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Human Cytosolic 5′-Nucleotidase I

CHARACTERIZATION AND ROLE IN NUCLEOSIDE ANALOG RESISTANCE*

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Nucleoside analogs are important in the treatment of hematologic malignancies, solid tumors, and viral infections. Their metabolism to the triphosphate form is central to their chemotherapeutic efficacy. Although the nucleoside kinases responsible for the phosphorylation of these compounds have been well described, the nucleotidases that may mediate drug resistance through dephosphorylation remain obscure. We have cloned and characterized a novel human cytosolic 5′-nucleotidase (cN-I) that potentially may have an important role in nucleoside analog metabolism. It is expressed at a high level in skeletal and heart muscle, at an intermediate level in pancreas and brain, and at a low level in kidney, testis, and uterus. The recombinant cN-I showed high affinity toward dCMP and lower affinity toward AMP and IMP. ADP was necessary for maximal catalytic activity. Expression of cN-I in Jurkat and HEK 293 cells conferred resistance to 2-chloro-2′-deoxyadenosine, with a 49-fold increase in the IC_{50} in HEK 293 and a greater than 400-fold increase in the IC_{50} in Jurkat cells. Expression of cN-I also conferred a 22-fold increase in the IC_{50} to 2′,3′-difluorodeoxycytidine in HEK 293 cells and an 82-fold increase in the IC_{50} to 2′,3′-dideoxycytidine in Jurkat cells. These data indicate that cN-I may play an important role in the regulation of physiological pyrimidine nucleotide pools and may also alter the therapeutic efficacy of certain nucleoside analogs.

Nucleoside analogs are used as chemotherapeutic agents in the treatment of hematologic malignancies and as anti-viral drugs. These compounds are taken up by cells through nucleoside transporters in the cell membrane or, in the case of more lipophilic drugs, through diffusion (1–3). Once in the cytoplasm, the nucleoside analogs are substrates for phosphorylation by the nucleoside kinases of the deoxyribonucleoside salvage pathway (reviewed in Ref. 4). The enzyme 2′-deoxycytidine kinase phosphorylates dAdo, dCyd, and dGuo as well as the analogs AraC,[1] dFdC, and CdA, whereas thymidine kinase 1 phosphorylates dThd and dUrd as well as the analogs d4T and 5-FU. Phosphorylation to the monophosphate form is considered the rate-limiting step of activation of nucleoside analogs (4).

Nucleoside analog monophosphates are rapidly phosphorylated to their triphosphate forms, which are believed to mediate their cytotoxic activities by several different mechanisms (reviewed in Ref. 5). For example, they can be directly incorporated into DNA, leading to chain termination, or they can inhibit DNA synthesis by direct inhibition of DNA polymerase. It has recently been demonstrated that certain of these metabolites, including CdATP, AraATP, and F-AraATP, can induce apoptosis in nonproliferating cells by interacting with cytochrome *c* and apoptosis protein-activating factor-1 (APAF-1) to cleave and activate the caspases responsible for the induction of apoptosis (6, 7). In contrast, the triphosphates of antiretroviral compounds, such as 2′,3′-dideoxycytidine (ddC) and d4T, inhibit the reverse transcriptase of the human immunodeficiency virus through chain termination. Cytotoxicity can occur when these compounds are incorporated into nuclear or mitochondrial DNA (reviewed in Ref. 8).

Resistance to nucleoside analogs is a significant clinical problem and can be caused by a number of factors affecting the metabolism of the drugs, including decreased numbers of nucleoside transporters, increased deamination of cytidine and adenosine analogs, loss of expression of activating kinases, modulation of the downstream apoptotic machinery, and increased activity of nucleotidases that catalyze the conversion of nucleotides back to nucleosides (reviewed in Ref. 5). The 5′-nucleotidases oppose the action of nucleoside kinases by dephosphorylating the monophosphate form of nucleosides and nucleoside analogs and, therefore, are likely to be important in determining the sizes and turnover rates of the deoxyribonucleotide pools. Thus, 5′-nucleotidases that catabolize the monophosphates of nucleoside analogs used in the clinic will lower their clinical efficacy.

To date, the substrate specificity of the cytosolic nucleotidases responsible for dephosphorylation and inactivation of nucleoside monophosphates has not been well defined. Among several potentially important 5′-nucleotidases, only human cytosolic 5′-nucleotidase II (cN-II) has been studied in detail. cN-II is a 65-kDa protein that is strongly activated by ATP and prefers IMP to AMP as a substrate (9–11). Although increased expression of cN-II has been shown to lead to drug resistance in

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF331801.

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[1] The abbreviations used are: AraC, 1-β-D-arabinofuranosylcytosine; dFdC, 2′,3′-difluorodeoxycytidine; CdA, 2-chloro-2′-deoxyadenosine; d4T, 2′,3′-didehydro-3′-deoxythymidine; 5-FU, 2,4-dihydroxy-5-fluoropyrimidine; AraATP, adenine-9-β-D-arabinofuranoside triphosphate; F-AraATP, 2-fluoroadenine-9-β-D-arabinofuranoside triphosphate; cN-I, cytosolic nucleotidase I; cN-II, cytosolic nucleotidase II; PCR, polymerase chain reaction; ddC, 2′,3′-dideoxycytidine; F-AraA, 2-fluoroadenine-9-β-D-arabinofuranoside; F-Ara-AMP, 2-fluoroadenine-9-β-D-arabinofuranoside monophosphate AraCMP, 1-β-D-arabinofuranosylcytosine monophosphate AraCMP, 1-β-D-arabinofuranosylcytosine monophosphate; CdAMP, 2-chloro-2′-deoxyadenosine monophosphate; MOPs, 4-morpholinepropanesulfonic acid; HEK cells, human embryonic kidney cells; AMPCP, α, β-methyleneadenosine-5′-diphosphate.

two cell lines (12, 13), its kinetic properties make it unlikely to participate in pyrimidine nucleotide dephosphorylation (10). Cytosolic 5′-nucleotidase I (cN-I) has been isolated from pigeon, rat, rabbit, dog, and human hearts (14–19). This 5′-nucleotidase is activated by ADP and prefers AMP to IMP as a substrate (20). Although recent kinetic data suggest that cN-I may participate in deoxyribonucleoside monophosphate catabolism (17), the role of this enzyme in nucleoside analog resistance has never been examined. Thus we hypothesize that cN-I might play a role in dephosphorylation of deoxyribonucleoside monophosphates and in inactivation of nucleoside analogs.

