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

Srinivas R. S. MULLAPUDI, Francis ALI-OSMAN, Jiang SHOU and Kalkunte S. SRIVENUGOPAL¹

Section of Molecular Therapeutics, Department of Neurosurgery, Box 169, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, U.S.A.

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 [Srivenuogal, 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 Mg²⁺ more efficiently than Mn²⁺ for phosphorylating human recombinant AGT (rAGT) protein. Both [γ -³²P]ATP and [γ -³²P]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. Phospho-aminoacid 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)-Sephacrose 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 genestein. 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 alkylating agents [1,2]. This protective function is due to the specific ability of AGT to remove alkyl groups bound to the *O*⁶ position of guanine and the *O*⁴ position of thymine and to restore the base integrity of the DNA, thereby preventing G → A and T → C transitions respectively. This dealkylation *in situ* occurs in a stoichiometric reaction in which the alkyl groups are transferred to a cysteine residue in the active site of AGT. Because the alkyl groups are covalently bound to the cysteine residue, AGT is inactivated 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) and chloroethylating agents [1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea] induce *O*⁶-methylguanine and *O*⁶-chloroethylguanine lesions respectively, which act as efficient substrates for AGT

[2,4]. With bifunctional alkylating agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea, the removal of chloroethyl adducts from guanine prevents the production of cytotoxic DNA inter-strand cross-links [1]. Because of these characteristics, human AGT has emerged as a key target for improving cancer therapy 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 myelotoxicity 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- β -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.

¹ To whom correspondence should be addressed (e-mail ksrivenu@mdanderson.org).

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

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol) and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (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/Tyr) (4:1) co-polymer 20–60 kDa in molecular mass, poly(Glu/Tyr)-Sephacrose, phosphatase inhibitor cocktail (100-fold concentrated mixture of sodium vanadate, sodium molybdate, sodium tartrate and imidazole), and all other chemicals were from Sigma Chemicals (St Louis, MO, U.S.A.).

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 benzamide, 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 10000 *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)₆GS-], 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* JM109 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 thioglycoside 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, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (100 μM ; 2.5 μCi), 5 mM MgCl_2 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 $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ 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 ^{32}P -labelled protein was extracted by electroelution in 50 mM NH_4HCO_3 /0.1% SDS at 4 °C for 4 h. The eluates were dialysed overnight against 50 mM NH_4HCO_3 , 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 $\times$ 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 ^{32}P label associated with the spots was detected by autoradiography.

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

Affinity chromatography of cell extracts was performed on poly(Glu/Tyr)-Sephacrose, 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)-Sephacrose in the cold at a flow rate of 40 μ l/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 μ g) 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 32 P-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 μ g) and the fraction bound to the poly(Glu/Tyr)-Sephacrose (6 μ g) were subjected to SDS/PAGE [8% (w/v) gel] with or without the addition of rAGT protein (250 μ g/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 - 32 P]ATP (10 μ Ci/ml). After incubation, the gel was washed with 5% (w/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.

In-gel kinase assays under non-denaturing conditions were performed similarly without SDS in the PAGE. However, the stacking and separating gels contained 0.5 mM DTT and the electrophoresis was performed at 4 °C. The gels were suspended directly in the kinase assay buffer without the denaturation and renaturation steps, and then processed for autoradiography as described above.

Immune kinase assay

To examine the association of AGT kinase with its substrate, HBT 228 cell extract (300 μ g 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 μ M ATP, 2 μ Ci of [γ - 32 P]ATP and 4 μ g 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 μ l reaction mixtures containing 40 mM Tris/HCl (pH 7.4), 1 mM DTT, 2 mM MnCl₂, 100 μ M ATP (unlabelled), 5 μ g of rAGT and 20–60 μ g 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 3 H-radiolabelled 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 - 32 P]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 - 32 P]ATP (Figure 1B, lane 1) but the addition of rAGT and [γ - 32 P]ATP to the cell extracts resulted in the efficient transfer of 32 P 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 μ g). The phosphorylation was maximal with 5 μ g of rAGT and 30 min of incubation.

The high level of incorporation of 32 P into various cellular proteins often interfered with the accurate assessment of AGT phosphorylation, particularly when cell extracts with more than 60 μ g 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 32 P-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 32 P counts associated with the rAGT protein bands were similar, showing that both procedures provided sensitive and reliable quantification of rAGT phosphorylation.

