Proteasomal Degradation of *N*-Acetyltransferase 1 Is Prevented by Acetylation of the Active Site Cysteine

A MECHANISM FOR THE SLOW ACETYLATOR PHENOTYPE AND SUBSTRATE-DEPENDENT DOWN-REGULATION*

Neville J. Butcher‡§, Ajanthy Arulpragasam, and Rodney F. Minchin‡

From the Laboratory for Cancer Medicine, Western Australian Institute for Medical Research, Royal Perth Hospital, Perth, Western Australia 6000

Many drugs and chemicals found in the environment are either detoxified by *N*-acetyltransferase 1 (NAT1, EC 2.3.1.5) and eliminated from the body or bioactivated to metabolites that have the potential to cause toxicity and/or cancer. NAT1 activity in the body is regulated by genetic polymorphisms as well as environmental factors such as substrate-dependent down-regulation and oxidative stress. Here we report the molecular mechanism for the low protein expression from mutant NAT1 alleles that gives rise to the slow acetylator phenotype and show that a similar process accounts for enzyme down-regulation by NAT1 substrates. NAT1 allozymes NAT1 14, NAT1 15, NAT1 17, and NAT1 22 are devoid of enzyme activity and have short intracellular half-lives (~4 h) compared with wild-type NAT1 4 and the active allozyme NAT1 24. The inactive allozymes are unable to be acetylated by cofactor, resulting in ubiquitination and rapid degradation by the 26 S proteasome. This was confirmed by site-directed mutagenesis of the active site cysteine 68. The NAT1 substrate *p*-aminobenzoic acid induced ubiquitination of the usually stable NAT1 4, leading to its rapid degradation. From this study, we conclude that NAT1 exists in the cell in either a stable acetylated state or an unstable non-acetylated state and that mutations in the NAT1 gene that prevent protein acetylation produce a slow acetylator phenotype.

Human arylamine *N*-acetyltransferase 1 (NAT1)[1] (EC 2.3.1.5) is a ubiquitous enzyme that catalyzes the *N*-acetylation of many arylamine and hydrazine xenobiotics. It also appears to be important in the catabolism of folic acid, where it acetylates *p*-aminobenzoylglutamate following cleavage of the folate molecule (1, 2). The gene that encodes NAT1 is located at 8p21–22 and exhibits genetic as well as functional polymorphisms (reviewed in Refs. 3 and 4). Numerous NAT1 alleles have been identified but only a small number result in

altered activity *in vivo* (5, 6). Recently, Fretland *et al.* (7) showed that the NAT1 allozymes that have reduced catalytic activity (*NAT1*14*, *NAT1*15*, *NAT1*17*, and *NAT1*22*) compared with wild-type (*NAT1*4*) are expressed at barely detectable levels in yeast. Because the rate of gene transcription did not appear to differ between alleles, they concluded that a decrease in protein translation and/or protein turnover was responsible for the very low levels of expression. Interestingly, NAT1-specific substrates such as *p*-aminobenzoic acid (PABA) and *p*-aminosalicylic acid down-regulate enzyme activity by a mechanism that leads to a decrease in NAT1 protein (8). Again, the loss of protein appeared to occur at a post-transcriptional level because the drugs did not affect NAT1 mRNA levels. More recently, NAT1 activity has been shown to be affected by oxidative stress such as exposure to hydrogen peroxide or *S*-nitrosothiols (9, 10). Despite these observations indicating that NAT1 is regulated at both an environmental and a genetic level, the molecular mechanisms responsible are poorly understood.

The acetyl-coenzyme A (acetyl-CoA)-dependent acetylation reaction catalyzed by NAT1 proceeds via a classical ping-pong bi bi mechanism (11, 12). Initially, the active site cysteine (Cys 68 for human NAT1) of the enzyme accepts an acetyl group from acetyl-CoA, forming a thiol ester. The second step involves the binding of substrate to the acetylated enzyme, transfer of the acetyl group to the substrate, and release of the acetylated product. The initial acetylation of NAT1 can proceed independent of the presence of substrate (13). Turnover of acetylated enzyme in the absence of substrate is relatively slow, probably because of the exclusion of water from the active site, which is buried below the surface of the protein (14). However, turnover is increased in the presence of substrate, suggesting that a change in equilibrium from an acetylated to a non-acetylated form may occur.

We hypothesized that NAT1 exists in the cell in either a stable acetylated state or an unstable non-acetylated state. Thus, substrates induce down-regulation by shifting the equilibrium of the protein toward the non-acetylated state. This mechanism could also explain the apparent instability of the allozymes expressed from alleles that give rise to the slow acetylator phenotype if these proteins exist predominantly in the non-acetylated state. To investigate this, we examined the half-life of a number of NAT1 allozymes previously reported to have little or no activity and compared these to the wild-type NAT1 4. Here we report that the mutant allozymes were not acetylated by acetyl-CoA. Moreover, these proteins were rapidly ubiquitinated in cultured cells. We also show that the NAT1 substrate PABA can induce ubiquitination of NAT1 4, leading to its degradation. We conclude that the acetylation

‡ Present address: School of Biomedical Sciences, University of Queensland, St. Lucia, Qld, Australia 4072.

§ To whom correspondence should be addressed. Tel.: 61-7-3365-3123; Fax: 61-7-3365-1766; E-mail: n.butcher@uq.edu.au.

[1] The abbreviations used are: NAT1, *N*-acetyltransferase 1; PABA, *p*-aminobenzoic acid; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; HRP, horseradish peroxidase; GST, glutathione *S*-transferase; NQO1, NAD(P)H:quinone oxidoreductase 1; HA, hemagglutinin.

status of the active site cysteine determines the stability of the NAT1 protein *in vivo* and propose that this is the molecular mechanism responsible for the slow acetylator phenotype of the alleles investigated in this study. In addition, substrate-dependent down-regulation of human NAT1 occurs because of enzyme deacetylation and subsequent degradation by the ubiquitin/26 S proteasome pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—HT-29 cells (human colon adenocarcinoma) were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, gentamicin (50 μg/ml), and benzylpenicillin (80 μg/ml). Cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

Isolation of PBMC, Total RNA Extraction, and NAT1 Genotyping of cDNA—Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Ficoll-Paque as described previously (6). Total RNA was extracted from PBMC using a High Pure RNA isolation kit (Roche Applied Science) and reverse transcribed using avian myeloblastosis virus-reverse transcriptase and oligo(dT)$_{15}$ (Promega). The *NAT1* open reading frame was amplified from cDNA using NAT1-specific primers and genotyped for *NAT1*14* and *NAT1*17* as described previously (6).