To determine the role of the human cN-I enzyme in normal nucleotide metabolism as well as in drug resistance, we have cloned the human cN-I cDNA, defined the substrate specificity of the recombinant cN-I protein, and investigated the role of this enzyme in nucleoside analog resistance.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

AraC, ddC, CdA, F-AraA, 5-FU, d4T, and AraCMP were purchased from Sigma. CdAMP was manufactured by Biolog Life Science Institute (Bremen, Germany). Compound 506U (AraG prodrug) was obtained from Glaxo-Wellcome (Research Triangle Park, NC), dFdC was a gift from Lilly, and F-AraAMP was obtained from Berlex (Richmond, CA).

Cloning of Human cN-I

Reverse transcription-PCR was performed using human heart poly(A) mRNA (CLONTECH, Palo Alto, CA). Primers were designed to overlap the 5′ (TAAGCTTGGTACCATGGAACCTGGGCAGCCCCGGG-AGCCCCAG) and 3′ (CTACTGTGCAGATGGGGCCTGCTTTGC) ends of the cDNA-coding region. The reverse transcription reaction was performed using avian myeloblastosis virus reverse transcription (Promega, Madison, WI), the PCR reaction was performed with Pfu Turbo (Stratagene, La Jolla, CA) in a buffer containing 10% Me_2SO followed by 10 cycles with *Taq* polymerase (Life Technologies, Inc.) to enable cloning into the TA vector (Invitrogen, Carlsbad, CA), and individual clones were sequenced.

Northern Blot Analysis

Total cellular RNA was extracted using TRI REAGENT (Molecular Research Center, Cincinnati, OH). Fifteen μg of total RNA was loaded on each lane and separated in 1% agarose, 100 mM MOPS buffer, and 2.0 M formaldehyde. RNA was transferred to a nylon membrane, UV-cross-linked, and baked at 80 °C for 1 h. Blots were prehybridized in 1% SDS, 0.1 M NaCl at room temperature for 1 h and at 42 °C in High Efficiency Hybridization System containing 50% formamide (Molecular Research Center, Cincinnati, OH) for 1 h. The ^{32}P-labeled 671-base pair probe (extending from base pair 437 to base pair 1107 of the cN-I cDNA sequence) was then added and hybridized overnight at 42 °C. The blots were washed for 30 min at 55 °C and for 30 min at 60 °C in 1× SSC (0.15 M NaCl and 0.015 M sodium citrate), 0.2% SDS and autoradiographed. The ^{32}P-labeled β-actin probe was hybridized as described above and washed twice for 15 min at room temperature in 2× SSC, 0.05% SDS and twice for 20 min at 50 °C in 0.1× SSC, 0.1% SDS.

Baculovirus Expression

cN-I was expressed in Sf9 insect cells using the baculovirus expression system (Life Technologies) according to the product protocol. Briefly, the cN-I construct was subcloned into the PDR120 transfer vector and transfected into DH10Bac cells. Bacmid was isolated from DH10Bac bacterial colonies, and baculovirus was produced by infecting Sf9 cells with bacmid DNA and harvesting virus from the media after cell lysis. For protein purification, Sf9 cells were collected 4 days after infection, pelleted, and frozen at −80 °C.

Purification of cN-I

Recombinant human cN-I was purified using a modification of previously published protocols for purification of the rabbit enzyme (16, 17). Sf9 cells (7.7 g) were resuspended in 40 ml of extraction buffer containing 40 mM NaHEPES, pH 7.0, 15% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 1× complete protease inhibitor mixture (Roche Molecular Biochemicals). The cells were homogenized 3 × 10 s with a tissue homogenizer (Tekmar Tissumizer, Cincinnati, OH) and centrifuged at 16,000 × *g* at 4 °C to pellet membranes. The pellet was re-homogenized, and the supernatants were combined. cN-I was precipitated with 40% ammonium sulfate, and the resulting pellet was solubilized in 2 ml of extraction buffer and dialyzed against 3 changes of buffer A (40 mM NaHEPES, pH 7.0, 15% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM $MgCl_2$) overnight.

After dialysis the sample was rehomogenized in 11 ml of buffer A and incubated for 1 h at 4 °C with 5 g of wet Whatman P11-cellulose phosphate (Whatman, Kent, ME). The phosphocellulose was centrifuged to remove unbound proteins, resuspended in buffer A, and transferred into a column. The column was washed with 100 ml of buffer A, and bound proteins were eluted with 100 ml each of 0.25 M NaCl, 0.4 M NaCl, 0.6 M NaCl, and 2 M NaCl/2.1 mM sodium phosphate. Fractions containing cN-I activity were eluted at 0.25 M NaCl and were further concentrated to ~15 ml using an Ultrafree-15 filter with 30-kDa cut-off range (Millipore, Bedford, MA). The sample was dialyzed overnight against buffer A and run on a DEAE-Sepharose column with buffer A adjusted to pH 7.5. The column was washed, and proteins were eluted with a 0–0.5 M NaCl gradient in buffer A. The fractions containing cN-I activity were concentrated to ~1 ml by spinning through an Ultrafree-15 filter unit with a Biomax-30 membrane (Millipore). The sample was then run on a Sephacryl S-300 column (1.6 × 90 cm) with buffer A, pH 7.0, containing 100 mM KCl. The fractions with cN-I activity were dialyzed overnight against buffer A containing 50% glycerol, and bovine serum albumin was added to a final concentration of 2 mg/ml. Aliquots of enzyme were stored at −80 °C until use.

