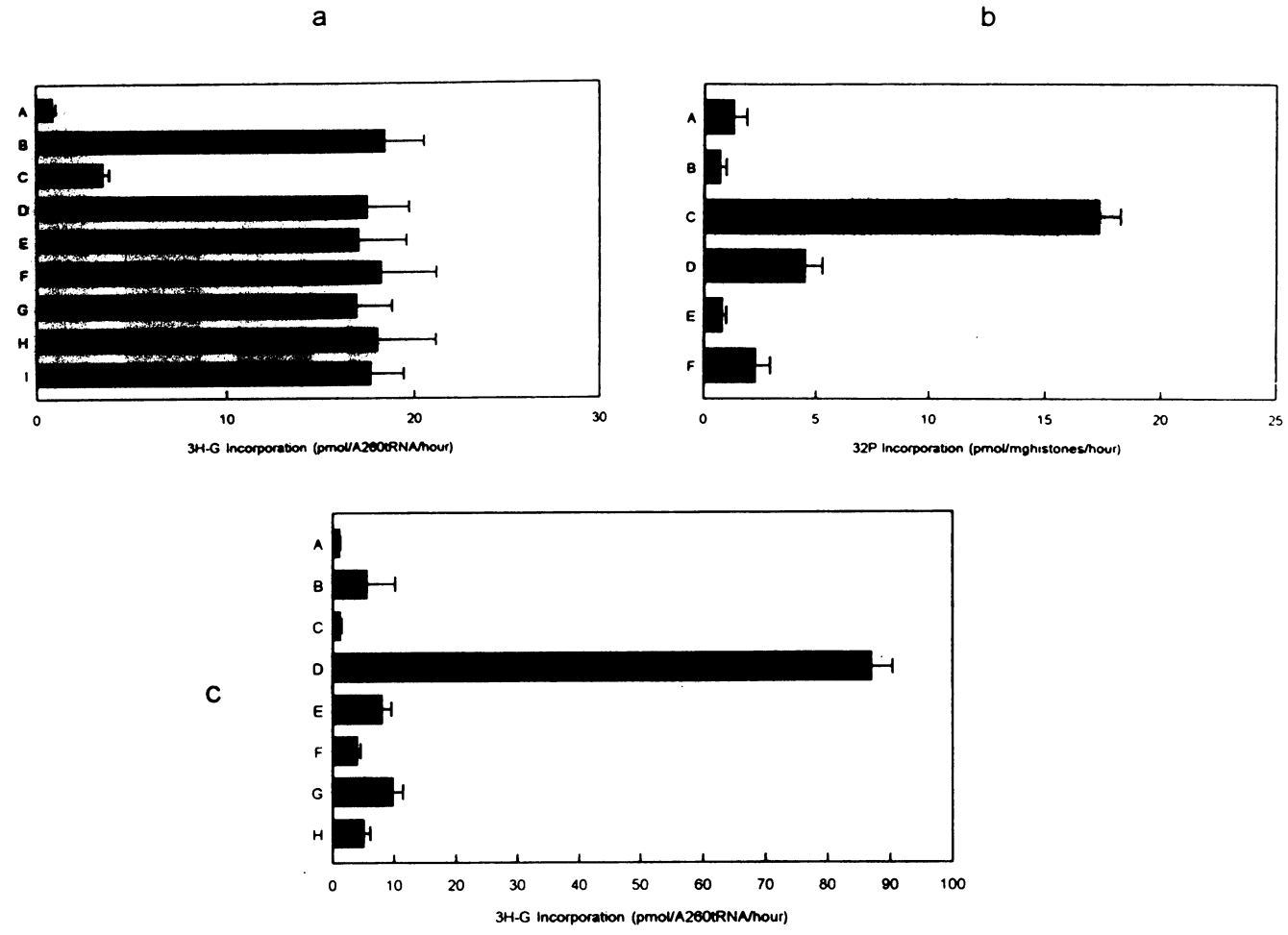

Figure 4. The effect of PKC modulators on purified TGRase and PKC activities. (**a**) The effect of PKC modulators on 5.0 μg TGRase activity. Bar A indicates non-specific binding of ^{3}H-guanine to protein within the sample (no tRNA substrate added). Bar B indicates the TGRase activity without modulators (positive control). Bar C indicates the TGRase activity with the addition of 10 μM TGRase inhibitor 7-methylguanine (negative control). Bars D–I indicate the TGRase activity with the addition of: known PKC activators 1 mM calcium chloride, 5.0 μg diolein, and 5.0 μg phosphatidyl serine (Bar D), 100 μM ATP (Bar E) and activators with ATP (Bar F); and known PKC inhibitors 50 μM H-7, 100 nM staurosporine, 25 μM sphingosine (Bars G, H and I). (**b**) The effect of modulators on the activity of 10 μU PKC. Bar A indicates non-specific binding of [γ-^{32}P]ATP to protein within the PKC sample (no histone) substrate). Bar B indicates the residual PKC activity without modulators (negative control). Bar C indicates the phosphorylation activity of PKC on 5.0 μg histones with the addition of known PKC activators 1 mM calcium chloride, 5.0 μg diolein and 5.0 μg phosphatidyl serine (positive control). Bars D, E and F indicate the phosphorylation activity of PKC on 5.0 μg histones with the addition of PKC activators and inhibitors 50 μM H-7, 100 nM staurosporine, 25 μM sphingosine, respectively. (**c**) The effect of PKC modulators on PKC-activated TGRase activity (10 μU PKC and 5.0 μg purified TGRase). Bar A indicates non-specific binding of ^{3}H-guanine to protein within the sample (no tRNA substrate added). Bar B indicates the non-PKC activated (base-line) TGRase activity without modulators (negative control). Bar C indicates the contaminating TGRase activity in the PKC sample. Bar D indicates the PKC-activated TGRase activity with PKC activators present (positive control). Bar E indicates the PKC-activated TGRase activity with PKC activators present and the addition of 10 μM TGRase inhibitor 7-methylguanine (negative control). Bars F, G and H indicate the PKC-activated TGRase activity with PKC activators present and the addition of known PKC inhibitors 50 μM H-7, 100 nM staurosporine and 25 μM sphingosine.

