Calcium-dependent Dimerization of Human Soluble Calcium Activated Nucleotidase
CHARACTERIZATION OF THE DIMER INTERFACE

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Mammals express a protein homologous to soluble nucleotidases used by blood-sucking insects to inhibit host blood clotting. These vertebrate nucleotidases may play a role in protein glycosylation. The activity of this enzyme family is strictly dependent on calcium, which induces a conformational change in the secreted, soluble human nucleotidase. The crystal structure of this human enzyme was recently solved; however, the mechanism of calcium activation and the basis for the calcium-induced changes remain unclear. In this study, using analytical ultracentrifugation and chemical cross-linking, we show that calcium or strontium induce noncovalent dimerization of the soluble human enzyme. The location and nature of the dimer interface was elucidated using a combination of site-directed mutagenesis and chemical cross-linking, coupled with crystallographic analyses. Replacement of Ile170, Ser172, and Ser226 with cysteine residues resulted in calcium-dependent, sulfhydryl-specific intermolecular cross-linking, which was not observed after cysteine introduction at other surface locations. Analysis of a super-active mutant, E130Y, revealed that this mutant dimerized more readily than the wild-type enzyme. The crystal structure of the E130Y mutant revealed that the mutated residue is found in the dimer interface. In addition, expression of the full-length nucleotidase revealed that this membrane-bound form can also dimerize and that these dimers are stabilized by spontaneous oxidative cross-linking of Cys30, located between the single transmembrane helix and the start of the soluble sequence. Thus, calcium-mediated dimerization may also represent a mechanism for regulation of the activity of this nucleotidase in the physiological setting of the endoplasmic reticulum or Golgi.

It was recently discovered that mammals, including rats (1) and humans (2), express proteins homologous to the nucleotidases used by blood-sucking insects, such as bed bugs (3), mosquitoes (4), and ticks (5). The mammalian enzymes exist as both membrane-bound forms in the endoplasmic reticulum and Golgi (1), as well as secreted, soluble forms (2). The physiological functions of these vertebrate nucleotidases are not yet well established, although they may play a role in protein glycosylation (1). In insect saliva, these soluble proteins are part of the anti-coagulation mixture injected at the site of skin puncture to keep the blood of the host from clotting, so that the insect can continue to feed. However, unlike the insect members of this family, the mammalian enzymes hydrolyze ADP very poorly (1, 2, 6), making control of platelet activation and subsequent blood coagulation an unlikely function for the mammalian enzymes, because ADP is a critical agonist for platelet activation. However, Dai et al. (7) were able to engineer the soluble human enzyme to hydrolyze ADP efficiently, by combining five point mutations based on their analysis of the crystal structure. Because of its substrate preference and subcellular localization, Failer et al. (1) postulated that the membrane-bound rat enzyme may function to support glycosylation reactions related to protein biosynthetic quality control in the endoplasmic reticulum.

These mammalian enzymes are termed calcium activated nucleotidases (CAN),3 as described in a recent review (8). Mammalian CAN enzymes were discovered less than 5 years ago, and there have been only a few publications characterizing these enzymes (1, 2, 6, 7, 9). However, the progress on the structure of the soluble form of this nucleotidase (SCAN) was rapid, culminating in the report of the crystal structure of the human enzyme in 2004 (7). Nonetheless, some questions remain, and alternative interpretations of the crystal structure have been presented (9), based on the lack of calcium ions during crystallization (7), because it was previously demonstrated that human SCAN is calcium-activated and undergoes a calcium-induced conformational change (6).

There have been no published reports of CAN dimerization. In fact, the size of human SCAN, determined by size exclusion chromatography, was reported to be consistent with a monomer in two previously published works from this laboratory (2, 6). In addition, sedimentation equilibrium experiments con-
Calcium Activated Nucleotidase Dimerization

ducted with 2 mM CaCl₂ in a buffer that also contained 150 mM NaCl indicated that SCAN was monomeric under these conditions (7). Thus, it was unexpected when the first data suggesting that this protein forms a dimer in solution were obtained.

In this study, we characterized the dimerization of human SCAN by size exclusion chromatography, analytical ultracentrifugation, chemical cross-linking, site-directed mutagenesis, and x-ray crystallography. It was observed that under appropriate solution conditions, this nucleotidase forms dimers in the presence of calcium and strontium, but not in the presence of magnesium ions. Dimers are also observed in the membrane-bound, full-length form of human SCAN expressed in mammalian COS cells. In COS cells, these dimers are partially disulfide-linked in the wild-type enzyme, mediated by a cysteine close to the N terminus that is not present in the soluble form of the enzyme.

The reason for the observed increased nucleotidase activities of a previously published mutation of SCAN (E130Y) (9) was not clear, given that this residue is far removed from the active site as identified by the crystal structure of the human enzyme co-crystallized with a nonhydrolyzable nucleotide analogue (7). However, a likely mechanism is suggested by the location of this residue in the dimer interface identified in the current study by cysteine replacement mutations combined with sulfhydryl-specific cross-linking, as well as crystallographic analyses.