Enzyme Kinetics

Radiochemical Assay and Estimation of Kinetic Parameters—Enzyme assays were performed in 50 mM Tris, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 5 mM β-glycerophosphate, 1 mg/ml bovine serum albumin, 100 μM AMPCP, and 1 mM ADP, with various concentrations of substrate. When AMP and IMP were used as substrates, 22 nCi of ^{14}C-labeled compounds were used in a 20-μl reaction volume. Alternatively, ^{14}C-labeled dCMP was used in an amount of 5.5 nCi in a 20-μl reaction volume. Enzyme concentrations were 69 pg/μl when assayed with dCMP, 552 pg/μl when assayed with AMP and 1.38 ng/μl when assayed with IMP. Enzymatic reactions were initiated by the addition of enzyme, run at 37 °C, and stopped by heating at 85 °C for 2 min. Reaction rates were determined to be linear within the protein concentrations and incubation times used. Fifteen μl of each sample was spotted onto cellulose chromatography sheets with fluorescent indicators (Kodachrome, Eastman Kodak Co. and Analtech, Newark, DE), and thin layer chromatography was performed in a solvent containing 60% butanol, 20% methanol, 19% dH_2O, and 1% ammonia. Bands were visualized under UV light, and spots corresponding to the nucleoside products were cut out and counted on a Packard scintillation counter. Specific activity was expressed as μmol/min/mg of substrate. The substrate saturation curves were obtained within 5 μM to 60 mM concentrations, and the effect of ADP, ATP, and GTP (activator saturation curves) was tested within concentrations ranging from 20 μM to 4 mM. Data was graphed using SigmaPlot 2000 (SPSS, Inc., Chicago, IL), and kinetic parameters were derived using either nonlinear regression (three parametric Hill equation) or linear regression (Hanes equation) procedures as previously described (10, 21).

Colorimetric Assay and Estimation of Substrate Specificity—Colorimetric assays were performed in 50 mM Tris, pH 7.0, buffer containing 100 mM KCl, 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 100 μM AMPCP, 1 mM ADP, and 5 mM each substrate. cN-I (34.5 ng/reaction) was incubated in 50 μl final volume for 10 min at 37 °C. The reaction was stopped with the addition of 1 ml of Chen reagent as previously described (22). With F-AraAMP and AraCMP, incubation time was increased to 40 min, and the concentration of cN-I was 10 times higher. For the inhibition experiments, the buffer included 5 mM AMP and 1 mM competing nucleoside analog monophosphate with a 10 min incubation and 34.5 ng of cN-I.

Cell Culture

Sf9 cells were maintained in Grace's insect medium (Life Technologies) supplemented with 10% fetal bovine serum (Sigma) and 1.8 mM L-glutamine (Life Technologies). HEK 293 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum, and Jurkat cells were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum. All media was supplemented with penicillin and streptomycin (Lineberger Cancer Research Center Tissue Culture Facility, University of North Carolina). Transfected HEK 293 and Jurkat E6–1 cells

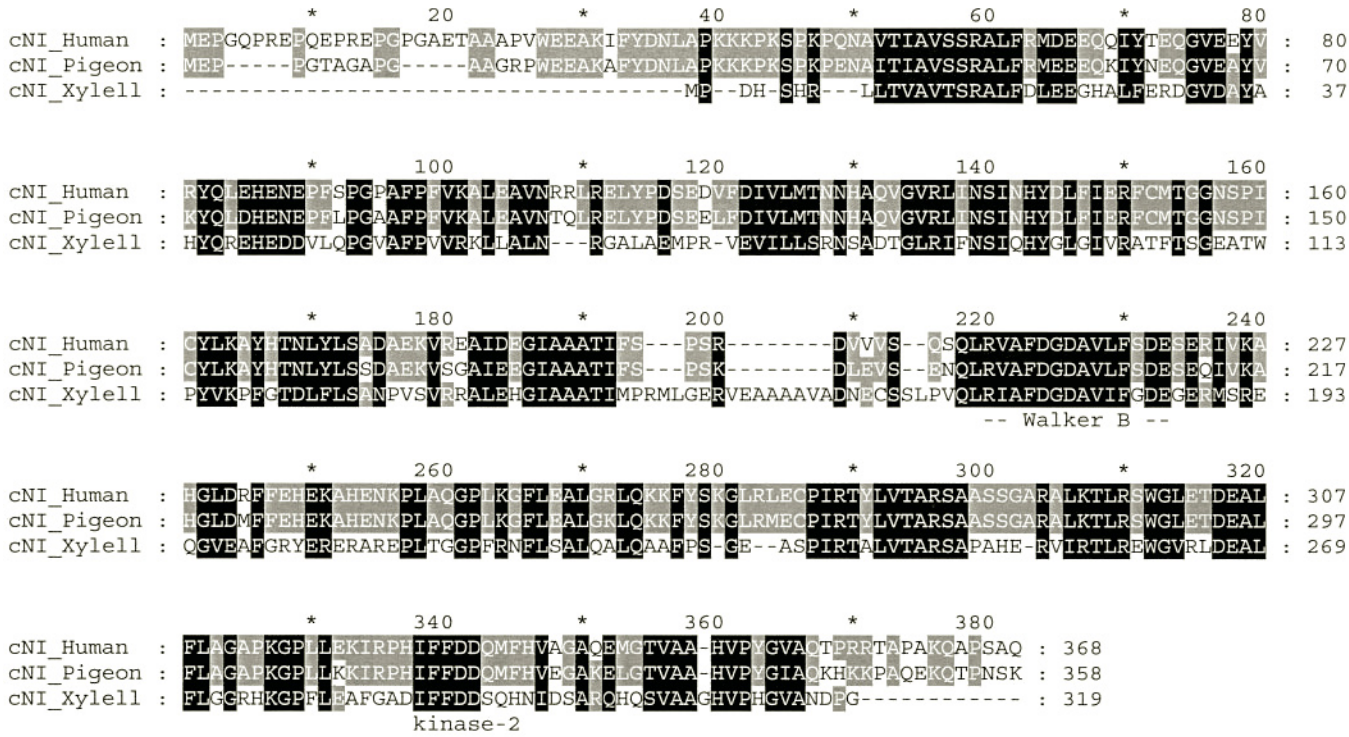

FIG. 1. **Alignment of human, pigeon, and *X. fastidiosa* cN-I amino acid sequences.** Similar conserved amino acids are *shaded*. The conserved Walker B and kinase 2 motifs are indicated below the alignment.

were maintained in the presence of G418 (Life Technologies).

