Genetic manipulation indicates that ARD1 is an essential $N^\alpha$-acetyltransferase in *Trypanosoma brucei*☆

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Abstract

$N^\alpha$-acetylation, the most common protein modification, involves the transfer of an acetyl group from acetyl-coenzyme A to the $N$-terminus of a protein or peptide. The major $N^\alpha$-acyltransferase in *Saccharomyces cerevisiae* is the ARD1-NAT1 complex. To investigate $N^\alpha$-acylation in *Trypanosoma brucei* we have cloned and characterised genes encoding putative homologues of *ARD1* and *NAT1*. Both genes are single copy and *ARD1*, the putative catalytic component, is expressed in both bloodstream-form and insect-stage cells. In either of these life-cycle stages, disruption of both *ARD1* alleles was only possible when another copy was generated via gene duplication or when *ARD1* was expressed from elsewhere in the genome. These genetic manipulations demonstrate that, unlike the situation in *S. cerevisiae*, *ARD1* is an essential gene in *T. brucei*. We propose that protein modification by *ARD1* is essential for viability in mammalian and insect-stage *T. brucei* cells. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

$N^\alpha$-acetylation, mediated by *N*-$\alpha$-acyltransferases, is the most common protein modification. *N*-$\alpha$-acetylation occurs during protein synthesis and involves the transfer of an acetyl group from acetyl-coenzyme A to the protein $\alpha$-NH$_2$ group (reviewed in [1]). Acetylation neutralises a positive charge and, like protein phosphorylation, may influence protein function by altering stability or interactions with other molecules [2].

ARD1 and NAT1 are sub-units of an *N*-$\alpha$-terminal protein acetyltransferase in the yeast *Saccharomyces cerevisiae*. The ARD1-NAT1 complex, both sub-units of which are required for enzyme activity, is the major *N*-$\alpha$-acyltransferase in these cells [3]. ARD1-NAT1 modifies about 20% of all yeast proteins [4,5] including 24 ribosomal proteins [6] and six 20-S proteosome sub-units [7]. Up to 80% of proteins are thought to be *N*-$\alpha$-acylated in other eukaryotes. *N*-$\alpha$-acyltrans-
ferase activity is lost in ardl or natI mutants [5] but, despite the fact that the ARD1-NAT1 complex modifies many proteins, such mutants are viable, displaying only a few specific phenotypes. For example, ardl mutants are unable to enter stationary phase or sporulate. These phenotypes suggested a defect in arrest of the mitotic cell cycle and lead to the designation ardl for arrest defective [8]. Yeast ardl mutations also cause a mating defect due to de-repression of the mating-type locus, HML [9]. In addition, ARD1 was among a number of genes shown to be necessary for gene silencing at telomeres [10]. natI (N*-acetyltransferase, also known as aalI) mutants exhibit identical phenotypes to ardl mutants [5,10,11]. Since the yeast ARD1-NAT1 complex modifies a large number of proteins it is not clear how these phenotypes occur, but the mechanism certainly appears to be indirect [12]. Another yeast acetyltransferase mutant (sas2) displays similar phenotypes [13].

ARD1 is a member of a large and diverse super-family of acetyltransferases that includes histone acetyltransferases [14] suggesting that it is the catalytic component of the ARD1-NAT1 complex. Crystal structure and mutagenesis studies with other members of this family reveal the acetyl CoA binding site and suggest a mechanism for catalysis (reviewed in [15]). In addition to ARD1-NAT1, there are at least two other N*-acetyltransferases in S. cerevisiae known as NAT3 and MAK3 [16]. Whether NAT2 [17] is an N*-acetyltransferase is not so clear [16]. The ARD1-NAT1 or NatA substrates are acetylated on Ser, Ala, Gly and Thr termini [6,16] following removal of the initiator methionine.

Trypanosoma brucei are evolutionary divergent [18] protozoan parasites of mammals, causing sleeping sickness in humans and Nagana in cattle. Antigenic variation, which allows T. brucei to persist in a mammalian host, relies upon gene silencing at telomeres (reviewed in [19]). Since the ARD1-NAT1 N*-acetyltransferase is required for telomeric gene silencing in yeast [10] we chose to study T. brucei genes related to ARD1 and NAT1. Also, although protein phosphorylation has been studied in some detail in T. brucei, very little is known about protein acetylation in this organism.

