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Characterization and importance of the dimer interface of human calcium-activated nucleotidase

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Abstract

Human calcium-activated nucleotidase (CAN) exists as both a membrane-bound form in the endoplasmic reticulum and pre-Golgi intermediate membranes, as well as a secreted, soluble form. Although the wild-type human enzyme hydrolyzes ADP poorly, engineered soluble human proteins (SCANs) hydrolyze ADP much more efficiently, making them potentially useful therapeutic proteins for treatment of human clotting pathologies. According to the crystal structure and the recently identified dimeric nature of the soluble nucleotidase, the dimer interface contains a central core of hydrophobic residues. Previously, we demonstrated that the mutation of glutamic acid 130 (located in the dimer interface) to tyrosine increased both the tendency to form dimers and the ADPase activity. In the present study, we investigated the importance of the dimeric state for enzymatic activity and biological function in this nucleotidase by mutating isoleucine 170, which is located in the center of the hydrophobic core of the dimer interface. The results of analytical ultracentrifugation, chemical cross-linking, and tryptophan fluorescence analyses demonstrated that mutation of isoleucine 170 to either positively or negatively charged amino acids (lys or glu) disrupted the calcium-dependent dimerization in soluble CAN. Furthermore, these mutations decreased maximal ADPase activity for both the soluble and membrane-bound enzymes. Although not as critical as the hydrophobic interactions centered at isoleucine 170, the role of hydrophilic interactions in dimer formation was also demonstrated. Thus, mutation of aspartic acid 228 to threonine (D228T) decreased both the tendency to form dimers and ADPase activity, while double mutation of D228T/K224N largely restored the ability to form dimers and the ADPase activity, further indicating that the nucleotidase activity of CAN is linked to its quaternary structure. Since ADPase activity of the soluble form is crucial for its potential development as a therapeutic protein, these findings have implications for engineering the soluble human calcium-activated nucleotidase for clinical applications. In addition, future comparison of monomeric (I170K and I170E mutants) and dimeric (wild-type) crystal structures of SCAN will advance our understanding of its enzymatic mechanism and aid in engineering efforts.

Keywords

soluble calcium-activated nucleotidase; site-directed mutagenesis; dimerization; analytical ultracentrifugation; cross-linking; tryptophan fluorescence

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In 2002, Smith et al. first described the cloning and expression of a unique human nucleotidase, and named it hSCAN-1, for human soluble calcium-activated nucleotidase 1 (1). In the same year, the rat homologue of this protein was also cloned, sequenced and characterized, and termed the Ca^{2+}-dependent endoplasmic reticulum nucleoside diphosphatase (2), in light of its existence as a membrane-bound form in the endoplasmic reticulum. In 2006, this protein was renamed calcium-activated nucleotidase (CAN) (3). CAN is a protein homologous to the apyrases expressed in blood-sucking insects. In hematophagus arthropods, these apyrases are used to hydrolyze ADP to inhibit platelet activation and clotting in response to ADP, allowing the insect to feed on the host's blood for an extended time. However, the mammalian homologues of these insect nucleotidases hydrolyze ADP poorly and may play a role in protein glycosylation (2), but the physiological functions are not yet well established. Dai et al (4) and our laboratory (5) have used site-directed mutagenesis to engineer the soluble human enzyme to hydrolyze the platelet agonist, ADP, much more efficiently. In addition, we showed that the soluble form of CAN (SCAN) exists as both a monomer and a dimer, depending on the concentrations of both protein and calcium ion (6). Our structural data also revealed that a central hydrophobic region anchors the dimer interface, with many of the residues in this hydrophobic core being well conserved between vertebrate and invertebrate members of this enzyme family.

In order to understand the functional importance of the dimeric state of CAN, mutations were made in attempts to deliberately disrupt the ability to form dimers. Thus, the hydrophobic residue at the very center of the dimeric interface, isoleucine 170, was mutated to either positively charged lysine or negatively charged glutamic acid, thereby introducing repulsive charges in the center of the dimeric interface. These mutations were made both in the bacterially expressed, soluble form of the CAN protein, as well as the mammalian COS cell expressed, full-length, membrane-bound form of the protein. Using enzyme activity, chemical cross-linking, analytical ultracentrifugation, and tryptophan fluorescence assays, it was demonstrated that this single mutation in the center of the dimer interface disrupted dimerization and impaired enzyme activity.

The dimeric interface inferred from the crystal structure of SCAN (6) also suggested several ionic interactions at the periphery of the central hydrophobic core as being important for the stabilization of the dimer. To demonstrate their importance for dimerization and SCAN activity, we chose one such interaction for mutation which, based on multiple sequence alignments, should be different in the mammalian and insect members of this nucleotidase family. Thus, the D228T mutation was constructed and was also shown to decrease the tendency to form dimers as well as decrease the maximal ADPase activity, demonstrating its involvement in formation of the dimeric interface needed for maximal nucleotidase activity and biological function of this calcium-activated nucleotidase.

Experimental Procedures

Materials

The QuikChange 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, *Not*I and *Eco*RI restriction endonucleases were obtained from New England Biolabs. The bacterial expression vector pET28a and the bacterial expression BL21(DE3) cells were purchased from Novagen. Glycerol and dialysis tubing were from Fisher Scientific. DSS cross-linker, B-PER bacterial extraction reagent and enhanced chemiluminescent reagents were purchased from Pierce and the pre-cast SDS-PAGE 4–15% gradient minigels were obtained from Bio-Rad laboratories. DMEM, Lipofectamine and, Plus transfection reagents, and antibiotics/antimycotics were purchased

from Gibco BRL/Invitrogen. Carbenicillin, kanamycin, nucleotides, isopropyl-β-D-thiogalactopyranoside (IPTG), glucose, DTT, and glutaraldehyde were from Sigma.

Site-directed mutagenesis and protein purification

The mutants were made with the Stratagene QuikChange site-directed mutagenesis kit. The presence of the correct mutation and lack of unwanted mutations were confirmed by DNA sequencing. The wild-type and mutant SCAN cDNA constructs were used to transform bacterial expression host BL21(DE3) cells, and after induction of expression with IPTG, bacterial inclusion bodies containing the SCAN proteins were prepared. 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 (7). The purified proteins were quantified by their absorbance at 280 nm and assayed for purity by SDS-PAGE.

Nucleotidase assays

Nucleotidase activity was determined by measuring the amount of inorganic phosphate released from nucleotide substrates at 37°C using a modification of the technique of Fiske and Subbarow (8) as previously described (9). Nucleotide hydrolyzing units are expressed in micromoles of P_i liberated per milligram of protein per hour. Generally, assays were conducted in 20 mM MOPS, pH 7.4, containing 5 mM $CaCl_2$ and 2.5 mM nucleotide substrate. In some experiments, slight modifications to the assays were made, which are described in the appropriate figure legends.