Development of Stable Cell Lines

The cN-I cDNA was subcloned into the pcDNA3 mammalian expression vector that contains a pCMV promoter (Invitrogen, Carlsbad, CA). HEK 293 cells were transfected using Fugene6 (Roche Molecular Biochemicals), and Jurkat E6–1 cells were transfected by electroporation using a Bio-Rad Gene Pulser. The transfected cells were selected in increasing concentrations of G418, up to 750 μg/ml for HEK 293 cells and up to 1 mg/ml for Jurkat cells. An empty pcDNA3 vector was used as a control for both cell lines. Individual clones were selected by serial dilution of cells transfected with the cN-I construct in 96-well plates. Colonies grown from single cells were tested to determine cN-I activity, and cells were maintained in the presence of G418. Due to low transfection efficiency, the Jurkat cells were pre-selected with 0.5 μM CdA for 2 days before the serial dilutions.

Generation of Polyclonal Antibody and Western Blot Procedure

A synthetic acetylated peptide corresponding to the C-terminal 16 amino acids of cN-I (acetyl-CQTPRRTAPAKQAPSAQ-OH) was synthesized and conjugated to keyhole limpet hemocyanin at the peptide synthesis facility at University of North Carolina. The peptide was used to produce rabbit polyclonal antisera to cN-I by Rockland Immunochemicals (Gilbertsville, PA). Antibody was purified on a Sulfolink column (Pierce) coupled to the original antigen peptide conjugated to bovine serum albumin, according to the manufacturer's instructions.

For Western blots, proteins (50 μg) were separated on a 10% polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore). Protein concentration was measured using the Bio-Rad Protein Assay Dye Reagent. Western blots were performed with cN-I antibody at a dilution of 1:1000 followed by horseradish peroxide-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech) at 1:5000. Bands were visualized using ECL (Amersham Pharmacia Biotech).

Drug Sensitivity Studies

To determine whether overexpression of cN-I affects the IC_{50} for various nucleoside analogs, HEK 293 and Jurkat E6–1 parental, pCDNA3-transfected and cN-I-transfected cells were plated in 96-well plates at a density of 2.5×10^3 cells/well for the HEK 293 cells and 2×10^4 cells/well for the Jurkat E6–1 cells. The cells were treated with concentrations of nucleoside analogs between 1 nM and 10 mM depending on the toxicity of the drug. Cells were incubated in 96-well plates in the presence of drug for 4 days. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed as previously described (23) to determine relative cell viability, with the absorbance measured at 595 nm.

RESULTS

Cloning of Human cN-I—A FASTA search performed with the cDNA sequence of the pigeon cN-I enzyme (24) identified the human cN-I gene (GenBank™ accession number: AL035404.20). Sequence analysis identified six exons with an open reading frame of 1107 bases localized on human chromosome 1 p33-p34.3. Reverse transcription-PCR using human heart poly(A) mRNA as a template produced the expected 1107-base pair cDNA. An A to G base change at position +729 in the cDNA sequence and 75,616 in the GenBank™ sequence was found in all sequenced PCR products from several different reactions and is therefore unlikely to be a PCR-related mutation. The base change does not affect the amino acid sequence. This sequence was scanned against the GenBank™ data base and was significantly related to three other sequences: (AL033526.24, a fragment of AL035404.20 containing exons 4–6 of the cN-I enzyme; AJ131243.1, *Columba livia* (pigeon) mRNA for cN-I, with 82% identity to the human sequence (24); and AB045992.1, *Macaca fascicularis* (macaque primate) brain cDNA, containing a stretch of 135 amino acids that is 97% identical to the first exon of human cN-I. The human cN-I cDNA encodes a 368 amino acid protein of 47.7 kDa that has 83% identity to the 40-kDa protein of the cloned pigeon cN-I enzyme.

The human cN-I amino acid sequence was scanned against the GenBank™ data base and, in addition to pigeon cN-I, was significantly related to *Xylella fastidiosa* (proteobacteria) 5′-nucleotidase F82601 (25). An alignment of the human, pigeon, and *X. fastidiosa* cN-I amino acid sequences is shown in Fig. 1. All three cN-I sequences contain a conserved classic Walker B motif (R/K)X_{1-4}G$X_{2-4}\phi X\phi_2$(D/E) (ϕ is a hydrophobic amino acid) (26) at amino acids 220 to 233. The Walker B motif is found in many ATP-binding proteins and may serve as part of the nucleotide binding pocket (26). All three cN-I sequences

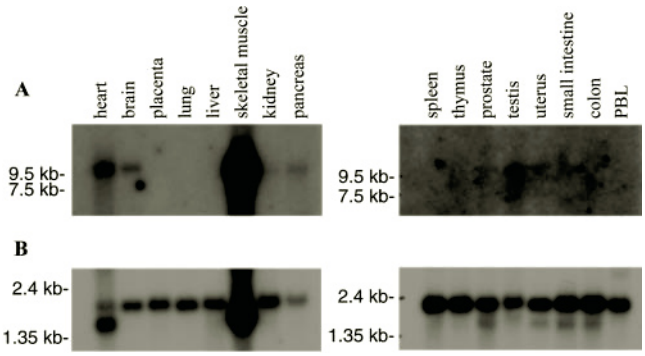

FIG. 2. **Expression of cN-I in human tissues.** *A*, Northern blot analysis was performed with two human tissue blots (CLONTECH MTN 7760–1 and 7759–1). The cN-I mRNA migrates at ~10 kilobases (*kb*). *B*, blots were reprobed with β-actin cDNA to control for loading. *PBL*, peripheral blood leukocyte.

