DNA repair protein O\(^6\)-alkylguanine-DNA alkyltransferase is phosphorylated by two distinct and novel protein kinases in human brain tumour cells

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We showed recently that human O\(^6\)-alkylguanine-DNA alkyltransferase (AGT), an important target for improving cancer chemotherapy, is a phosphoprotein and that phosphorylation inhibits its activity [Srivenugopal, Mullapudi, Shou, Hazra and Ali-Osman (2000) Cancer Res. 60, 282–287]. In the present study we characterized the cellular kinases that phosphorylate AGT in the human medulloblastoma cell line HBT228. Crude cell extracts used Mg\(^{2+}\) more efficiently than Mn\(^{2+}\) for phosphorylating human recombinant AGT (rAGT) protein. Both \([\gamma-\text{P}]\text{ATP}\) and \([\gamma-\text{P}]\text{GTP}\) served as phosphate donors, with the former being twice as efficient. Specific components known to activate protein kinase A, protein kinase C and calmodulin-dependent kinases did not stimulate the phosphorylation of rAGT. Phosphoamino acid analysis after reaction in vitro with ATP or GTP showed that AGT was modified at the same amino acids (serine, threonine and tyrosine) as in intact HBT228 cells. Although some of these properties pointed to casein kinase II as a candidate enzyme, known inhibitors and activators of casein kinase II did not affect rAGT phosphorylation. Fractionation of the cell extracts on poly(Glu/Tyr)-Sepharose resulted in the adsorption of an AGT kinase that modified the tyrosine residues and the exclusion of a fraction that phosphorylated AGT on serine and threonine residues. In-gel kinase assays after SDS/PAGE and non-denaturing PAGE revealed the presence of two AGT kinases of 75 and 130 kDa in HBT228 cells. The partly purified tyrosine kinase, identified as the 130 kDa enzyme by the same assays, was strongly inhibited by tyrphostin 25 but not by genistein. The tyrosine kinase used ATP or GTP to phosphorylate the AGT protein; this reaction inhibited the DNA repair activity of AGT. Evidence that the kinases might physically associate with AGT in cells was also provided. These results demonstrate that two novel cellular protein kinases, a tyrosine kinase and a serine/threonine kinase, both capable of using GTP as a donor, phosphorylate the AGT protein and affect its function. The new kinases might serve as potential targets for strengthening the biochemical modulation of AGT in human tumours.

Key words: base-excision repair, cancer chemotherapy, drug resistance, O\(^6\)-methylguanine, post-translational modification.

INTRODUCTION

O\(^6\)-Alkylguanine-DNA alkyltransferase (AGT), also known as O\(^6\)-methylguanine-DNA methyltransferase (‘MGMT’; EC 2.1.1.63), is a unique ubiquitous DNA repair protein that attenuates the mutagenic and lethal effects of a variety of anti-cancer alkylating agents [1,5]. This is because the O\(^6\)-alkylguanines are covalently bound to the cysteine residue in the active site of AGT. Because the alkyl groups are transferred to a cysteine residue in the active site of AGT, AGT is inactive after each reaction and is degraded through the ubiquitin–proteasome pathway [3]. The anti-mutagenic activity of AGT against endogenous and environmental carcinogens also imparts to AGT a primary role in conferring tumour resistance on many anti-cancer alkylating agents [4]. This is because the clinically active methylating agents (temozolomide, dacarbazine and procarbazine) activate the AGT gene by using alkylating agents that generate O\(^6\)-alkylguanines. Thus powerful pseudosubstrates for AGT, such as O\(^6\)-benzylguanine (BG), which effectively deplete cellular AGT and enhance drug-induced cytotoxicity, have been developed [1,5]. BG is currently the subject of clinical trials to enhance the efficacy of chloroethylnitrosoureas against human brain tumours and other cancers [6]. In addition, because myelo-toxicity is an important limitation in using AGT-targeted alkylating drugs, gene therapy strategies are being used to transfer the AGT gene into haemopoietic cells to protect them against toxicity and to improve the therapeutic index [7].

We recently provided the first evidence that AGT exists as a phosphoprotein in human brain tumour cells and showed that it is phosphorylated at serine, threonine and tyrosine residues [8]. We also showed that phosphorylation negatively regulates AGT, inhibiting its enzymic activity, and that the AGT protein has consensus motifs for many established kinases such as casein kinase II (CK II) [8]. A thorough knowledge of the role of phosphorylation in regulating the biological functions of AGT is

Abbreviations used: AGT, O\(^6\)-alkylguanine-DNA alkyltransferase; CK II, casein kinase II; DRB, 5,6-dichloro-1-\(\beta\)-D-ribofuranosylbenzimidazole; poly(Glu/Tyr), random co-polymer of glutamic acid and tyrosine in a 4:1 ratio; rAGT, human recombinant AGT; BG, O\(^6\)-benzylguanine; DTT, dithiothreitol.

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crucial not only for understanding the basic physiological role of AGT but also for devising new therapeutic strategies for human tumours. To this end, in the present study we identified and characterized the cellular kinases that phosphorylate the AGT protein and found that two kinases, a tyrosine kinase and a serine/threonine kinase with novel properties, are involved in the process.