Tryptophan fluorescence as a measure of Ca-induced dimerization

Protein intrinsic fluorescence measurements were carried out as previously described (7). The intensity of the intrinsic SCAN tryptophan fluorescence as a function of divalent Ca ion concentration was measured using a Hitachi F-2000 fluorescence spectrophotometer. A 0.85 μM (32 μg/mL) concentration of SCAN in 20 mM MOPS, pH 7.4 was excited at 295 nm, and fluorescence emission was recorded at its maximum (340 nm), before and after stepwise addition of Ca^{2+}.

Chemical cross-linking with glutaraldehyde or disuccinimido suberate (DSS), lysine -specific cross-linking reagents

Stock solutions of glutaraldehyde and DSS were freshly prepared before use (glutaraldehyde in water and DSS in dry DMSO). Purified SCAN proteins (0.3 mg/ml) were incubated in 20 mM MOPS (pH 7.4) in the presence or absence of 5 mM $CaCl_2$, for 15 min at 22°C in the presence or absence of glutaraldehyde (500 μM) or DSS (250 μM). Cross-linking was stopped by adding an excess (25 mM) of lysine and incubating for 5 min at room temperature. Finally, SDS sample buffer was added, the samples were boiled for 5 min, run on SDS-PAGE, and analyzed by Coomassie staining of the gel and densitometry.

Analytical ultracentrifugation analyses

Sedimentation velocity experiments were carried out at 20°C in a Beckman XL-I ProteomeLab analytical ultracentrifuge using absorbance optics, as previously described (6). 400 μl samples of 0.85 μM SCAN or SCAN mutants in 20 mM MOPS (pH 7.4) were adjusted to contain a total concentration of 0, 0.2, or 2 mM $CaCl_2$, centrifuged overnight at 48,000 rpm, and scanned at a wavelength of 230 nm. Sedimentation velocity data were fitted using the program SEDFIT (10) to determine sedimentation coefficient distribution plots.

Circular dichroism analyses

Far-UV circular dichroism spectra were collected between 190 and 260 nm for wild-type and mutant SCAN in 20 mM MOPS (pH 7.4) at 25°C using an AVIV model 215 spectrophotometer with a 0.5 mm path length quartz cell. For each sample, three spectra were collected and averaged. The data were converted to mean residue ellipticity and the secondary structure content was analyzed using the program K2D (see Table 1), as previously described (7). For comparison, the secondary structure content of dimeric SCAN was determined from the crystal structure (pdb 2H2N, (6)), using the PROMOTIF summary in PDBsum (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum, (11)).

Full-length CAN expression

Mammalian COS cell expression was carried out as previously described (12). In these experiments, empty pcDNA3 vector was used as a control, and the CAN C30S mutant was used as the background for mutations rather than full-length wild-type CAN, due to the ability of C30 to form intermolecular disulfides via oxidation to cystine, which would interfere with some of the subsequent analyses. The crude cell membrane preparations were obtained as previously described (12).

Results

We previously identified the likely surface of the SCAN nucleotidase protein involved in dimer formation induced by the presence of calcium ions (6). We also suggested that the reason for the increased ADPase activity of a mutant involving this region (E130Y) was due to more efficient dimer formation (6). However, because calcium is required both for SCAN enzymatic activity and for SCAN dimerization, it is difficult to accurately assess the contribution of SCAN dimerization to its enzymatic activity. In the present study, we investigated the possibility of making a SCAN protein incapable of forming dimers by introduction of like charges into the hydrophobic center of the dimeric interface. Thus, the I170K and I170E mutations were expressed in this soluble nucleotidase, with the expected outcome being the disruption of dimer formation (see Figure 1 for the spatial arrangements of the mutations made in the dimeric interface region in relationship to the monomer active sites and the Ca^{2+} residing at the center of the 5-bladed beta propeller secondary structural elements in each monomer). After refolding from bacterial inclusion bodies and purification, the specific activities of these mutations were determined, and expressed as a fraction of the wild-type activity (see Figure 2A). SCAN has much higher activity using GDP, UDP, and IDP as substrates, compared to ADP, ATP, and CDP. Therefore, the concentrations of SCAN proteins used in the GDP, UDP, and IDP assays are much lower than those used in the ADPase, ATPase and CDPase activity assays. Specifically, the concentrations of SCAN protein used in the ADPase, ATPase and CDPase assays were 118–125 nM (at which concentration significant dimerization would occur (6)), whereas for the GDP, UDP, and IDP substrates, the concentration of SCAN protein used in the assays was 0.77 nM, a concentration at which almost all SCAN protein will exist as monomers (6). Therefore, one would predict decreased activity in the I170K and I170E mutants relative to the wild-type enzyme using ADP, ATP and CDP as substrates if these mutants prohibit dimer formation and the activity of the dimeric protein is greater than the activity of the monomeric protein. In contrast, one would predict a much smaller decrease in the GDPase, UDPase, and IDPase activities for these mutants, since these assays are performed using very low protein concentrations which should result in virtually all SCAN enzymes being monomers. The data in Figure 2A are consistent with this hypothesis.

We hypothesized that, in addition to the hydrophobic core of the dimeric interface, several hydrophilic residues on the periphery of the dimeric interface are also important for the stability and properties of the SCAN dimer. In order to assess this, mutation of one such residue, D228T,

was evaluated. This mutation was chosen since D228 is predicted to electrostatically interact with K224 on the other monomer in the dimer (see Figure 1), and this pair of amino acids involved in this interaction is different in the insect enzymes. For example, D228 is a threonine residue, while K224 is an asparagine residue in the bed bug enzyme (13). Therefore, we constructed and evaluated both the single mutant, D228T, and the double mutant, D228T/K224N, with the double mutant expected to mimic the hydrophilic interaction between these two residues between the two monomers in the bed bug insect enzyme. As expected, this double mutant rescued the decreased enzymatic activities observed in the D228T single mutant (see Figure 2B). Also as expected, the decrease of hydrolysis of GDP, UDP and IDP observed for the D228T mutant is much less than the decrease observed for the hydrolysis of ADP, CDP and ATP (see Figure 2B), consistent with the results obtained for the I170K and I170E mutations, and with this residue being involved in dimer stabilization.

