Site-Directed Mutagenesis of Human Soluble Calcium-Activated Nucleotidase 1 (hSCAN-1): Identification of Residues Essential for Enzyme Activity and the Ca^{2+}-Induced Conformational Change†

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ABSTRACT: Human soluble calcium-activated nucleotidase 1 (hSCAN-1) is the human homologue of soluble apyrases found in blood-sucking insects. This family of nucleotidases is unrelated in sequence to more well-studied nucleotidases, and very little is known about the enzymatic mechanism. By multiple sequence alignment, eight regions that are highly conserved in the hSCAN-1 family were identified and named. To identify amino acids important for catalytic activity and enzyme specificity, seven point mutations were constructed, expressed in bacteria, refolded, purified, and characterized. Substitution of glutamic acid 130 with tyrosine resulted in dramatically increased nucleotidase activities, while mutagenesis of aspartic acid 151 to alanine and aspartic acid 84 to alanine completely abolished activity. Mutagenesis of arginine 133 and arginine 271 resulted in enzymes with very little nucleotidase activity. Mutagenesis of aspartic acid 175 to alanine and glycine 122 to glutamic acid had smaller negative effects on enzyme activities. Previously, our laboratory showed that calcium triggers a conformational change in hSCAN-1 necessary for nucleotidase activity. Here we show that several mutants (D84A, R133A, and D151A) that lost most of their activity were unable to undergo the conformational change induced by Ca^{2+}, as shown by Cibacron blue binding, limited proteolysis, and tryptophan fluorescence. We conclude that aspartic acid residues 84 and 151, as well as arginine residue 133, are essential for the Ca^{2+}-induced conformational change that is necessary for enzyme activity. Aspartic acid 175 and glutamic acid 130 are important for determining substrate specificity. In addition, we show that Sr^{2+}, unlike Mg^{2+} and other divalent cations, can substitute for Ca^{2+} to induce the conformational change necessary for enzyme activity. However, Sr^{2+} cannot substitute for Ca^{2+} to support nucleotide hydrolysis, presumably because Sr^{2+} cannot substitute for Ca^{2+} in its second role as a nucleotide cosubstrate. The ramifications of our results on the interpretation of a recently published crystal structure are discussed. This information will facilitate future engineering of this enzyme designed to enhance its ability to hydrolyze ADP and thus increase its potential for therapeutic use in the treatment of pathological ischemic events triggered via activation of platelets by ADP.

Apyrases (EC 3.6.1.5) are nucleoside di- and triphosphate hydrolyzing enzymes that have been implicated in the maintenance of hemostasis and inhibition of platelet aggregation through the hydrolysis of extracellular adenosine diphosphate (*1*−*7*). These enzymes also play an important role in regulating neurotransmission mediated by activation of purinergic receptors by nucleotides (*8*).

Recently, a human soluble apyrase related to the hematophagous bed bug *Cimex lectularius* apyrase has been cloned (*9*) and characterized (*10*). Since its enzymatic activity is strictly dependent on calcium, it has been named human soluble calcium-activated nucleotidase 1 (hSCAN-1).[1] This enzyme belongs to a family of enzymes that consist primarily of those apyrases cloned from a variety of hematophagous insects. (*11*, *12*). The rat homologue of hSCAN-1 has also been recently described (*13*). Although analogous to the eNTPDase apyrases with regard to their enzymatic properties, these enzymes, related to the blood-sucking insect anticoagulant enzymes, are not homologous to the eNTPDase apyrase family of enzymes with regard to their amino acid sequences.

Nucleotidases modulate physiological and pathological effects of released nucleotides, including ATP and ADP. Recently, a novel pharmaceutical strategy for treatment of patients with heart and vascular diseases was proposed utilizing exogenously added NTPDases for inhibition of platelet reactivity (*5*). hSCAN-1, a soluble mammalian nucleotidase, appears to be an attractive alternative candidate for this therapeutic purpose. Therefore, identification of its active site, as well as protein structural elements required

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[1] Abbreviations: hSCAN-1, human soluble calcium-activated nucleotidase-1 (GenBank accession number AF328554); eNTPDase, ectonucleotide triphosphate diphosphohydrolase; IPTG, isopropyl-β-D-thiogalactopyranoside; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; NCBI, National Center for Biotechnology Information; BLAST, basic local alignment search tool.

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for nucleotidase activity and specificity, is an important and potentially therapeutically useful goal.

In this work, eight highly conserved regions of amino acid sequence were identified in the hSCAN-1 family of apyrases. Five mutants were constructed by changing residues in those highly conserved areas of the protein sequences. Two additional mutants were designed using a different rationale, targeting amino acids that are conserved in all the vertebrate sequences, while not conserved as the same amino acid in the invertebrate sequences. The hypothesis tested is that by mutating amino acids conserved in all the vertebrate sequences to amino acids occurring in the invertebrate sequences, the substrate specificity of the human enzyme may be made more similar to the invertebrate enzymes. This hypothesis and goal have direct therapeutic potential, since ADP released by activated platelets triggers blood clotting. The invertebrate enzymes hydrolyze ADP efficiently, while the vertebrate enzymes examined thus far do not. To have utility as an anticoagulant protein in humans, it is therefore desirable to engineer a human protein that hydrolyzes ADP more efficiently. Thus, two of the goals of the current study are to identify residues essential for activity and to identify residues responsible for the enzymatic differences between the invertebrate and vertebrate members of this family of enzymes.

At the time this study was first submitted for publication, there were are no systematic studies reporting amino acids residues that are important for the enzymatic activity of the hSCAN-1 class of nucleotidases. However, during the review process, a crystal structure for hSCAN-1 was published (the protein was termed "human apyrase/human platelet function inhibitor" by those authors (*14*)). Those authors reported a single Ca^{2+} ion present in the crystal at the center of the β-propeller secondary structure dominating the 3-D structure of this enzyme. We hypothesize that the divalent cation found in that crystal is a Sr^{2+} ion bound to the site that induces the conformational change necessary for enzyme activity, which is detected both by changes in tryptophan fluorescence and sensitivity to limited proteolysis.

MATERIALS AND METHODS

Materials. The QuickChange site-directed mutagenesis kit and *Escherichia coli* competent bacteria were purchased from Stratagene. The DNA Core Facility at the University of Cincinnati produced the synthetic oligonucleotides and sequenced all the cDNA constructs. Plasmid purification kits and Ni−NTA agarose were purchased from Qiagen Inc. *Nhe*I and *Not*I restriction endonucleases were obtained from Invitrogen. The bacterial expression vector pet28a and the bacterial expression BL21 cells were purchased from Novagen. Glycerol and dialysis tubing were from Fisher. B-PER bacterial extraction reagent and Enhanced Chemiluminescent reagents were purchased from Pierce. The Expressway in vitro expression system was obtained from Invitrogen Corporation. Cibacron blue gel matrix (Affi-gel blue gel) and the pre-cast SDS−PAGE 4−15% gradient minigels were obtained from Bio-Rad laboratories. Ampicillin, kanamycin, nucleotides, isopropyl-β-D-thiogalactopyranoside (IPTG), glucose, DTT, and other reagents were from Sigma. Trypsin was purchased from Boehringer Mannheim GmbH.