EXPERIMENTAL

Reagents

[γ-32P]ATP (3000 Ci/mmol) and [γ-32P]GTP (3000 Ci/mmol) were purchased from ICN (Costa Mesa, CA, U.S.A.). Phosphorylated amino acid standards, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), poly(Glu)phorylated amino acid standards, 5,6-dichloro-1-

Cell culture and preparation of extracts

The cell line HBT228, which was used in this study and in our previous study of AGT phosphorylation [8], was established from a human medulloblastoma specimen in our laboratory. HBT228 cells in their exponential growth phase were harvested by treatment with trypsin and washed with Tris-buffered saline, pH 8.0. The cells were suspended in buffer A, which contained protease and phosphatase inhibitors and consisted of 40 mM Tris/HCl (pH 7.4), 1 mM dithiothreitol (DTT), 0.05% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM PMSF, 0.5 mM benzamidine, 0.1 mM NaF, 0.1 mM sodium pyrophosphate and the phosphatase inhibitor cocktail diluted 1:100. The cell extracts were prepared by sonication followed by centrifugation at 20,000 g for 10 min. The supernatants served as the enzyme source for all the kinase studies described here. Protein content was estimated by the method of Bradford [9].

Expression and purification of human recombinant AGT (rAGT)

rAGT protein was expressed by using the pQE vector (Qiagen), which adds 12 amino acids, including a stretch of six histidine residues [MRGHS(H)GS-], to the N-terminus of the AGT protein. Previous studies have shown that this polypeptide tag does not alter the properties of the AGT protein [10]. Escherichia coli JM110 transformed with the pQE-AGT construct was grown in Terrific broth containing 400 mM sorbitol and 2.5 mM betaine hydrochloride. Protein expression was induced by the addition of 0.4 mM isopropyl thiogalactoside for 4 h. Sorbitol and betaine supplementation resulted in the production of higher levels of soluble AGT and a minimal formation of inclusion bodies [11]. Native AGT was purified after lysis with lysozyme and metal-chelation chromatography with Talon resin (Clontech, Palo Alto, CA, U.S.A.) as described previously [12]. The preparation was homogeneous, producing a single 24 kDa protein band on SDS/PAGE. The dialysed protein was stored frozen at a concentration of 1 mg/ml.

Assay for phosphorylation of rAGT by cell extracts

Purified rAGT protein (5 μg) was phosphorylated in a 50 μl reaction mixture containing buffer A, [γ-32P]ATP or [γ-32P]GTP (100 μM; 2.5 μCi), 5 mM MgCl₂ and HBT228 cell extract (50 μg of protein) or 5–15 μg of partly purified tyrosine kinase. The reactions were performed at 30 °C for 30 min; AGT phosphorylation was quantified by two methods described below.

SDS/PAGE procedure

The reactions were terminated by the addition of 10 μl of sample buffer [250 mM Tris/HCl (pH 6.8)/8 % (w/v) SDS/20 % (v/v) 2-mercaptoethanol/40 % (v/v) glycerol/0.004 % Bromophenol Blue] followed by boiling for 2 min. The samples were subjected to SDS/PAGE on 15 % (w/v) gels. The bottom portion of each gel, approx. 2.5 cm below the tracking dye, was excised to remove the radioactivity associated with the free nucleotides; the gels were stained, destained thoroughly, dried and autoradiographed. For quantification of AGT phosphorylation, the rAGT protein bands on the dried gels were cut out and the radioactivity was counted. In some experiments the AGT bands on the autoradiographs were quantified by densitometry as well.

Selective purification of rAGT by Talon resin and SDS/PAGE

At the end of incubation, the kinase reactions were diluted with 300 μl of 40 mM sodium phosphate buffer, pH 7.6, containing 0.5 M NaCl. Next, 20 μl of Talon resin [50 % (w/v) suspension in PBS] was added to the samples, which were kept on ice for 10 min to allow the histidine-tagged rAGT protein to bind to the resin. The resin pellets were recovered by centrifugation (10000 g for 3 min) and washed twice with the phosphate buffer with 0.3 M NaCl. The bound proteins were eluted with 40 μl of 2 % (w/v) SDS in 50 mM Tris/HCl (pH 6.8)/40 mM EDTA. The samples were boiled and subjected to SDS/PAGE, gels were stained, dried and autoradiographed, and the AGT phosphorylation was quantified as described for the SDS/PAGE procedure.

Phosphoamino acid analysis

To identify the amino acids phosphorylated in the rAGT protein in the reactions in vitro, the phosphorylation reactions with [γ-32P]ATP or [γ-32P]GTP as phosphate donors were scaled up 4-fold and the samples were subjected to SDS/PAGE as described above. The gels were stained with Coomassie Blue to locate the AGT protein bands, which were excised. The 32P-labelled protein was extracted by electrodialution in 50 mM NH₄HCO₃/0.1 % SDS at 4 °C for 4 h. The eluates were dialysed overnight against 50 mM NH₄HCO₃, freeze-dried and hydrolysed in 6 M HCl at 110 °C in a sealed tube for 2 h. The HCl was then removed by repeated freeze-drying. The hydrolysates were dissolved in distilled water and mixed with phosphorylated amino acid standards (1 mg/ml phosphoserine/1 mg/ml phosphothreonine/1 mg/ml phosphotyrosine). A 1–3 μl aliquot of these samples was spotted on 20 cm × 20 cm thin-layer cellulose chromatography plates (Aldrich Co., Milwaukee, WI, U.S.A.) and subjected to ascending chromatography in isobutyric acid/butan-1-ol/pyridine/acetic acid/water (65:2:5:3:29, by vol.) [13]. After drying, the standard phosphoamino acids were located by staining with ninhydrin, and the 32P label associated with the spots was detected by autoradiography.