To further test the hypothesis that the I170K and I170E mutants have lower ADPase activities due to decreased ability to form dimers, the concentrations of SCAN proteins used in the assays were varied from well below the dimerization constant concentration to greater than the dimerization constant, yielding the results shown in Figure 3. As can be seen, there is almost no increase in specific activity of the I170K/E mutant proteins as a function of protein concentration, whereas the wild-type protein activity increases approximately 5-fold at a protein concentration of approximately 0.1 μM, consistent with dimerization causing the increase in activity in the wild-type enzyme, which does not occur in the I170 mutants (K_D for dimerization of wt SCAN is approximately 0.17 μM (6)). The D228T and D228T/K224N mutant ADPase activities increased to nearly the same extent and at similar protein concentrations as did the wild-type enzyme, suggesting that these mutants were able to form dimers under these conditions.

Another way to assess the ability of the SCAN proteins to form dimers is to measure the Ca-dependent chemical cross-linking of the protein. As shown in Figure 4A, when cross-linking is performed with the lysine-specific cross linker glutaraldehyde, a clear cross-linked dimer is observed for the wild-type in the presence of Ca^{2+}, which is greatly attenuated in the I170K mutant, and completely abolished in the I170E mutant. In addition, there is a smaller decrease in cross-linking efficiency for the D228T mutant, and this decrease is almost abolished by introduction of the K224N mutation (i.e., in the D228T/K224N mutant) in this relatively crude assay of dimerization tendency. Using another lysine specific cross-linking reagent, DSS, similar, but not identical, results were obtained for these mutants (Figure 4B). The reason for the decreased DSS cross-linking efficiency in the D228T/K224N double mutant (58%) relative to the D228T single mutant (103%) is not clear, but may be due to the fact that the K224 surface lysine residue which is absent in the double mutant (but present in the D228T single mutant) is one of the lysine residues involved in some of the cross-linking induced by DSS (which is able to cross-link lysine residues separated by larger distances than glutaraldehyde).

Cross-linking efficiency was somewhat higher with DSS than glutaraldehyde (i.e., there was more wild-type dimer formed, compare Figure 4B with Figure 4A), but still lower than expected, and this less than expected cross-linking was consistent with low efficiency of SCAN cross-linking observed under all conditions using several lysine-specific cross-linkers. We conclude that the lysine residues present in each monomer of the dimer are not in locations and orientations that are favorable for intermolecular cross-linking to occur. Nonetheless, the DSS cross-linking data showed a larger decrease in I170K cross-linking relative to wild-type than that observed with glutaraldehyde, with DSS cross-linking being almost completely abolished by both the I170K and I170E mutations, as is expected from other results. In addition, it should be noted that introduction of a lysine residue (i.e., the I170K mutation) could increase the chemical cross-linking efficiency by a lysine-specific reagent of any small amount of dimer

that may form in this mutant, giving rise to the differences in glutaraldehyde cross-linking results observed between the I170E and I170K mutants in Figure 4A.

More quantitative and definitive data regarding the dimerization ability of the mutant SCAN proteins were obtained using sedimentation velocity analytical ultracentrifugation (see Figure 5). As shown in the figure, the addition of 2 mM Ca^{2+} results in nearly quantitative dimerization of the wild-type SCAN. However, under identical buffer and protein concentration conditions, no dimers are observed for either the I170K or I170E mutations (see Figure 5A). Mutations of a hydrophilic residue (D228T) on the periphery of the dimeric interface also affects dimer formation of this enzyme, but is less critical for dimer formation, as shown in Figure 5B. Hence, in 2 mM Ca^{2+}, there is only a slight shift towards the monomer for the D228T mutant relative to the wt SCAN, but there is an obvious shift towards the monomer in 0.2 mM Ca for the D228T mutant. This shift towards the monomer in 0.2 mM Ca is virtually abolished in the double mutant, D228T/K224N (Figure 5B).

Increases in tryptophan fluorescence have been used as an indirect measure of SCAN dimerization, and quantitatively agree with measurements of dimer dissociation constants calculated from AUC equilibrium sedimentation data (6). As can be seen in Figure 6, under conditions where the wild-type enzyme nearly quantitatively dimerizes, the I170K and I170E mutant proteins do not dimerize, as monitored by the absence of an increase in tryptophan fluorescence (in contrast to the wt enzyme). Ca^{2+} can induce increases in tryptophan fluorescence characteristic of dimer formation in the D228T mutant, but the EC_{50} for calcium is 0.3 mM in this mutant compared 0.06 mM in wild type, while the EC_{50} for calcium is an intermediate 0.11 mM in the double mutant D228T/K224N (Figure 6). Thus, dimer formation in mutations of hydrophilic residues on the periphery of the dimeric interface is not as severely affected as was observed for mutations of I170 in the hydrophobic core of the dimer interface, but the dependence of dimerization on Ca^{2+} concentration is changed.

To exclude the possibility that the point mutations resulted in delocalized global changes in SCAN structure, circular dichroism (CD) experiments were performed to verify that the secondary structural elements in the mutants are not substantially different from that of the wild-type enzyme (the secondary structure of SCAN is dominated by beta sheets (4,6,7)). As can be seen in Table 1, the mutants displayed no substantial differences from the wild-type enzyme in the quantity of secondary structural elements, and these results are consistent with previously published circular dichroism SCAN results (7), theoretical calculations based on the amino acid sequence (based on the PROF computer program), as well as the crystal structure of SCAN (4,6), all of which indicate a secondary structure dominated by beta sheets. CD spectra were not performed in the presence of Ca^{2+}, since we previously showed that Ca^{2+} (and therefore dimerization) did not substantially change the CD spectra (and therefore the secondary structure) of wild-type SCAN (7).

The effect of mutation of I170 was also assessed in the full length, membrane-bound form of the CAN enzyme expressed in COS cells. As can be seen in Figure 7, compared to the C30S mutant background in which the I170 mutations were made, both the I170K and I170E mutations have decreased nucleotidase activities. It was previously found that glutamic acid residue 130 is in the dimer interface (6), and that its mutation to tyrosine greatly increased ADPase activity of the soluble SCAN enzyme (5). However, the effect of this mutation on the membrane-bound, full-length SCAN was not reported previously. Thus, ADPase and GDPase activities of the E130Y CAN mutant expressed as the membrane- bound form in mammalian COS cells were also investigated in this study (see Figure 7). In stark contrast to the I170K and I170E mutants, both ADPase and GDPase activities of the E130Y mutant are increased, with a larger increase in ADPase, similar to that found previously for the same mutation expressed in the soluble SCAN. The C30S mutant CAN background was used for these

experiments, rather than the wild-type full-length CAN, since we previously showed that the C30 residue, located in the "stalk" region between the globular, soluble part of the enzyme and the single transmembrane domain, can become oxidized and form a covalent dimer of full-length CAN proteins (6). The C30S CAN enzyme has wild-type activity and properties, and was used as a background for these mutations to avoid this oxidative covalent dimerization complication. It should be noted that the dimerization status of the full-length, membrane-bound CAN enzyme is complicated by the likelihood that the interaction between monomers in a dimer involves both interactions between the transmembrane helices as well as the dimeric interface of the soluble portion of the protein which is disrupted by mutation of I170 and enhanced by the E130Y mutation.