We have cloned and characterised T. brucei genes encoding homologues of both components of the ARD1-NAT1 N*-acetyltransferase. We show that, unlike the situation in yeast, ARD1 is essential for growth in both bloodstream-form and insect-stage T. brucei cells.

2. Materials and methods

2.1. Cells

T. brucei strain MiTat 427 bloodstream-form cells were maintained and cloned as previously described [20]. Bloodstream-form trypanosomes were differentiated to the insect-stage by transferring 1 × 10⁷ cells into DTM with 3 mM citrate/cis-aconitate [21] at 27°C. Electroporation was as previously described [20] except that cells were pulsed once at 1.4 kV and 25 μF in 2 mm gap cuvettes using a Gene pulser II (Bio-Rad). Plasmid DNA, prepared using anion-exchange columns (Qiagen), was digested with PstI/ApaI (pARD-BLE), SmaI-ApaI (pARD-NEO) and SalI (pTUB-HYG-ARD) prior to electroporation. Medium containing 2 μg ml⁻¹ phleomycin (Cayla), 2 μg ml⁻¹ G418 (MBI Fermentas) and 2.5 μg ml⁻¹ hygromycin B (Sigma) were added to bloodstream-form cultures 6 h after electroporation to select for BLE, NEO or HYG expression, respectively. Following drug addition bloodstream-form cultures were distributed in 12 or 24-well plates. For insect-stage cultures, 2.5 μg ml⁻¹ phleomycin, and/or 15 μg ml⁻¹ G418 were added 24 h after electroporation to select for BLE and/or NEO expression, respectively, and cultures were maintained in flasks.

2.2. ARD1 and NAT1 cloning

Sequences of portions of the putative ARD1 and NAT1 genes from T. brucei were from the EST database [22,23]. We designed primers based on these sequences and, using the polymerase chain reaction (PCR) we amplified fragments from genomic DNA using the primer pairs, ARD-a (5’-gaattctttgacgcaatgcaggtgc-3’) and ARD-b (5’-gaattcggacgctagacgaggtg-3’) and NAT-a (5’-
generate pAHA. The processing signals were removed from pARD signals was excised from pBEAHA5 by aldolase splice acceptor and polyadenylation oligonucleotides were synthesised by Gibco-BRL. All oligonucleotides were sequenced using an ABI Prism 377 automated sequencer according to the dye-terminator kit (Applied Biosystems) and an ing was performed using a Thermo Sequenase pNAT5 and pNAT3, respectively. DNA sequencing was performed using a T. brucei genomic library, consisting of ApaI-PstI fragments in pBluescript (Stratagene). Clones that hybridised with the NAT1 probe were isolated from a T. brucei genomic library, consisting of KpnI-BglII fragments in pBluescript. The ARD1 clone was designated pARD and clones containing the 5’ and 3’-ends of NAT1 were designated pNAT5 and pNAT3, respectively. DNA sequencing was performed using a Thermo Sequenase dye-terminator kit (Applied Biosystems) and an ABI Prism 377 automated sequencer according to the manufacturer instructions. All oligonucleotides were synthesised by Gibco-BRL.

2.3. Plasmid constructions

ARD1 targeting constructs were generated as follows. Briefly, a SacI site was destroyed in pARD to generate pARD-SacΔ. A SacI fragment was then removed from pARD-SacΔ and replaced with BLE, a BamHI fragment from pbRn5 [20], or NEO, a Smal-BamHI fragment from pHD30neo [24], to generate pARD-BLE and pARD-NEO, respectively. Since the putative ARD1 splice acceptor site, as determined by comparing our sequence to the EST sequence, was disrupted, BLE and NEO were inserted along with actin splice acceptor signals [24].

To generate pTUB-HYG-ARD, ARD1 was linked to a hygromycin resistance gene (HYG) and both genes were inserted into a tubulin-targeting construct. Briefly, a HYG gene flanked by aldolase splice acceptor and polyadenylation signals was excised from pBEAHA5 [25] as a SpeI-SalI fragment and cloned into pBluescript to generate pAHA. The ARD1 coding region and processing signals were removed from pARD- SacΔ as a SmaI-KpnI fragment and cloned into a SalI site downstream from the HYG cassette in pBS-AHA generating pHYG-ARD. The tubulin target was a SalI fragment cloned into pBluescript generating pTUB. Following the destruction of a SalI site in pHYG-ARD, a XhoI-SpeI fragment containing both HYG and ARD1 was inserted into a MluI site in pTUB generating pTUB-HYG-ARD.