Computer Analyses of Sequences. The amino acid sequence of hSCAN-1 was compared to the nonredundant database of sequences using the basic BLASTP search program available through the NCBI at http://www.ncbi.nlm.nih.gov/BLAST/. A multiple protein sequence alignment of hSCAN-1, along with the sequences most closely related to it, was performed using the CLUSTALW program, and the resulting aligned sequences were shaded using the BOXSHADE program. These programs are available through http://www.ch.embnet.org/software/clustalw.html and http://www.chembner.prgsoftware/box_form.html. The "PlotSimilarity" computer program is part of Genetics Computer Group (GCG) sequence analysis programs software suite (SeqWeb) at http://trinity.cchmc.org:9092/gcg-bin/seqweb.cgi.

Site-Directed Mutagenesis of hSCAN-1. The mutagenesis methodology was described previously (*15*) and used the QuikChange site-directed mutagenesis kit as described (*16*). The sense oligonucleotides used for mutagenesis with the substitution sites bolded and underlined are as follows: D84A, 5′-GTTATCGCAGACCTG**GCC**ACAGAGTCAAGGGC-3′; G122E, 5′-GACAAAGACCAT**GAG**GTCCTGGAGTCCCAC-3′; E130Y, 5′-GTCCCACCTGGCG**TAT**AAGGGGAGAGGCATG-3′; R133A, 5′-GCGGAGAAGGGG**GCA**GGCATGGAGCTATC-3′; D151A, 5′-CTCTACTCCGTG**GCT**GACCGGACGG-3′; D175A, 5′-CTGTCCGACGGC**GCT**GGCACCGTGGAG-3′; R271A, 5′-CCTGCCGCGC**GCC**GCCAGCCAGG-3′. The complementary antisense oligonucleotides also necessary for the mutagenesis are not shown. The presence of the correct mutation and lack of unwanted mutations were confirmed by automated DNA sequencing.

Expression of hSCAN-1 Mutants in E. coli BL21 Bacterial Cells. The wild-type and mutant hSCAN-1 cDNA constructs were used to transform an expression host, BL21 cells (*10*), and after induction of expression with IPTG, bacterial inclusion bodies containing the hSCAN-1 proteins were purified as described previously (*10*, *17*).

Refolding and Purification of hSCAN-1 Mutants. Mutant proteins were purified as described for wild-type hSCAN-1 (*10*). Thus, the inclusion body proteins were denatured and refolded, purified via their N-terminal hexahistidine tags, thrombin-cleaved to remove the N-terminal tag, and further purified by anion exchange chromatography to yield purified proteins.

Cell-Free in Vitro hSCAN-1 Expression. A small-scale expression of each hSCAN-1 mutant was carried out in a cell-free system using the ExpresswayTM in vitro system according to the manufacturer's protocol. In short, 20 μL of *E. coli* lysate, 25 μL of reaction cocktail, and 1 μg of hSCAN-1 mutant cDNA (in the pet28a expression vector) were combined and brought to a total reaction volume of 50 μL with DNase- and RNase-free water. The mixture was incubated for 2 h at 37 °C with shaking. The calcium-activated GDPase activity of expressed wild-type and mutant hSCAN-1 was measured in the presence of 5 mM $CaCl_2$ in 20 mM MOPS buffer, pH 7.4. Expression was confirmed by Western blot analysis (using an affinity-purified anti-carboxy-terminal antibody for hSCAN-1 (*9*)).

Protein Assay. Protein concentrations were determined using the Bio-Rad G-250 dye binding technique according to the modifications of Stoscheck (*18*), using bovine serum albumin as the standard.

Nucleotidase Assays. Nucleotidase activities were determined by measuring the amount of inorganic phosphate (P_i)

released from nucleotide substrates in the presence of 5 mM Ca^{2+} at 37 °C using a modification of the technique of Fiske and Subbarow (*19*), as described previously (*20*). The enzyme assay was initiated by the addition of a final concentration of 2.5 mM nucleotide. The units used for enzyme activity are μmol of Pi/((mg of protein)•h). Prior to assay, hSCAN-1 protein was diluted into 50 mM Tris-HCl, pH 6.8, containing 0.1% Tween 20. The Tween detergent was included to minimize the adsorption to surfaces noted for dilute protein solutions of hSCAN-1.

Cibacron Blue Binding Assays. hSCAN-1 proteins in 50 mM Tris-HCl, 2 mM $CaCl_2$ (pH 7.4) or 50 mM Tris-HCl, 2 mM EDTA (pH 7.4) were incubated at room temperature on a rotator for 15 min with 40 μL of Cibacron blue gel slurry (Affi-gel blue, BioRad). The Cibacron blue gel beads containing bound proteins were then washed three times with the respective buffers above. Bound proteins were eluted by boiling the Affi-gel blue gel slurry for 5 min in a reducing SDS−PAGE sample buffer. SDS−PAGE was performed on 4−15% acrylamide gradient gels as described (*21*).

Limited Proteolysis Assays. Proteins at a final concentration of 0.1 mg/mL in 50 mM Tris-Cl, pH 7.4, were incubated with 0.002 mg/mL trypsin (1:50 ratio of protease to hSCAN-1 protein) in the presence of 5 mM $CaCl_2$, 5 mM $MgCl_2$, or 2 mM EDTA for 10 min at 22 °C, basically as described earlier (*22*). An equal volume of reducing SDS−PAGE sample buffer was added, and the samples were immediately boiled for 5 min prior to SDS−PAGE analysis.

Effect of Divalent Cations on Tryptophan Fluorescence. The intensity of the intrinsic hSCAN-1 tryptophan fluorescence as a function of divalent cation concentration was measured using a Hitachi F-2000 fluorescence spectrophotometer. A 0.85 μM (32 μg/mL) concentration of hSCAN-1 in 20 mM MOPS, pH 7.1, was excited at 295 nm, and fluorescence emission was recorded at its maximum (340 nm) before and after stepwise addition of divalent cations.

Effect of Divalent Cations on Rates of Thermal Denaturation. Purified hSCAN-1 protein at 0.0015 mg/mL was preincubated at 37 °C in 20 mM MOPS/0.05% Tween 20 or in the same buffer containing 2 mM EDTA, 2 mM $CaCl_2$, or 2 mM $SrCl_2$. After preincubation for 0, 2, 16, 20, and 24 h at 37 °C under these conditions, the residual GDPase activity was assayed in the presence of 5 mM Ca^{2+}.