Discussion

The calcium-activated nucleotidase (CAN) is a Ca^{2+} dependent protein, with calcium needed for both dimerization (6) and nucleotidase activities (7). Previously, we showed the dependence of SCAN dimerization on protein concentration, and found that the ADPase activity of SCAN increased as the protein concentration increased (6). However, even at low protein concentrations, there will be a small amount of dimer present, and, conversely, at high concentrations, some monomer present, and thus, there is no facile way to measure and compare quantitatively the specific activities of the monomeric and dimeric forms of wild-type SCAN directly. Therefore, in this study, we generated mutations intended to abolish or substantially weaken the ability to form dimers, but otherwise have no effects on SCAN/CAN structure and activity. Two of the mutations made, I170K and I170E, would result in a pair of like, repulsive charges being brought into close proximity in the core of the interface between the mutant SCAN dimers (see Figure 1). Thus, the expected and desired outcome is that the dimerization of these mutants would be greatly attenuated or abolished even in the presence of calcium, and that the effects of their inability to dimerize on nucleotidase activities could be measured and evaluated.

Direct (analytical ultracentrifugation analysis) and indirect (chemical cross-linking, tryptophan fluorescence, and activity assays) measures of SCAN dimerization all indicated that the I170K and I170E mutations disrupt the ability to form dimers, but do not globally modify the structure of the protein, as evidenced by maintenance of secondary structure (as assessed by circular dichroism measurements, Table 1). Thus, these mutants are essentially identical to the wild-type SCAN as monomeric proteins, but are deficient in the ability to form dimers. This indicates that both monomer and dimer are active, but that the dimer is a more active ADPase than the monomer. The same also holds true for the D228T and D228T/K224N mutants, designed to disrupt and restore a hydrophilic interaction on the periphery of the inferred dimeric interface (see Figure 1), which we hypothesized would also be important for dimer formation and stability.

The dimer interface is far removed from the active site of SCAN according to the crystal structure of human SCAN (see Figure 1 and reference (6)), but results with these dimerization deficient mutations indicate the specific activity of the SCAN protein for hydrolysis of ADP increases about five times upon dimerization. The reason for this increase in enzymatic activity upon dimerization is not known. However, it seems likely that the substrate binding site on each monomer must be influenced by the conformational change induced by dimerization. It is interesting to note that although both Dai et al (4) and our own laboratory (6) obtained SCAN protein crystals readily and solved the structure of SCAN by X-ray crystallography, all crystals were obtained at high protein concentrations in the presence of Ca^{2+} or Sr^{2+}, the only 2 divalent cations capable of inducing SCAN dimerization (5). Therefore, no SCAN protein crystals have been obtained under conditions where the protein is monomeric. Thus, comparison of the crystal structures of wild-type (dimeric) and I170K/E mutants (monomeric) in the future may

directly address the question as to what changes occur in the active site as a result of dimerization of the protein via a distant site. This should increase our limited knowledge of the hydrolytic mechanism of this enzyme. It should also aid in the design of modified forms of human SCAN for use as anti-coagulant and anti-thrombotic therapeutic proteins. For example, combining mutations at the active site of the enzyme that increase ADPase activity as described by Dai et al (4) with mutations at the dimeric interface that increase dimer formation, protein stability, and ADPase activity (e.g., E130Y), should yield very stable and very active human enzymes that may prove useful for treating thrombotic pathologies, including heart attacks and ischemic strokes, via hydrolysis of the platelet agonist, ADP.

In the integral membrane protein expressed form of this nucleotidase (full length CAN expressed in COS cells), both the I170K and I170E mutations show decreased nucleotidase activities, but do not exhibit the property of a substantially greater loss of ADPase than GDPase activities, as is observed in the soluble form of these mutants (compare Figure 2A and Figure 7). The reason for this is not clear, but undoubtedly partially resides in the stabilization of dimer formation by the interactions of the trans-membrane domains in the full-length enzyme that are not present in the soluble form.

The soluble form of the human SCAN E130Y mutant dimerizes more efficiently and has higher ADPase activity than the wild-type enzyme (5,6). Since the main function of the CAN enzyme in humans seems to be that of the ER membrane-bound, intracellular form, rather than the secreted soluble form, we are also interested in elucidating the nature and significance of dimerization of the full-length, membrane-bound form of the enzyme. In addition to the soluble dimeric interface surrounding I170, the dimer form of the ER, membrane-bound CAN may be stabilized by a disulfide bond between CAN monomers involving cysteine 30 and by transmembrane helix interactions. However, our results clearly show that the E130Y mutant of the full-length, membrane-bound form also significantly increases its ADPase activity in the absence of C30 (i.e., in the C30S background), indicating that the dimerization interface of the soluble portion of SCAN is also present and important in the membrane-bound form, and that the dimeric state, the activity, and presumably the function of the membrane-bound CAN enzyme is also modulated by the concentration of Ca^{2+} present in the lumen of the endoplasmic reticulum. Thus, the increase in ADPase activity upon dimerization of membrane-bound CAN suggests that the dimerization of this membrane-bound protein is not just an intellectual curiosity of the soluble form, but that it may have physiological importance in the endoplasmic reticulum by modulation of the (currently unknown) intracellular function of human CAN.

Previously, we noted that the E130Y SCAN mutation increased ADPase activity dramatically (5). Later, we demonstrated that the mechanism by which this mutation increases ADPase activity is via its positive effect on calcium-induced SCAN dimer formation (6). In that same work, we also identified the likely dimer interface surface on the SCAN monomer by substitution of cysteine residues at various locations and measuring the ability of the mutated proteins to be cross-linked in a calcium-dependent fashion. One such mutation, I170C, was found to be very efficiently cross-linked, and is located in the center of a hydrophobic surface which was identified as the dimer interface, based on our crystal structure, as well as several biochemical analyses (6). In the present study, we demonstrate that dimerization, and the increase in ADPase activity upon dimerization, can be abolished by means of a single mutation which introduces a charged residue at this key position (the I170K/E mutations), indicating the importance of this hydrophobic patch on the surface of the SCAN monomer for dimer formation and activation of ADPase activity. The present study enables future comparisons of the crystal structures of wild-type (dimeric) and I170K/E mutants (monomeric) to directly address how dimerization changes the active site to increase ADPase activity, thereby increasing the potential utility of SCAN as an anti-coagulant and anti-thrombotic agent. In

addition, based on comparisons of multiple sequence alignments of the mammalian versus insect SCAN proteins, as well as inspection of the crystal structure, we identified in this study a potential salt bridge interaction at the periphery of the dimeric interface which we now show is also involved in dimer formation (D228 – K224, disrupted by the D228T mutation). When a second mutation (K224N) was introduced to mimic the hydrophilic interaction that presumably occurs at this position in the insect SCAN enzyme (i.e., the D228T/K224N mutant), the dimerization ability of the mutated human SCAN was largely restored, suggesting that such peripheral hydrophilic interactions are present and functionally significant in both human and insect SCAN dimers.

