RESEARCH COMMUNICATION

C-Terminally truncated human *O*⁶-alkylguanine-DNA alkyltransferase retains activity

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A cDNA encoding the human *O*⁶-alkylguanine-DNA alkyltransferase (ATase; EC 2.1.1.63; methylated-DNA: protein-cysteine methyltransferase) has been manipulated to generate a C-terminally deleted protein which retains full methyltransferase activity. The elimination of 22 amino-acid residues from the C-terminus was achieved by endonuclease-*Sac*I digestion of the 623 bp cDNA coding sequence and ligation of a *Sac*I/*Hind*III linker containing an in-frame stop codon. The truncated protein was characterized by its reduced molecular mass in immunoblots probed with an antiserum against the full-length protein and by fluorography after incubation with [³H]methylated calf thymus DNA. The rate of methyl transfer was virtually identical for the full-length and truncated ATases. The construction of such a truncated, yet still functional, ATase, with a molecular mass of 19.7 kDa should facilitate a detailed n.m.r. structural study and help to determine the functional significance of the C-terminal domain of mammalian ATases.

INTRODUCTION

The presence of *O*⁶-methylguanine in DNA is believed to be a critical lesion in carcinogenesis. For example, *N*-methyl-*N*-nitrosourea (MNU) causes specific mutation of the central guanine residue in the 12th codon (GGA) of the rat *H-ras* proto-oncogene, leading to its activation in mammary carcinomas [1]. Repair of this lesion is effected by *O*⁶-alkylguanine-DNA alkyltransferase (ATase, EC 2.1.1.63; methylated-DNA: protein-cysteine methyltransferase), a protein which dealkylates *O*⁶-alkylguanine and *O*⁴-alkylthymine residues in DNA by stoichiometric transfer of the alkyl group to its active-site cysteine residue in an autoinactivating reaction [2]. The cDNA sequences encoding the human [3–6], rat [7–9], murine [10–12] and Chinese-hamster [13] ATases have now been cloned, over-expressed in *Escherichia coli* and partially characterized. Each has a predicted molecular mass of approx. 22 kDa and they show considerable amino acid sequence similarity to their counterparts in yeast [14] and bacteria [15,16], as well as to the C-terminal region of the *ada* protein [2,17], the DNA-repair protein induced during the adaptive response of *E. coli* (and some other bacteria) to methylating carcinogens such as MNU.

A major feature of the ATase proteins is a core region of about 70 amino acids which is highly conserved in both pro- and eukaryotes. The N-terminal region is more variable if all available sequences are considered, but within mammals it remains highly conserved. The mammalian ATases differ markedly from the other available sequences by the presence of a hexapeptide (SFTRQV) insert and a C-terminal tail of approx. 28 amino acid residues [12,13].

As part of a programme to investigate structure/function aspects of the ATase proteins by n.m.r. spectroscopy, we felt that it would be useful to generate an active truncated form of the human protein, having a molecular mass of less than 20 kDa, in order to facilitate an n.m.r. structural study. Furthermore, as the

C-terminal tail is a mammalian ATase feature, it was considered that it might be possible to assess the functional contribution of this region by its deletion. We report here the generation of a novel 3'-truncated human ATase cDNA which encodes a C-terminally deleted ATase (22 amino acids), which apparently retains full functional activity.

MATERIALS AND METHODS

Truncation of the human ATase cDNA

Removal of 70 bp from the 3' end of the human cDNA (hAT cDNA) was achieved by *Sac*I digestion of the 623 bp coding sequence [6]. This eliminated the region encoding the 23 most C-terminal amino acid residues plus the stop codon. A *Sac*I/*Hind*III linker containing an in-frame stop codon was ligated to the truncated cDNA; the re-creation of the *Sac*I site also resulted in the codon for serine being reintroduced immediately 5' to the stop codon and thus the net loss of coding sequence was 66 bp, that is, 22 amino acids. This truncated cDNA (hAT-C) was subsequently cloned into pUC 8.0 and transformed into competent *E. coli* DH5α. The resultant clones were screened for plasmids containing the insert and for expression of a functionally active ATase (see below).

Analysis of the truncated cDNA protein product

Bacterial clones were confirmed to contain the truncated ATase construct by *Bam*HI/*Hind*III digestion and agarose-gel electrophoresis of plasmid DNA to determine insert size. Clones of transformed *E. coli* were expanded overnight and extracts prepared by sonication in buffer I [50 mM-Tris/HCl (pH 8.3) 3 mM-dithiothreitol/1 mM-EDTA] and assayed for ATase activity using [³H]MNU-methylated calf thymus DNA substrate as described previously [18].

A 1 litre culture of a clone containing the hAT-C construct in the appropriate orientation and a clone harbouring a full-length

Abbreviations used: MNU, *N*-methyl-*N*-nitrosourea; ATase, *O*⁶-alkylguanine-DNA alkyltransferase (methylated-DNA: protein-cysteine methyltransferase, EC 2.1.1.63).