Affinity chromatography of HBT228 cell extracts on poly(Glu/Tyr)-Sepharose

Affinity chromatography of cell extracts was performed on poly(Glu/Tyr)-Sepharose, which has been reported to bind protein tyrosine kinases selectively [14,15]. The affinity matrix (2.5 ml bed volume; 12 mg/ml binding capacity) was equilibrated with buffer A. Freshly prepared HBT228 cell extract (30 mg of
protein) was passed through the poly(Glu/Tyr)-Sepharose in the cold at a flow rate of 40 μl/min. Fractions of 1.2 ml from the beginning of chromatography were collected. The beads were washed with 20 ml of buffer A containing 0.1 M NaCl. The bound proteins were eluted with buffer A containing 0.3 M NaCl and 0.1% (v/v) Triton X-100. Protein concentrations in the unbound and bound fractions were determined; both sets of fractions were tested for protein tyrosine kinase activity by using rAGT and poly(Glu/Tyr) (5 μg) as substrates.

To analyse the amino acids phosphorylated in the rAGT protein after its reaction with the unbound fraction, we compared the stability of the phosphate groups linked to aliphatic and aromatic amino acids in acidic and alkaline solutions [16]. For this, three aliquots of rAGT were phosphorylated by the unbound fraction; the samples were then subjected to SDS/PAGE. The resolved proteins were then transferred electrophoretically to PVDF membranes. Three membrane strips containing 3P-labelled rAGT bands were then cut out; one was not treated (control), another was treated with 0.2 M HCl at 60 °C for 30 min and the third was treated with 1 M NaOH at 75 °C for 30 min. The strips were then washed with Tris-buffered saline, pH 8.0, and autoradiographed.

In-gel AGT kinase assay

This assay was performed as described previously [17], with minor modifications. HBT228 cell extracts (30 μg) and the fraction bound to the poly(Glu/Tyr)-Sepharose (6 μg) were subjected to SDS/PAGE [8% (w/v) gel] with or without the addition of rAGT protein (250 μg/ml) to the separating gel just before polymerization. After electrophoresis, the SDS was removed from the gel by being washed twice with 20% (v/v) propan-2-ol in 50 mM Tris/HCl, pH 8.0, for 1 h and in 50 mM Tris/HCl, pH 8.0, containing 5 mM 2-mercaptoethanol for 1 h at room temperature. The proteins in the gel were denatured with 6 M guanidinium chloride and 50 mM DTT for 3 h, then renatured with five changes of 50 mM Tris/HCl, pH 8.0, containing 0.04% (v/v) Tween 20 at 4 °C over 24 h. For the kinase reaction, the gels were incubated at room temperature for 5 h in a buffer containing 50 mM Tris/HCl, pH 7.6, 2 mM DTT, 10 mM MgCl2, 2 mM MnCl2 and [γ-32P]ATP (10 μCi/ml). After incubation, the gel was washed with 5% (v/v) trichloroacetic acid containing 1% (w/v) sodium pyrophosphate until the radioactivity of the solution became negligible. The gel was then dried on Whatman 3 MM filter paper and autoradiographed.

Immune kinase assay

To examine the association of AGT kinase with its substrate, HBT 228 cell extract (300 μg of protein) was precleaned with Protein A–agarose and immunoprecipitated with polyclonal [18] or monoclonal [19] antibodies against the human AGT protein, as described previously [8]. The immune complexes were washed with Tris-buffered saline and incubated at 30 °C for 30 min with kinase assay buffer containing 40 mM Tris/HCl (pH 7.6), 5 mM MgCl2, 0.5 mM DTT, 5% (v/v) glycerol, 20 μM ATP, 2 μCi of [γ-32P]ATP and 4 μg of rAGT substrate. The reaction was terminated by the addition of SDS/PAGE sample buffer; the mixture was then boiled for 5 min and subjected to SDS/PAGE [12.5% (w/v) gel]. The gels were dried and autoradiographed.

Assay of AGT activity after phosphorylation by cellular tyrosine kinase

To determine the effect of tyrosine phosphorylation on AGT activity, 60 μl reaction mixtures containing 40 mM Tris/HCl (pH 7.4), 1 mM DTT, 2 mM MnCl2, 100 μM ATP (unlabelled), 5 μg of rAGT and 20-60 μg of partly purified tyrosine kinase (dialysed bound fraction) were incubated at 30 °C for 1 h. The controls contained all reaction components except the tyrosine kinase. EDTA was then added to 3 mM to stop the kinase reaction; the reaction mixtures were diluted 1:1 with buffer B [40 mM Tris/HCl (pH 7.4)/0.5 mM EDTA/1 mM DTT/10% (v/v) glycerol]. AGT activity in these samples was determined by measuring the transfer of the 3H-radio labelled methyl group from the O6 position of guanine in the DNA to AGT protein, as described previously [8].