In nature, many proteins are found to exist in both monomeric and dimeric forms. In some proteins, the assembly of dimer or oligomers contributes to both conformational and thermal stabilities. Through changes between oligomeric states, protein functions are often regulated. For example, a hormone-sensitive lipase is 40 times more active as a dimer than as a monomer (14). Thus, the function of the membrane-bound form of CAN may be modulated by conditions that modulate dimerization, including membrane fluidity or change in protein-protein interactions (affecting dimerization via modulation of interactions of CAN transmembrane helices) and Ca^{2+} ion concentration variations in the endoplasmic reticulum lumen (affecting dimerization via the soluble dimerization interface disrupted in this work). In any event, engineering of the human SCAN as a potential anti-coagulant and thrombolytic therapeutic protein should include strategies for increasing the strength of the dimeric interaction, thereby increasing the enzyme's ability to hydrolyze ADP, which is the agonist used to trigger platelet activation and blood clotting.

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Abbreviations

CAN, Calcium Activated Nucleotidase (Gen Bank accession number AF328554); SCAN, Soluble Calcium Activated Nucleotidase; DMEM, Dulbecco's modified Eagle medium; DSS, disuccinimidyl suberate; MOPS, 3-[N-morpholino]propanesulfonic acid; ER, endoplasmic reticulum; AUC, analytical ultracentrifugation; CD, circular dichroism.

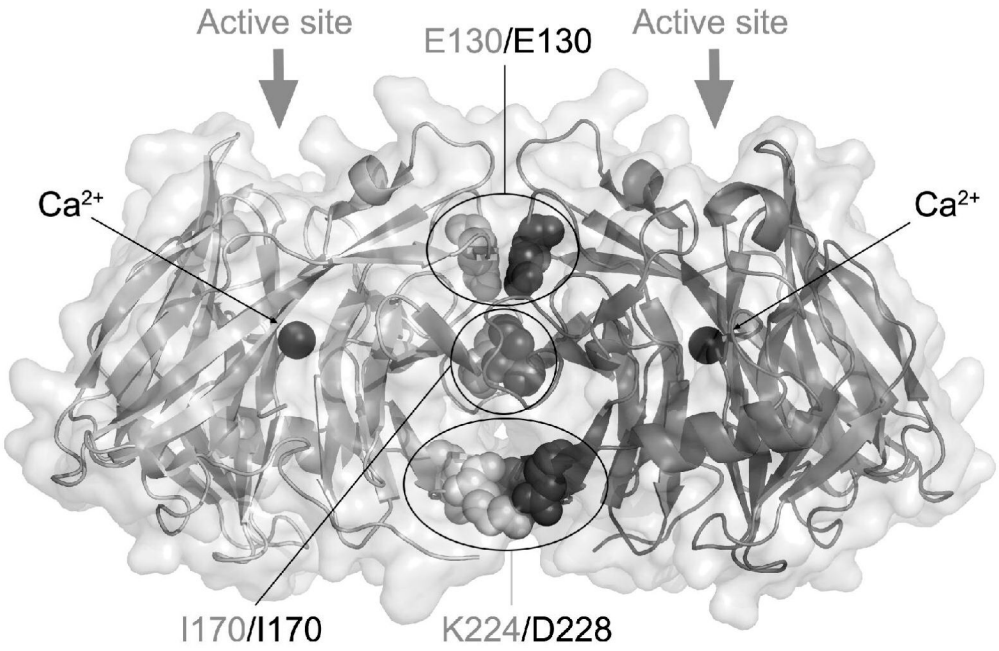

Figure 1. Location of mutations within the SCAN dimer interface

The SCAN dimer crystallized in the presence of $CaCl_2$ is illustrated (pdb 2H2N). Residues mutated in this study are shown as spheres, shaded to indicate individual monomers. The second K224/D228 pair is hidden in the background behind the labeled pair. For each monomer, the central Ca^{2+} ion is shown as a black sphere, and the entrance to the active site is illustrated by a large gray arrow.