2.4. DNA and RNA analysis

Southern and northern blotting were carried out according to standard protocols. DNA and RNA were isolated using DNA-Stat-60 and RNAzolB, respectively (Biogenesis). All post hybridisation washes were at 65°C in 0.2 × SSC and 0.2% SDS. The ARD1 probe was a SacI fragment, the 3’ probe was a KpnI fragment and the BLE and NEO probes were the entire coding regions. For PCR assays, temperature was cycled 22 times through 94, 58 and 72°C for 30, 30 and 45 s, respectively, in the presence of 40 ng of genomic DNA. Taq DNA polymerase, a forward primer, ArdF (5’-cagcccgttccggagtga-3’), and three reverse primers, ArdR (5’-gggagatttctcaagcttcg-3’), BleR (5’-tcacacctggtcctggac-3’), and NeoR (5’-tcacacctggtcctggac-3’).

3. Results and discussion

3.1. N* acetyltransferase homologues in T. brucei

Expressed sequence tags of putative T. brucei ARD1 and NAT1 homologues were identified by El-Sayed and colleagues [22,23]. Based on these sequences, we designed oligonucleotides to PCR amplify portions of the putative ARD1 and NAT1 genes from the MiTat 427 strain of T. brucei (see Materials and methods). The PCR products were used as probes to isolate full-length ARD1 and NAT1 genes from T. brucei genomic libraries (see Materials and methods). Sequencing revealed that TbARD1 encodes a 239 amino acid polypeptide with a predicted molecular mass of ~27.5 kDa while TbNAT1 encodes a 711 amino acid polypeptide with a predicted molecular mass of
82.2 kDa. The predicted protein sequences are aligned for comparison with ARD1 and NAT1 from S. cerevisiae and the putative homologues from Drosophila while comparisons with putative homologues from other species are tabulated (Fig. 1).

Despite the evolutionary divergence of these organisms [18], ARD1-related proteins from human, Drosophila, nematode, yeast, slime-mould and trypanosome are highly conserved (Fig. 1A) suggesting that all these sequences represent N-terminal protein acetyltransferase. Of particular significance, motif A (Q/RxxGxG/A where x represents a variable amino acid), which forms part of the acetyl CoA binding loop [15], is found in all of these putative ARD1 homologues (Fig. 1A, always RxxGxA in ARD1). Comparison of the predicted amino acid sequences of NAT1-related proteins from T. brucei, yeast and Drosophila reveals significant similarity between and throughout the three proteins (Fig. 1B) suggesting that TbNAT1 is an N-terminal acetyltransferase subunit.

3.2. Nα-acetyltransferase gene organisation and expression

To determine the organisation of the ARD1 and NAT1 genes we carried out Southern analysis. Representative digests are shown for ARD1 (Fig. 2A) and NAT1 (Fig. 2B). This analysis suggested that both genes are present as a single copy per haploid genome. Although both ARD1 and NAT1 are required for enzyme activity in yeast, ARD1 appears to be the catalytic component of the complex (see above) so subsequent experiments focussed on the ARD1 gene. Pulsed-field gel electrophoresis indicated that ARD1 is...
located on one of the largest (~6 Mbp) chromosomes in the MITat 427 strain (data not shown). To investigate ARD1 expression we extracted RNA from bloodstream-form and insect-stage cells. A northern blot was hybridised with an ARD1 probe (Fig. 2C) revealing ARD1 mRNA in both cell types. ARD1 mRNA appears to be slightly more abundant in insect-stage cells.

3.3. Attempts to disrupt ARD1 in bloodstream-form T. brucei

*S. cerevisiae ard1 and nat1 null mutants are viable and display similar phenotypes [5,9–11]. We wanted to address the role of ARD1 in *T. brucei and therefore attempted targeted disruption of the ARD1 gene. *T. brucei is diploid and specific loci can be targeted by homologous recombination [26] so two constructs, designed to replace both ARD1 alleles, were created using the resistance markers BLE and NEO, coding for the Sh BLE protein and neomycin phosphotransferase, respectively. Our approach should result in the removal of all but the last 71 nucleotides of the ARD1 coding region. This remaining segment will lie immediately downstream of the marker gene stop codon (see Fig. 3A). The constructs do not contain promoters but rely upon endogenous polycistronic transcription for selectable marker expression.