Cloning of NAT1 Alleles—The protein coding regions of *NAT1*14* and *NAT1*17* were amplified by PCR from DNA of heterozygous individuals using forward primer 5′-CCTTGCTTGGATCCTCATGGACAT-TGAAGC-3′ (BamHI) and reverse primer 5′-ACTCCGAATTCTAAAT-AGTAAAA-3′ (EcoRI) and cloned into the mammalian expression vector pEF1/His (Invitrogen). The protein coding regions of *NAT1*22* and *NAT1*24* were amplified from plasmids generously provided by Dr. H. Lin (University of California) using the above primers and cloned into pEF1/His. Plasmid containing wild-type NAT1 (pEF1/His-*NAT1*4*) was kindly provided by Prof. E. Sim (University of Oxford). The *NAT1-C68Y* construct was prepared by amplifying the open reading frame of *NAT1*4* as two fragments using the above forward primer and reverse primer 5′-ATGATTGACCTGGAGGTACCATCCAC-3′ (KpnI) and forward primer 5′-GTGGATGGTACCTCCAGGTC-3′ (KpnI) and the above reverse primer. The PCR fragments were digested with KpnI, ligated together, amplified by PCR with the primers used to amplify *NAT1*14*, and cloned into the BamHI and EcoRI sites of pEF1/His vector. *NAT1*4*, *NAT1*14*, *NAT1*17*, *NAT1*22*, *NAT1*24*, and *NAT1-C68Y* were amplified by PCR from the pEF1/His constructs using forward primer 5′-TCCGGAATTCAATGGACATTGAAGC-3′ (EcoRI) and reverse primer 5′-CGCGGGATCCTAAATAGTAAAAAATC-3′ (BamHI) and cloned into the mammalian expression vector p3XFLAG-CMV-7.1 (Sigma). The truncated variant *NAT1*15* was prepared by PCR as above using pEF1/His-*NAT1*4* as template and reverse primer 5′-CGCGGGATCCTAGTATTTGCTGTC-3′ (BamHI). Constructs were verified by DNA sequencing.

Determination of Intracellular NAT1 Half-life—HT-29 cells were seeded at a density of 2×10^5 cells/well (24-well plate) and incubated overnight at 37 °C. Cells were transfected in the absence of fetal bovine serum with 1 μg of plasmid DNA using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. After a 4-h incubation at 37 °C, the culture medium was aspirated and replaced with 1 ml/well fresh Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and the cells incubated for a further 20 h at 37 °C. Cells were then treated with cycloheximide (10 μg/ml) and harvested at times 0, 4, and 8 h post-treatment. Cells were washed twice with 1 ml of PBS, resuspended in 0.8 ml of 20 mM Tris/1 mM EDTA buffer (pH 7.4) containing 1 mM dithiothreitol, and disrupted on ice using a cell sonicator (Branson Sonifier B250; duty cycle 100%, output = 5; 3 × 5 s bursts). Cell lysates were then centrifuged for 3 min at 16,000 × g (4 °C) and the supernatant retained for Western blot and assay of NAT1 activity. Protein concentration was determined by the method of Bradford (15).

NAT1 Activity Assay—NAT1 activities were quantified by HPLC as described previously using the NAT1-selective substrate PABA (8).

Western Blot Analysis—Cell lysates were electrophoresed on 12% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted using a polyclonal NAT1-specific antibody (kindly provided by Prof. E. Sim, University of Oxford) as described previously (6) or an anti-FLAG-M2 HRP antibody (A 8592; Sigma) according to the manufacturer's instructions. NAT1 was visualized using Supersignal West Pico chemiluminescent substrate (Pierce). Protein content was estimated from Western blots by densitometry using ImageQuant Ver. 1.1 (Amersham Biosciences).

Synthesis of [³H]Acetyl-CoA—Coenzyme A (5 mg) was dissolved in 0.5 ml of ammonium hydroxide (100 mM). On ice, [³H]acetic anhydride (18.5 GBq/mmol; Amersham Biosciences) was added dropwise with mixing until the reaction was complete, as assessed by thiol assay using 3-carboxy-4-nitrophenyl disulfide (Sigma). The reaction mixture was purified on a Sep-Pak C18 cartridge (Waters Corp.) and eluted in 0.5 ml of methanol. The methanol was evaporated at 37 °C under a gentle stream of N_2 and the [³H]acetyl-CoA residue reconstituted in 0.5 ml of water. Radiochemical purity assessed by HPLC was >85%, and there was a single major peak that co-chromatographed with authentic acetyl-CoA. The major contaminant was acetic acid.

Expression and Purification of Recombinant NAT1 in Escherichia coli—The open reading frame of *NAT1*4*, *NAT1*24*, the slow acetylator alleles *NAT1*14*, *NAT1*15*, *NAT1*17*, and *NAT1*22* and the inactive *NAT1-C68Y* were cloned into the bacterial expression vector pGEX-6P-2 and introduced into the *E. coli* strain BL21-CodonPlus(DE3)-RP (Stratagene) by electroporation. Cultures (4 liters of Luria Bertani containing 100 μg/ml chloramphenicol, 50 μg/ml tetracycline, and 100 μg/ml ampicillin) were grown at 37 °C to an optical density of 0.8 and then induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 4 h at room temperature. Bacteria were harvested (4000 × g, 10 min, 4 °C) and the pellets resuspended in 40 ml of lysis buffer (50 mM Tris, 1 mM EDTA, 0.5 M NaCl pH 8.0 containing 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml DNase I) and lysed using a French press. The cell lysates were centrifuged at 15 000 × g for 30 min at 4 °C and the soluble fraction collected. The GST fusion proteins were purified using glutathione-agarose beads (G 4510; Sigma) and the relative amounts of protein estimated by SDS-PAGE and Coomassie Blue staining.