Figure 1 Assay for the phosphorylation of human rAGT protein by the endogenous kinases present in HBT228 cell extracts

(A, B) The rAGT substrate (5 µg) was incubated with or without cell extract (20 µg of protein) in the presence of Mg^{2+} and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 30 min, followed by SDS/PAGE as described in the Experimental section. The migration of proteins as detected by Coomassie Blue staining is shown in (A). This gel was dried and exposed to film to obtain the pattern of ^{32}P -labelled proteins shown in (B). The bands in the left-most lane in (A) are the molecular-mass standards. The effect of protein concentration on rAGT phosphorylation in this assay is shown in (C). The locations of rAGT bands are marked.

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 $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ 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 µM respectively. To ensure that the $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ included in the assay mixtures was not converted into $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ 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 $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ into $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ 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 β -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,

Figure 2 Choice of bivalent cations, phosphate group donors and the effect of compounds required for the stimulation of specific protein kinases on rAGT phosphorylation in cell extracts

(A) Autoradiogram showing the effect of increasing MgCl_2 or MnCl_2 concentration on rAGT phosphorylation by HBT228 cell extracts. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was the phosphate donor. Reactions were assayed by pulling down rAGT with Talon resin followed by SDS/PAGE. (B) Autoradiogram showing the ability of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ to serve as a direct phosphate donor for rAGT phosphorylation in cell extracts. The reactions contained a constant level of radiolabel ($2.5\text{ }\mu\text{Ci}$ of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$) with the corresponding unlabelled nucleotides at the concentrations specified. Phosphorylation was assayed by rAGT pull-down and SDS/PAGE. (C) The effect of specific components required for the activities of protein kinase A, protein kinase C and calmodulin-dependent kinases on rAGT phosphorylation. The basic reaction mixtures containing 5 mM Mg^{2+} and $100\text{ }\mu\text{M } [\gamma\text{-}^{32}\text{P}]\text{ATP}$ and cell extract (lane 1) were supplemented with $100\text{ }\mu\text{M cAMP}$ (lane 2), $200\text{ }\mu\text{M CaCl}_2$, $10\text{ }\mu\text{g/ml}$ diolefin and $400\text{ }\mu\text{g/ml}$ phosphatidylserine (lane 3) and $200\text{ }\mu\text{M CaCl}_2$ and $40\text{ }\mu\text{g/ml}$ calmodulin (lane 4). rAGT pull-down followed by SDS/PAGE assay was applied to detect ^{32}P incorporation.

namely the use of Mn^{2+} and GTP [24,25] and the ability to mediate serine/threonine phosphorylation. Therefore, to determine whether the AGT phosphorylation that we saw was mediated by CK II, we studied the effect of CK II activators and inhibitors on the reaction. The univalent cation K^+ and spermidine, both known to enhance CK II activity [24,26], did not affect the phosphorylation of rAGT (Figure 4A). Heparin inhibited the reaction only slightly over a concentration range of $4\text{--}6\text{ }\mu\text{M}$ (Figure 4B), which is 28–40-fold that required for maximal inhibition of CK II [27]. DRB, which exerts a strong and specific inhibition of CK II activity at doses of $2.5\text{--}10\text{ }\mu\text{M}$ [28], did not curtail AGT phosphorylation (Figure 4C). These results indicate that CK II or its variants [24] are unlikely to be involved in the cellular phosphorylation of AGT.

Resolution of the two kinase activities that phosphorylate AGT

To determine whether HBT228 cell extracts contain more than one AGT kinase, we performed chromatography of the extracts on Sepharose linked to a random polymer of glutamic acid and tyrosine, which is an exclusive substrate for protein tyrosine kinases [15]. We hypothesized that the kinase associated with the tyrosine phosphorylation of AGT would bind to this affinity

Figure 3 Identification of the amino acids phosphorylated in the rAGT protein by cell extracts in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$

The rAGT protein phosphorylated in the presence of labelled ATP or GTP was subjected to acid hydrolysis, mixed with phosphorylated amino acid standards [phosphoserine (p-ser), phosphothreonine (p-thr) and phosphotyrosine (p-tyr)] and analysed by one-dimensional TLC as described in the Experimental section. The chromatogram was first sprayed with ninhydrin to locate the phosphorylated amino acids, then autoradiographed to detect the ^{32}P -labelled amino acids.