Effect of Divalent Cations on hSCAN-1 Refolding. Small scale (1 mL) refolding of wild-type hSCAN-1 inclusion bodies was performed by dilution of 0.05 mL of 0.06 mg/mL guanidine-HCL denatured inclusion body protein (*10*) into a solution containing 50 mM Tris-Cl, 250 mM NaCl, 5% glycerol, pH 8.0 (buffer A), to yield a final protein concentration of 3 μg/mL. Refolding was allowed to proceed for 3 days at 4 °C. To compare the effects of the presence or absence of divalent cations on the refolding efficiency, EDTA, $MgCl_2$, $CaCl_2$, or $SrCl_2$ was added to buffer A to a final concentration of 2 mM in separate refolding samples.

RESULTS

Eight Highly Conserved Regions Were Identified in the Amino Acid Sequence of the hSCAN-1 Family. By multiple sequence alignment and analysis using the "PlotSimilarity" computer program, eight highly conserved regions were found in the hSCAN-1 family of nucleotidases. We have designated them as "nucleotidase conserved regions" 1−8 (NCR1−8) (see Figures 1 and 2). The degree of amino acid conservation in different regions of the sequence is shown in a quantitative manner in Figure 2. Table 1 lists these 12 proteins, six of which are from vertebrate species and six of which are from invertebrate species. Some of them are known to be nucleotidases/apyrases by analysis of their protein products. Examination of the alignments and quantification of sequence conservation revealed that certain conserved sequence motifs may be important for the structure and function of these proteins. Thus, amino acid residues present in the NCRs are likely to be involved in functionally important motifs, including the catalytic site(s), the Ca^{2+} binding sites that modulate the enzymatic activity of these apyrases (*10*), and the regions important for the conformational changes accompanying Ca^{2+} binding. In addition, regions where the invertebrate sequences are conserved but differ from the vertebrate sequences are likely to be critical for determining the different nucleotide specificities of these subgroups of apyrases. These species variations in nucleotide specificity are very likely to be important for determining the different biological functions of the vertebrate and invertebrate members of this family of apyrases.

Of the seven point mutations made and characterized, five were chosen from the NCR regions (D84A, D151A, D175A, R133A, and R271A). Table 2 lists the conserved amino acids chosen for mutagenesis and shows these residues in the context of the surrounding hSCAN-1 protein sequence. Consistent with their degree of conservation, our results indicate that all five mutations made in these NCRs have substantial effects on enzyme activities of hSCAN-1. All seven mutations characterized in this present study are indicated by the positions of filled circles in Figures 1 and 2.

Biochemical Characterization of Mutant hSCAN-1 Proteins. To characterize the bacterially expressed hSCAN-1 mutants and to compare them with wild-type hSCAN-1, we analyzed nucleotidase activity of the refolded and purified proteins using a variety of substrates (final nucleotide concentrations of 2.5 mM), all in the presence of 5 mM calcium ion. The relative GDPase and ADPase activities for the mutant proteins are given in Figure 3. The results indicate that the D84A and D151A mutants are very nearly devoid of GDPase and ADPase activities, whereas the R133A and R271A mutants display only 1.8% and 3% of wild-type GDPase activity and 0.3% and 3.2% of wild-type ADPase activity, respectively. Both GDPase and ADPase activities were decreased to lesser extents in the G122E and D175A mutants. The G122E mutant displayed 83% and 62% of wild-type GDPase and ADPase activity, respectively. The D175A mutant retained 48% and 27% of wild-type GDPase and ADPase activity, respectively. Most interestingly, the E130Y mutant has higher enzyme activity than that of the wild-type, about 2-fold increased for GDPase and 5-fold increased for ADPase. Therefore, we investigated this mutant further. Figure 4 shows the nucleotide substrate profile of the E130Y mutant compared to the wild-type hSCAN-1. Clearly, the hydrolysis rates for all substrates are significantly increased compared to the wild-type enzyme. A striking difference is seen in the ADPase activity, where a 5-fold increase is observed over the wild-type activity.

The nucleotidase profile of the partially active D175A mutation was also measured, as shown in Figure 5. All the