NAT1 Acetylation Assay—Recombinant GST·NAT1 fusion proteins (10 μg) bound to GSH-agarose beads (Sigma) were incubated with 6.7 μM [³H] acetyl-CoA (specific activity = 9.25 GBq/mmol) in PBS at 37 °C for 2 h. The beads were recovered by filtration on 0.45 μm nitrocellulose membranes (Millipore), washed twice with 5 ml of ice-cold PBS, and radioactivity determined by liquid scintillation spectroscopy. GST bound to GSH-agarose beads was included to measure nonspecific binding. Specific binding was calculated by subtracting nonspecific binding from total binding.

MG132 Treatment and Immunoprecipitation—HT-29 cells were seeded at a density of 1×10^6 cells/well (6-well plate) and co-transfected with 1 μg of pFLAG-NAT1 plasmid and 1 μg of pcDNA-HA-ubiquitin plasmid using LipofectAMINE 2000. After overnight incubation at 37 °C, cells were treated with MG132 (20 μM, Calbiochem) or vehicle (Me₂SO) for 8 h and then washed twice with 1 ml of PBS and harvested by scraping into 0.8 ml of 20 mM Tris/1 mM EDTA buffer (pH 7.4) containing 1 mM dithiothreitol and protease inhibitor mixture (P 8340; Sigma). Cells were disrupted on ice by sonication and the supernatants cleared by centrifugation for 3 min at 16,000 × g (4 °C). Anti-FLAG-M2 monoclonal antibody (10 μg, F 3165; Sigma) was added, and the supernatants were rotated at 4 °C for 2 h. Protein G-Sepharose 4B (P 3296; Sigma) was then added and the lysates incubated for a further 1 h at 4 °C. Immunoprecipitates were collected by centrifugation and the beads washed three times with PBS. The recovered proteins were separated on 12% SDS-polyacrylamide gels and immunoblotted using either anti-FLAG-M2 HRP antibody (Sigma) or anti-HA antibody (H 6908; Sigma) according to the manufacturer's instructions.

RESULTS

NAT1 Protein levels Are Diminished in PBMC from Slow Acetylator Individuals—Previously, we and others have shown that individuals heterozygous for either the *NAT1*14B* or *NAT1*17* slow acetylator alleles had approximately half the NAT1 activity of individuals who were homozygous wild-type (*NAT1*4*) (5). Here, we show by Western blot that PBMC lysates from individuals heterozygous for either *NAT1*14B* or *NAT1*17* have lower levels of immunodetectable NAT1 protein compared with individuals who are homozygous wild-type (Fig. 1*A*). Moreover, the lower protein levels correlated with a lower enzyme activity (Fig. 1*B*), suggesting that the slow acetylator phenotype results from a reduction in the amount of NAT1. To determine whether the *NAT1*14B* and *NAT1*17* alleles were expressed *in vivo*, cDNA isolated from PBMC obtained from homozygous and heterozygous individuals was genotyped as described previously (6). Genotyping showed the presence of both variant forms of mRNA, indicating that each variant
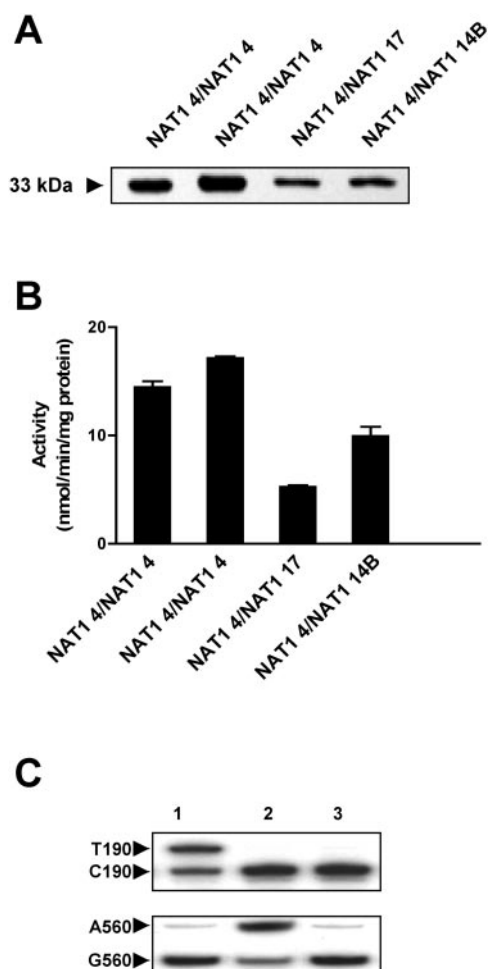
FIG. 1. **NAT1 expression in PBMC from individuals with different NAT1 genotypes.** *A*, Western blot analysis of NAT1 from two wild-type homozygotes (*NAT1*4/NAT1*4*) and two heterozygotes (*NAT1*17* and *NAT1*14B*) showing a decrease in immunodetectable protein in the heterozygotes. *B*, enzymatic activity in lysates prepared from PBMC obtained from the same individuals shown in *panel A. C*, genotyping of mRNA extracted from PBMC obtained from a *NAT1*4/NAT1*17* heterozygote (*lane 1*), a *NAT1*4/NAT1*14B* heterozygote (*lane 2*), and a *NAT1*4* homozygote (*lane 3*). The results show the presence of both RNA species in the two heterozygote individuals.

FIG. 2. **Expression of His-tagged NAT1 proteins in HT-29 cells.** *A*, HT-29 cells were transiently transfected with each expression vector or empty vector (*control*), and protein levels were determined by Western blot 24 h later. Endogenous NAT1 (*33 kDa*) is shown by the *lower band*. The tagged proteins chromatographed with a molecular mass of 36 kDa and were expressed at levels much greater than the endogenous protein. *B*, NAT1 activity in cell lysates for each NAT1 protein was determined using PABA as substrate. Control cells were transfected with empty vector. Data are mean ± S.E., $n = 3$. * indicates activities significantly greater than control samples ($p < 0.05$), by one-way analysis of variance.

allele was expressed *in vivo* (Fig. 1*C*).