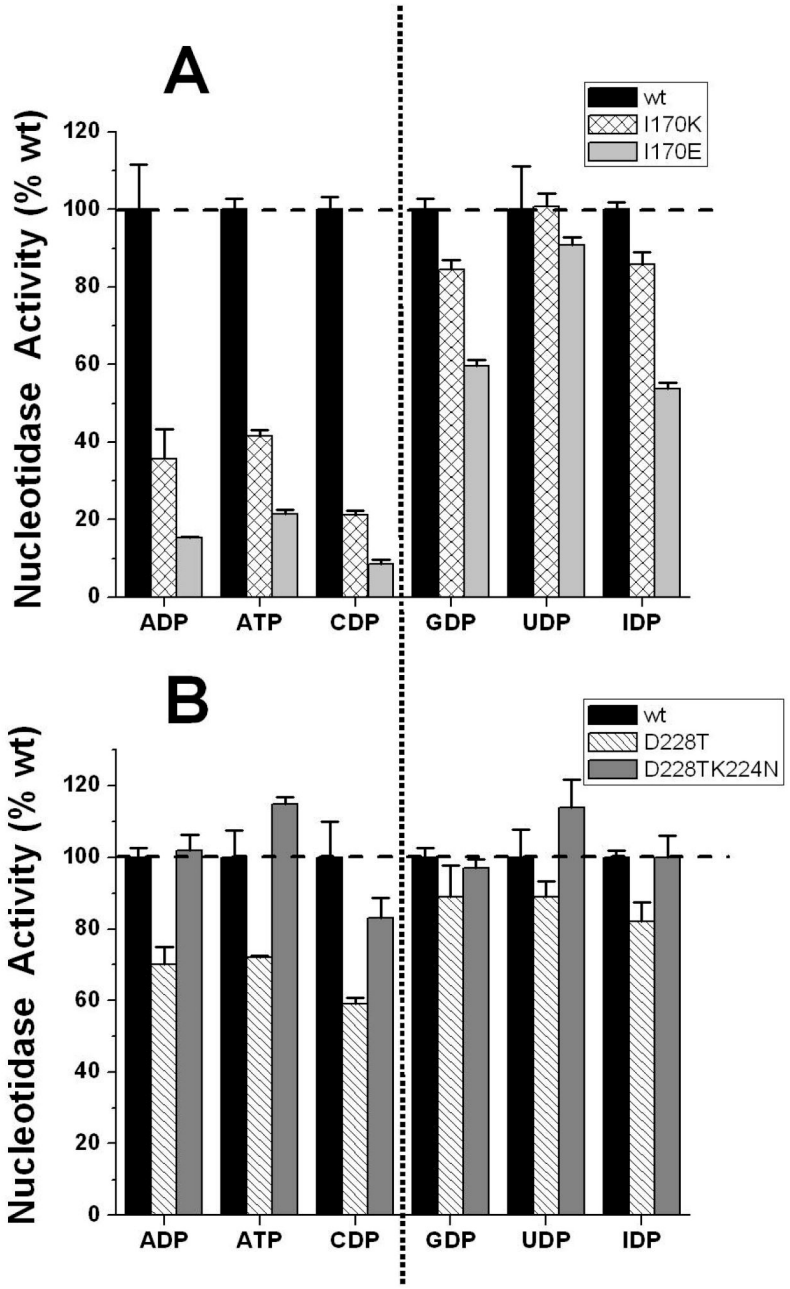

Figure 2. Enzymatic activities of SCAN mutants

Panel A - Nucleotidase activities of I170K, I170E, and wild-type SCAN, expressed as a fraction of the wild-type activities. For ADP, ATP and CDP substrates, the assays were performed at a final protein concentration of 118–125 nM; for the GDP, UDP and IDP substrates, the final protein concentration in the assays was 0.77 nM. For the GDPase, UDPase and IDPase assays, SCAN protein was first diluted into 50 mM Tris-HCl pH 6.8 containing 0.1% Tween 20 detergent, in order to prevent adsorption to sample tubes prior to assays at this very low protein concentration. Nucleotidase activity was determined by measuring the amount of inorganic phosphate released from nucleotide substrates at 37°C, at a final nucleotide concentration of 2.5 mM, in the presence of 5 mM $CaCl_2$. **Panel B - Nucleotidase activities**

of D228T, D228T/K224N and wild-type SCAN, expressed as a fraction of the wild-type activities. The assays were performed as described for Panel A, at a final nucleotide concentration of 2.5 mM, in the presence of 5 mM $CaCl_2$. Error bars are standard errors of the means in both Panel A and B. The average of the means for the wild-type SCAN activities reported in Panels A and B are (in μmol/mg/hr): ADPase, 2,520; ATPase, 306; CDPase, 1,980; GDPase, 114,000; UDPase, 120,000; and IDPase, 102,000.

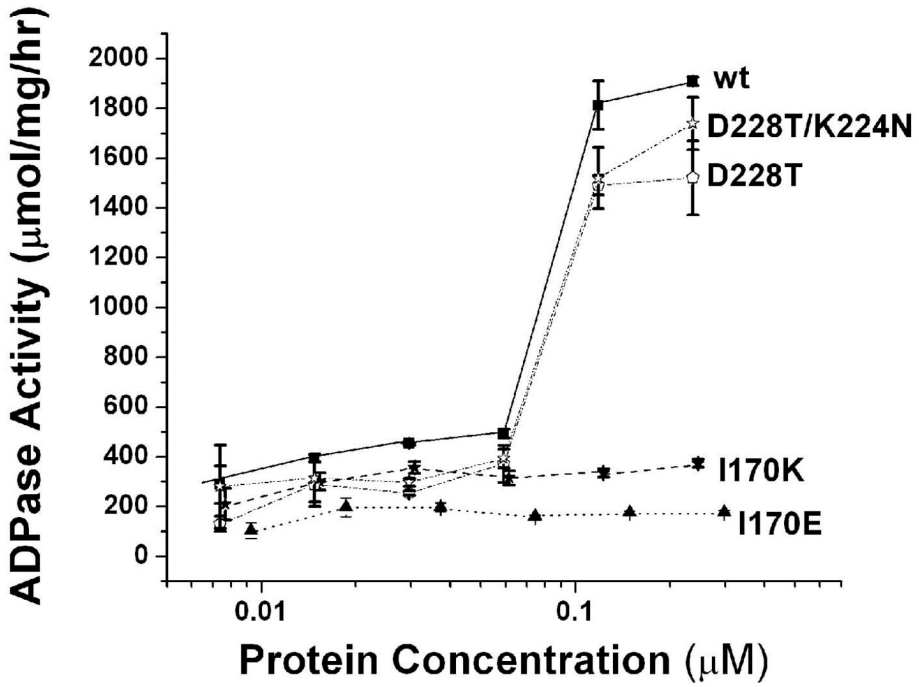

Figure 3. ADPase activities at 22°C of I170K, I170E, and wild-type SCAN as a function of protein concentration

Nucleotidase activity was determined by measuring the amount of inorganic phosphate released from nucleotide substrates at 22°C, at a final ADP concentration of 5 mM, in a buffer containing 5 mM $CaCl_2$. Error bars are the standard errors of the means.