The identified dimer interface has a more extensive buried surface area and better shape complementarity than other protein-protein interfaces observed in the crystal structure. Human SCAN has a five-bladed β-propeller fold (7). Our results indicate that the interface of the dimer is localized primarily within the 2d and 3d elements of the β-propeller and that the dimer interface is dominated by hydrophobic interactions.

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 cDNA constructs. Plasmid purification kits and nickel-nitrilotriacetic acid-agarose were purchased from Qiagen. The bacterial expression vector pET28a and the bacterial expression BL21(DE3) cells were purchased from Novagen. Glyceraldehyde and dialysis tubing were from Fisher. The chemical cross-linkers DSS, glutaraldehyde, BMH, and BMOE were purchased from Pierce, as were B-PER bacterial extraction reagents and the enhanced chemiluminescent reagents used for Western blots. The pre-cast SDS-PAGE 4–15% gradient mini-gels enhanced chemiluminescent reagents used for Western blots. The pre-cast SDS-PAGE 4–15% gradient mini-gels were obtained from Bio-Rad. Ampicillin, kanamycin, nucleotides, isopropyl-β-D-thiogalactopyranoside, glucose, DTT, and other reagents were from Sigma. Sephacryl S-100 used for size exclusion chromatography was obtained from Amersham Biosciences.

Site-directed Mutagenesis—A series of mutants of human SCAN have been designed and made to introduce cysteine residues at various surface locations, thus allowing analysis of the ability of these mutant proteins to be intermolecularly cross-linked into dimers. There is only one native cysteine (Cys²⁵⁷) in the sequence of human SCAN, and to simplify the interpretation of experiments, this residue (Cys²⁵⁷) was mutated to serine (C257S), to serve as the starting point for the cysteine substitution mutants. This “cysteine-less” C257S mutant has wild type-like activity and characteristics. Thus, most of the following mutations were made in this C257S background using the primers listed, with the substitution sites in bold type and underlined: C257S, 5'-CCATGAAGTCCTGCC-ACGTGGATGCAACGC-3'; S109C, 5'-CTGACCCTGTCA-AGCTGGATGCAACGC-3'; I170C, 5'-CAAGGCCCCTGG-CTTGCTGGATGCAACGC-3'; S278C, 5'-CCAGGACGCTACTTCGAGAAGGACGA-CCACG-3'; A287C, 5'-GGGAGCGCTACTTCGAGAAGGACGA-CCACG-3'; S226C, 5'-GTGCTGGCTAACTGTCGATTAAGACCTGGAGAAGGACGA-CCACG-3'; and E130Y, 5'-GTCACCCTGGGCGATTAAGACCTGGAGAAGGACGA-CCACG-3' (mutated in wild-type background). The complementary antisense oligonucleotides also necessary for the mutagenesis are not shown. The mutagenesis methodology was described previously (10) and used the QuikChange site-directed mutagenesis kit as described (11). The presence of the correct mutation and lack of unwanted mutations were confirmed by automated DNA sequencing.

Expression, Refolding, and Purification of SCAN Mutants in E. coli BL21(DE3) Bacterial Cells—The wild-type and mutant SCAN cDNA constructs were used to transform bacterial expression host BL21(DE3) cells (6), and after induction of expression with isopropyl-β-D-thiogalactopyranoside, bacterial inclusion bodies containing the SCAN proteins were isolated, and the protein was refolded and purified as described previously (6, 12). The only modification was that DTT was added during some stages of the purification of the refolded cysteine mutant proteins to avoid cysteine oxidation. Thus, after eluting from nickel-nitrilotriacetic acid beads that bind the N-terminal His₅ tag, the proteins were processed in buffer which includes 0.2 mM DTT. Even so, mutations that tend to efficiently oxidize spontaneously, such as S226C, still formed some intermolecular dimers during purification. DTT was also added to a final concentration of 0.2–0.3 mM after protein elution from the anion exchange cartridge. Prior to cross-linking experiments, residual DTT was eliminated or minimized by dialysis or by dilution and subsequent protein concentration using Amicon 30-kDa molecular mass cut-off concentrators. Protein concentrations were determined using the Bio-Rad G-250 dye binding technique according to the modifications of Stoscheck (13) or using calculated molar extinction coefficients for the 37,764–Da wild-type SCAN protein of 81,360 cm⁻¹ M⁻¹ at 280 nm and 82,850 cm⁻¹ M⁻¹ for the E130Y mutant, estimated by the ProtParam tool on the ExPASy server (us.expasy.org/tools/protparam.html).

Nucleotidase Assays—Nucleotidase activities were determined by measuring the amount of inorganic phosphate (Pi) released from nucleotide substrates in the presence of 5 mM Ca²⁺ at 37 °C using a modification of the technique of Fiske and Subbarow (14), as described previously (15). The enzyme assays were initiated by the addition of a final concentration of 2.5 mM nucleotide, except for the experiments measuring the depend-
ence of activity on SCAN protein concentration (see Fig. 7), where a final concentration of 4 mM ADP was used. The units used for enzyme activity are μmol of P$_i$ generated per mg of protein/h. Prior to assay, CAN protein was diluted into 50 mM Tris-HCl, pH 6.8, containing 0.1% Tween 20 to avoid adsorption to surfaces previously noted to occur when handling very dilute solutions of SCAN.