Expression and Activity of NAT1 Proteins in HT-29 Cells—To determine protein expression and activity of the variant NAT1 proteins, each allele in the pEF1/His vector was transiently transfected into the human colon carcinoma cell line, HT-29. Western blots using a NAT1-specific antibody (Fig. 2*A*) showed that His-tagged wild-type NAT1, as well as all of the variant His-NAT1 proteins (36 kDa), were expressed at levels greater than endogenous NAT1 (33 kDa). Cells expressing the slow acetylator His-NAT1 proteins had NAT1 activities that were not significantly different ($p > 0.05$) from the endogenous NAT1 activity of HT-29 cells (5.7 ± 0.1 nmol/min/mg protein), indicating that these variant proteins are catalytically inactive, at least when PABA is used as substrate (Fig. 2*B*). Cells expressing His-NAT1 4 and His-NAT1 24 had enzymatic activities of 122 ± 1.2 and 130 ± 2.3 nmol/min/mg protein, respectively, which was more than 20-fold higher than the endogenous activity of HT-29 cells.

Stability of NAT1 Proteins in HT-29 Cells—To determine the stability of the different NAT1 allozymes *in vivo*, HT-29 cells were transiently transfected with the various pEF1/His-NAT1 constructs, treated with cycloheximide for up to 8 h, and ana-
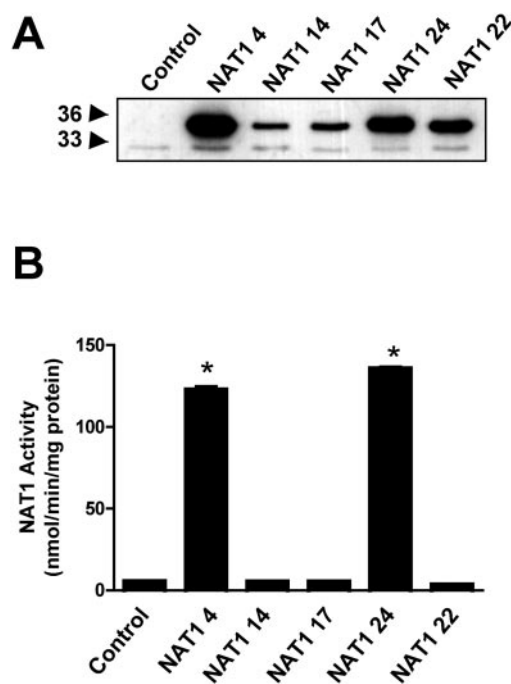
lyzed by Western blotting using a NAT1-specific antibody (Fig. 3*A*). The His-tagged NAT1 proteins were ~3 kDa larger than endogenous NAT1, so the endogenous protein did not interfere with the protein stability measurements. Endogenous NAT1 4, His-NAT1 4, and the rapid acetylator protein His-NAT1 24 had similar stabilities with no noticeable degradation occurring over the 8-h time period of the experiment (Fig. 3*B*). In fact, no significant degradation of His-NAT1 4 occurred over a 24-h time period of cycloheximide treatment, indicating that the half-life of the wild-type protein is in excess of 24 h (data not shown). These results show that the active allozymes were stable *in vivo* and that the His tag did not decrease protein stability. By contrast, all the slow acetylator (inactive) proteins were considerably less stable, having half-lives of between 2 and 4 h (Fig. 3*B*). The truncated slow acetylator protein NAT1 15 lacks the epitope for the NAT1-specific antibody used in these studies, so it was cloned into an N-terminal FLAG-tag vector so that its half-life could be determined using a FLAG-specific antibody. To ensure the FLAG tag did not affect protein stability, FLAG-*NAT1*4* and FLAG-*NAT1*17* also were constructed for comparison (Fig. 3*A*, *lower panels*). The half-lives of FLAG-NAT1 4 and FLAG-NAT1 17 were similar to their His-tagged counterparts, indicating that the tag had no appreciable effect on protein stability (Fig. 3*B*). The half-life of FLAG-NAT1 15 was less than 4 h (Fig. 3*B*), which was similar to that observed for the other slow acetylator NAT1 proteins. The results of the FLAG-tag experiments also show that the loss of NAT1 protein observed using the NAT1-specific antibody raised against the last 11 amino acids of NAT1 (16) was not because of loss of the epitope because FLAG-NAT1 17 (N-terminal tag) and His-NAT1 17 both had similarly short half-lives compared with wild-type.
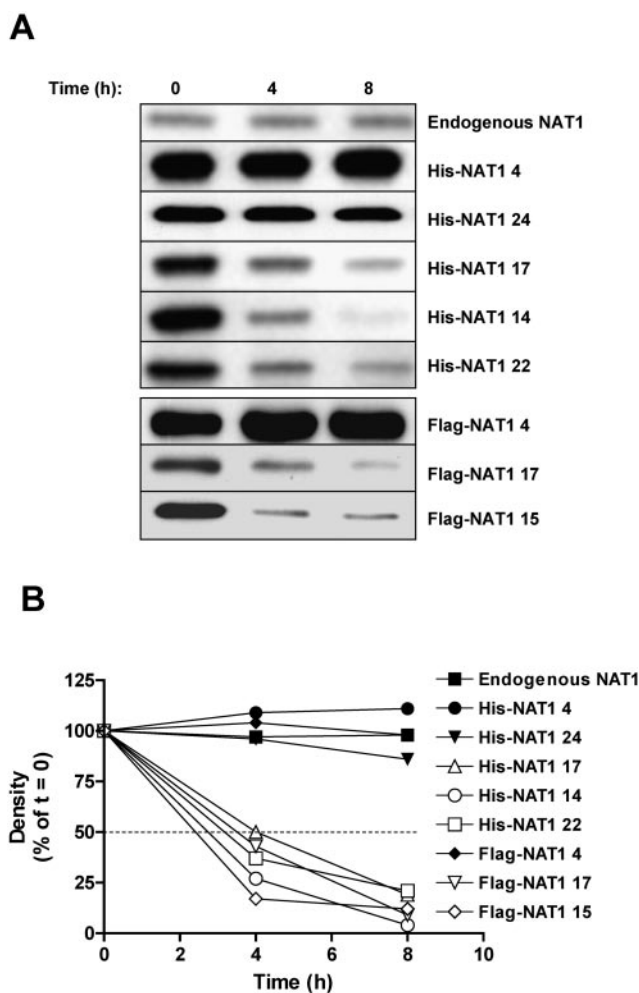
Stability of Human N-Acetyltransferase 1