We transformed bloodstream-form *T. brucei cells with each construct. Of approximately 60 drug-resistant clones obtained, four were selected for further manipulation (two BLE-resistant clones and two NEO-resistant clones). We attempted to generate NEO and BLE double drug-resistant cells from these four clones. Double drug-selection was applied to eliminate cells in which the first selectable marker had been replaced by the second. Only two clones survived NEO and BLE selection. These results suggested that, relative to replacement of the first allele, cells survived replacement of a second ARD1 allele at a 30-fold lower frequency.

Genomic DNA from single and double drug-resistant clones was analysed by Southern blotting and in a quantitative, competitive PCR assay (see Materials and methods). Some of the Southern blots are shown in Fig. 3B. Wild-type cells (Fig. 3B, lane 1) contain both ARD1 alleles, while cells
expressing either NEO or BLE have a single ARD1 allele (Fig. 3B, lane 2 and 3 and data not shown) and NEO or BLE genes integrated as expected (Fig. 3B, lanes 2 and 3). The double drug-resistant cells, however, have all three genes intact (Fig. 3B, lane 4). One possible explanation to account for the persistence of ARD1 in double-drug-resistant cells is that constructs designed to target the second ARD1 allele had in fact integrated elsewhere in the genome leaving the native ARD1 allele intact. This does not appear to be the case however since the fragments detected by the 3′ probe display the expected change in size brought about by integration of the constructs at the ARD1 locus (Fig. 3B, lane 4). Our results indicate that the ARD1 locus has been duplicated in these cells, presumably a relatively rare event (~1 in every 30 integration events), which explains why these cells were obtained at a reduced frequency (see above). An additional copy of the locus may have been generated during parental clone expansion or may have been induced by the second integration event. Our data are more consistent with a tandem duplication induced during integrative recombination (data not shown). The results of this study are consistent with disruption of the ARD1 gene being a lethal event in these cells.

3.4. ARD1 is essential for growth in bloodstream-form cells

The possibility that our genetic manipulations disrupted some other ARD1-proximal, essential gene had to be ruled out to demonstrate that this phenotype is a direct consequence of ARD1 disruption. To determine whether ARD1 is essential we chose to attempt disruption of both wild-type alleles while expressing ARD1 from an independent locus. ARD1 was linked to a gene (HYG) conferring hygromycin B resistance and the entire cassette was targeted to the tubulin (TUB) array (see Fig. 4A). This construct was introduced into cells maintaining a single copy of ARD1. Of over 100 HYG-resistant clones obtained we chose two for further analysis. These two clones were subsequently transformed with a second ARD1-targeting construct. In these experiments HYG-selection would not be expected to prevent replacement of the ARD1 ectopic copy (Fig. 4A) so only BLE and NEO drug-selection were applied at this point. We obtained more than 50 clones from these experiments. In twice the number of experiments using cells lacking an ectopic copy of ARD1 we had previously obtained only two clones.
A quantitative, competitive PCR assay (see Materials and methods) was used to assess the location of ARD1 genes and selectable-markers in ten of these drug-resistant clones (data not shown). Although the ARD1 targeting construct could have replaced the ectopic copy, the native allele presented a longer target sequence at one end. Indeed, all ten clones appeared to have lost the second native ARD1 allele, indicating a dramatic increase (~50-fold) in the efficiency of native ARD1 disruption in the presence of an ectopic copy of the gene. Southern blot analysis of two of these clones is shown in Fig. 4B. A polymorphic HindIII site exists at the ARD1 locus so Southern analysis of HindIII digested genomic DNA with an ARD1 probe produces two fragments corresponding to the ARD1 alleles (Fig. 4, lanes 1 and 5). In the single drug-resistant clones one allele of ARD1 has been disrupted (Fig. 4B, lanes 2 and 6). Clones with an ectopic copy of ARD1 produce an additional band showing the construct had integrated as expected (Fig. 4B, lanes 3 and 7). Finally, transformation with the second ARD1 targeting construct results in the disruption of the remaining native ARD1 allele while the ectopic copy remains intact (Fig. 4B, lanes 4 and 8). In combination with the results shown in Fig. 3, these results strongly suggest that ARD1 is indeed an essential gene in bloodstream-form T. brucei.