**Size Exclusion Chromatography**—Human wild-type SCAN protein was analyzed on a 28-cm-long, 11.5-ml bed volume Sephacryl S-100 column equilibrated in 20 mM MOPS, pH 7.4, with or without 2 mM CaCl$_2$, at a flow rate of 0.28 ml/min. The cross-linking reactions were carried out in 20 mM MOPS, 200-μl aliquots of various concentrations of SCAN protein in the column buffer were applied to the column, and the elution positions of the 280-nm peaks were noted.

**Chemical Cross-linking with Glutaraldehyde or DSS, a Hydrophobic, Lysine-specific Cross-linker**—Cross-linking stock solutions were always freshly prepared (in dry Me$_2$SO for DSS, in water for glutaraldehyde). Purified SCAN proteins (0.1 mg/ml) were incubated in 20 mM MOPS (pH 7.4) in the presence or absence of 5 mM CaCl$_2$, for 10 min at 22 °C in the presence or absence of DSS (250 μM) or glutaraldehyde (500 μM). Cross-linking was stopped by adding an excess (10 μM) of lysine and incubating for 5 min at room temperature. Finally, nonreducing sample buffer was added, and the samples were boiled for 5 min, run on SDS-PAGE, and analyzed either by Coomassie staining of the gel or by Western blot. When analyzed by Western blot, an affinity-purified anti-CAN anti-peptide antibody raised against the C-terminal sequence was used, and the bands were detected by enhanced chemiluminescence, as described previously (2).

**Analysis of Cysteine Substitution Mutants by Cross-linking Using the Hydrophobic, Sulfhydryl-specific Reagents BMH and BMOE**—Stocks of 20 mM BMH and BMOE were made in dry Me$_2$SO and stored at −80 °C. The final concentrations of these reagents used for cross-linking were varied from 6 to 50 μM. The cross-linking reactions were carried out in 20 mM MOPS, pH 7.4, at 22 °C, at a final protein concentration of 0.1 mg/ml, both in the presence and absence of 5 mM CaCl$_2$, BMH or BMOE (or Me$_2$SO used as a vehicle control) was added and incubated for 10 min at 22 °C. The cross-linking reactions were stopped by adding an excess of cysteine and incubating for 5 min prior to adding nonreducing SDS-PAGE sample buffer. After heating the samples for 10 min at 60 °C, SDS-PAGE was performed as described above.

**Calcium-induced Change in the Tryptophan Fluorescence of SCAN as a Function of Protein Concentration**—The increase in tryptophan fluorescence (excitation at 295 nm, emission at 340 nm) was measured after a single addition of CaCl$_2$ to a final Ca$^{2+}$ concentration of 2 mM in 20 mM MOPS buffer, pH 7.4 (6). The protein concentration was varied from a low near the sensitivity limit of the instrument, to a high at approximately the concentration where inner filter effects and other nonideal fluorescence effects are observed. To allow comparison with previous studies and to the current analytical ultracentrifugal studies, the experiments were performed in the presence and absence of 50 mM NaCl.

**Analytical Ultracentrifugation Analysis**—Sedimentation velocity and equilibrium experiments were performed at 20 °C in a Beckman XL-I ProteomeLab analytical ultracentrifuge equipped with an absorbance optical system as described before (16). For these experiments, two different buffers were used; Buffer A (20 mM MOPS, pH 7.4) is identical to that used previously to monitor absorbance and fluorescence changes induced by Ca$^{2+}$ (6) and very similar to that used for nucleotidase activity assays, whereas Buffer B (20 mM MOPS, pH 7.4, 50 mM NaCl) is designed to minimize thermodynamic nonideality caused by charge-charge interactions during sedimentation (Table 1). For sedimentation velocity experiments, 0.85 μM wild-type SCAN was analyzed in the absence and the presence of a divalent cation (2 or 20 mM MgCl$_2$, 2 mM CaCl$_2$ or SrCl$_2$) or in the presence of monovalent cation (150 or 300 mM NaCl) at 48,000 rpm. Sedimentation coefficient distributions were determined using the program Sedfit (17). For sedimentation equilibrium experiments, the wild-type and E130Y mutant SCANS were analyzed in the absence and the presence of 0.2 mM or 2 mM CaCl$_2$. Samples at three concentrations (0.25, 1.25, and 2.50 μM) in Buffer A were centrifuged at three different speeds (15,000, 18,000, and 26,000 rpm), whereas samples in Buffer B at two protein concentrations (1.25 and 2.50 μM) were centrifuged at five different speeds (15,000, 18,000, 21,000, 25,000, and 36,000 rpm). For each calcium concentration, a global analysis of the resultant nine or ten data sets was performed using the program WinNONLIN (Jeff Lary, University of Connecticut, Storrs, CT).