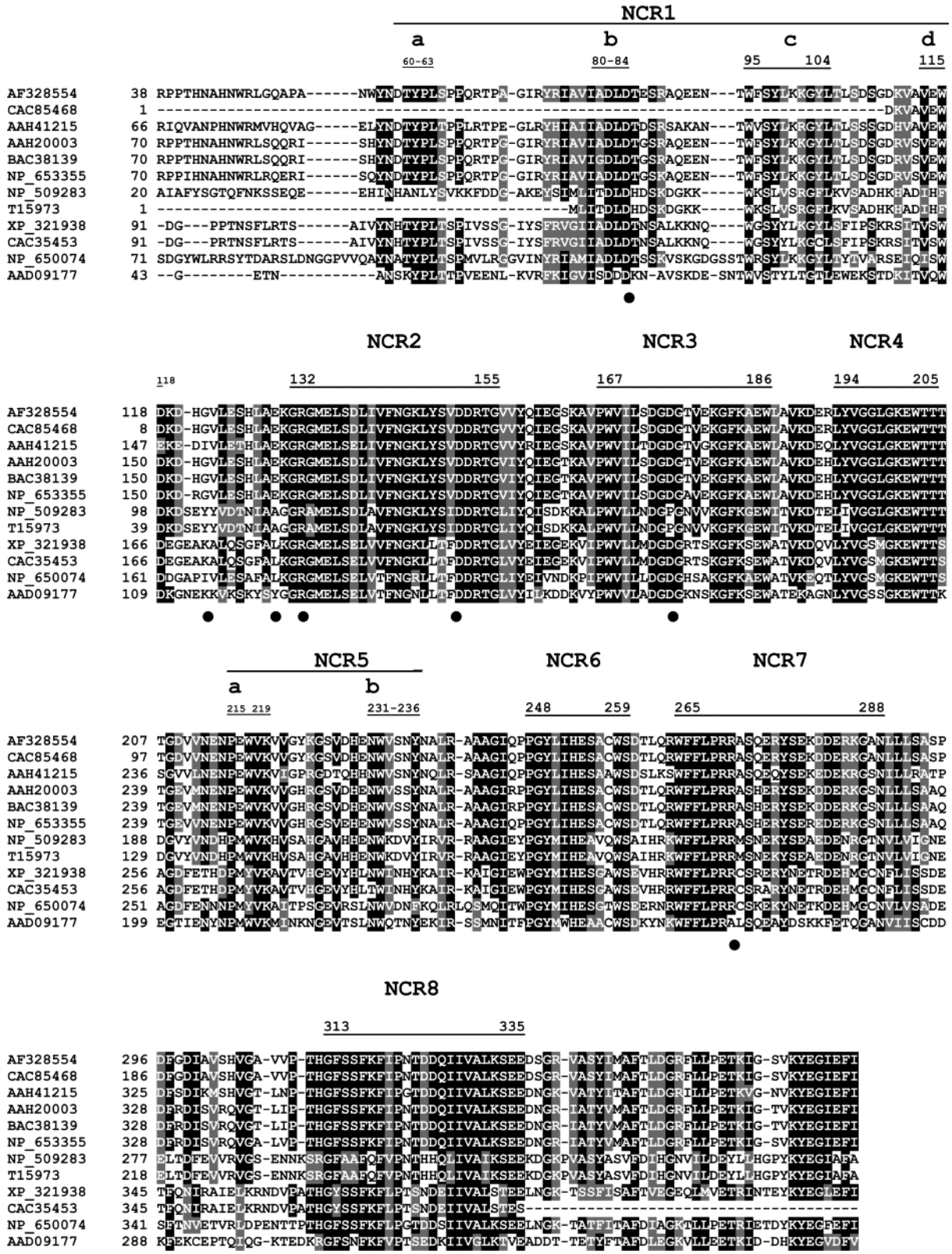

FIGURE 1: Multiple sequence alignment of hSCAN-1 with related apyrases. The soluble human apyrase amino acid sequence (top line) was aligned with the 11 most similar sequences in the NCBI protein database (See Table 1 for information concerning the proteins used in the alignment) using the CLUSTALW alignment algorithm. Residues were shaded to indicate various levels of conservation using the BOXSHADE program. Completely conserved amino acids are indicated by the darkest shading. Alignment gaps in the sequences are represented by a dash (−). Several regions of completely conserved acidic amino acids are evident and have been named using the nucleotidase conserved region (NCR) nomenclature. The extreme N-terminal portions of some of the proteins are not shown, since there is very little homology in this region. Thus, the sequence of the hSCAN-1 protein shown (top line) begins with the arginine residue presumed to be the cleavage site releasing the soluble protein from the N-terminal signal peptide (*9*). The positions of the seven point mutations described in this study are indicated below the sequence alignments by filled circles.

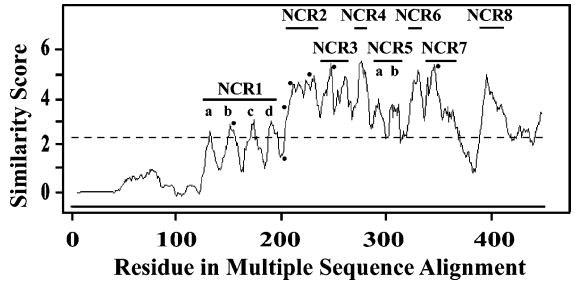

FIGURE 2: PlotSimilarity computer analysis of hSCAN-1 and 11 highly homologous proteins. The 12 highly homologous protein sequences belonging to the hSCAN-1 family (see Table 1) were analyzed using default parameters of the "PlotSimilarity" program, which is part of the Genetics Computer Group (GCG) sequence analysis suite of programs (SeqWeb). The dashed line indicates the average amino acid similarity over the entire multiple sequence alignment. The highly conserved regions are labeled in the same manner as in Figure 1. Note that the residue numbers for the plotted similarity values do not match the mutation residue numbers due to extended nonconserved amino terminal cytoplasmic sequences of some of the family members used in the analysis. The positions of the seven point mutations described in this study are indicated by filled circles.

Table 1: hSCAN-1 and Eleven Other Related Proteins

accession no.	name (reference)	species	no. amino acids
AF328554	soluble calcium-activated nucleotidase, SCAN-1	*Homo sapiens*	371
CAC85468	putative apyrase	*Homo sapiens*	261
AAH41215	similar to Ca^{2+}-dependent ER apyrase	*Xenopus laevis*	400
AAH20003	shapy-pending protein	*Mus musculus*	403
BAC38139	unnamed protein product	*Mus musculus*	403
NP653355	apyrase	*Rattus norvegicus*	403
NP509283	apyrase	*C. elegans*	355
T15973	hypothetical protein	*C. elegans*	296
XP321938	ENSANGP00000013982	*Anopheles gambiae*	436
CAC35453	Ag9 protein	*Anopheles gambiae*	392
NP650074	CG5276-PA	*Drosophila*	419
AAD09177	apyrase	*Cimex lectularius*	364

Table 2: Location of hSCAN-1 Mutations Described in This Work[a]

mutation	sequence	location
D84A	IADL**D**TES	NCR1b
G122E, E130Y	H**G**VLESHLA**E**K	
R133A, D151A	G**R**GMELSDLIVFNGKLYSV**D**DRTG	NCR2
D175A	PWVILSDG**D**GTVEKGFKAEW	NCR3
R271A	WFFLPR**R**ASQERYSEKDDERKGAN	NCR7

[a] The amino acid residues mutated in this work (bolded and underlined) and the sequences of the surrounding regions of HSCAN-1 are shown.

nucleotidase activities were decreased to various extents for this mutant, with ITPase activity affected the least (78% of the wild-type activity). Most other nucleotidase activities were decreased to 25−45% of that of the wild-type, while the CDPase activity was reduced to 16% of the wild-type. Thus, the substitution of aspartic acid 175 with alanine not only decreased enzymatic activity, but also changed the substrate specificity.

Conformational Analysis of Mutants. Cibacron blue has been used as a nucleotide analogue for many enzymes and has been shown to be useful for characterizing mutations of NTPDase3, a member of a different class of ecto-nucleotidases (*23*−*25*). In addition, it was previously demonstrated that hSCAN-1 is strictly dependent upon calcium for activity and that calcium induces a conformation change (*10*). Figure 6 shows that in the presence of calcium, wild-type hSCAN-1 protein binds efficiently to Cibacron blue, but in the absence of Ca^{2+} (in EDTA), it binds to a much lesser extent. This effect of Ca^{2+} was specific, since Mg^{2+} did not promote the binding of the wild-type enzyme to the Cibacron blue matrix (data not shown). The numbers shown below the hSCAN-1 protein bands in Figure 6 represent the relative amounts of protein bound to the affinity matrix, normalized to the wild-type enzyme in the presence of Ca^{2+} (lane 3, assigned a density of 100%). Untreated wild-type sample (second lane from the left) was applied directly to the gel and serves as an estimate of recovery. By densitometry, 80% of the starting wild-type enzyme was recovered after elution from the beads

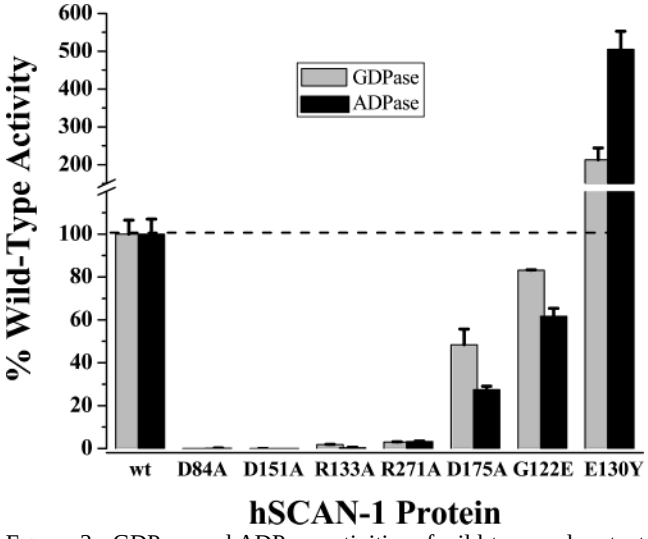

FIGURE 3: GDPase and ADPase activities of wild-type and mutant hSCAN-1. Values plotted are the percent of the wild-type GDPase (light gray bars) and ADPase (black bars) activities. The error bars are the standard errors for three separate assays. The GDPase and ADPase activities of all mutants are statistically different from the wild-type activities (student T-test, $p < 0.05$).