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 ^{32}P 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 $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ or $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to phosphorylate rAGT protein (Figure 6A) and the poly(Glu/Tyr) substrate (results not shown). At $100\text{ }\mu\text{M}$ nucleotide, the ^{32}P incorporated into the AGT protein with GTP was approx. 50% less than with ATP. Further, unlabelled GTP ($50\text{--}100\text{ }\mu\text{M}$) significantly inhibited the homogenate-catalysed modification of AGT when $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ 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. Phospho amino acid analysis of rAGT after phosphorylation by the bound fraction (0.3 M NaCl eluate) showed that only tyrosine was phosphorylated (Figure 6C). These findings demonstrate conclusively that a tyrosine kinase is involved in AGT protein modification and that it was separated from its serine/threonine counterpart by chromatography on poly(Glu/Tyr)-Sepharose.

Tyrosine phosphorylation of AGT was probed with two inhibitors, genestein and tyrphostin 25, which are specific to protein tyrosine kinases. These inhibitors, which curtail the activity of a wide range of tyrosine kinases by competing with the ATP-binding site and/or the substrate-binding site, were tested over a concentration range of $20\text{--}40\text{ }\mu\text{M}$, which is commonly used for these compounds [29]. As shown in Figure 7(B), the

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)-Sephacel 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 μ M), whereas tyrphostin 25 inhibited the reaction strongly in a dose-dependent manner with 90 % inhibition at 40 μ M (Figure 7A).

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

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 - 32 P]GTP or [γ - 32 P]ATP (Figure 8A). In different experiments, the incorporation of 32 P 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 μ M (results not shown), and phosphorylated the

Figure 6 Characterization of rAGT-phosphorylating activity bound to poly(Glu/Tyr)-Sephacel

(A) Phosphorylation of rAGT protein by the fraction bound to the column (0.3 M NaCl eluate) in the presence of 100 μ M [γ - 32 P]ATP or [γ - 32 P]GTP. (B) Inhibition of rAGT phosphorylation by unlabelled GTP. Phosphorylation reactions contained 100 μ M [γ - 32 P]ATP and the bound kinase fraction. (C) Phospho amino acid analysis of rAGT modified by the bound fraction. rAGT was phosphorylated in the presence of [γ - 32 P]ATP; it was then subjected to acid hydrolysis. The hydrolysate was mixed with phospho amino acid standards [phosphoserine (p-ser), phosphothreonine (p-thr) and phosphotyrosine (p-tyr)] and analysed by TLC as described in the Experimental section.

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 32 P label incorporated

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

Genestein and tyrphostin 25 were added to the reactions; rAGT phosphorylation was quantified by protein pull-down followed by SDS/PAGE assay. Results are means $\pm$ 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 μ M [γ - 32 P]ATP or [γ - 32 P]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 32 P-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.

into rAGT by the unbound kinase fraction remained stable in hot acid but was lost after treatment with hot NaOH, indicating the absence of tyrosine phosphorylation. Collectively, the results of the rAGT phosphorylation in unfractionated cell extracts (Figure 3) and the data from poly(Glu/Tyr) chromatography (Figures 5 and 6) demonstrate that two classes of protein kinase, a serine/threonine type and a protein tyrosine kinase

that binds to (poly) Glu/Tyr-Sepharose, are involved in phosphorylating the AGT protein in human brain tumour cells.

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 re-naturation 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

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 μ g) 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

Figure 10 Effect of BG treatment on rAGT phosphorylation and evidence for physical association between AGT and its kinase(s)

(A) rAGT protein (5 μ g) was exposed to 20 μ M BG for 10 min at room temperature. The substrates with and without BG treatment were then subjected to phosphorylation by HBT228 cell extracts. (B) Immune kinase assays. AGT protein present in HBT228 cell extract was immunoprecipitated with preimmune serum (lane 1) or AGT antiserum (lane 2) and AGT-specific monoclonal antibodies. Washed immunocomplexes were supplemented with rAGT substrate and [γ - 32 P]ATP for kinase assays followed by SDS/PAGE as described in the Experimental section. No phosphorylation of rAGT was detected in complexes isolated with preimmune serum.