**Full-length CAN Expression in Mammalian COS Cells**—COS-1 cells were transfected with 4 μg of plasmid DNA encoding wild-type or mutant CAN proteins/100-mm plate using Lipofectamine and PLUS reagents as described previously (10). Transfection with an empty pcDNA3 vector was also performed as a control. Approximately 48 h post-transfection, the COS-1 cells were harvested, and the crude cell membrane preparations were obtained as described (10). In some experiments, the alkylating agent, N-ethylmaleimide, was added to the buffer used to homogenize the crude COS cell membranes (final concentration, 2 mM) to prevent oxidation of free sulfhydryls to disulfides during membrane preparation.

**Crystallization and Structure Determination of Wild-type and E130Y Human SCAN**—Crystallization was performed using a sitting drop vapor diffusion method at 293 K in 96-well plates. The crystallization conditions for both wild-type and E130Y SCAN were very similar to that of the wild-type protein crystallized with strontium (7). For wild-type SCAN, 1 μl of protein solution (5.9 mg/ml in 20 mM MOPS, pH 7.4, and 0.1 mM NaCl) was mixed with 1 μl of reservoir solution (8% polyethylene glycol 4000, 0.2 M ammonium sulfate, 0.1 M sodium acetate, pH 4.8, and 10 mM CaCl$_2$) and equilibrated against 100 μl of reservoir solution. For the E130Y mutant, 1 μl of protein solution (4.9 mg/ml in 20 mM MOPS, pH 7.4 and 0.1 M NaCl) was mixed with 1 μl of reservoir solution (16% polyethylene glycol 4000, 0.2 M ammonium sulfate, 0.1 M sodium acetate, pH 4.8, and 10 mM CaCl$_2$) and equilibrated against 100 μl reservoir solution. The crystals were cryoprotected by transfer to a mother-liquor solution containing 15% glycerol and flash frozen in a cold nitrogen stream maintained at 110 K. For the wild-type
CaCl$_2$. Additional experiments were carried out in the presence of 0.2 mM Ca$^{2+}$ to investigate the monomer-dimer equilibrium for wild-type SCAN and the E130Y super-activity mutant (9) in the absence and presence of Ca$^{2+}$, we also performed sedimentation equilibrium experiments (Figs. 3 and 4). The apparent $K_D$ values for formation of the dimer were calculated by global fitting of the sedimentation equilibrium data and are listed in Table 1. The data for wild-type and E130Y mutant SCAN in the presence of 0.2 mM Ca$^{2+}$ at five different speeds are shown in Fig. 3, indicating that the fitted curves using the parameter values in Table 1 correspond closely to the experimental data. Human SCAN in buffer A (20 mM MOPS, pH 7.4, similar to the buffer used for activity assays) dimerized with an apparent $K_D$ of 0.170 mM in the presence of 2 mM CaCl$_2$. Additional experiments were carried out in the presence of 50 mM NaCl (buffer B) to ensure thermodynamic ideality and mitigate potential electrostatic effects. Human SCAN also dimerizes in a calcium-dependent manner in buffer B (20 mM MOPS, pH 7.4, containing 50 mM NaCl), although the dimerization was weaker (apparent $K_D$ of 0.563 mM in the presence of 2 mM CaCl$_2$).

**RESULTS**

Unexpectedly, the apparent size of SCAN, as indicated by the elution position during size exclusion chromatography, varies as a function of the protein concentration loaded onto the column. This was true in the presence of 2 mM CaCl$_2$ (Fig. 1), but not in its absence (where SCAN eluted at the same position, regardless of amount loaded, as indicated in Fig. 1 by the boxed data point artificially set at 0.001 μM). This suggested the possibility of oligomer formation and prompted analyses of this phenomenon by other techniques.