RESULTS AND DISCUSSION

Assay for phosphorylation of AGT in vitro and its validation

Our previous study provided evidence for the presence of a protein kinase in HBT228 cell extracts that phosphorylated AGT and evidence that endogenous AGT is a poor substrate for this modification [8]. We therefore developed an electrophoretic assay for the reliable detection and quantification of AGT phosphorylation by using human AGT protein expressed in E. coli (rAGT) as the substrate and cell-free HBT228 extracts as the enzyme source (Figures 1A and 1B). The purified rAGT, which is expected to lack major post-translational modifications, migrated as a discrete 24 kDa protein (Figure 1A, lane 1). After combining with the cell extract, the rAGT migrated to a region unoccupied by many proteins (Figure 1A, lane 3). More importantly, the incubation of cell extracts with [γ-32P]ATP alone did not result in the phosphorylation of 22-26 kDa endogenous proteins, although larger proteins were phosphorylated (Figure 1B, lane 2). Phosphorylation of a 20 kDa band was observed in some experiments; however, this did not interfere with the assay of rAGT phosphorylation. rAGT by itself was not phosphorylated in the presence of [γ-32P]ATP (Figure 1B, lane 1) but the addition of rAGT and [γ-32P]ATP to the cell extracts resulted in the efficient transfer of 3P label to rAGT, thereby demonstrating the utility of rAGT as a substrate for cellular protein kinases (Figure 1B, lane 3). Figure 1(C) shows that the level of rAGT phosphorylation increased linearly with the amount of enzyme protein (15-45 μg). The phosphorylation was maximal with 5 μg of rAGT and 30 min of incubation.

The high level of incorporation of 32P into various cellular proteins often interfered with the accurate assessment of AGT phosphorylation, particularly when cell extracts with more than 60 μg of protein were used. To overcome this problem we exploited the strong affinity of the hexahistidine tag in the rAGT for Talon metal chelator resin. rAGT protein, including the 32P-rAGT present in the reaction mixtures, was selectively purified and subjected to SDS/PAGE. This method provided reproducible recovery of the AGT protein (more than 90%), and eliminated almost all of the background due to the phosphorylation of other endogenous proteins. When duplicate reactions, one subjected to purification with Talon resin and the other without, were processed by SDS/PAGE, the 32P counts associated with the rAGT protein bands were similar, showing that both procedures provided sensitive and reliable quantification of rAGT phosphorylation.
Characterization of the AGT kinase reaction

The cell extracts showed a strict requirement for bivalent cations for phosphorylating rAGT, with Mg\(^{2+}\) and Mn\(^{2+}\) promoting the reaction (Figure 2A). Ca\(^{2+}\) could not replace Mg\(^{2+}\) or Mn\(^{2+}\). The optimal concentrations ranged from 5 to 10 mM for Mg\(^{2+}\) and from 2.5 to 5 mM for Mn\(^{2+}\); Mg\(^{2+}\) was more effective than Mn\(^{2+}\) in activating the reaction. Higher concentrations of Mn\(^{2+}\) ions were inhibitory (Figure 2A). Previously we reported that the endogenous AGT protein present in HBT228 extracts was maximally phosphorylated at 10 mM Mg\(^{2+}\) [8], the slightly lower Mg\(^{2+}\) optimum (5 mM) observed in the present study might have been due to the higher concentration of the substrate and the greater number of phosphorylation sites present in rAGT than in endogenous AGT. To examine further the identity of the kinase involved, the Mg\(^{2+}\)-containing reaction mixtures were supplemented with components that stimulate specific protein kinases, namely protein kinase A, protein kinase C and calmodulin-dependent protein kinases. None of these components affected the level of rAGT phosphorylation (Figure 2C), suggesting that these three kinases are not involved in AGT phosphorylation.

Next we characterized the specificity of the phosphate donors. As shown in Figure 2(B), the cell extracts incorporated \(^{32}\)P into rAGT with either \([\gamma-\text{P}]\text{ATP}\) or \([\gamma-\text{P}]\text{GTP}\) as the phosphate group donor. With equivalent nucleotide concentrations, the band density of \(^{32}\)P-labelled rAGT on autoradiograms and the amount of radioactivity present in the rAGT protein revealed that GTP was half as efficient as ATP in supporting AGT phosphorylation (Figure 2B). Under the conditions used, the optimal concentrations of ATP and GTP required for the reaction were 100 and 50 \(\mu\)M respectively. To ensure that the \([\gamma-\text{P}]\text{GTP}\) included in the assay mixtures was not converted into \([\gamma-\text{P}]\text{ATP}\) by the action of nucleoside diphosphate kinase or other salvage enzymes [20] in the crude extracts used, the fate of labelled GTP was followed over the course of AGT phosphorylation. Using TLC with 0.375 M potassium phosphate, pH 3.5, as the ascending solvent to separate the nucleotides and their derivatives (ATP, ADP, GTP, GDP and P\(_i\)) [21], we found no conversion of \([\gamma-\text{P}]\text{GTP}\) into \([\gamma-\text{P}]\text{ATP}\) during the reaction. Thus the observed modification of AGT by cell extracts must have resulted from the direct use of GTP as a phosphate donor.