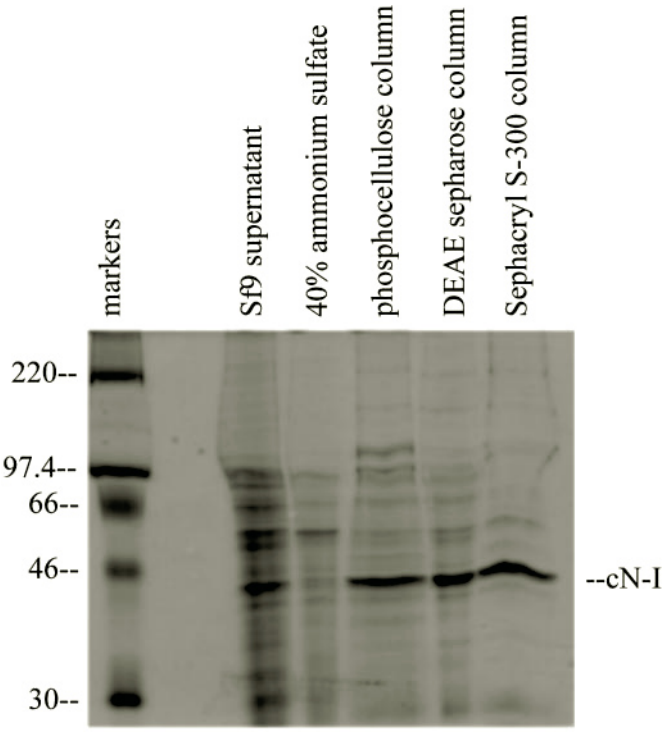

FIG. 3. **Purification of recombinant human cN-I.** Recombinant human cN-I was expressed in Sf9 insect cells using Life Technologies, Inc. baculovirus expression system. The Sf9 cells were homogenized, and the membranes were pelleted. The supernatant was precipitated with 40% ammonium sulfate. The sample was further purified on a phosphocellulose column, a DEAE-Sepharose column, and a Sephacryl-300 gel filtration column. The following amount of protein was loaded onto each lane: supernatant, 20 μg; ammonium sulfate precipitation, 20 μg; phosphocellulose column, 10 μg; DEAE-Sepharose column, 5 μg; and Sepharcryl-300 column, 5 μg. Protein was run on a 10% SDS-polyacrylamide electrophoresis gel and stained with Coomassie Blue.

also contain a conserved kinase-2 motif near the C terminus, IFFDD (at amino acids 338 to 342 on the alignment). Although the sequences of kinase-2 domains are highly variable, they usually consist of four hydrophobic residues followed by an aspartate that interacts with Mg^{2+}, Ca^{2+}, or other divalent metal ions (26). Mg^{2+} is necessary for cN-I activity (16, 18–20), and this conserved domain may be essential for enzyme function. There are several additional putative nucleotide binding domains that are conserved only in the human and pigeon cN-I enzymes: two kinase-2 domains are located at amino acids 31–35 and 313–317, and a putative kinase 3a domain (26) is located at amino acids 299–304. Three putative nucleoside monophosphate binding motifs (NMP-2), which may bind the pentose sugar (26), are located at amino acids 68–73, 177–182, and 313–318 on the alignment. Neither 5′-nucleotidase signature 1 nor signature 2 (PROSITE), derived from conserved ecto-5′-nucleotidase domains, was found in the cN-I sequence.

Tissue Expression—Northern blot analysis was performed on two human tissue blots (CLONTECH) to determine the tissue-specific expression of cN-I. Data presented in Fig. 2 show that skeletal muscle expressed a very high level of the ~10-kilobase cN-I transcript, whereas a lower level of expression was found in heart, brain, and pancreas. Upon overexposure, expression could also be seen in kidney, testis, and uterus.

Purification of Recombinant cN-I—Recombinant human cN-I expressed in Sf9 insect cells was purified from cell supernatants by ammonium sulfate precipitation followed by phosphocellulose chromatography, DEAE-Sepharose chromatography, and gel filtration chromatography (Fig. 3). This purification procedure yielded a protein that was ~90% pure and had a specific activity of 14.4 μmol/min/mg when assayed with 200 μM dCMP and 1 mM ADP.

Kinetics Parameters—The relative V_{max} and apparent K_m values for AMP, dCMP, and IMP were determined in both the presence and absence of ADP as an activator. Table I summarizes the data and shows that the enzyme exhibited the highest V_{max} in the presence of AMP, followed by IMP and dCMP. On the other hand, the highest substrate affinity was obtained for dCMP. The high ratio of V_{max}/K_m for dCMP demonstrated that it was the most efficient substrate for human recombinant cN-I.

ADP was required for the full activity of recombinant human cN-I. ADP both increased the V_{max} and decreased the K_m for all three substrates (Table I). The Hill coefficient in the presence of ADP was close to 1 for all three substrates, whereas in the absence of ADP, it increased to 2.6 or 2.7 (Table I). Such kinetic behavior suggests that ADP functions as an allosteric activator of cN-I. When cN-I activity was measured at increasing ADP concentrations (from 20 μM to 2 mM) and at a fixed concentration of dCMP, half-maximal activity ($A_{0.5}$) was reached at a concentration of 89 μM (Fig. 4 and Table II). The steep initial slope of the curve indicates that ADP at concentrations below 200 μM is a critical physiological regulator of cN-I activity. GTP also served as an activator for cN-I, although not as strong as ADP, and there was only slight activation by ATP (Fig. 4 and Table II).