A strong band at ~60 kDa was observed in the P-11 sample and in an inactive TGRase-containing Mono-Q fraction sample. A faint band in the same location also appeared in the TGRase-active Mono-Q fraction. There was also a faint band in the P-11 sample and in the TGRase-active and inactive Mono-Q fractions at ~34.5 kDa. These bands matched up well with stained bands in the gel. A non-denaturing PAGE exhibited a simple banding pattern for the same sample (data not shown). In the non-denaturing gel, Mono-Q fractions exhibiting TGRase activity exhibited a single visible band between 30 and 45 kDa. This correlated well with the denaturing SDS–PAGE results. However, the subsequent fraction from the Mono-Q isolation step (which exhibited good ^{32}P-incorporation but limited TGRase-activity) exhibited a large molecular weight band between 100 and 110 kDa in the non-denaturing gel, and not the two smaller bands seen in the SDS–PAGE experiment. This suggested a possible subunit interaction as evidenced by banding differences under non-denaturing versus denaturing conditions. Therefore, a study using native and denaturing chromatography was performed.

Comparisons were made between native Superose-6 chromatographs of the sequential TGRase-active and ^{32}P-incorporating TGRase inactive Mono-Q fractions (Fig. 7). Compared with identifiable protein standards, the ^{32}P-incorporating TGRase inactive Mono-Q fraction maintains it's major peak at ~100–110

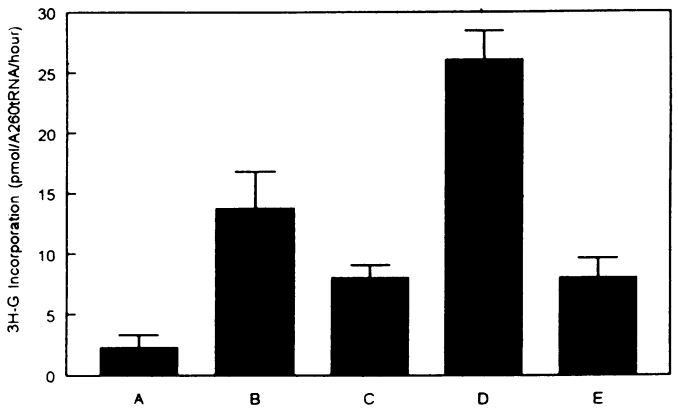

Figure 5. The modulation of purified TGRase activity by phosphorylation. Bar A indicates the non-specific binding of ^{3}H-guanine to protein within the 5.0 µg TGRase sample (no tRNA substrate added). Bar B indicates the residual TGRase activity in this sample. Bar C indicates the TGRase activity with the addition of 0.40 mU alkaline phosphatase. Bar D indicates the TGRase activity with the addition of 10 µU protein kinase C (PKC). Bar E indicates the TGRase activity with the addition of competing PKC and alkaline phosphatase enzymes, 10 µU and 0.40 mU respectively.