Identification of the amino acids phosphorylated in the kinase reactions in vitro

The amino acyl residues of rAGT that were phosphorylated by cell extracts were identified by acid hydrolysis and one-dimensional TLC. Figure 3 shows that serine, threonine and tyrosine residues in rAGT underwent phosphorylation in the presence of ATP or GTP as donors. In a previous study we demonstrated that the AGT protein in HBT228 cells is also phosphorylated at serine, threonine and tyrosine residues [8]. Taken together, our results strongly suggest that the kinases characterized in the present study are the enzymes that act on the AGT protein intracellularly.

Many of the properties described here for AGT phosphorylation, such as the promotion of the reaction by GTP as well as ATP, the activation by Mn\(^{2+}\) or Mg\(^{2+}\) and the resulting tyrosine modification, are intriguing but did not permit the easy identification of the kinase involved. Generally, the well-characterized serine/threonine kinases phosphorylate the hydroxy groups on the \(\beta\)-carbon of serine and threonine, whereas the phenolic hydroxy group of tyrosine is modified by protein tyrosine kinases. However, a third group of enzymes, designated dual-specificity protein kinases, which phosphorylate serine and threonine residues as well as tyrosine residues, have been described in Drosophila, yeast and humans [22,23]. Our results on the characteristics of AGT phosphorylation argue for the involvement of either (1) at least two kinases, one phosphorylating the aliphatic residues and one the aromatic residues, or (2) a single dual-specificity enzyme modifying serine, threonine and tyrosine residues.

Effect of CK II inhibitors and activators on AGT phosphorylation

The ubiquitous and well-characterized CK II [24] shares many of the characteristics that we observed for AGT phosphorylation,
Phosphorylation by HBT228 cell extracts. SDS/PAGE assay was applied to detect 32P incorporation. Tyrosine phosphorylation of AGT would bind to this affinity matrix, whereas the serine/threonine-modifying activity would not bind. The fractionation resulted in two distinct peaks of AGT kinase activity (Figure 5). The first peak of activity (fractions 5–15) was in the unbound fraction; the second peak of activity was in the bound fraction and was eluted with 0.3 M NaCl. The pooled and dialysed unbound and bound kinase fractions were used for further characterization.

Properties of the partly purified tyrosine kinase

Measuring 32P incorporation into poly(Glu/Tyr) showed that the tyrosine kinase (bound fraction) was purified approx. 30-fold by the chromatography step. The bound fraction was able to use [γ-32P]GTP or [γ-32P]ATP to phosphorylate rAGT protein (Figure 6A) and the poly(Glu/Tyr) substrate (results not shown). At 100 μM nucleotide, the 32P incorporated into the AGT protein with GTP was approx. 50% less than with ATP. Further, unlabelled GTP (50–100 μM) significantly inhibited the homogenate-catalysed modification of AGT when [γ-32P]ATP was used as the phosphate donor (Figure 6B). Because most kinases require millimolar levels of GTP for inhibition, this result confirmed the use of GTP as the phosphate donor by the AGT tyrosine kinase and revealed its unique property. The kinase was characterized further by identifying the specific amino acid phosphorylated in AGT when [γ-32P]ATP was used. Phosphoamino acid analysis of rAGT confirmed the use of GTP as the phosphate donor (Figure 6B). Because most kinases require millimolar levels of GTP for inhibition, this result confirmed the use of GTP as the phosphate donor by the AGT tyrosine kinase and revealed its unique property.
Figure 4 Effect of CK II activators and inhibitors on the phosphorylation of rAGT by cell extracts

(A) The phosphorylation of rAGT by HBT228 cell extracts with the supplementation of 40 mM KCl (second lane) and 1 mM spermidine (third lane) was determined. (B) Heparin was added to the cell extracts at the concentrations specified; the samples were then maintained at room temperature for 5 min. This was followed by the addition of rAGT substrate and determination of the performance of kinase reactions as described in the Experimental section. (C) Cell extracts were preincubated with DRB at the concentrations indicated; the phosphorylation of rAGT was then determined. Reaction mixtures were subjected to SDS/PAGE without rAGT purification in all these experiments.

Figure 5 Chromatography of the cell extracts on poly(Glu/Tyr)-Sepharose resolves the two kinase activities acting on rAGT protein

HBT228 cell extracts were fractionated and the bound proteins were eluted with 0.3 M NaCl, as described in the Experimental section. Similar results were obtained in three separate experiments.

effect of genestein on AGT phosphorylation was minimal (25% inhibition at 40 μM), whereas tyrphostin 25 inhibited the reaction strongly in a dose-dependent manner with 90% inhibition at 40 μM (Figure 7A).