Analytical ultracentrifugation analyses of pure, human SCAN protein indicate that this soluble nucleotidase forms dimers in a divalent cation-dependent fashion. Fig. 2 depicts sedimentation coefficient distribution plots from four sedimentation velocity experiments, demonstrating a significant shift in the sedimentation coefficient of human SCAN protein from 3.0 to 4.7 S in the presence of 2 mM Ca$^{2+}$ or Sr$^{2+}$. The sedimentation shift occurs specifically with these two divalent cations; no shift occurs in the presence of either 2 or 20 mM Mg$^{2+}$ or in 150 or 300 mM Na$^+$. The estimated mass of the 4.7 S peak is consistent with a SCAN dimer (the calculated monomeric mass is 37,764 Da for the monomer and 75,528 Da for the dimer). To investigate the monomer-dimer equilibrium for wild-type SCAN and the E130Y super-activity mutant (9) in the absence and presence of Ca$^{2+}$, we also performed sedimentation equilibrium experiments (Figs. 3 and 4). The apparent $K_D$ values for formation of the dimer were calculated by global fitting of the sedimentation equilibrium data and are listed in Table 1. The data for wild-type and E130Y mutant SCAN in the presence of 0.2 mM Ca$^{2+}$ at five different speeds are shown in Fig. 3, indicating that the fitted curves using the parameter values in Table 1 correspond closely to the experimental data. Human SCAN in buffer A (20 mM MOPS, pH 7.4, similar to the buffer used for activity assays) dimerized with an apparent $K_D$ of 0.170 mM in the presence of 2 mM CaCl$_2$. Additional experiments were carried out in the presence of 50 mM NaCl (buffer B) to ensure thermodynamic ideality and mitigate potential electrostatic effects. Human SCAN also dimerizes in a calcium-dependent manner in buffer B (20 mM MOPS, pH 7.4, containing 50 mM NaCl), although the dimerization was weaker (apparent $K_D$ of 0.563 mM in the presence of 2 mM CaCl$_2$).
The monomer and dimer fractions of wild-type and E130Y SCAN under various conditions are illustrated in Fig. 4. Wild-type SCAN forms negligible levels of dimer in the absence of Ca\(^{2+}\) (upper left panel). However, the super-active mutant, E130Y, does form low levels of dimer even in the absence of added Ca\(^{2+}\), at micromolar protein concentrations (lower left panel). At an intermediate Ca\(^{2+}\) concentration (0.2 mM, center panels), the discrepancy in the relative tendency to form dimers between the wild-type enzyme and the E130Y super mutant is obvious (apparent \(K_D\) values of 41.9 \(\mu\)M for wild-type SCAN and 1.69 \(\mu\)M for E130Y (see Table 1), with excellent fitting statistics.

The E130Y mutant results suggest that residue Glu130 is important for dimer formation and stabilization and that therefore this might be a part of a dimer interface. To further guide our experimental approach for investigating the dimeric contact areas, we analyzed protein-protein crystal packing interfaces of the human SCAN crystal structure (7) to identify possible sites of dimerization. Residue Glu130 participates in a crystal packing interaction, suggesting a potential site for the dimer interface. This region is a reasonable candidate for the dimer interface, based on its having larger buried surface area and better shape complementarity than other crystal packing interfaces, as described below.

Based on all of the analyses described above, several cysteine substitution mutations were made to experimentally test the putative dimer interface. The GDPase and ADPase specific activities for several mutants are listed in Table 2, including: potential dimer interface mutations (I170C, S172C, and S226C), negative control mutations outside the putative interface (S109C, S278C, and A287C), and the E130Y super-active mutant. None of these cysteine mutations had substantial deleterious effects on the nucleotidase activities of the refolded and purified proteins. All of these mutations were screened for their ability to be intermolecularly cross-linked in a calcium-dependent manner using cysteine-specific reagents. Mutants with cysteine residues that are in the proper orientation in the dimer interface regions are expected to be cross-linked under conditions promoting dimerization (i.e. in the presence of Ca\(^{2+}\)), but not under conditions favoring monomers (in the absence of Ca\(^{2+}\)). The results shown in Fig. 5 are consistent with the computationally predicted dimer interface region; the I170C, S172C, and S226C, negative control mutations outside the putative interface (S109C, S278C, and A287C), and the E130Y super-active mutant. None of these cysteine mutations had substantial deleterious effects on the nucleotidase activities of the refolded and purified proteins. All of these mutations were screened for their ability to be intermolecularly cross-linked in a calcium-dependent manner using cysteine-specific reagents. The presence of many lysine residues in SCAN, lysine-specific intermolecular cross-linking was found to be less efficient than expected using either glutaraldehyde or DSS.
Calcium Activated Nucleotidase Dimerization

However, the divalent cation specificity of the dimer formation was demonstrated by cross-linking the wild-type SCAN using the lysine-specific cross-linking agent, DSS. Fig. 6 shows that DSS cross-linking occurs in the presence of Ca$^{2+}$ or Sr$^{2+}$, but not in the presence of Mg$^{2+}$. Thus, these lysine cross-linking data are consistent with the sedimentation velocity data shown in Fig. 2 and with the earlier observation that of all the divalent cations tested, only Ca$^{2+}$ and Sr$^{2+}$ can induce the changes seen in the absorbance and fluorescence of human SCAN protein (9).

To help integrate the present dimerization findings with previously published data demonstrating a fairly large increase in tryptophan fluorescence upon Ca$^{2+}$ binding, the effect of Ca$^{2+}$ on the tryptophan fluorescence was measured as a function of SCAN concentration, both in the presence and absence of 50 mM NaCl. As is evident from Fig. 7, the relative percentage increase in fluorescence upon the addition of 2 mM Ca$^{2+}$ increases with increasing protein concentration, and this effect is shifted to higher protein concentrations by the inclusion of 50 mM NaCl in the 20 mM MOPS buffer used in this and previous SCAN fluorescence studies (6, 9). The dependence of the fluorescence increase on SCAN protein concentration indicates that the fluorescence change is to a large degree because of SCAN dimerization. Furthermore, the protein concentration dependence of the fluorescence changes in both 0 and 50 mM NaCl are consistent with the dimerization $K_D$ values determined by analytical ultracentrifugation (Table 1). To determine whether dimerization, as monitored by the protein concentration dependence of the Ca$^{2+}$ induced fluorescence increase, affects the activity of SCAN, the ADPase activity (in 5 mM Ca$^{2+}$) was measured as a function of protein concentration, and the data obtained from the average of three such experiments is shown in Fig. 7. The ADPase specific activity is shown to increase with increasing SCAN protein concentration in a fashion similar to that observed for the Ca$^{2+}$-induced increase in fluorescence (Fig. 7).