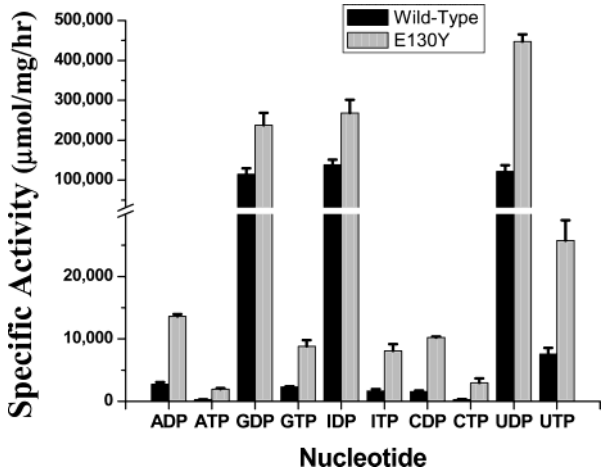

FIGURE 4: Nucleotidase activity profile of wild-type hSCAN-1 and the E130Y mutant. Assays were done using a final concentration of 2.5 mM nucleotide in a buffer containing 5 mM $CaCl_2$. Activity units are micromoles of Pi liberated per milligram of protein per hour at 37 °C. The break in the *y*-axis denotes a greater than 10-fold change in scale. The error bars represent the standard error of the mean ($n = 3$). All activities of the E130Y mutant are statistically different from the wild-type activity (student T-test, $p < 0.05$).

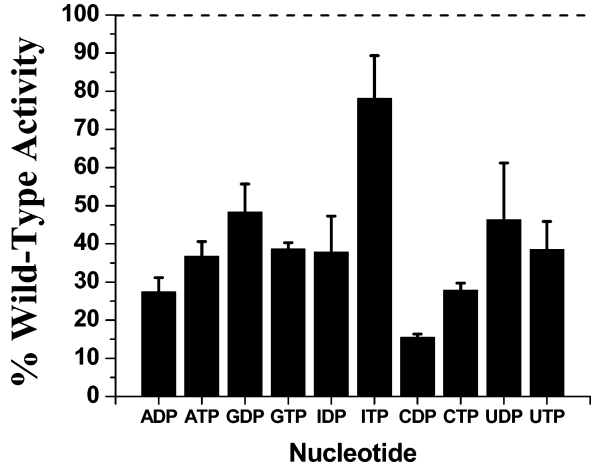

FIGURE 5: Substrate preference of hSCAN-1 mutant D175A. Assays were done using a final concentration of 2.5 mM nucleotide in a buffer containing 5 mM $CaCl_2$. Values plotted are the percent of the wild-type nucleotidase activities. The error bars represent the standard error of the mean ($n = 3$).

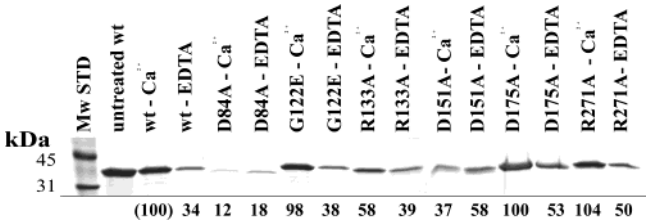

FIGURE 6: Cibacron blue (Affi-gel blue) binding assay of wild-type hSCAN-1 (wt) and mutants. Equal amounts of each sample were incubated with the Cibacron blue gel in the buffer as described in Materials and Methods. The "untreated wt" in lane 2 represents an identical amount of wild-type hSCAN-1 applied directly to the gel without incubation with the affinity beads. After the affinity matrix beads were washed, bound proteins were eluted with SDS−PAGE sample buffer and loaded onto the SDS−PAGE gel as described in the methods. The numbers below the hSCAN-1 stained protein bands are the relative amounts of protein bound to the affinity matrix, normalized to the wild-type enzyme in the presence of Ca^{2+} (lane 3, assigned a density of 100%).

incubated with wild-type enzyme in the presence of Ca^{2+} (compare lane 3 with lane 2 in Figure 6). The superactive E130Y mutant behaved like the wild-type enzyme in this Cibacron blue assay (data not shown). In contrast, the D84A, R133A, and D151A mutants bound poorly to the Cibacron blue nucleotide analogue affinity matrix in the presence of calcium, which suggests an alteration in their nucleotide binding pockets or possibly a change in their overall conformation. The G122E, D175A, and R271A mutants maintained the ability to bind to Cibacron blue in the presence of calcium, despite their decreased enzyme activity.

Limited Tryptic Digestion Analysis of Wild-Type hSCAN-1 and Mutants. Limited proteolysis is a general method for detecting changes in conformational structure of proteins and has been used to analyze mutations of the NTPDase class of ecto-nucleotidases (*23*, *24*). Thus, all hSCAN-1 mutations were subjected to limited trypsin proteolysis in the presence and absence of Ca^{2+}, an ion known to induce a conformational change accompanied by a change from an inactive to an active nucleotidase in wild-type hSCAN-1 (*10*). Analysis of the mutants with decreased enzymatic activities revealed that they were also more susceptible to limited proteolysis. The susceptibilities to limited proteolysis in the presence of

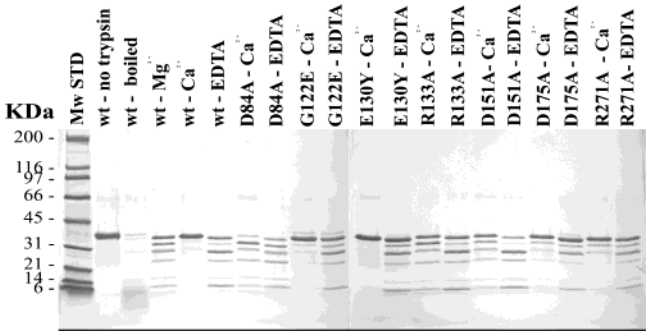

FIGURE 7: Limited tryptic digestion of wild-type and mutant hSCAN-1. Limited proteolysis was performed at a 1:50 protease/protein ratio for 10 min at 22 °C as described in Materials and Methods. The lane labeled "wt - no trypsin" is undigested (control) wild-type enzyme. The lane labeled "wt - boiled" represents wild-type enzyme that was boiled for 5 min prior to the protease treatment, simulating susceptibility to trypsin of the unfolded, denatured enzyme. After trypsin treatment, reducing SDS sample buffer was added to all samples, which were then immediately boiled for 5 min prior to SDS−PAGE analysis.