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 - 32 P]ATP and rAGT substrate. As shown in Figure 10(B), rAGT became phosphorylated in complexes isolated with anti-

Figure 11 Conservation of a tyrosine phosphorylation site in mammalian AGTs and the effect of tyrosine phosphorylation on AGT activity

(A) Human and mammalian AGT protein sequences were searched for tyrosine kinase recognition sites on the PROSITE database (www.expasy.ch/tools/scnpsite.html [36]). The search resulted in a single tyrosine kinase motif in all AGTs, which is shown. (B) Tyrosine phosphorylation of AGT inhibits its activity. The activity of rAGT protein was quantified after its phosphorylation by the partly purified tyrosine kinase (bound fraction) as described in the Experimental section. Results are means $\pm$ S.E.M. for four separate experiments.

AGT polyclonal (lane 2) or monoclonal antibodies (lane 3) but not with the preimmune serum (lane 1). These results demonstrate that one or both kinases described in this study associate physically with their substrate under physiological conditions. The failure to detect the phosphorylation of endogenous AGT (21–22 kDa) in these experiments might relate to its rarity in the immunoprecipitates. Although the biological significance of this protein–protein interaction remains to be explored in detail, it is tempting to speculate that the kinases might induce rapid changes in the phosphorylation of AGT and permit the acute regulation of its activity.

Effect of tyrosine phosphorylation of AGT protein on its activity

We showed previously that the phosphorylation of the endogenous AGT by the kinases present in HBT228 cell extracts resulted in a significant inhibition of AGT activity [8]. However, at present it is unclear whether overall phosphorylation (serine, threonine and tyrosine) or a specific phosphorylation is responsible for the down-regulation of AGT function. To obtain further insight into the importance of tyrosine modification, we performed a computer-assisted motif search [36] of human and other mammalian AGT proteins. All these AGTs were found to have a common tyrosine kinase recognition site around residues 111–118 (residues 107–114 for human AGT) (Figure 11A). Molecular models [37] and evidence from a recent crystal structure [38] have located Tyr-114, the potential phosphorylation site in human AGT, in the DNA-binding domain of the AGT protein near its active site. In this context, the remarkable conservation of this tyrosine and the stretch of amino acids that

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 Mn^{2+} 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.

This work was supported by National Institutes of Health grant CA 74321 and grants from the M. D. Anderson Biomedical Research Support Committee, the National Childhood Cancer Foundation and the Pediatric Brain Tumor Foundation of the United States, all to K.S.S.

REFERENCES

- Pegg, A. E., Dolan, M. E. and Moschel, R. C. (1995) Structure, function, and inhibition of O⁶-alkylguanine-DNA alkyltransferase. *Prog. Nucleic Acid Res. Mol. Biol.* **51**, 167–223
- Mitra, S. and Kaina, B. (1993) Regulation of repair of alkylation damage in mammalian genomes. *Prog. Nucleic Acid Res. Mol. Biol.* **44**, 109–142