A

B

FIG. 3. **Stability of NAT1 proteins in HT-29 cells.** *A*, HT-29 cells were transiently transfected with 1 μg of plasmid DNA (pEF-*NAT1*4*, pEF-*NAT1*14*, pEF-*NAT1*17*, pEF-*NAT1*22*, pEF-*NAT1*24*, or empty vector), incubated for 24 h, and treated with cycloheximide (10 μg/ml). Cell lysates were prepared at 0, 4, and 8 h for Western blotting. A NAT1-specific antibody that identifies the last 11 amino acids was used for the His-tagged proteins. Because NAT1 15 lacks the epitope, this allele was FLAG-tagged and a FLAG antibody was used for detection (*lowest panel*). NAT1 4 and NAT1 17 also were FLAG-tagged as controls to ensure the tag did not affect stability. *B*, densitometry scanning was used to quantify the amount of NAT1 protein at each time point. Density was normalized to *t* = 0.

Acetylation Capacity of NAT1 Allozymes—To determine whether the inactive NAT1 proteins produced by the slow acetylator alleles were acetylated by acetyl-CoA, GST·NAT1 fusion proteins were incubated with [³H]acetyl-CoA, and the radioactivity associated with the proteins was determined. The active allozymes GST·NAT1 4 and GST·NAT1 24 bound ~100 pmol [³H]/nmol protein, whereas all the inactive proteins bound less than 10% of that seen with the wild-type enzyme (Fig. 4). The lack of binding was not because of a lack of protein expression, determined by SDS-PAGE analysis (Fig. 4). Taken together with the protein stability data above, these results suggest that NAT1 in the non-acetylated state is rapidly degraded in the cell.

Stability of NAT1-C68Y Mutant in HT-29 Cells—To further investigate the effect of acetylation on NAT1 stability, the active site cysteine 68 was mutated to a tyrosine to prevent formation of a thiol ester with acetyl-CoA. This construct (pEF1/His-*NAT1-C68Y*) was transfected into HT-29 cells, and stability was determined using cycloheximide treatment. As expected, the expressed protein did not acetylate PABA (data
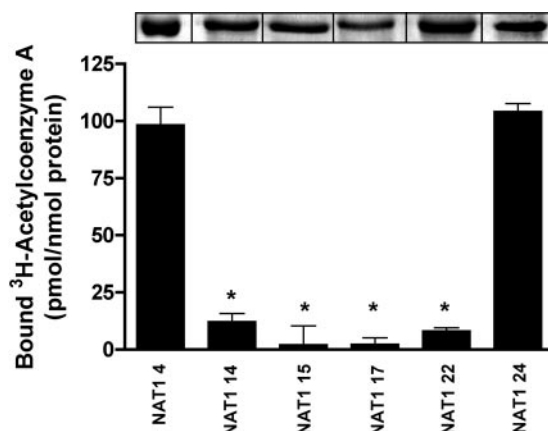

FIG. 4. **Acetylation capacity of GST·NAT1 fusion proteins.** GST·NAT1 proteins were incubated with [³H]acetyl-CoA and radioactivity associated with the proteins quantified by liquid scintillation counting. Specific binding was calculated by subtracting nonspecific binding (GST-bound beads) from total binding. Data are presented as mean ± S.E., *n* = 3. * indicates significantly different ($p < 0.05$) from NAT1 4. Shown *above the graph* is an SDS-PAGE gel stained with Coomassie Blue showing the presence of each expressed protein. NAT1 15 chromatographed at a lower molecular mass compared with the other proteins and has been digitally shifted for clarity.

not shown), and when expressed as a GST fusion it did not bind [³H]acetyl-CoA (Fig. 5*A*). The level of expression and stability of His-NAT1-C68Y was similar to that observed for the other inactive His-NAT1 proteins (Fig. 5, *B* and *C*). These results further support the postulation that the non-acetylated form of NAT1 is susceptible to rapid degradation in the cell.