Ca^{2+} or EDTA for wild-type and mutant hSCAN-1 proteins are shown in Figure 7. In the presence of Ca^{2+}, the wild-type enzyme is resistant to limited trypsin proteolysis; however, in EDTA or in Mg^{2+}, wild-type enzyme was rapidly cleaved into several smaller fragments. In contrast, the inactive mutations D84A, R133A, and D151A, in both calcium and EDTA, show approximately the same pattern of digestion as the wild-type hSCAN-1 digested in EDTA. This suggests that these mutants are all folded like the wild-type enzyme, since the same residues are exposed for cleavage, leading to the same proteolytic pattern. However, these three mutants are not stabilized toward proteolysis by Ca^{2+}, unlike the wild-type enzyme. Control experiments using wild-type enzyme that was boiled prior to proteolysis with trypsin (to denature and unfold the enzyme) resulted in very rapid degradation of hSCAN-1 into very small fragments (seen as a smear on the gel below 6 kDa in lane 3), dissimilar to the pattern seen with the wild-type enzyme digested in Mg^{2+} or EDTA (see lanes 3, 4, and 6 in Figure 7). The G122E, D175A, and R271A mutants displayed a decreased protective effect of calcium on proteolysis by trypsin compared to the wild-type, whereas the E130Y mutant behaved like the wild-type enzyme in this assay.

Response of Wild-Type and Mutant hSCAN-1 Tryptophan Fluorescence to Divalent Cations. To correlate the Cibacron blue and limited proteolysis results to earlier findings that calcium-induced conformational changes in hSCAN-1 could be detected by increase in tryptophan fluorescence, we measured the fluorescence response to various divalent cations for wild-type and mutant hSCAN-1 proteins. The wild-type enzyme had an EC_{50} for Ca^{2+} of 61.0 ± 6.6 μM, while the G122E mutant had an EC_{50} of 57 μM, and the highly active E130Y mutant had a statistically significantly lower EC_{50} of 28 ± 1.7 μM. The other less active mutants did not display the sigmoidal increase in tryptophan fluorescence in the presence of Ca^{2+} characteristic of the wild-type enzyme, so EC_{50} values were not obtained for these mutants.

Both Ca^{2+} and Sr^{2+} can induce the conformational change as measured by the sigmoidal increase in tryptophan fluorescence (Figure 8). The wild-type enzyme has an EC_{50}

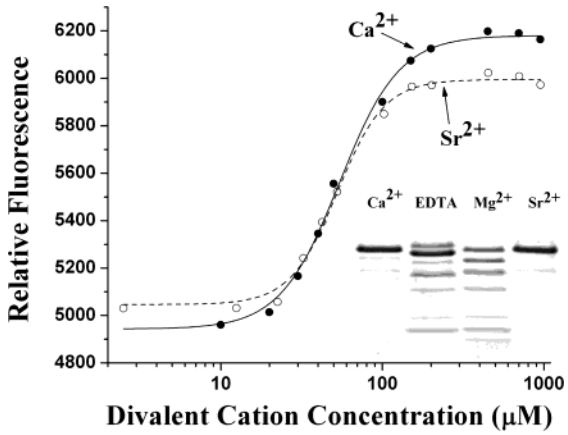

FIGURE 8: Sr^{2+} can substitute for Ca^{2+} to induce a conformational change in wild-type hSCAN-1. The intrinsic tryptophan fluorescence (excitation at 295 nm, emission at 340 nm) of wild-type hSCAN-1 was measured as a function of calcium (●) or strontium (○) ion concentration. The inset of the figure is an SDS−PAGE gel analysis of a limited proteolysis experiment utilizing wild-type hSCAN-1 digested in the absence or presence of divalent cations indicated in the figure. This demonstrates that 2 mM Ca^{2+} or Sr^{2+}, unlike Mg^{2+} or EDTA, can protect hSCAN-1 from digestion by trypsin.

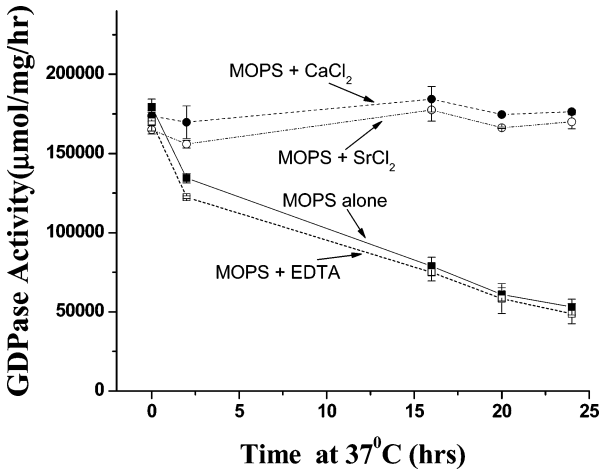

FIGURE 9: The effect of calcium, strontium, and EDTA on the rate of thermal inactivation at 37 °C of hSCAN-1 Ca-GDPase activity. Purified hSCAN-1 (0.0015 mg/mL) samples were preincubated in 20 mM MOPS containing 0.05% Tween 20 (■), with the same buffer also containing 2 mM EDTA (□), or with the same buffer containing 2 mM $CaCl_2$ (●) or 2 mM $SrCl_2$ (○). The error bars represent the standard deviations for each determination ($n = 3$). After the times indicated in the figure, the residual GDPase activity was assayed in the presence of 5 mM Ca^{2+}. The results demonstrate the protective effect of Ca^{2+} and Sr^{2+} and suggest that there is no tightly bound, high-affinity divalent cation integral to the structure of this protein, since EDTA does not destabilize the activity to thermal denaturation.

61.0 ± 6.6 μM for Ca^{2+} and 50.5 ± 15.2 μM for Sr^{2+}. Among the ions tested (Ca^{2+}, Sr^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Co^{2+}), Sr^{2+} was the only divalent cation besides Ca^{2+} that protected the protein from proteolysis by trypsin (see inset of Figure 8, data using some cations not shown). However, unlike Ca^{2+}, Sr^{2+} was unable to support nucleotidase activity effectively, since for the wild-type hSCAN-1, Sr-GDPase was only 1.5% ± 0.1% of the Ca-GDPase activity measured under identical assay conditions. It is possible that this small amount of activity in the presence of Sr^{2+} is due to a small amount of Ca^{2+} contamination in the buffers used.

Effect of the Presence and Absence of Divalent Cations on the Thermal Stability of hSCAN-1. To measure the effect of added divalent cations on the thermal stability of the enzyme, as well as to attempt to remove any divalent cations that might be tightly bound to the protein after refolding and purification, the stability of enzyme activity was monitored as a function of time at 37 °C (see Figure 9). In all samples, residual GDPase activity was assayed in the presence of 5 mM Ca^{2+}. As can be seen, Ca^{2+} and Sr^{2+} protect the enzyme from thermal inactivation. It is also evident that EDTA does not increase the rate of inactivation, suggesting the lack of a tightly bound divalent cation that is integral to the structure of the protein. This was true even when using 20 mM EDTA instead of the 2 mM EDTA used in Figure 9 (data not shown). These experiments were performed at elevated temperature, since under all conditions tested the enzyme activity was stable for more than 1 week at room temperature.