To assess the potential in vivo relevance of calcium-dependent SCAN dimerization, the ability of COS cell-expressed, membrane-associated, full-length CAN protein to form dimers was also investigated. The COS-cell expressed, membrane-bound CAN can also be cross-linked with lysine-specific reagents such as glutaraldehyde, as shown in lanes 2 and 3 of Fig. 8. In addition, it was noted that the full-length CAN wild-type protein spontaneously formed disulfide-linked dimers, as evidenced by the dimeric size noted for some of the wild-type CAN protein on Western blots after nonreducing SDS-PAGE (lane 5). Thus, in Fig. 8 two bands are visible upon immunoblotting of the wild-type protein after nonreducing SDS-PAGE, a lower band of ~45 kDa (a glycosylated monomer) and an upper band of ~90 kDa (a dimer; Fig. 8, lane 5). This disulfide-linked dimer forms naturally in the COS cell by oxidation, rather than being formed artificially during COS membrane preparation, because unlike a similar dimer noted for nucleoside triphosphate diphosphohydrolase 3 nucleotidase (19), the disulfide-linked CAN dimer was not eliminated by inclusion of the alkylating reagent, N-ethylmaleimide, in the membrane preparation buffers (data not shown).

There are only two cysteine residues present in full-length human CAN: Cys$^{257}$, which according to the crystal structure is relatively inaccessible in a cleft in the soluble portion of the molecule, and Cys$^{390}$, which is located just after the transmembrane signal sequence, but before the start of the soluble sequence, and thus is removed by signal peptide cleavage and not present in the soluble form. To determine whether this Cys$^{390}$ residue is responsible for the spontaneous disulfide cross-linking of the full-length CAN, it was mutated to serine. The resultant C30S CAN enzyme has wild-type-like nucleotidase activity.

### TABLE 1

| Buffer                  | Dimerization constant (95% CI) in μM | Wild-type SCAN | E130Y SCAN |
|-------------------------|-------------------------------------|----------------|------------|
| 20 mM MOPS 7.4 + 0.2 mM CaCl₂ | 0.992 (0.670–1.44) |                |            |
| 20 mM MOPS 7.4 + 2 mM CaCl₂   | 0.170 (0.109–0.259) |                |            |
| 20 mM MOPS 7.4 + 20 mM CaCl₂  | 0.142 (0.086–0.228) |                |            |
| 20 mM MOPS 7.4 + 2 mM SrCl₂    | 0.0306 (0.0146–0.0595) |              |            |
| 20 mM MOPS 7.4/50 mM NaCl + 0 mM CaCl₂ | 116.0 (33.3–906.0) | 11.8 (9.34–14.9) |            |
| 20 mM MOPS 7.4/50 mM NaCl + 0.2 mM CaCl₂ | 41.9 (31.4–56.5) | 1.69 (1.33–2.13) |            |
| 20 mM MOPS 7.4/50 mM NaCl + 2 mM CaCl₂ | 0.563 (0.384–0.799) | 0.179 (0.107–0.283) |            |

### TABLE 2

| Human SCAN protein | Residue number in crystal structure | Surrounding sequence (mutated residue in bold and underlined) | Mutation in putative dimer interface | Ca$^{2+}$-GDPase activity (μmol/mg/h) | Ca$^{2+}$-ADPase activity (μmol/mg/h) |
|--------------------|------------------------------------|-------------------------------------------------------------|-------------------------------------|-------------------------------------|--------------------------------------|
| Wild type          | 69                                 | TLSDGIRDKEV                                                   | No                                  | 122,000                             | 2,150                                |
| S109C              | 90                                 | SHLAEKGRG                                                   | Yes                                 | 72,000                              | 1,220                                |
| E130Y              | 130                                | VPWELDSIDID                                                | Yes                                 | 247,000                             | 13,600                               |
| I170C              | 132                                | WVLIDDEGGD                                                 | Yes                                 | 62,000                              | 850                                  |
| S172C              | 186                                | CYKGGVSYHNE                                               | Yes                                 | 69,000                              | 1,430                                |
| C257S              | 217                                | HESAWSDEFT                                                | Yes                                 | 110,000                             | 2,030                                |
| S278C              | 238                                | QERYSEKDD                                               | No                                  | 91,000                              | 1,640                                |
| A287C              | 247                                | ERRGDSLLL                                                 | No                                  | 90,000                              | 1,110                                |
|                    |                                    |                                                              |                                      | 71,000                              | 1,570                                |
activity (not shown). As seen in the nonreducing SDS-PAGE Western portion of Fig. 8 (lane 6), elimination of this cysteine residue resulted in loss of formation of the disulfide dimer of COS cell-expressed CAN, establishing Cys\textsuperscript{30} as the residue involved in the formation of this naturally occurring covalent dimer. However, the C30S mutant can be cross-linked to form a dimer by glutaraldehyde just like the wild-type protein (lane 3), which indicates that noncovalent dimer formation in the membrane-bound, full-length CAN is not dependent on the presence of this disulfide bond but is likely stabilized by the Cys\textsuperscript{30} disulfide bond. It is also clear that the Cys\textsuperscript{30} intermolecular disulfide bond is not required for CAN activity or expression.