Role of the Ubiquitin/26 S Proteasome Pathway in NAT1 Degradation—To determine whether the ubiquitin/26 S proteasome pathway was involved in the rapid degradation of the slow acetylator (inactive) NAT1 proteins, the effect of the 26 S proteasome-specific inhibitor MG132 on the intracellular half-life of the unstable FLAG-NAT1 17 was investigated. HT-29 cells were transiently transfected with pFLAG-*NAT1*17* and treated with cycloheximide for up to 8 h. In the absence of MG132, FLAG-NAT1 17 was rapidly degraded (Fig. 6*A*, *left panel*). However, when MG132 (20 μM) was added concurrently with the cycloheximide, the stability of the variant protein increased significantly with little protein loss occurring over the 8-h time period of the experiment (Fig. 6*A*, *right panel*). Furthermore, when the experiment was conducted in the absence of cycloheximide, treatment with MG132 (20 μM) for 8 h resulted in the appearance of higher molecular mass bands, consistent with ubiquitination of FLAG-NAT1 17 (Fig. 6*B*). Higher molecular mass bands also were observed for the other inactive NAT1 variant proteins but less so for the active variant protein FLAG-NAT1 24 and the wild-type protein FLAG-NAT1 4 (Fig. 6*B*). We next co-expressed each of the FLAG-tagged allozymes and FLAG-NAT1-C68Y with a HA-tagged ubiquitin construct to identify ubiquitination of the NAT1 proteins. Following treatment with MG132 and immunoprecipitation with anti-FLAG antibody, Western blotting with anti-HA antibody showed specific polyubiquitination of the unstable NAT1 proteins (Fig. 6*C*), whereas the stable NAT1 24 and NAT1 4 proteins were polyubiquitinated to a much lesser extent. Moreover, when cells expressing FLAG-NAT1 4 and HA-ubiquitin were treated with PABA, significantly more polyubiquitination of the immunoprecipitated protein was evident (Fig. 6*D*). Taken together, these results show that the unstable NAT1 allozymes and the non-acetylated stable NAT1 protein are targeted to the 26 S proteasome pathway by polyubiquitination.
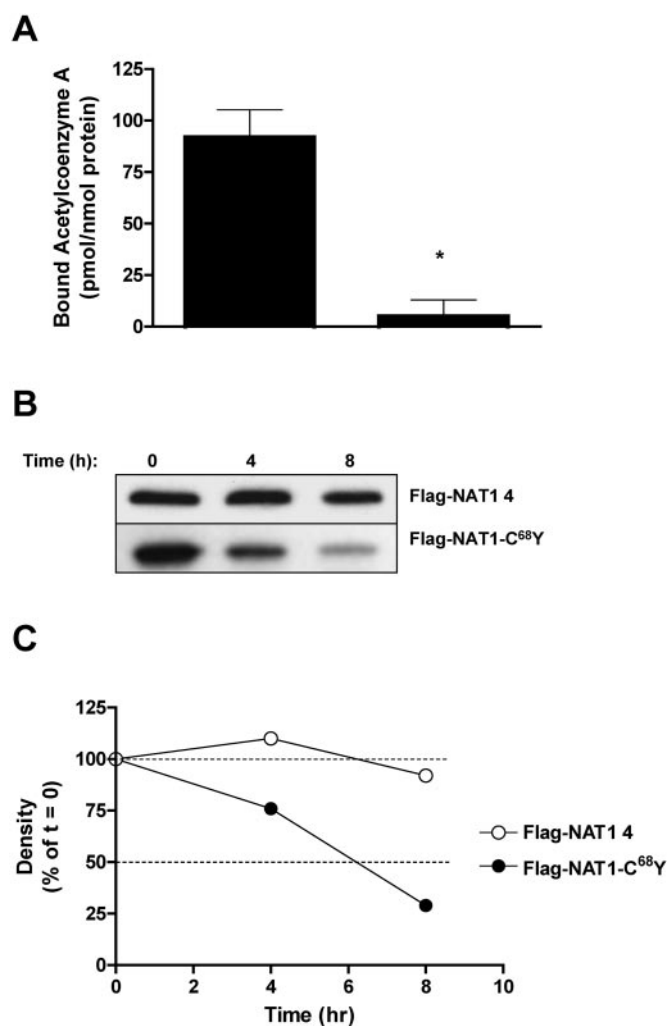
FIG. 5. **Stability of the NAT1·C68Y mutant.** *A*, binding of [³H]acetyl-CoA to GST·NAT1 4 and GST·NAT1-C68Y expressed in bacteria. Conditions were as described in Fig. 4. Data are presented as mean ± S.E., *n* = 3. * indicates significantly different (*p* <0.05) from NAT1 4. *B*, Western blot analysis of His-NAT1 4 and His-NAT-C68Y 24 h after transient transfection of HT-29 cells and treatment with cycloheximide (10 *μg*/ml). Each band was quantified by densitometry scanning and normalized to *t* = 0 (*C*).

DISCUSSION

Point mutations in the NAT1 locus result in a slow acetylator phenotype that is observed in ~5% of Caucasians and up to 25% in a Lebanese population (6, 17–19). Numerous genotypes have been reported, but those mutations that affect activity have only recently been described (6, 7, 19). Expression of 13 common and rare alleles in yeast by Fretland *et al.* (7) reveals two possible causes for the slow acetylator phenotype in humans. First, they observed negligible expression of some alleles and suggested translational efficiency may be important. Second, they observed more rapid heat inactivation of some mutant proteins and suggested intracellular stability also may be important. In this study, we have shown that mutant proteins are rapidly polyubiquitinated and targeted for degradation via the ubiquitin/26 S proteasome pathway. Moreover, degradation correlates with the non-acetylated state of the allozyme. For two of the alleles (*NAT1*14* and *NAT1*17*), protein instability also was demonstrated in PBMC from heterozygous individuals. From these studies, we propose the reaction model shown in Fig. 7. NAT1 is acetylated *in vivo* to a stable form. Hydrolysis of the acetyl-NAT1 is relatively slow but can be enhanced by substrate that removes the acetyl moiety from the protein.

Mutant proteins that do not catalyze the formation of an acetyl thiol ester exist in the non-acetylated state, which is ubiquitinated and targeted for degradation. This model explains the low protein levels seen with slow acetylator individuals and the instability of mutant proteins reported here and elsewhere (7). Moreover, it provides a plausible mechanism for the substrate-specific down-regulation of NAT1 reported earlier (8) and confirmed in the present study.

The present observations are restricted to the specific slow acetylator alleles studied (*NAT1*14, NAT1*15, NAT1*17*, and *NAT1*22*). However, very low levels of translated protein were also observed in yeast for *NAT1*19* (7), suggesting that this mutant protein also may undergo ubiquitination. There are clear similarities between NAT1 instability and the NAT2 slow acetylator phenotype described for some allelic variants. In humans homozygous for *NAT2*5A* or *NAT2*6A*, there is a decrease in immunodetectable protein present in the liver (20). It will be important to determine whether NAT2 protein levels *in vivo* can also be regulated by ubiquitination or down-regulated by selective substrates.