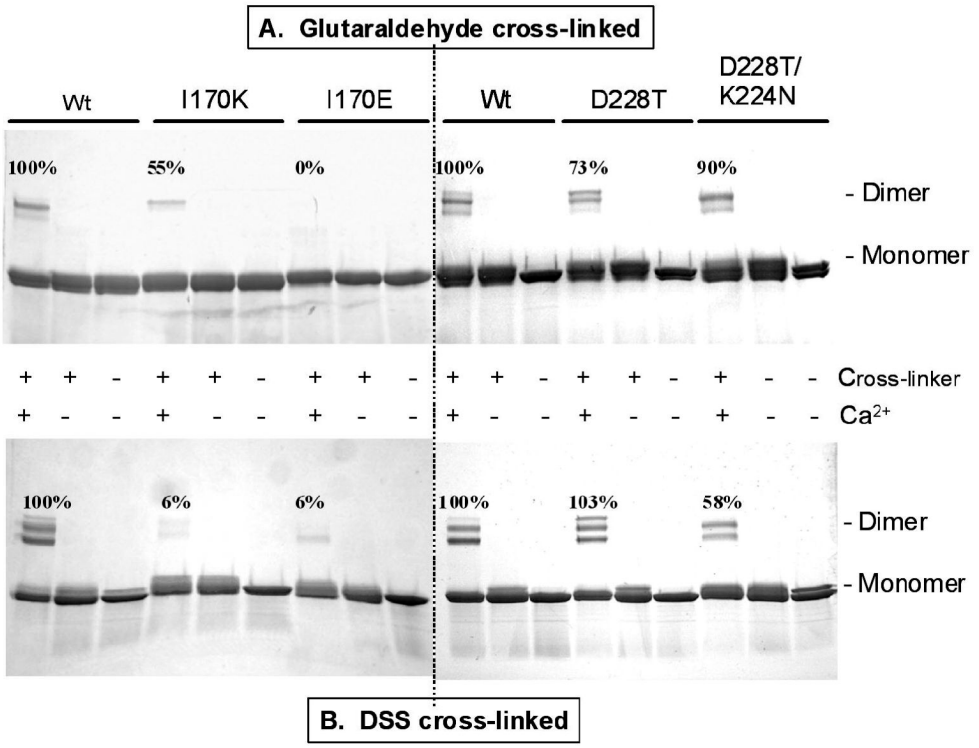

Figure 4. SDS-PAGE analysis of calcium dependent dimerization of wild-type and mutant SCAN proteins after lysine-specific cross-linking. Panel A (top), glutaraldehyde was used for cross-linking. Panel B (bottom), DSS was used for cross-linking

The samples were run on SDS-PAGE and stained, and the migration positions of monomers and dimers are indicated in the figure. Multiple bands in cross-linked samples indicate that the SCAN monomers are being cross-linked into dimers by reaction of the cross-linking agents with different lysine residues on the surface of the monomers, leading to multiple dimeric species that migrate differently on SDS-PAGE due to differences in their effective hydrodynamic radii as a result of differences in their SDS denatured shapes. As annotated in the figure, the relative amounts of cross-linked dimers for each set of three samples were determined by densitometry, by comparing the amounts of mutant dimers relative to the wild-type dimers formed under identical conditions, as calculated from the integrated density of all the dimeric species present. Note the greatly attenuated calcium dependent cross-linking in the I170K and I170E mutants.

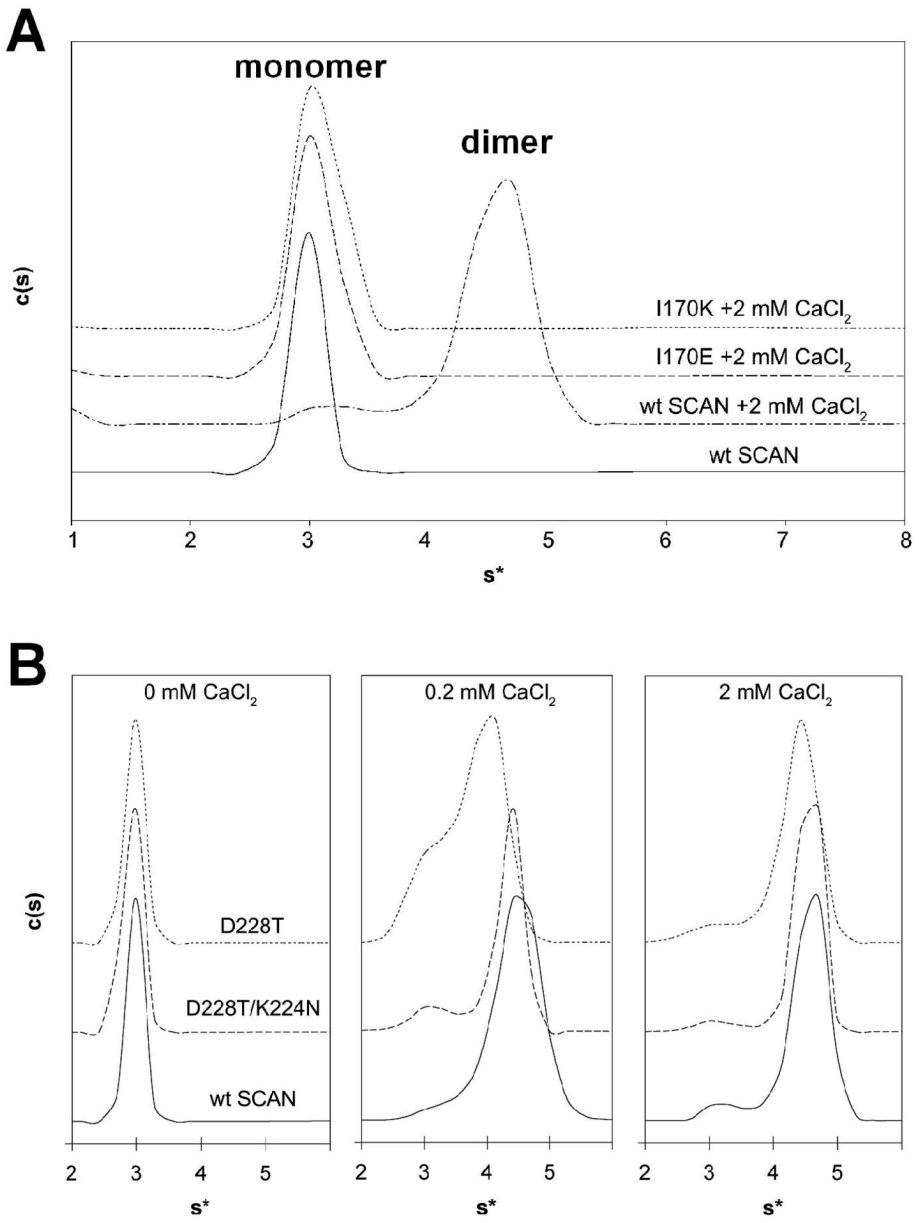

Figure 5. AUC sedimentation velocity analysis of dimerization of wild-type and mutant SCAN proteins

Panel A - Sedimentation coefficient distribution plots for wild-type SCAN and I170K and I170E mutants. Samples of wild-type SCAN in the absence of $CaCl_2$ and wild-type SCAN, I170E SCAN, and I170K SCAN in the presence of 2 mM $CaCl_2$ were centrifuged overnight at 48,000 at 20°C. **Panel B - Sedimentation coefficient distribution plots for wild-type SCAN and D228T and D228T/K224N mutants.** Each sample was analyzed in the presence of 0, 0.2, or 2 mM $CaCl_2$, with the data at each calcium concentration plotted separately for clarity. All samples in both panels were measured at a protein concentration of 0.85 μM in 20 mM MOPS buffer (pH 7.4).