The data in Table III summarizes the substrate specificity with a larger range of substrates. In general, higher specific activities were observed with purine substrates and pyrimidine ribonucleoside monophosphates, and lower specific activities were measured with pyrimidine deoxyribonucleoside monophosphates. The nucleoside analog CdAMP was a good substrate for cN-I, with a relative activity 39% that of AMP. The other analogues used in clinic, F-AraAMP and AraCMP, were not dephosphorylated by cN-I.

Since F-AraAMP and AraCMP appear to be poor substrates for cN-I, we tested the possibility that they may function as competitive inhibitors. Table IV demonstrates that, in an assay buffer containing 5 mM AMP, cN-I activity was inhibited 70% by 1 mM F-AraAMP and 25% by 1 mM AraCMP, indicating that both compounds negatively affect the activity of the enzyme.

Effect of cN-I Expression on Drug Sensitivity—Clonal populations of HEK 293 cells and Jurkat E6–1 cells stably expressing cN-I were isolated by serial dilution. The Jurkat clone used for this study had 7-fold higher cN-I activity (9.8 nmol/mg/min) than parental cells, and the HEK 293 clone had 147-fold higher cN-I activity (128 nmol/mg/min) than parental cells when activity was measured in the presence of 200 μM dCMP and 1 mM ADP. Under the assay conditions used, ecto-5′- nucleotidase

TABLE I
Substrate specificity of recombinant human cN-I

The V_{max} and apparent K_m of cN-I for AMP, IMP, and dCMP were determined by varying the concentration of substrate both in the presence and absence of ADP as an activator. The V_{max}, apparent K_m, and Hill coefficients were calculated using non-linear regression (10, 21). Experimental values are shown ± S.E. for two or three (*) experiments.

Substrate	ADP	V_{max}	Relative V_{max}	Apparent K_m	V_{max}/K_m	Hill coefficient
	mM	*μmol/mg/min*	*%*	*mM*		
AMP	1*	364.5 ± 17.1	100	1.87 ± 0.23	53	1.25 ± 0.13
	0	7.7 ± 0.8	2	2.96 ± 0.07		2.62 ± 0.46
dCMP	1	49.5 ± 0.1	14	0.012 ± 0.001	1133	1.40 ± 0.01
	0	18.1 ± 0.6	5	0.193 ± 0.008		2.74 ± 0.06
IMP	1*	155.0 ± 11.0	43	2.47 ± 0.62	17	1.00 ± 0.10
	0	33.2 ± 2.6	9	12.11 ± 1.00		2.69 ± 0.18

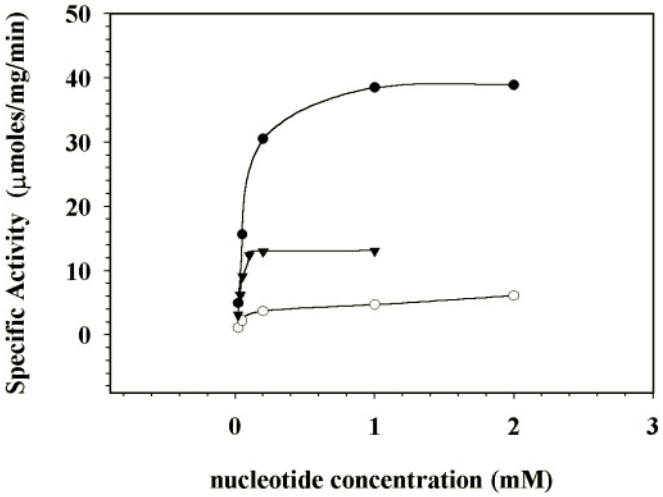

FIG. 4. **The effects of ADP, ATP and GTP concentration on the activity of cN-I.** cN-I specific activity was measured at varying concentrations of ADP (●), ATP (○), and GTP (▼) and at a fixed concentration (20 μM) of dCMP substrate. Representative plots are shown.

TABLE II
Activation of cN-I by ADP, ATP, and GTP

The specific activity of cN-I was measured at varying concentrations of ADP, ATP, and GTP and a fixed concentration (20 μM) of dCMP substrate. The V_{max} and $A_{0.5}$ were calculated using linear regression (21) and are presented ± S.E. for two (GTP) or three (ADP, ATP) experiments.

Activator	V_{max}	$A_{0.5}$
	μmol/mg/min	*μM*
ADP	40.6 ± 3.6	89 ± 11
ATP	5.6 ± 0.5	122 ± 14
GTP	15.1 ± 1.5	33 ± 1

and cN-II could not have contributed significantly to measured cN-I activity. Northern and Western blot analyses showed cN-I mRNA and protein only in the overexpressing cell lines (Fig. 5, *A* and *B*).

HEK 293 and Jurkat E6–1 cells were treated with eight nucleoside analogs for 4 days, and cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. In the presence of CdA, overexpression of cN-I led to a 49-fold increase in IC_{50} in the HEK 293 cell line and a greater than 400-fold increase in IC_{50} in the Jurkat cell line (Fig. 6 and Table V). The IC_{50} for the Jurkat cells expressing cN-I was greater than 100 μM, the highest concentration used in this assay, so the exact IC_{50} and fold increase due to expression of cN-I could not be determined. Both cell lines showed smaller increases in IC_{50} to both AraC and F-AraA and no change in IC_{50} with compound 506U (AraG prodrug) or d4T (Table V). Overexpression of cN-I conferred a 22-fold increase in IC_{50} to dFdC and a 5.2-fold increase in IC_{50} to 5-FU in the

TABLE III
Substrate specificity of recombinant human cN-I

Phosphate generation by cN-I was measured for each nucleoside monophosphate shown using Chen reagent (22). Specific activity was calculated in μmol of phosphate generated/min/mg of cN-I protein ± S.D. for three experiments. All substrate concentrations were 5 mM.