NAT1 possesses a catalytic triad active site (14, 21). Structurally, it is similar to many cysteine proteases such as the caspases, transglutaminases, and deubiquitinases. The active site is located within a cleft that may serve to exclude water from the catalytic domain protecting the acetyl thiol ester at Cys-68 from hydrolysis. Recently, Mushtaq *et al.* (22) showed that the C-terminal sequence of NAT from *Salmonella typhimurium* controls hydrolysis of acetyl-CoA. Normally, the protein is stably acetylated, and therefore net hydrolysis of acetyl-CoA is negligible. In the absence of the C terminus, the acetyl protein is rapidly deacetylated and thus able to continually catalyze the conversion of acetyl-CoA to coenzyme A. When the C terminus was removed and expressed in bacteria, the resulting truncated NAT was toxic, possibly because of depletion of intracellular acetyl-CoA. If a similar mechanism occurs in humans, then rapid degradation of non-acetylated protein may be a protective mechanism against excessive hydrolysis of acetyl-CoA.

The results from the present study indicated that the slow acetylator phenotype is due to an inactive protein that is rapidly degraded within the cell, at least for NAT1 14, NAT1 15, NAT1 17, and NAT1 22. They also demonstrated that substrate-dependent down-regulation is due to rapid degradation of the enzyme via the ubiquitin/26 S proteasome pathway. The different mutations in *NAT1* that give rise to an inactive protein change single amino acids located at different sites throughout the enzyme. For NAT1 15, the mutation introduces a stop codon and the protein is truncated at amino acid 186. The common feature for each of the slow acetylator enzymes is their inability to stably exist as an acetyl protein. The NAT1-C68Y mutant confirmed the difference in stability of the acetylated and non-acetylated proteins. Moreover, addition of substrate (PABA), which would be expected to shift the equilibrium between acetylated and non-acetylated enzyme toward the non-acetylated state, induced polyubiquitination and subsequent protein degradation. These data suggest that acetylation of Cys-68 may prevent access of ubiquitinases to target lysine residues. Because NAT1 15 was ubiquitinated in a similar manner as the other mutant proteins, the lysine residue(s) most likely reside in the first 186 N-terminal amino acids. In this region, there are seven lysines, two (Lys-13 and Lys-14) are adjacent to a putative D-box (R*XX*L) that has been described as necessary for the degradation of many cell cycle-dependent proteins (23). However, preliminary mutagenesis of
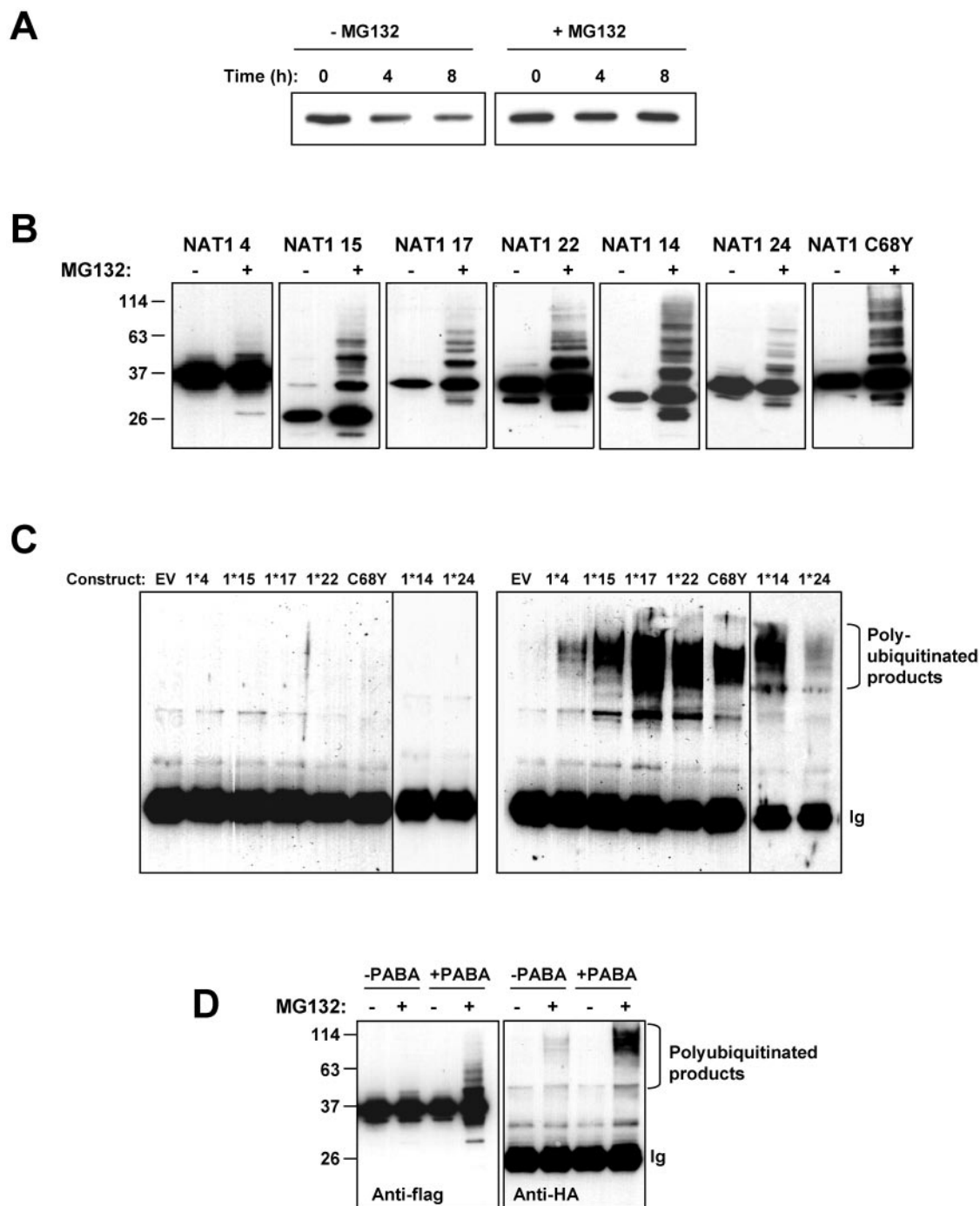
FIG. 6. **Ubiquitination of NAT1 proteins.** *A*, effect of proteosomal inhibitor on the stability of NAT1 17. HT-29 cells were transfected with FLAG-*NAT1*17* and then treated concurrently with cycloheximide (10 µg/ml) and MG132 (20 µM) or vehicle (Me₂SO). Cell lysates were prepared at the indicated times and Western blots performed using anti-FLAG-M2 HRP antibody. In the absence of MG132, NAT1 17 showed rapid degradation, whereas in the presence of the inhibitor it was stable over 8 h. *B*, formation of higher molecular mass products of the NAT1 allozymes in the presence of MG132. HT-29 cells were transfected with FLAG-tagged *NAT1* plasmids and then treated with MG132 (+) or vehicle (−) for 8 h. Cell lysates were prepared and subjected to Western blot with anti-FLAG-M2 HRP antibody. The molecular mass of unmodified FLAG-NAT1 was 36 kDa except for the truncated NAT1 15, which was 26 kDa. *C*, formation of polyubiquitinated products of NAT1. HT-29 cells were co-transfected with HA-ubiquitin plasmid and FLAG-tagged NAT1 plasmids. After 8 h of treatment with MG132, cell lysates were prepared and immunoprecipitated with anti-FLAG antibody, Western blotted, and probed with anti-HA antibody. Polyubiquitinated products are shown. *D*, effect of PABA on polyubiquitination of NAT1 4. HT-29 cells co-transfected with HA-ubiquitin plasmid and FLAG-*NAT1*4* were treated with MG132 or vehicle for 8 h in the absence or presence of PABA (1 mM). Cell lysates were prepared and subjected to Western blot with either anti-FLAG-M2 HRP antibody (*left panel*) or immunoprecipitated with anti-FLAG antibody and then subjected to Western blot with anti-HA antibody (*right panel*). Polyubiquitinated products are shown.