- 3 Srivenugopal, K. S., Yuan, X.-H., Friedman, H. S. and Ali-Osman, F. (1996) Ubiquitination-dependent proteolysis of O⁶-methylguanine-DNA methyltransferase in human and murine tumour cells following inactivation with O⁶-benzylguanine or 1,3-bis(2-chloroethyl)-1-nitrosourea. *Biochemistry* **35**, 1328–1334
- 4 Gerson, S. L. and Willson, J. K. V. (1995) O⁶-alkylguanine-DNA alkyltransferase, a target for the modulation of drug resistance. *Hematol.-Oncol. Clin. N. Am.* **9**, 431–450
- 5 Dolan, M. E. and Pegg, A. E. (1997) O⁶-benzylguanine and its role in chemotherapy. *Clin. Cancer Res.* **3**, 837–847
- 6 Spiro, T. P., Gerson, S. L., Liu, L., Majka, S., Haaga, J., Hoppel, C. L., Ingalls, S. T., Pinda, J. M. and Willson, J. K. (1999) O⁶-benzylguanine: a clinical trial establishing the biochemical modulatory dose in tumour tissue for alkyltransferase-directed DNA repair. *Cancer Res.* **59**, 2402–2410
- 7 Davis, B. M., Roth, J. C., Liu, X., Xu-Welliver, M., Pegg, A. E. and Gerson, S. L. (1999) Characterization of the P140K, PVP(138–140)MLK, and G156A O⁶-methylguanine-DNA methyltransferase mutants: implications for drug resistance gene therapy. *Hum. Gene Ther.* **10**, 2769–2778
- 8 Srivenugopal, K. S., Mullapudi, S. R. S., Shou, J., Hazra, T. K. and Ali-Osman, F. (2000) Protein phosphorylation is a regulatory mechanism for O⁶-alkylguanine-DNA alkyltransferase in human brain tumour cells. *Cancer Res.* **60**, 282–287
- 9 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- 10 Edara, S., Kanugula, S., Goodtsova, K. and Pegg, A. E. (1996) Resistance of the human O⁶-alkylguanine-DNA alkyltransferase containing arginine at codon 160 to inactivation by O⁶-benzylguanine. *Cancer Res.* **56**, 5571–5575
- 11 Blackwell, J. R. and Horgan, R. (1991) A novel strategy for production of a highly expressed recombinant protein in an active form. *FEBS Lett.* **295**, 10–12
- 12 Xu-Welliver, M., Leitao, J., Kanugula, S. and Pegg, A. E. (1996) Alteration of the conserved residue tyrosine-158 to histidine renders human O⁶-alkylguanine-DNA alkyltransferase insensitive to the inhibitor O⁶-benzylguanine. *Cancer Res.* **59**, 1514–1519
- 13 Boyle, W. J., Van der Geer, P. and Hunter, T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* **201**, 110–149
- 14 Braun, S., Abdel Ghany, M., Letteri, J. A. and Racker, E. (1986) Partial purification and characterization of protein tyrosine kinases from normal tissues. *Arch. Biochem. Biophys.* **247**, 424–432
- 15 Adamikova, D., Resnick, R. J. and Tomaska, L. (1996) Enrichment of yeast protein tyrosine kinase activity by substrate affinity chromatography. *Yeast* **12**, 833–838
- 16 Martensen, T. M. (1984) Chemical properties, isolation, and analysis of O-phosphates in proteins. *Methods Enzymol.* **107**, 3–23
- 17 Kameshita, I. and Fujisawa, H. (1989) A sensitive method for detection of calmodulin-dependent protein kinase II activity in sodium dodecyl sulfate-polyacrylamide gels. *Anal. Biochem.* **183**, 139–143
- 18 Ostrowski, L. E., Pegram, C. N., von Wronski, M. A., Humphrey, P. A., He, X., Shiota, S., Mitra, S., Brent, T. P. and Bigner, D. D. (1991) Production and characterization of antipeptide antibodies against human O⁶-methylguanine-DNA methyltransferase. *Cancer Res.* **51**, 3339–3344
- 19 Brent, T. P., von Wronski, M., Pegram, C. N. and Bigner, D. D. (1990) Immunoaffinity purification of human O⁶-alkylguanine-DNA alkyltransferase using newly developed monoclonal antibodies. *Cancer Res.* **50**, 58–61
- 20 Kornberg, A. (1980) DNA Replication. W. H. Freeman, San Francisco
- 21 Cui, T., Sugrue, R. J., Xu, Q., Lee, A. K. W., Chan, Y.-C. and Fu, J. (1998) Recombinant dengue virus type 1 NS3 protein exhibits specific viral RNA binding and NTPase activity regulated by the NS5 protein. *Virology* **246**, 409–417
- 22 Lindberg, R. A., Quinn, A. M. and Hunter, T. (1992) Dual-specificity protein kinases: will any hydroxyl do? *Trends Biochem. Sci.* **17**, 114–119