ATase encoding plasmid were pelleted, and cell-free sonicated material was subjected to double-stranded DNA-cellulose chromatography as previously described for other recombinant ATases [12,13].

Equal amounts of hAT and hAT-C protein, based on activity measurements, were analysed by immunoblotting, carried out as detailed in Rafferty *et al.* [13]. Polyclonal antibodies against the full-length human ATase were raised in Half-Lop rabbits (S. M. Lee, J. A. Rafferty, C.-Y. Fan, P. M. Potter, R. H. Elder & G. P. Margison, unpublished work; details are available from R. H. E. on request).

Samples for fluorography were incubated with substrate DNA before separation by SDS/PAGE. ^{14}C -labelled Rainbow markers (Amersham International) were included as standards. Proteins were transferred to nitrocellulose (Hybond C; Amersham International) for 1 h at 100 V as described by Towbin *et al.* [19]. After it had been dried, the membrane was soaked in scintillant (Optiphase Hisafe; National Diagnostics), blotted dry, wrapped in clingfilm (Saran Wrap) and exposed to Fuji RS film at -70°C for up to 10 days.

RESULTS AND DISCUSSION

Bacterial clones were shown to contain the truncated cDNA on the basis of insert size after *Bam*HI/*Hind*III digestion of plasmid DNA. Extracts of *E. coli* harbouring either the hAT-C, or hAT plasmid were then assayed for ATase activity. The results of these assays showed that the truncated cDNA encoded a protein which was active as a methyltransferase and could be expressed in *E. coli* at levels similar to that of the full-length recombinant protein (results not shown).

Actual confirmation of the production of a truncated product was obtained by its purification on DNA-cellulose and subsequent analysis of the purified protein by immunoblotting (Fig. 1a) and fluorography (Fig. 1b). The immunoblot in Fig. 1(a) shows that polyclonal antibodies raised against full-length human ATase also recognize the truncated form, indicating that the two denatured polypeptides share common epitopes. Fluorography demonstrated both its molecular mass and the activity of the truncated ATase. The expected reduction in molecular mass of approx. 2 kDa for the truncated cDNA product was confirmed by this procedure; the observed molecular mass for the truncated ATase was 21 kDa compared with 23 kDa for the full-length protein (Fig. 1b). An anomalously high molecular mass is a characteristic of mammalian ATases under denaturing-gel conditions, probably attributable to their high proline content [13]. Furthermore, as for the full-length protein, the binding of the transferred methyl group to hAT-C is through a covalent bond, since the methylated protein was resistant to boiling in the presence of a high concentration of dithiothreitol and SDS.

The kinetics of methyl transfer from $[^3\text{H}]\text{MNU}$ -treated calf thymus DNA by samples of the purified full-length and truncated proteins were compared (Fig. 2) and the rates of methyl transfer were found to be essentially identical, indicating that the removal of the majority of the C-terminal tail has no measurable effect on the rate of reaction of the enzyme.

In conclusion, the C-terminal truncation described herein results in an active ATase, capable of methyl transfer from MNU-treated calf thymus DNA at a rate indistinguishable from that of the full-length protein. Epitopes present in the full-length hAT protein are retained in the truncated product, at least in the denatured form. Thus the C-terminal tail, from serine-184 onwards, is not essential for the DNA binding or alkyltransferase

Fig. 1. Analysis of hAT and hAT-C proteins separated by SDS/PAGE followed by (a) immunoblotting and (b) fluorography

(A) Immunoblot of hAT and hAT-C (1 pmol) probed with a rabbit anti-hAT antiserum. Lane 1, hAT-C; lane 2, hAT. (b) Fluorography of hAT and hAT-C after incubation with $[^3\text{H}]$ methylated calf thymus DNA. Lane 1, hAT-C; lane 2, hAT; lane 3, ^{14}C -labelled markers (46, 30, 21.5, 14.3, 6.5 kDa).

activity of the human protein, as had been proposed [20]. Given the high degree of sequence similarity in this region of the polypeptide chain, it is not unreasonable to suggest that the same is also likely to be true for the other mammalian ATases. N.m.r. studies of proteins are commonly restricted to the molecular-mass range below 15–16 kDa, and 20 kDa as a likely upper limit at present. This would clearly preclude studies of the mammalian ATases, and therefore the truncation of the hAT protein to a molecular mass of below 20 kDa is a useful step for n.m.r. and other studies. However, assignment for spectra for molecules of this size (19.7 kDa) is still extremely difficult, and it is likely that further truncation will be necessary. In this regard it is encouraging that the truncated protein retained activity and it is therefore possible that a further reduction in molecular mass can be achieved without a concomitant loss of activity.

Fig. 2. Kinetics of $[^3\text{H}]$ methyl transfer from substrate DNA to hAT (●) and hAT-C (▽)

Duplicate samples of hAT and hAT-C (100 fmol) were incubated with substrate DNA at 37°C for periods of up to 1 h and assayed for activity as described in [18].

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