these residues did not appear to affect ubiquitination.[2] Further mutagenesis work is underway to identify the crucial lysine(s) involved in NAT1 degradation. Under conditions where sub-

strates are not present, NAT1 will exist in equilibrium between an acetylated and non-acetylated state. From the reaction mechanism (Fig. 7), the equilibrium is determined by the concentration of acetyl-CoA within the cell. The very long half-life of NAT1 4 (>24 h) suggests that the majority of the protein is
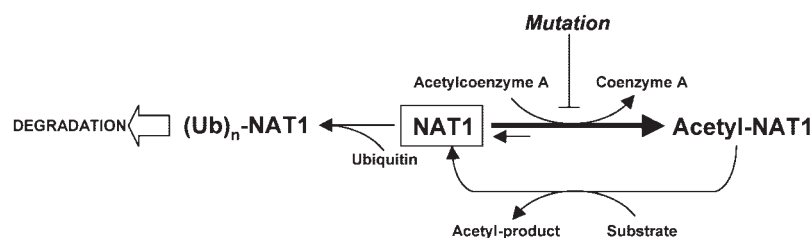
FIG. 7. **Model of the intracellular regulation of NAT1 by acetylation of cysteine 68.** NAT1 undergoes acetylation on cysteine 68 to form a stable thiol ester. Based on stability studies that show the deacetylation of NAT1 is a slow process (25), the equilibrium favors the formation of acetyl-NAT1 in the absence of substrate. Deacetylation of NAT1 occurs when substrate is present, shifting the equilibrium to the non-acetylated state. This form of NAT1 is rapidly ubiquitinated and degraded. Mutations that prevent acetylation (*e.g.* NAT1-C68Y) or result in a shift in the equilibrium to the non-acetylated state produce an unstable protein that is rapidly degraded. The difference in stability of the acetylated and non-acetylated protein accounts for the low NAT1 protein levels in heterozygous individuals as well as the substrate-dependent down-regulation of NAT1.

acetylated *in vivo*. A shift in equilibrium because of a change in cofactor levels will lead to an increase or decrease in the amount of NAT1 present in the cell. In this manner, NAT1 may behave as a sensor of acetyl-CoA levels. However, further investigation is required to determine the relationship, if any, between acetyl-CoA and NAT1 levels. Recently, Dupret and co-workers (9, 10) have shown that oxidation of the active site cysteine of NAT1 reversibly inhibits enzymatic activity. However, it is not known whether this modification affects protein stability *in vivo* by altering the acetylation status of NAT1.

The differential stability of wild-type NAT1 and mutant NAT1 proteins is similar to that observed for wild-type human NAD(P)H:quinone oxidoreductase 1 (NQO1) and the mutant protein NQO1 2. Anwar *et al.* (24) showed that the molecular chaperone Hsp70, which catalyzes protein folding, associates with the wild-type protein but not with the mutant protein NQO1 2. It has been proposed that the amino acid change in NQO1 2 disrupts the central parallel β-sheet, resulting in a decreased affinity for cofactor. Anwar *et al.* suggest that the lack of association of Hsp70 with NQO1 2 may be because of its inability to bind cofactor. It is plausible that a similar mechanism exists for the mutant NAT1 proteins, where their inability to bind the cofactor acetyl-CoA (demonstrated in the present study) prevents their association with chaperone proteins such as Hsp70 and leads to incomplete protein folding and subsequent ubiquitination and degradation. This also may explain how mutations located throughout the NAT1 gene that change single amino acids spatially distinct from the active site all lead to the production of an inactive, rapidly degraded protein. Further study is required to determine whether Hsp70 or any other chaperone proteins interact with NAT1.

In summary, the present study provides experimental evidence to support a molecular mechanism that may explain the low level of protein expression from mutant NAT1 alleles. In addition, it also may explain how substrates for NAT1 can induce protein down-regulation and, therefore, modification of NAT1 activity *in vivo*. The role of the ubiquitin/26 S protea-some pathway in regulating NAT1 levels has not previously been described for this or related protein families.

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Proteasomal Degradation of *N*-Acetyltransferase 1 Is Prevented by Acetylation of the Active Site Cysteine: A MECHANISM FOR THE SLOW ACETYLATOR PHENOTYPE AND SUBSTRATE-DEPENDENT DOWN-REGULATION

Neville J. Butcher, Ajanthy Arulpragasam and Rodney F. Minchin

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