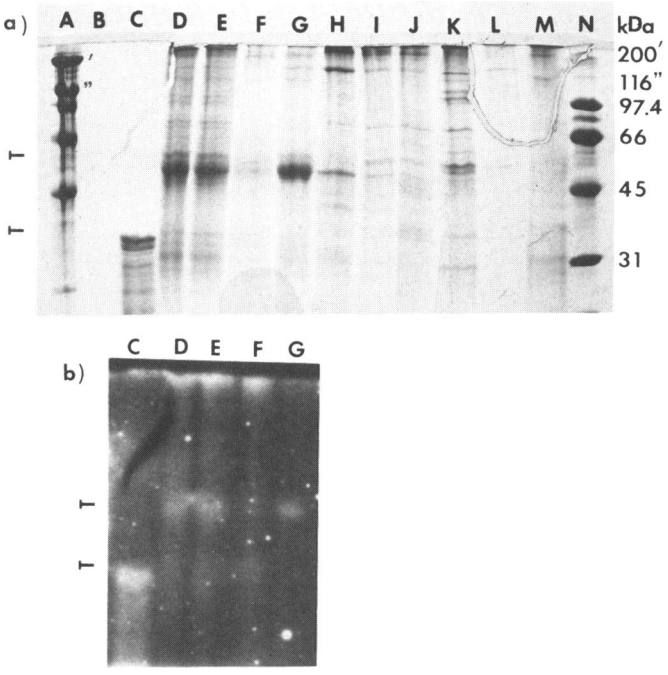

Figure 6. Electrophoretic and autoradiographic analysis of protein samples under denaturing conditions. **(a)** SDS–PAGE gel separation of reaction mixtures: lane A, high molecular weight standards; lane B, protein kinase C (PKC) (not visible under Coomassie staining); lane C, 10 µg histone fraction IV with 10 µU PKC; lanes D and E, 40 µg TGRase active P-11 column sample with 10 µU PKC; lanes F–M, 10 µg samples of successive fractions from a Mono Q column with 10 µU PKC (measurable TGRase activity observed in fraction corresponding to lane F) and lane N, low molecular weight standards. **(b)** Autoradiograph of gel shown in (a). PKC catalyzed phosphorylation shown by ^{32}P incorporation in lane C (positive control); lanes D, E and G (major band) at ~60 kDa and lanes D, E and F (minor band) at ~34.5 kDa. Arrows point to bands on the SDS–PAGE gel corresponding to those seen on the autoradiograph.

kDa (Fig. 7a). When this fraction was denatured in 4 M guanidinium chloride a definitive shift in its peak retention time occurred (Fig. 7b). The 100–110 kDa decreased substantially, while the bulk of the protein in this peak appeared to shift to the right and plateau between 65 and 30 kDa. The TGRase-active Mono-Q fraction appeared to have the major peak at ~30–38 kDa, with a possible minor peak between 55 and 65 kDa (Fig. 7c). The peak overlaps observed in the chromatographs could be due to the presence of subunit complexes. This data supports the possibility that TGRase is a large 104 kDa complex and it dissociates into subunits of 60 and 34.5 kDa, as was indicated by the electrophoretic data.

Studies are currently under way to address the effects of phosphorylation on the kinetic parameters of K_m and V_{max} for TGRase, and the stoichiometry of phosphorylation for the TGRase 60 and 34.5 kDa subunits.

DISCUSSION

The post-transcriptional modification of tRNA anticodons with queuosine has been implicated in aspects of neoplastic transformation, growth control, stress compensation and differentiation. The enzyme, TGRase, which is responsible for this modification, has been under scrutiny since its discovery in 1973. In this time, a few laboratories have been able to purify TGRase to homogeneity from wheat germ (32), *E.coli* (33) and rabbit reticulocytes (34). However, other literature documents describe only partial purifications and limited storage life times (4). In our hands, TGRase isolated from rat, rabbit and human tissues has always shown instability and loss of assayable activity with short term storage (1–4 weeks).

Recently, we discovered a close link between PKC activity and cellular uptake of queuine in cultured human fibroblasts (27), whereby PKC maintains optimal activity of a specific queuine transport mechanism. Stimulation of PKC activity by short-term exposure to TPA, growth factors, diacylglycerol and calcium have been shown to stimulate cellular uptake of queuine. Inhibition of PKC activity by H-7, staurosporine, sphingosine or chronic exposure to TPA inhibited queuine uptake in human fibroblast cultures. However, there was always a basal rate of queuine uptake available to these cultured cells. A combination of decreased uptake and decreased TGRase activity could lead to the profound queuosine-hypomodification observed in cultured human fibroblast tRNA with chronic exposure to phorbol esters (21). A partial block on queuine uptake alone would not have demonstrated this prononuced tRNA-undermodification effect.