Characterization of the AGT kinase activity excluded from poly(Glu/Tyr)-Sepharose

In contrast with the tyrosine-kinase-bound fraction, the unbound fraction was devoid of kinase activity towards the poly(Glu/Tyr) polymer but still phosphorylated the rAGT protein strongly in the presence of [γ-32P]GTP or [γ-32P]ATP (Figure 8A). In different experiments, the incorporation of 32P in the presence of GTP was 30–55% of that observed with ATP. This kinase remained largely insensitive to heparin, with approx. 30% inhibition at 8 μM (results not shown), and phosphorylated the rAGT exclusively on serine and threonine residues but not on tyrosine residues, as revealed by the differential chemical stability of phosphate groups linked to aliphatic and aromatic amino acids (Figure 8B). Whereas phosphate groups linked to serine and threonine are stable in acid but labile in alkaline solutions, phosphate on tyrosine is stable both in acid and alkali [16]. As shown in Figure 8(B, lower panel), the 32P label incorporated
Physicochemical characterization of AGT kinases present in HBT228 extracts

The protein fraction showing tyrosine kinase activity (the bound fraction) was subjected to SDS/PAGE along with the unbound fraction and the HBT228 cell extract to assess protein purification (Figure 9A). There were approximately five major and six minor protein bands of 25–170 kDa in the bound fraction (Figure 9A, lane 3). The unbound fraction, showed little change from the cell extract in the protein staining pattern. These results and those of the in-gel kinase assays showing a molecular mass of 175 kDa for the AGT tyrosine kinase (described in the next section) suggest that AGT tyrosine kinase is a minor component in the bound fraction and also point to its low abundance in cells.

Despite our demonstration of the involvement of intrinsic serine/threonine and tyrosine kinases in the phosphorylation of AGT, the question of whether these activities found in unfractionated and partly fractionated extracts comprise a single kinase or multiple kinases in each category has not been answered. To determine the number of kinases acting on AGT and their molecular masses, we performed in-gel kinase activity assays by subjecting HBT228 cell extract and the bound fraction to electrophoresis under denaturing (Figure 9B) and non-denaturing (Figure 9C) conditions. These assays were performed without or with rAGT protein in the polymerized gel to reveal and distinguish between the kinase autophosphorylation and the phosphorylation specific to AGT protein respectively. In the SDS/PAGE-based assay with protein denaturation and renaturation before phosphorylation in situ, the rAGT-containing gel (Figure 9B, right panel) showed two intense bands of phosphorylation in the cell homogenate (lane 1) and one band of similar intensity in the tyrosine kinase fraction (lane 2) that co-migrated with the upper band in the homogenate, thereby demonstrating the presence of two AGT-specific protein kinases in HBT228 extracts, one of which binds to poly(Glu/Tyr)-Sepharose. In the gel without rAGT (Figure 9B, left panel), the same band pattern was evident, but the bands were 30–45-fold less intense than those observed with rAGT, indicating that the AGT kinases underwent autophosphorylation. Prolonged autoradiography of this gel revealed three additional faint bands, indicating the presence of other autophosphorylating kinases in the extract. The molecular masses of the two AGT kinases observed in Figure 9B were computed as approx. 130 kDa and 75 kDa.

In-gel kinase assays were also performed under non-denaturing conditions (Figure 9C) to determine the native structure of the AGT kinases and to gain some insight into their protein composition. The phosphorylation band pattern obtained after subjecting the cell extracts to electrophoresis again revealed two AGT kinases (Figure 9C, right panel, lane 1), and the bound fraction contained a single kinase (lane 2) that co-migrated with the second band found in the cell extracts. The ability of both kinases to undergo autophosphorylation was again evident from the pattern in the gel that was polymerized without rAGT protein (Figure 9C, left panel). From the foregoing and from the properties of the AGT kinases described so far, we draw the following conclusions. A single tyrosine kinase of 130 kDa and a single serine/threonine kinase of 75 kDa participate in phosphorylating the AGT protein in HBT228 cells. Both kinases are apparently monomeric. The tyrosine kinase, despite having a higher mass (130 kDa), migrated faster on native gels than its that binds to (poly) Glu/Tyr-Sepharose, are involved in phosphorylating the AGT protein in human brain tumour cells.

Figure 7 Effect of the tyrosine kinase inhibitors tyrphostin 25 (A) and genistein (B) on rAGT phosphorylation catalysed by the fraction bound to poly(Glu/Tyr)-Sepharose

Genistein and tyrphostin 25 were added to the reactions; rAGT phosphorylation was quantified by protein pull-down followed by SDS/PAGE assay. Results are means ± S.E.M. for four independent experiments.