To analyze the molecular details of the dimerization interface, wild-type and E130Y SCAN proteins were crystallized in the presence of 5 mM CaCl\textsubscript{2}, and their structures were solved by molecular replacement using the published coordinates of SCAN crystallized with strontium (7) (Fig. 9 and Table 3). Both structures (Protein Data Bank codes 2H2N and 2H2U) closely resemble the previous SCAN structures (Protein Data Bank codes 1S18 and 1S1D), with root mean square deviations of 0.44 and 0.53 Å over 314 residues for wild type and E130Y, respectively. The nucleotidase activity of SCAN as a function of protein concentration, using 4 mM ADPase as substrate, is also shown (filled squares).
Calcium Activated Nucleotidase Dimerization

TABLE 3
SCAN crystallographic data collection and refinement statistics

| Source | Wild type (Protein Data Bank 2H2N) | E130Y (Protein Data Bank 2H2U) |
|--------|-----------------------------------|--------------------------------|
| Rigaku RA-Micro007 | APS SER-22ID |
| 1.54178 | 0.97182 |
| P1 | P1 |
| 43.09, 52.37, 77.43 | 42.89, 52.41, 77.81 |
| 75.06, 74.52, 79.47 | 100.94, 106.51, 99.32 |
| 31.08-2.30 (2.38-2.30) | 50.00-2.40 (2.49-2.40) |
| 99.7 (99.3) | 86.8 (83.8) |
| 150511/27727 | 36128/24181 |
| 5.43 (5.13) | 1.70 (1.50) |
| 4.9 (4.9) | 3.2 (2.81) |
| 5.8 (17.8) | 6.5 (45.1) |
| 31.08-2.30 | 50.00-2.40 |
| 26332/1395 | 19868/1092 |
| 16.8/24.9 | 21.3/29.9 |
| 0.027 | 0.012 |
| 2.136 | 1.488 |
| 317/315 | 315/317 |
| 22/24 | 24/22 |
| 228 | 35 |
| 2 | 2 |
| 1 | 0 |
| 486 residues, 89.0% | 466 residues, 85.3% |
| 60 residues, 11.0% | 76 residues, 13.9% |
| 0 residues, 0% | 4 residues, 0.7% |

The values in parentheses refer to the highest resolution shell.

**Rmerge** = \[ \sqrt{\Sigma |I_i - \langle I_i \rangle|/\Sigma I_i} \]

**Rfree** = \[ \sqrt{\Sigma |I_i - \langle I_i \rangle|/\Sigma I_i} \]

The side chain of Tyr^{130} in the E130Y mutant was clearly visible in simulated annealing electron density omit maps, adopting a preferred rotamer position (Fig. 9B). As described above, residue 130 is found in the dimer interface. However, comparison of the wild-type and E130Y dimer interfaces reveals only subtle differences. The E130Y mutant shows a very slight increase in buried surface area in the dimer interface (from 688 to 706 Å²), although the surface complementarity is slightly worse compared with wild type (S_c = 0.723 versus 0.685). However, the E130Y mutant shows a 12% increase in the number of hydrophobic contacts within 5 Å. Other slight changes in the E130Y dimer include loss of the salt bridge observed in wild-type SCAN, gain of two long range electrostatic interactions, and only seven hydrogen bonds compared with 12 for the wild-type enzyme.

Based on the site-directed mutagenesis results and the crystal structures of wild-type and E130Y SCAN, we present the structural model of the SCAN dimer in Fig. 9C. Indicated in the figure are the locations of the mutations in the dimer interface region (I170C, S172C, and S226C), which are cross-linked to dimers in a Ca^{2+}-dependent fashion, as well as the S109C, S278C, and A287C mutants, which are located distant to the dimer interface and are not cross-linked to dimers in a Ca^{2+}-dependent fashion. In addition, the location of Glu^{130}, whose mutation to tyrosine dramatically increases enzyme activity (9) and facilitates SCAN dimerization (Fig. 4), is shown.