Our data suggests that PKC activity is essential in maintaining the activity of TGRase, as well as activation of the cellular uptake mechanism for it's substrate, queuine. Therefore, PKC activity appears to support the complete modification of tRNA with queuosine. We have seen several stored TGRase preparations isolated from rat liver with various low levels of activity, undergo reactivation on exposure to PKC isolated from rat brain. Inactivation of PKC in combined TGRase and PKC assays with H-7, staurosporine or sphingosine resulted in a large decrease in the activity level of TGRase as compared with the PKC activated control. Neither the PKC inhibitors nor the PKC activators exerted effects on directly on TGRase. Therefore, PKC appears to be phosphorylating TGRase as is shown in the combined PKC and TGRase ^{32}P-incorporation assay and the autoradiographic experiments.

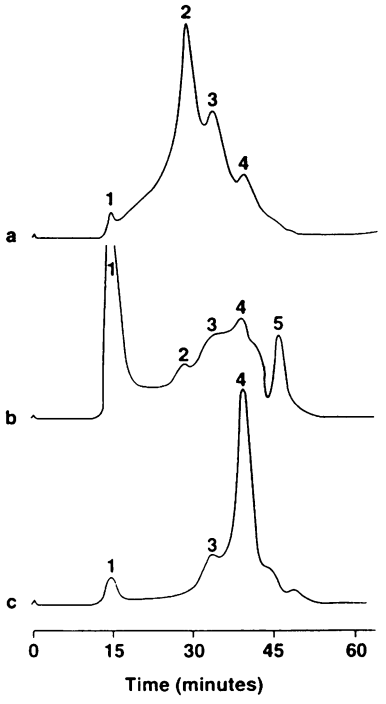

Figure 7. Superose-6 FPLC separation of Mono Q fractions exhibiting TGRase activity and major site of PKC-catalyzed phosphorylation under native and denaturing conditions. (**a**) Chromatograph of major PKC-phosphorylated fraction (800 µg) under native conditions. (**b**) Chromatograph of major PKC-phosphorylated fraction under denaturing conditions (4 M guanidinium chloride). (**c**) Chromatograph of 800 µg TGRase-active fraction under native conditions. Peak 1 represents the void volume; peaks 2, 3 and 4 represent regions of ~104, 60 and 34.5 kDa, respectively and Peak 5 represents the elution of the guanidinium salt.

It is proposed that the TGRase enzyme exists as a heterodimer of ~104 kDa, with a PKC-substrate regulatory subunit of 60 kDa and a catalytic subunit of 34.5 kDa (a weaker PKC substrate). Previous purifications of TGRase from various mammals [rabbit (34) and bovine (35)] have shown these isolates to be heterodimers of almost identical size. The PKC-catalyzed phosphorylation of the dimer is suggested to reduce the affinity between the two subunits and cause the release of a catalytically more active 34.5 kDa subunit. It is proposed that unphosphorylated TGRase remains in the low activity dimer state. Phosphatase activity or down-regulation of PKC activity, therefore, would promote the dephosphorylation of TGRase and minimize its activity, resulting in queuosine-undermodified tRNA.

This work suggests that TGRase activity is directly stimulated by activated PKC, and that competing phosphatase activity down-regulates the activity of TGRase. Any agent or treatment regiment that could lead to a down-regulation in PKC activity, such as chronic exposure to phorbol-ester tumor promoters, would have an inhibitory effect on both queuine uptake into the cell and TGRase activity leading to queuosine-undermodified tRNA. PKC control over the queuosine modification of tRNA ties tRNA metabolism directly to a list of molecular events known to occur in the signal transduction pathway's control of gene expression and tumor promotion.

ACKNOWLEDGEMENTS

The authors acknowledge the support of the National Cancer Institute in funding this work and the previous work on queuine transport (27,35) (NIH/NCI R29-CA45213). The authors thank Drs James Yuan and Werner Langgut for their insightful critical analysis and suggestions regarding this manuscript, and our families for tolerating our ridiculous hours in performing this research.