Substrate	Specific activity	Relative activity
	μmol/mg/min	*% AMP*
AMP	122 ± 3	100
dAMP	111 ± 15	91
CMP	104 ± 10	85
dIMP	102 ± 11	84
UMP	95 ± 12	78
dGMP	92 ± 13	75
IMP	75 ± 6	61
GMP	70 ± 10	57
CdAMP	48 ± 11	39
TMP	16 ± 4	13
dCMP	12 ± 1	10
dUMP	9 ± 1	7
F-AraAMP	0	0
AraCMP	0	0

TABLE IV
Inhibition of AMP dephosphorylation by F-AraAMP and AraCMP

Phosphate generation was measured using 5 mM AMP as a substrate and 1 mM of competitor. Specific activity represents the mean ± S.D. for three assays.

Competitor	Specific activity	% Inhibition
	μmol/mg/min	
None	121 ± 6	
F-AraAMP	36 ± 2	70
AraCMP	91 ± 2	25

HEK 293 cells and an 82-fold increase in IC_{50} to ddC in the Jurkat cell line (Fig. 6 and Table V). Higher levels of cN-I in the HEK 293 cells could not explain all the differences in drug sensitivity because the Jurkat cells showed a larger increase in IC_{50} to ddC, F-AraA, and CdA, indicating that cell type-specific differences are also important.

DISCUSSION

Interest in cN-I and cN-II originally stemmed from research to determine which nucleotidase was responsible for increased adenosine formation from AMP in the heart in response to ischemia, hypoxia, and increased workloads. During conditions of metabolic stress, net catabolism of ATP leads to increased ADP and AMP levels (27). Adenosine, produced by dephosphorylation of AMP, can help restore ATP levels by decreasing ATP utilization and increasing blood flow and, therefore, O_2 and substrate supply to the heart (28). Adenosine also contributes to ischemic preconditioning, a cardioprotective mechanism that can prevent damage from a longer period of ischemia (29). The kinetic characteristics of cN-I and cN-II have been studied to determine the contribution of each enzyme to this process. From the characteristics of the purified cN-I enzyme and the

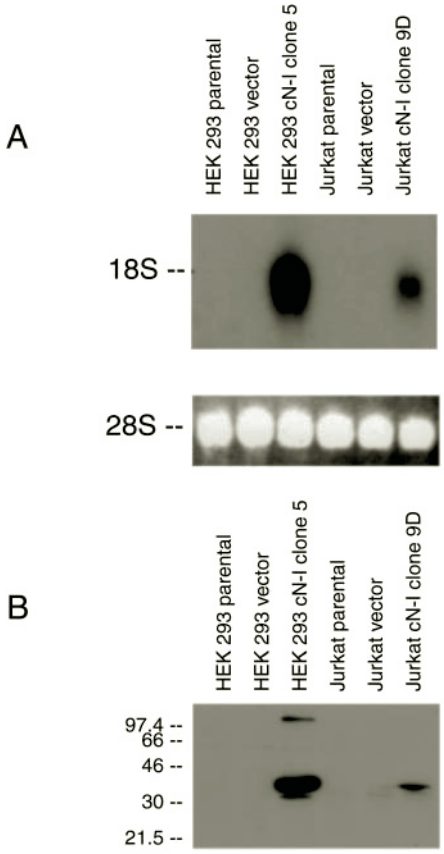

FIG. 5. **Expression of cN-I in stably transfected cell lines.** HEK 293 and Jurkat E6–1 cells were stably transfected with the cN-I construct in a pcDNA3 vector, and clonal cell lines were obtained for each. *A*, Northern blot analysis with cN-I cDNA (*top*) and ethidium bromide-stained membrane showing 28 S rRNA (*bottom*). *B*, Western blot analysis with anti-cN-I antibody.

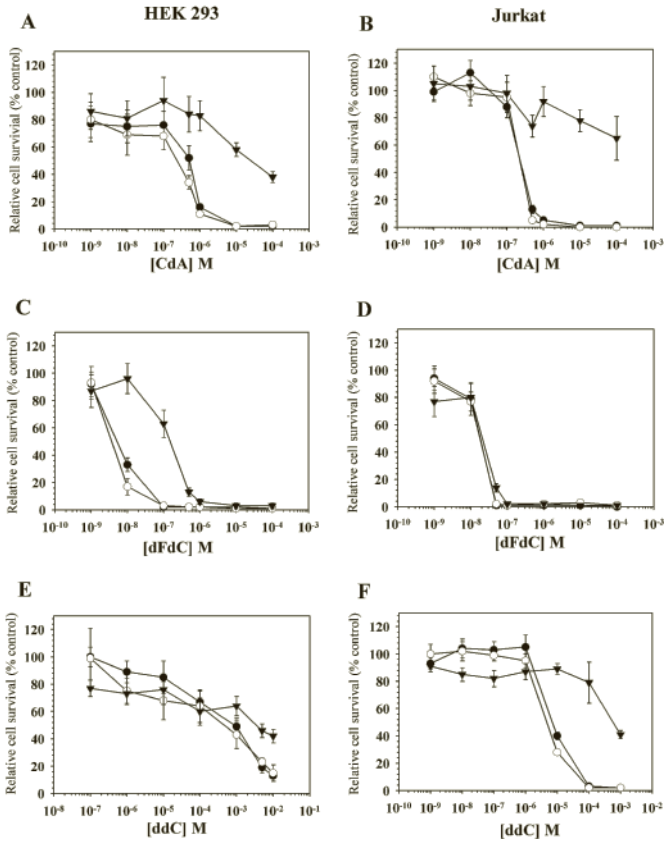

FIG. 6. **Drug resistance in cell lines stably expressing cN-I.** Parental, pcDNA3-transfected, and cN-I transfected HEK 293 and Jurkat E6–1 cell lines were treated with nucleoside analogs for 4 days. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays and compared with control cells that did not receive nucleoside analog treatment. Representative graphs are shown from single experiments done with nine replicates with parental (●), vector-transfected (○), and cN-I-transfected (▼) cells.

cloned pigeon cN-I enzyme, cN-I has been identified as the enzyme responsible for most of the adenosine production in hypoxic and ischemic heart tissue (15, 19, 24, 30).