- 23 Becker, W. and Joost, H. G. (1999) Structural and functional characteristics of Dyrk, a novel subfamily of protein kinases with dual specificity. *Prog. Nucleic Acid Res. Mol. Biol.* **62**, 1–17
- 24 Guerra, B., Boldyreff, B., Sarno, S., Cesaro, L., Issinger, O. G. and Pinna, L. A. (1999) CK2: a protein kinase in need of control. *Pharmacol. Ther.* **82**, 303–313
- 25 Gatica, M., Hinrichs, M. V., Jedlicki, A., Allende, C. C. and Allende, J. E. (1993) Effect of metal ions on the activity of casein kinase II from *Xenopus laevis*. *FEBS Lett.* **315**, 173–177
- 26 Leroy, D., Hériché, J. K., Filhol, O., Chambaz, E. M. and Cochet, C. (1997) Binding of polyamines to an autonomous domain of the regulatory subunit of protein kinase CK2 induces a conformational change in the holoenzyme. A proposed role for the kinase stimulation. *J. Biol. Chem.* **272**, 20820–20827
- 27 Hathaway, G. M., Lubben, T. H. and Traugh, J. A. (1980) Inhibition of casein kinase II by heparin. *J. Biol. Chem.* **255**, 8038–8041
- 28 Critchfield, J. W., Coligan, J. E., Folks, T. M. and Butera, S. T. (1997) Casein kinase II is a selective target of HIV-1 transcriptional inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6110–6115
- 29 Al-obeidi, F. A., Wu, J. J. and Lam, K. S. (1998) Protein tyrosine kinases: structure, substrate specificity, and drug discovery. *Biopolymers* **47**, 197–223
- 30 Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D. and Maniatis, T. (1995) Signal-induced site-specific phosphorylation targets I κ B α to the ubiquitin-proteasome pathway. *Genes Dev.* **9**, 1586–1597
- 31 Montagnoli, A., Fiore, F., Eytan, E., Carrano, A. C., Draetta, G. F., Hershko, A. and Pagano, M. (1999) Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. *Genes Dev.* **13**, 1181–1189
- 32 Hannan, R. D., Hempel, W. M., Cavanaugh, A., Arino, T., Dimitrov, S. I., Moss, T. and Rothblum, L. (1998) Affinity purification of mammalian RNA polymerase I: identification of an associated kinase. *J. Biol. Chem.* **273**, 1257–1267
- 33 Caedenas, M. E., Walter, R., Hanna, D. and Gasser, S. M. (1993) Casein kinase II copurifies with yeast DNA topoisomerase II and reactivates the dephosphorylated enzyme. *J. Cell Sci.* **104**, 533–543
- 34 Goldenring, J. R., Casanova, J. E. and Delorenzo, R. J. (1984) Tubulin-associated calmodulin-dependent kinase: evidence for an endogenous complex of tubulin with a calcium-calmodulin-dependent kinase. *J. Neurochem.* **43**, 1669–1679
- 35 Srivenugopal, K. S. and Ali-Osman, F. (1997) Coordinate up-regulation of cyclin-dependent kinase 4 and its inhibitor p16^{INK4} in human glioma cells following chloroethylnitrosourea-induced DNA damage. *Int. J. Oncol.* **11**, 1251–1256
- 36 Hoffman, K., Bucher, P., Falquet, L. and Bairoch, A. (1999) The PROSITE database, its status in 1999. *Nucleic Acids Res.* **27**, 215–219
- 37 Vora, R. A., Pegg, A. E. and Ealick, S. E. (1998) A new model for how O⁶-methylguanine-DNA methyltransferase binds DNA. *Proteins* **32**, 3–6
- 38 Wibely, J. E. A., Pegg, A. E. and Moody, P. C. E. (2000) Crystal structure of the human O⁶-alkylguanine-DNA alkyltransferase. *Nucleic Acids Res.* **28**, 393–401
- 39 Feinstein, E., Einat, M., Gokkel, E., Marcelle, C., Croce, C. M., Gale, R. P. and Canaan, E. (1989) Nucleotide sequence analysis of human abl and bcr-abl cDNAs. *Oncogene* **4**, 1477–1481
- 40 Firmsbach-kraft, I., Byers, M., Shows, T., Dalla-Favera, R. and Krolewski, J. J. (1990) Tyk2, prototype of a novel class of non-receptor tyrosine kinase genes. *Oncogene* **5**, 1329–1336
- 41 Winston, J. T., Koepp, D. M., Zhu, C., Elledge, S. J. and Harper, J. W. (1999) A family of F-box proteins. *Curr. Biol.* **21**, 1180–1182
- 42 Jans, D. A. and Huber, S. (1996) Regulation of protein transport to the nucleus: central role of phosphorylation. *Physiol. Rev.* **76**, 651–685

Received 11 July 2000; accepted 8 August 2000