To further test the possibility that there could be a tightly bound divalent cation integral to the structure of the enzyme, the protein was refolded from denatured bacterial inclusion bodies by dilution into a refolding buffer (buffer A) to which was added either nothing, 2 mM EDTA, 2 mM Ca^{2+}, 2 mM Mg^{2+}, or 2 mM Sr^{2+}. After 3 days of refolding at 4 °C, all samples had activities that were not statistically different from each other (data not shown). Like the lack of increase in the rate of thermal inactivation in the presence of EDTA, these results are not consistent with the presence of a tightly bound divalent cation integral to the structure of hSCAN-1.

DISCUSSION

The hSCAN-1 amino acid sequence contains no previously described or characterized nucleotide-binding amino acid sequence motifs (*9*). However, multiple sequence alignment of hSCAN-1 with the 11 most homologous amino acid sequences demonstrates that invariant amino acids are found distributed throughout the amino acid sequence, often clustered in several highly conserved regions of residues (Figures 1 and 2). We have named these regions of sequence conservation NCRs for nucleotidase conserved regions. Although some of the proteins in this multiple sequence alignment have not been characterized, on the basis of the sequence similarities observed, they are all very likely to be nucleotidases (apyrases).

By site-directed mutagenesis studies, arginine residues have been identified as required for enzymatic activity in the ecto-NTPDase family of apyrases, and acidic amino acid residues such as aspartic acid and glutamic acid are thought to be involved in metal ion coordination or phosphate binding in the active sites of the NTPDases, or both (*16*, *20*, *23*). As can be seen in Figure 1, several invariant regions of amino acids are found in the hSCAN-1 family of apyrases, and these regions include conserved aspartic acid residues (D151 and D152 in NCR2) and glutamic acid residues (in NCR2 and NCR3). Due to their strong conservation, acidic nature, and negative charge, these residues may be involved in the catalytic cycle, the divalent cation-binding site, or both.

The E130Y mutation was designed and characterized due to the differences between the sequences of vertebrate and invertebrate hSCAN-1 family members at this position. This residue is conserved as a glutamic acid residue in the

vertebrate sequences but is a tyrosine residue in the invertebrate bed bug enzyme. The bed bug apyrase has been characterized on the protein level (*11*) and likely functions as an anticoagulant, efficiently hydrolyzing the ADP that is normally used by its host to trigger platelet activation and clotting. This insect enzyme thus allows blood feeding to continue by inhibiting host blood clotting. We hypothesized that this residue at position 130 has an effect on the nucleotide specificity of these enzymes since, like most invertebrate members of this apyrase family, the bed bug enzyme hydrolyzes ADP much more efficiently than human hSCAN-1 (*11*). The large increase in enzyme activity observed for the E130Y mutant (Figure 4) is consistent with this residue being important for nucleotide hydrolysis. The ADPase activity for this mutant is 5 times higher than the wild-type activity, and the ATPase activity is seven times that of wild-type hSCAN-1 activity. This suggests that the mutation of the human enzyme at this site will be a good starting point for future engineering of hSCAN-1 to be used therapeutically as an antithrombotic agent. Related to this potential use as a therapeutic anticoagulant protein, it has been found that NTPDase apyrases such as CD39 (NTPDase1) inhibit platelet release and aggregation through metabolic depletion of ADP, a major agent responsible for formation of the thrombus, and that CD39 reduces ischemia-induced norepinephrine release in the heart through the hydrolysis of ATP (*26*, *27*).

Cibacron blue, a reactive triazine dye and putative nucleotide analogue, has been previously shown to bind to and inhibit the enzymatic activity of several ecto-apyrases (*20*, *28*, *29*). Therefore, a Cibacron blue affinity matrix was used to investigate whether the loss of enzymatic activities of several hSCAN-1 mutations might be due to more delocalized changes in the nucleotide binding pocket, as opposed to changes in amino acids directly involved in the catalytic mechanism. Efficient binding of Cibacron blue by wild-type hSCAN-1 is dependent on Ca^{2+} (see Figure 6), suggesting that the Ca^{2+}-induced conformational change (*10*) is important for efficient binding of this nucleotide analogue. As shown in Figure 6, one mutant that lost activity bound very poorly to the Cibacron blue affinity matrix (D84A), and the R133A and D151A mutants having little nucleotidase activity also displayed decreased ability to bind to Cibacron blue. This suggests that there may be some delocalized conformational alterations in these mutants affecting the nucleotide binding/hydrolysis domain.

hSCAN-1 is a calcium binding protein with Ca^{2+} triggering a conformational change, changing the enzyme from an inactive form to an active nucleotidase (*10*). Several of the mutants characterized in this study apparently lost activity due to their failure to undergo a conformational change induced by calcium. This is evidenced by the lack of Ca^{2+} protection from proteolysis by trypsin (Figure 7), by the reduced ability to bind efficiently to Cibacron blue in Ca^{2+} (Figure 6), and by the lack of an increase in tryptophan fluorescence as a function of Ca^{2+} concentration that is observed for the wild-type enzyme (Figure 8). In such mutants (D84A, D151A, and R133A) there exists two possible explanations for the results obtained: either the mutations reduced or eliminated the ability to bind Ca^{2+} at the approximately 60 μM affinity Ca^{2+} site, which is necessary for enzyme activity (*10*), or the mutations reduced or eliminated the ability to undergo the required conformational change once Ca^{2+} is bound at this site. These two possibilities cannot be distinguished by the methods reported in this study or by the methods used in the previous work (*10*), since all these techniques detect Ca^{2+} binding indirectly by measuring either some conformational property of the enzyme or effects on nucleotidase activity. In fact, direct measurement of Ca^{2+} binding to site(s) having a K_D in the hundreds of micromolar range is experimentally problematic due to the high concentrations of Ca^{2+} and Ca^{2+} indicators needed.

Taken together, the data presented in Figures 6 and 7 strongly suggest that mutations D84A, D151A, and R133A do not respond normally to calcium, indicating that these mutated amino acids occur in regions that are important for either calcium binding or the conformational changes that occur as a result of Ca^{2+} binding. Interestingly, the R271A mutation resulted in a loss of most of the enzyme activity, but this mutant can still respond to calcium in the Cibacron blue binding and, to a lesser extent, in the limited trypsin digestion assays. This suggests that this mutation does not cause a calcium binding or conformational change defect but instead may indicate that this residue could be part of the catalytic mechanism for hSCAN-1 nucleotide hydrolysis.