To determine whether the ectopic copy of ARD1 was expressed, total cellular RNA was isolated from bloodstream-form cells and analysed by northern blotting (Fig. 4C). Relative to wild-type cells (lane 1), ARD1 mRNA levels appear to drop in cells which contain only one native copy of the gene (lane 2) while ARD1 mRNA expression is restored following integration of the ectopic copy (lane 3). The ectopic copy of ARD1 produces a larger mRNA transcript, possibly due to the use of alternative mRNA processing signals at the tubulin locus (lanes 3 and 4). Finally, the smaller of the two transcripts, derived from the native ARD1 allele, is specifically depleted, when the last native ARD1 allele is disrupted (lane 4). The signal in lane 4 represents mRNA derived from the ARD1 ectopic copy.

3.5. ARD1 is essential for growth in insect-stage T. brucei

ARD1 is expressed in bloodstream-form and insect-stage T. brucei cells. We therefore sought to determine if the gene is also essential in the insect-stage. Differentiation of bloodstream-forms to insect-stage cells can be efficiently achieved in vitro if bloodstream-form trypanosomes are subjected to a temperature drop to 27°C in a medium containing glycerol, citrate and cis-aconitate [21]. For this experiment wild-type T. brucei bloodstream-form cells and clones with a single ARD1 allele or with one allele plus an ectopic copy of ARD1 were differentiated. ARD1 was targeted for disruption in all three cultures. Once again, drug-resistant cells were obtained at a reduced frequency in cells with only one copy of ARD1 remaining. This result suggests that, similar to the situation in bloodstream-form cells, ARD1 is essential for viability in insect-stage cells. To survive drug selection, we predict that the ARD1
locus was rearranged in these cells, such that the selectable marker is integrated but the ARD1 gene remained intact. To test this we used the quantitative, competitive PCR assay (Fig. 5) in which primers specific for native ARD1 alleles, BLE or NEO compete for an ARD1-flanking primer (see Fig. 5A). As predicted, this assay shows that cells lacking an ectopic copy of ARD1 always maintain a native copy (lanes 2 and 3) even when that copy is targeted for replacement (lane 3). It was only possible to disrupt both native copies of ARD1 in cells with an ectopic copy of the gene (lane 4). These results indicate that ARD1 is essential in insect-stage T. brucei.

3.6. The role of ARD1 in T. brucei

N^*-acetylation is the most common protein modification and the ARD1–NAT1 complex is the major N^*-acetyltransferase in S. cerevisiae. Here we have identified and characterised putative ARD1 and NAT1 homologues from T. brucei, an evolutionary divergent [18] protozoan parasite. ARD1, the putative catalytic component of the complex, is conserved between trypanosomes and humans and database searches reveal ARD1 and NAT1 homologues in a variety of other organisms. Similar to the situation in yeast [3], ARD1 is likely to be responsible for N^*-acetylation of a large number of proteins in T. brucei, many of which are common to bloodstream and insect-stage cells. Consistent with this, ARD1 is expressed in both cell types. In contrast to the situation in yeast, however, where ard1 mutants are viable [8], ARD1 appears to be essential in bloodstream-form and insect-stage T. brucei. In fact, S. cerevisiae strains lacking any of the three confirmed N^*-acetyltransferases (ARD1-NAT1, NAT3 and MAK3) are viable [16]. Although telomeric gene silencing in yeast, which requires the ARD1-NAT1 complex, is not thought to affect native genes, many telomere-proximal genes involved in antigenic variation in T. brucei are transcriptionally repressed. All but one telomere appears to be active in bloodstream-form cells while none appear to be active in insect-stage cells. If telomeric gene silencing mechanisms are conserved between yeast and T. brucei, then disruption of ARD1 in T. brucei may activate telomeric genes, with lethal consequences. Whatever the mechanism, our results strongly suggest that ARD1 is an essential gene in T. brucei, so ARD1 may provide a target for novel chemotherapy agents against these parasites. Further studies on ARD1 and its substrates should reveal the role of N^*-acetylation in evolutionary divergent organisms. Since ARD1-NAT1 is known to modify a large number of proteins in yeast, however, it may be difficult to determine which ARD1 substrate(s) influence specific cellular functions. From our results, we propose that N^*-terminal protein acetylation by ARD1 is essential for viability in mammalian and insect-stage T. brucei.

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