Figure 8 Characterization of the protein kinase excluded from poly-(Glu/Tyr)-Sepharose

(A) Phosphorylation of rAGT protein by the unbound fraction in the presence of 100 μM [γ-32P]ATP or [γ-32P]GTP. (B) The differential chemical stabilities of the phosphate groups reveal the modification of serine and threonine, but not tyrosine, residues in the rAGT modified by the unbound fraction. Upper panel: rAGT was phosphorylated in triplicate, resolved by SDS/PAGE, transferred to PVDF membranes by electrotransfer and autoradiographed to detect by the unbound fraction. Upper panel: rAGT was phosphorylated in triplicate, resolved by SDS/PAGE along with the unbound fraction and the HBT228 cell extract to assess protein purification (Figure 9A). There were approximately five major and six minor protein bands of 25–170 kDa in the bound fraction (Figure 9A, lane 3). The unbound fraction, showed little change from the cell extract in the protein staining pattern. These results and those of the in-gel kinase assays showing a molecular mass of 175 kDa for the AGT tyrosine kinase (described in the next section) suggest that AGT tyrosine kinase is a minor component in the bound fraction and also point to its low abundance in cells.

Despite our demonstration of the involvement of intrinsic serine/threonine and tyrosine kinases in the phosphorylation of AGT, the question of whether these activities found in unfractionated and partly fractionated extracts comprise a single kinase or multiple kinases in each category has not been answered. To determine the number of kinases acting on AGT and their molecular masses, we performed in-gel kinase activity assays by subjecting HBT228 cell extract and the bound fraction to electrophoresis under denaturing (Figure 9B) and non-denaturing (Figure 9C) conditions. These assays were performed without or with rAGT protein in the polymerized gel to reveal and distinguish between the kinase autophosphorylation and the phosphorylation specific to AGT protein respectively. In the SDS/PAGE-based assay with protein denaturation and renaturation before phosphorylation in situ, the rAGT-containing gel (Figure 9B, right panel) showed two intense bands of phosphorylation in the cell homogenate (lane 1) and one band of similar intensity in the tyrosine kinase fraction (lane 2) that co-migrated with the upper band in the homogenate, thereby demonstrating the presence of two AGT-specific protein kinases in HBT228 extracts, one of which binds to poly(Glu/Tyr)-Sepharose. In the gel without rAGT (Figure 9B, left panel), the same band pattern was evident, but the bands were 30–45-fold less intense than those observed with rAGT, indicating that the AGT kinases underwent autophosphorylation. Prolonged autoradiography of this gel revealed three additional faint bands, indicating the presence of other autophosphorylating kinases in the extract. The molecular masses of the two AGT kinases observed in Figure 9B were computed as approx. 130 kDa and 75 kDa.

In-gel kinase assays were also performed under non-denaturing conditions (Figure 9C) to determine the native structure of the AGT kinases and to gain some insight into their protein composition. The phosphorylation band pattern obtained after subjecting the cell extracts to electrophoresis again revealed two AGT kinases (Figure 9C, right panel, lane 1), and the bound fraction contained a single kinase (lane 2) that co-migrated with the second band found in the cell extracts. The ability of both kinases to undergo autophosphorylation was again evident from the pattern in the gel that was polymerized without rAGT protein (Figure 9C, left panel). From the foregoing and from the properties of the AGT kinases described so far, we draw the following conclusions. A single tyrosine kinase of 130 kDa and a single serine/threonine kinase of 75 kDa participate in phosphorylating the AGT protein in HBT228 cells. Both kinases are apparently monomeric. The tyrosine kinase, despite having a higher mass (130 kDa), migrated faster on native gels than its that binds to (poly) Glu/Tyr-Sepharose, are involved in phosphorylating the AGT protein in human brain tumour cells.

Figure 7 Effect of the tyrosine kinase inhibitors tyrphostin 25 (A) and genistein (B) on rAGT phosphorylation catalysed by the fraction bound to poly(Glu/Tyr)-Sepharose

Genistein and tyrphostin 25 were added to the reactions; rAGT phosphorylation was quantified by protein pull-down followed by SDS/PAGE assay. Results are means ± S.E.M. for four independent experiments.

Figure 8 Characterization of the protein kinase excluded from poly-(Glu/Tyr)-Sepharose

(A) Phosphorylation of rAGT protein by the unbound fraction in the presence of 100 μM [γ-32P]ATP or [γ-32P]GTP. (B) The differential chemical stabilities of the phosphate groups reveal the modification of serine and threonine, but not tyrosine, residues in the rAGT modified by the unbound fraction. Upper panel: rAGT was phosphorylated in triplicate, resolved by SDS/PAGE, transferred to PVDF membranes by electrotransfer and autoradiographed to detect [32P]-labelled rAGT. Lower panel: the membrane was cut into three strips; one strip was untreated, the second was treated with acid, and the third was treated with alkali and then autoradiographed as described in the Experimental section.
Figure 9  Protein staining pattern of fractions from poly(Glu/Tyr)-Sepharose chromatography and in-gel kinase activity assays after SDS/PAGE and non-denaturing PAGE