It should be noted that, in the present study, hSCAN-1 protein solutions were diluted in buffers containing 0.1% Tween 20, unlike our previous study (*10*). During the course of this work, we observed that the apparent hydrolysis rates of hSCAN-1 for substrates was increased 2−4 times when diluting with a buffer containing 0.1% Tween 20 compared to the same buffer lacking a detergent. Thus, the specific activities reported in this work for wild-type hSCAN-1 (e.g., greater than 110 000 μmol/(mg·h) for GDPase) are significantly higher than those we reported earlier (e.g., approximately 30 000−40 000 μmol/(mg·hr) for GDPase (*10*)). This is most likely due to the detergent's ability to eliminate the adsorptive loss of protein to the walls of the plastic and glass tubes used when the hSCAN-1 solutions are diluted to the low protein concentrations needed for the GDPase nucleotidase assays. This explanation is consistent with the finding that Tween 20 enhances another enzyme's activity by favoring the release of the enzyme from the walls of the incubation vessel (*30*).

During the review process for this work, a paper was published (*14*) describing a crystal structure of a protein that is essentially identical to the hSCAN-1 protein described in this study. Those authors show that the basic structure is a five-bladed β-propeller structure surrounding a central Ca^{2+} ion. Their structure is consistent with the secondary structure determination of 44% β-sheet and 46% random coil as previously determined by circular dichroism in our laboratory (*10*). However, there are some inconsistencies between the conclusions based on the crystal structure determination and the current results. First, the crystals analyzed in the structure paper (*14*) were grown in the presence of Sr^{2+} and in the absence of added Ca^{2+}, and a Ca^{2+} ion was identified in the center of the five-bladed β-propeller structure. The binding of Ca^{2+} to such a centralized location in the structure could easily be envisioned to result in the characteristic delocalized conformational changes that were previously described by our laboratory that occur in response to Ca^{2+} (*10*). However, a Ca^{2+} binding site with an affinity of 60 μM (as determined

by UV difference absorbance and tryptophan fluorescence (see Figure 8 and ref *10*) would not be occupied under the crystal growth conditions (on the basis of the affinity, bound Ca^{2+} should dissociate in less than a second in the absence of added Ca^{2+}). There is an alternative possibility that the hSCAN-1 protein could have an additional high-affinity binding site for Ca^{2+} that would be integral to its structure. There is precedence for such a high-affinity site in a protein that seems to be fairly homologous to hSCAN-1, the diisopropylphosphatase (DFPase) enzyme of *Loligo vulgaris* (*31*, *32*). Besides being also able to hydrolyze phosphates, this enzyme is homologous to hSCAN-1 in structure—it has a high affinity "structural" Ca^{2+} that was determined to reside in the center of a similar β-propeller 3-D structure (a six-bladed β-propeller structure (*32*) for the DFPase, rather than the five-bladed β-propeller structure determined for hSCAN-1 (*14*)). Removal of this high-affinity, structural Ca^{2+} by incubation with EDTA results in irreversible inhibition and denaturation of the DFPase (*31*). The DFPase also has an additional lower-affinity Ca^{2+} binding site needed for activation of phosphatase activity (*31*), analogous to what we described previously for hSCAN-1 (*10*). However, in contrast to the DFPase, we have no evidence to suggest the presence of a high-affinity "structural" Ca^{2+} binding site in hSCAN-1. Arguing against the existence of such a high-affinity site are the findings that extended treatment of hSCAN-1 with EDTA does not result in an increased rate of inactivation (see Figure 9) and that efficient refolding occurs in the absence of divalent cations, both of which would not be expected if such a high-affinity structural Ca^{2+} binding site existed in hSCAN-1. Thus, the simplest interpretation of all the data suggests that the Ca^{2+} ion identified in the crystal structure (*14*) may be in fact a Sr^{2+} ion and that it occupies the approximately 60 μM "conformational" Ca^{2+} binding site necessary for enzyme activation (Sr^{2+} can substitute for Ca^{2+} to induce the conformation change associated with the increase in tryptophan fluorescence and resistance to limited proteolysis (Figure 8)). However, given our results that Sr^{2+} cannot effectively replace Ca^{2+} to support nucleotidase activity (the Sr-GDPase activity is only 1.5% of the Ca-GDPase activity), the enzyme whose crystal structure was determined most likely did not have a Ca-nucleotide substrate bound to it and thus was not in a fully active state. This explains the absence of a Ca^{2+} ion complexed to the nucleotide analogue as a cosubstrate, as demonstrated by the relatively large distance between the nucleotide analogue and the single "Ca^{2+}" ion found in the crystal structure. These hypotheses and conclusions may also explain the inability of the authors of the crystal structure paper to identify amino acid residues close enough to carry out the nucleophilic attack to hydrolyze the phosphate moiety of the nucleotide—the cosubstrate Ca^{2+} ion is missing from the structure, and therefore, the arrangement of amino acids around the substrate would be slightly changed relative to the actively hydrolyzing enzyme.

In summary, we show that several conserved residues in the nucleotidase conserved regions (NCRs) are important for hSCAN-1 to maintain normal nucleotidase activity and to mediate substrate specificity. Amino acid residues D84, R133, D151, and R271 were identified as essential for hSCAN-1 enzymatic activity. The differential ability of three of these mutations to bind to a Cibacron blue affinity matrix, as well as changes observed in the mutants with respect to tryptophan fluorescence and susceptibility to limited proteolysis, suggest that these three mutations (D84A, R133A, and D151A) do not mediate their effects via modification of amino acids directly involved in the catalytic mechanism but rather cause inactivation by not allowing the conformational change induced by Ca^{2+} that is required for nucleotidase activity. We hypothesize that there are at least two functional roles/binding sites for Ca^{2+} in this enzyme, one for maintaining an enzymatically competent conformation of the protein and another for serving as a cosubstrate with the nucleotide. Both are required for nucleotidase activity. Sr^{2+} can substitute for Ca^{2+} in the first functional role/binding site (conformational role) but not in the second functional role/binding site (cosubstrate role). Therefore, Sr^{2+} alone does not support enzyme activity. If Sr^{2+} can replace Ca^{2+} at the conformational Ca^{2+} binding site but cannot substitute for Ca^{2+} as a cosubstrate with the nucleotide, this suggests alternative interpretations for the role of the single Ca^{2+} found in the recently determined crystal structure. In addition, we find that the amino acid residue at position 130 is very important for enzyme activity and substrate specificity. The finding that this single point mutation increases nucleotidase activities substantially is both scientifically important and of potentially therapeutic utility and suggests that this residue is one of the crucial amino acids accounting for the different enzymology observed for the vertebrate versus the invertebrate members of this enzyme family. Combination of this mutation with those discussed by Dai et al. that increase the ADPase activity of this enzyme dramatically (*14*) should lead to designer versions of the hSCAN-1 enzyme having even better enzymatic characteristics and potential for therapeutic applications. These applications would include treatment of ischemic events triggered via activation of platelets by ADP, including myocardial infarctions and ischemic strokes.

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