Although cN-I has been purified and characterized from rabbit, rat, pigeon, dog, and human hearts, there is only limited information on its role in pyrimidine metabolism (17, 20). As has been shown for the purified cN-I enzymes, recombinant human cN-I showed the highest specific activity with AMP as a substrate. In addition, the recombinant enzyme showed high relative activity with all naturally occurring nucleoside monophosphates except the pyrimidine dexoyribonucleoside monophosphates. These results are consistent with those obtained for the pigeon enzyme (20), and although the relative activities are slightly higher for the human enzyme, the order of relative catalytic rate is nearly identical. Although the enzyme had a lower specific activity with pyrimidine deoxyribonucleoside monophosphates, when catalytic efficiency was assessed by the V_{max}/K_m ratio, dCMP was 21 times more efficient as a substrate than AMP. Similar results were reported for the rabbit cN-I enzyme (17). Although the K_m values for TMP and dUMP were not determined, based on the rabbit enzyme data they are also likely to be efficient substrates of cN-I. Thus, dCMP and other pyrimidine deoxyribonucleoside monophosphates are likely to be important physiological substrates of human cN-I.

Our study demonstrates that ADP is a critical regulator of cN-I activity, and this feature is compatible with an important role of this enzyme in adenosine generation in the heart during ischemia (30). Interestingly, the activation of recombinant human cN-I by ADP was 6–20 times stronger than for the cN-I purified from pigeon, rabbit, or dog hearts (16, 18, 20). This striking difference may reflect species differences or result from either copurification of an activator (ADP or GTP) or from a potential posttranslational modification of a native protein that renders cN-I less dependent on ADP. Our data suggest that ADP is a key regulator of human cN-I within the range of 10–200 μM. ADP levels in guinea pig heart increase from 38 μM to 72 μM under conditions of mild hypoxia and to 238 μM under conditions of severe hypoxia (31). In rat heart, control ADP

TABLE V
Fold increase in IC_{50} of nucleoside analogs in two cell lines overexpressing cN-I

HEK 293 and Jurkat cell lines were treated with varying concentrations of nucleoside analogs for 4 days. Percent cell viability was calculated compared with control cells that did not receive nucleoside analog treatment. The IC_{50} values were calculated from graphs of the data. The average fold increase in IC_{50} ± S.E. due to expression of cN-I is shown for two or three (*) experiments, each containing nine replicate platings. All values represent the fold increase compared with parental cells unless otherwise indicated (‡), because transfection with empty vector slightly increased the sensitivity of the cells to nucleoside analogs. Hence, a comparison of the cN-I-transfected cells to vector-transfected cells might overestimate the increase in IC_{50}.

Drug	HEK 293	Jurkat
AraC	3.7 ± 1.3	2.7 ± 0.3
CdA	49.3 ± 16.8*	>446.4
dFdC	21.6 ± 6.3	1.3 ± 0.0
F-AraA	2.3 ± 0.2	8.0 ± 3.0
ddC	3.7 ± 0.4	81.6 ± 43.0*
5-FU	5.2 ± 1.6	0.7 ± 0.1*‡
506U (AraG)	0.7 ± 0.3‡	1.6 ± 0.6*
d4T	1.0 ± 0.0	1.0 ± 0.0

concentrations of 64 μM increase to 106 μM under hypoxic conditions (32). These fluctuations in ADP levels are precisely within the range of $A_{0.5}$ value for this activator and further signify the important role of this enzyme in adenosine generation in the heart during ischemia (15, 17, 24) and potentially also in working skeletal muscle. Interestingly, recombinant human cN-I is also significantly activated by GTP, and an increase in GTP levels, for example during cellular proliferation, may also play a role in activation of the enzyme.

The data presented in Table V show that increased activity of cN-I can lead to resistance to several nucleoside analogs, including CdA, dFdC, and ddC, and to a smaller extent, to 5-FU, AraC, and F-AraA. The change in IC_{50} values for these compounds in the presence of cN-I seems to correlate with the *in vitro* activity of cN-I with CdAMP, F-AraAMP, and AraCMP (Tables III and IV). Thus, the relatively high activity with CdAMP leads to strong resistance to CdA in cells overexpressing cN-I and the lack of *in vitro* activity with either F-AraAMP or AraCMP correlates with a much smaller increase in resistance to the respective nucleosides. Unfortunately, we were not able to extend this comparison to other nucleoside monophosphates since they are not commercially available.

The strong dependence of cN-I activity on increased ADP concentrations may also be relevant to resistance to nucleoside analogs in tumors that experience hypoxia. Hypoxic tumors have poorer clinical prognosis, are often resistant to both radiation and chemotherapy (33), and are more likely to metastasize (34). The nucleoside analogs 5-FU and dFdC are used clinically in the treatment of solid tumors (35, 36), and we have shown that elevated expression of cN-I increases the IC_{50} for 5-FU 5.2-fold and the IC_{50} for dFdC 21.6-fold in the HEK cells (Table V). Although the effect of hypoxia on drug resistance in the context of cN-I activity remains to be investigated, this study provides support for the hypothesis that increased ADP concentrations in ischemic tumors may facilitate dephosphorylation of several nucleoside-based drugs and lower their pharmacological efficacy.

Although cN-I was not detected in peripheral blood leukocytes (Fig. 2), aberrant expression in malignant cells or enhanced expression as a result of prior chemotherapy exposure could play a role in resistance to nucleoside analogs in leukemias and lymphomas, where these drugs are extensively used. Future studies will determine whether cN-I levels are increased in leukemic cells from patients who are resistant to nucleoside analog therapy.

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