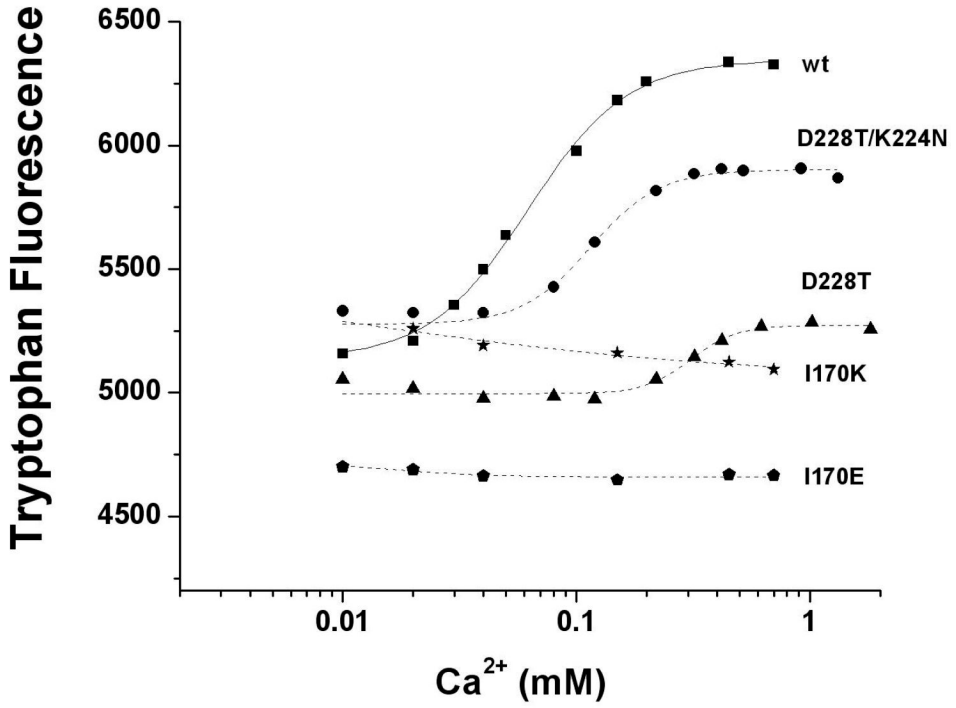

Figure 6. Dimerization, as monitored by calcium-induced increases in tryptophan fluorescence, does not occur in I170K or I170E SCAN mutants, and does occur in D228T and D228T/K224N mutants, but requires higher concentrations of calcium

The tryptophan fluorescence emission intensity was measured at 340 nm after excitation at 295 nm. The data points from wild-type (wt), D228T and D228T/K224N were fitted with a sigmoidal function to obtain EC_{50} values for calcium ion, which are 0.062 mM, 0.3 mM and 0.11 mM for wild-type, D228T, and D228T/K224N SCAN, respectively.

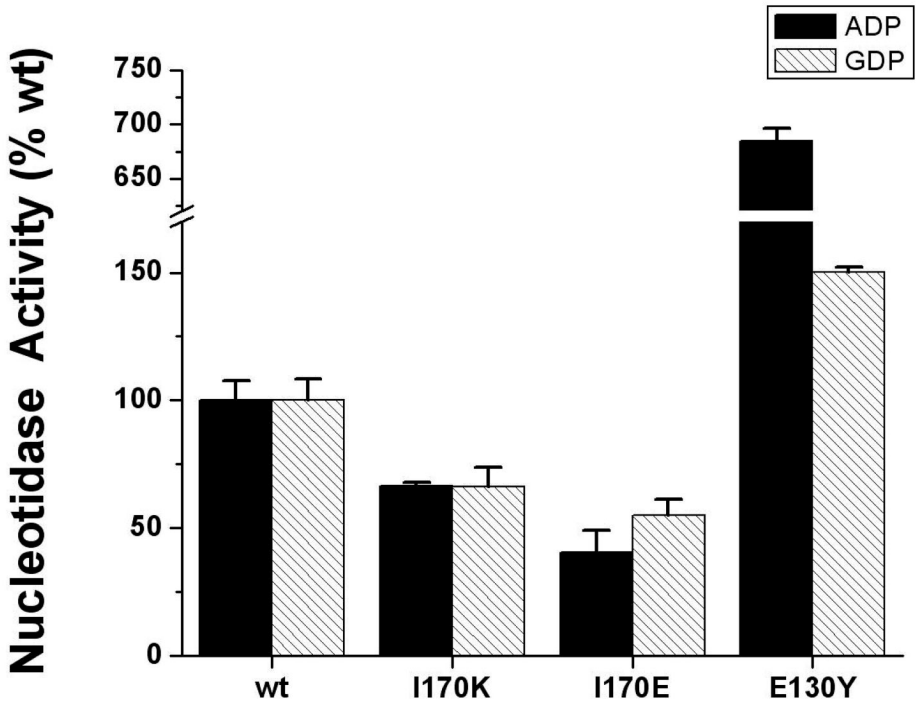

Figure 7. The Ca^{2+} dependent GDPase and ADPase activities of CAN mutations expressed as the membrane-bound forms in mammalian COS cells

The C30S full length CAN mutant was used as the "wild-type" background for the mutations. The malachite green phosphate assay (15) was employed in order to increase the sensitivity of the nucleotidase activity detection for the small amount of ADPase activity present. This assay requires a lower concentration of nucleotide to be used to yield a usable blank value. All assays were performed at 37°C, using a final nucleotide concentration of 2.5 mM for GDPase and 0.23 mM for ADPase, in the presence of 5 mM $CaCl_2$. Displayed data are corrected for the small background activities measured for COS cells transfected with the vector alone (pcDNA3), as well as for small differences in expression levels relative to those of "wild-type" (C30S) CAN, as determined by Western blotting. The average activity under these conditions for the crude COS cell membranes expressing the C30S "wild-type" CAN used as a background for these mutations was 137 μmol/mg/hr for GDPase and 0.77 μmol/mg/hr for ADPase.

Table 1

Circular Dichroism (CD) secondary structure analyses of purified SCAN proteins used in this study

The CD data, obtained in 20 mM MOPS buffer, pH 7.4, was analyzed by the K2D program (http://www.embl-heidelberg.de/~andrade/k2d/), and the results are given in the Table below, expressed as the % of the protein in alpha helix, beta sheet, and random coil secondary structures. The theoretical values, calculated from the wild-type SCAN crystal structure (crystals grown in the presence of Ca/Sr ions, and therefore dimeric) are: 6.4 % helix (combined α-helix and 3_{10} helix), 50.5 % β-sheet, and 43.2 % random coil. The PROF computer program predicted secondary structures based on the amino acid sequence of wt SCAN are: 11.5% α-helix, 44.5 % β-sheet, and 44% random coil.

SCAN protein	% α-helix	% β-sheet	% random coil
wt	11	42	47
I170K	10	44	46
I170E	9	45	46
D228T	12	39	50
D228T/K224N	11	40	48