(A) Coomassie Blue staining of the SDS-containing gel after electrophoresis of equivalent protein amounts (40 μg) from unfractionated HBT228 cell extract (lane 1), unbound fraction (lane 2) and bound fraction (lane 3). (B) In-gel kinase assay after SDS/PAGE. HBT228 cell extract (lane 1) and the tyrosine kinase fraction bound to poly(Glu/Tyr)-Sepharose (lane 2) were subjected to SDS/PAGE [8% (w/v) gel] on gels that were polymerized in the absence (left panel) or presence (right panel) of rAGT protein. The fractionated proteins were denatured and renatured in situ followed by the kinase reaction as described in the Experimental section. The autoradiographic pattern of the gels exposed for same length of time (10 h) is shown. Kinase activity bands representing autophosphorylation (left panel) and AGT phosphorylation (right panel) are indicated with arrows. (C) In-gel kinase assay after electrophoresis on gels without SDS. HBT228 cell extract (lane 1) and the tyrosine kinase fraction (lane 2) were subjected to SDS/PAGE [8% (w/v) gel] on gels that were polymerized in the absence (left panel) or presence (right panel) of rAGT protein. Both gels were subjected to kinase reactions in situ then washed and autoradiographed for 6 h to obtain the patterns shown.

low-molecular-mass serine/threonine counterpart (75 kDa). This opposite migration pattern of the AGT kinases on SDS/PAGE and non-denaturing PAGE (Figures 9B and 9C) is intriguing and provides an important clue to their amino acid composition: it seems that AGT tyrosine kinase is a more acidic neutral protein than the relatively basic serine/threonine kinase.

Effect of AGT inactivation on its phosphorylation

We have shown previously that AGT inactivated by BG becomes a substrate for ubiquitin conjugation, which is subsequently degraded by the proteasome [3]. It seems that for many proteins the two post-translational modifications, namely phosphorylation and ubiquitination, are closely linked, with the former often functioning as a signal for the selective destruction of proteins through the ubiquitin pathway [30,31]. It was therefore of interest to compare the phosphorylation of the BG-inactivated AGT with that of active protein. Figure 10(A) shows that BG-treated and untreated rAGT were phosphorylated to the same extent by HBT228 cell extracts. However, the results of this study do not preclude a role for phosphorylation in AGT degradation. For example, in cells, the phosphorylation of AGT on specific residues might facilitate the interaction of inactivated AGT with the enzymes catalysing ubiquitin conjugation [30].

Physical association of AGT with its kinases in vivo

Many of the enzymes in nucleic acid metabolism, such as RNA polymerase I, topoisomerase II and other cellular proteins, have been reported to exhibit tight and specific association with their protein kinases, as shown by their co-purification and their ability to form stable complexes [32–34]. To examine the possibility of AGT associating with its kinase(s), we exploited the immune kinase assay procedure, which is routinely used to study the phosphorylation associated with cyclin-dependent cell cycle kinases [35]. AGT and associated proteins were immunoprecipitated from HBT228 cell extracts and incubated with [γ-32P]ATP and rAGT substrate. As shown in Figure 10(B), rAGT became phosphorylated in complexes isolated with anti-
make up the tyrosine kinase motif in mammalian AGTs suggests a crucial role for this post-translational modification. The presence of an endogenous tyrosine kinase that phosphorylated AGT protein (in the bound fraction) provided us with an opportunity to study the specific effect of tyrosine phosphorylation on AGT activity. As shown in Figure 11(B), the reaction of rAGT with the partly purified tyrosine kinase significantly decreased the demethylation activity of rAGT (by more than 50%, P < 0.05). The degree of inhibition was correlated with the amount of tyrosine kinase used for phosphorylation. One possible mechanism by which tyrosine phosphorylation might down-regulate AGT activity is by interference with DNA binding; however, this needs to be proved. Nevertheless, our results suggest that tyrosine phosphorylation might have a major role in regulating AGT activity in human cells.

Conclusions

The identification and characterization of the cellular protein kinases involved in the phosphorylation of AGT are crucial for understanding the cellular regulation of AGT. In the present study we investigated the AGT kinases present in a human brain tumour cell line and identified two cytosolic protein kinases, a tyrosine kinase of 130 kDa and a serine/threonine kinase of 75 kDa, that phosphorylate AGT. Both of these kinases seem to be new and previously undescribed, as is evident from their molecular masses and novel biochemical properties. The serine/threonine kinase resembled the classical CK II in its ability to use Mn²⁺ and GTP in addition to ATP as a phospho donor; however, it differed in size from CK II and was insensitive to established CK II activators and inhibitors. The AGT tyrosine kinase was also able to use GTP as well as ATP as the phosphate donor; this is unique because very few tyrosine kinases have been reported to use both GTP and ATP to phosphorylate their substrates. Several non-receptor cytosolic tyrosine kinases, such as c-Abl, Tyk2 and Jak1, with molecular masses in the range 120–140 kDa, have been described previously [39,40]; however, the exact identities of the AGT tyrosine kinase and its serine/threonine kinase will require the purification of their proteins to homogeneity and/or their cDNA cloning. Protein phosphorylation seems to be a key post-translational modification for human AGT, with the potential to affect many of its structural and functional aspects such as protein turnover [41], subcellular localization [42] and catalytic activity, as demonstrated here and previously [8]. A greater understanding of the AGT kinases is therefore likely to shed new light on the cellular regulation of AGT under normal and pathological conditions as well as providing important clues on the molecular response of AGT to DNA damage. In addition, given the importance of AGT in preventing carcinogenesis and the continuing efforts at inhibiting AGT activity to improve chemotherapy, our findings also open up the possibility that AGT kinases might be targeted for the modulation of AGT activity in chemoprevention and cancer therapy.

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