REFERENCES

1 Harada, F. and Nishimura, S. (1972) *Biochemistry* **11**, 301–308.
2 Nishimura, S. (1983) *Prog. Nucleic Acids Res. Mol. Biol.* **28**, 49–80.
3 Katze, J.R. and Farkas, W.R. (1979) *Proc. Natl. Acad. Sci., USA* **76**, 3217–3275.
4 Shindo-Okada, N., Okada,N., Ohgi, T., Goto, T. and Nishimura, S. (1980) *Biochemistry* **19**, 345–400.
5 Schachner, E. and Kersten, H. (1984) *J. Gen. Microbiology* **130**, 135–144.
6 Beier, H., Zech, U., Zubrod, E. and Kersten, H. (1987) *Plant Mol. Biol.* **8**, 345–353.
7 White, B.N., Tener, G.M., Holden, J. and Suzuki, D.T. (1973) *J. Mol. Biol.* **74**, 635–651.
8 Owenby, R.K., Stulberg, M.P. and Jacobsen, K.B. (1979) *Mech. Aging Dev.* **11**, 91–103.
9 Farkas, W.R. and Jacobsen, K.B. (1980) *Insect Biochem.* **10**, 183–188.
10 Singhal, R.P., Kopper, R.A., Nishimura, S. and Shindo-Okada, N. (1981) *Biochem. Biophys. Res. Commun.* **99**, 120–126.
11 Noguchi, S., Nishimura, Y., Hirota, Y. and Nishimura, S. (1982) *J. Biol. Chem.* **257**, 6544–6550.
12 Reisser, T., Eicher, A. and Langgut, W. (1993) *Biochem. Biophys. Res. Commun.* **197**, 1319–1325.
13 Reisser, T., Langgut, W. and Kersten, H. (1994) *Eur. J. Biochem.* **221**, 979–986.
14 Schachner, E., Aschhoff, H.-J. and Kersten, H. (1984) *Eur. J. Biochem.* **139**, 481–487.
15 Katze, J.R., Beck, W.T., Cheng, C.S. and McCloskey, J.A. (1983) *Recent Results Cancer Res.* **84**, 140–159.
16 Okada, N., Shindo-Okada, N., Sato, S., Itoh, Y.H., Oda, K.I. and Nishimura, S. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4247–4251.
17 Emmerich,B., Zubrod, E., Weber, H., Maubach, P.A., Kersten, H. and Kersten, W. (1985) *Cancer Res.* **45**, 4308–4314.
18 Huang, B.-S., Wu, R.-T. and Chien, K.-Y. (1992) *Cancer Res.* **52**, 4696–4700.
19 Baranowski, W., Dirhamer, G., Jakowicki, J.A. and Keith, G. (1994) *Cancer Res.* **54**, 4468–4471.
20 Shindo-Okada, N., Terada, M. and Nishimura, S. (1981) *Eur. J. Biochem.* **115**, 423–428.
21 Elliott, M.S., Katze, J.R. and Trewyn, R.W. (1984) *Cancer Res.* **44**, 3215–3219.
22 Elliott, M.S., Trewyn, R.W. and Katze, J.R. (1985) *Cancer Res.* **45**, 1079–1085.
23 Farkas, W.R. (1979) *J. Biol. Chem.* **255**, 6832–6835.
24 Reyniers, J.P., Pleasants, J.R., Wostman, B.S., Katze, J.R. and Farkas, W.R. (1981) *J. Biol. Chem.* **256**, 11591–11594.
25 Katze, J.R. (1978) *Biochem. Biophys. Res. Commun.* **84**, 527–535.
26 Elliott, M.S. and Katze, J.R. (1986) *J. Biol. Chem.* **261**, 13019–13025.
27 Elliott, M.S. and Crane, D.L. (1990) *Biochem. Biophys. Res. Commun.* **171**, 393–400.
28 Parker, P.J. and Aillrich, A. (1987) *J. Cell Phys.* **5**, 53–56.
29 Howes, N.K. and Farkas, W.R. (1978) *J. Biol. Chem.* **253**, 9082–9087.
30 Walden, T., Reyniers, J.P., Hiatt, V. and Farkas, W.R. (1982) *Proc. Soc. Exp. Biol. and Med.* **170**, 328–332.
31 Laemmli U.K. (1970) *Nature.* **227**, 680–685.
32 Walden, T.L., Howes, N. and Farkas, W.R. (1982) *J. Biol. Chem.* **257**, 13218–13222.
33 Okada, N. and Nishimura, S. (1979) *J. Biol. Chem.* **254**, 3061–3066.
34 Howes, N.K. and Farkas, W.R. (1978) *J. Biol. Chem.* **253**, 9082–9087.
35 Slany, R. and Langgut, W., personal communication.
36 Elliott, M.S. and Crane, D.L. (1990) *Biochem. Biophys. Res. Commun.* **171**, 384–392.