The most likely tryptophan residues involved in generating the increase in fluorescence upon dimerization (Fig. 7) are those at or near the interface. Dimerization would be expected to create a more hydrophobic local environment, thereby increasing the fluorescence yield of the affected tryptophans. Analysis of the crystal structure suggests that residue Trp^{128} is directly in the dimer interface, whereas Trp^{55} would also be expected to show an increase in its fluorescence yield because it would become less solvent accessible upon SCAN dimerization (the tryptophan numbers above are the crystal structure residue numbers of SCAN, and correspond to Trp^{168} and Trp^{95} in the amino acid sequence of CAN).
FIGURE 9. Crystal structures of wild-type and E130Y human SCAN dimers. A, crystal structure of the dimer of human SCAN crystallized in ammonium sulfate (see "Experimental Procedures"). The dimer interface is located along a 2-fold noncrystallographic symmetry axis such that residues primarily from the 1d, 2d, and 3d strands from each protomer interact across the interface. B, chemical nature of the dimer interface in the E130Y mutant, shown looking through molecule A at the surface of molecule B. The side chains and dimer interface surface are colored according to the chemical nature of the residues that interact in the dimer: yellow for hydrophobic, green for polar, blue for positive, and red for negative residues. Inset, a zoomed view of the region around the E130Y mutation, showing a simulated annealing omit map for residue Tyr130 in both protomers. C, a schematic of full-length dimeric SCAN in the membrane-associated form, including residues discussed in this study. Residues implicated in formation of the dimer interface are shown as colored spheres, as follows: Glu130, red; Ile170, blue; Ser172, cyan; and Ser226, purple. Residues mutated to cysteine that did not display calcium-dependent cross-linking are shown in gray (Ser109, Ser278, and Ala137). The putative position of Cys20, the residue in full-length SCAN that forms an intermolecular disulfide bridge near the membrane, is also indicated in yellow, and the endoplasmic reticulum (ER) membrane is depicted as a gray rectangle.

DISCUSSION

In this study we examined the characteristics of dimer formation observed for the soluble and membrane-bound forms of the human CAN protein. The fact that this protein forms dimers at all was unexpected, because we had previously characterized SCAN as a monomeric enzyme by size exclusion gel chromatography (2, 6), and Dai et al. (7) also indicated that SCAN was monomeric utilizing sedimentation equilibrium experiments. The reasons for these apparent discrepancies only become evident upon examination of the experimental details of the previous studies. In the work examining the soluble, secreted SCAN expressed by mammalian COS cells, the size exclusion chromatography used to examine the apparent size of the protein was performed in a buffer containing 5 mM Ca2+ and 100 mM NaCl (2). The concentration of the SCAN protein applied to the column was very low because of the very small amount of CAN produced by COS cell expression, and the presence of 100 mM NaCl would also attenuate any dimer formation. The apparent Kd for dimerization of wild-type SCAN protein in saturating Ca2+ (2 mM) is 0.170 μM in the absence of NaCl and 0.563 μM in the presence of 50 mM NaCl (Table 1), and we estimate that the concentration of SCAN protein in the sample applied to the column in that earlier study was subnanomolar. Therefore, the large majority of the protein should have been monomeric, as was observed. Also, in our earlier work examining the apparent size of the bacterially expressed SCAN (6), size exclusion chromatography was performed in the absence of Ca2+, under which conditions we show in the present study that wild-type SCAN does not form dimers, even at very high protein concentrations (Figs. 2 and 4). Dai et al. performed their equilibrium sedimentation experiments in a buffer containing 2 mM CaCl2. However, the buffer also contained 150 mM NaCl, and thus the dimerization Kd would be well above the 0.563 μM measured in 50 mM NaCl, leading to the likelihood of their samples being mostly monomeric under those conditions. Thus, these apparent discrepancies with past findings are readily explained in light of our current results.

The dimerization of SCAN is supported by the presence of Ca2+ and Sr2+, but not by Mg2+ (Fig. 2). In this respect, the dimerization mimics the conformational change observed by monitoring the UV absorbance or tryptophan fluorescence of SCAN as a function of Ca2+ (or Sr2+) concentration (6). Ca2+ and Sr2+ are also the only divalent cations that protect SCAN from limited proteolysis and support binding to Cibacron blue (nucleotide analogue) matrices (9). As seen in Fig. 7, 6–8% of the 35–40% total fluorescence increase observed upon the addition of 2 mM Ca2+ is independent of protein concentration, and presumably is due to local conformational changes upon calcium binding. Interestingly, the remaining ~30% increase shows a protein concentration dependence that is consistent with the dimerization constants determined by analytical ultracentrifugation. Specifically, the EC50 values from the fluorescence data in Fig. 7 are 0.074 or 0.217 μM in the absence or presence of NaCl, respectively; assuming the transition is due to dimerization, these
Calcium Activated Nucleotidase Dimerization

would be equivalent to $K_{D}$ values of 0.148 or 0.434 $\mu$M, which are within the 95% confidence level limits of the dimerization $K_{D}$s determined by sedimentation (0.170 or 0.563 $\mu$M; Table 1). Therefore, it appears that the concentration-dependent fluorescence change in SCAN upon calcium binding is due to SCAN dimerization. In addition, it seems likely that the 6–8% increase in fluorescence upon the addition of Ca$^{2+}$ that is independent of protein concentration may represent a conformational change in the monomer that is necessary to allow dimer formation. Also, the dependence of the ADPase activity increase on SCAN protein concentration is very similar to the dependence of the Ca$^{2+}$-induced increase in tryptophan fluorescence on protein concentration (Fig. 7), indicating that that the dimeric form of SCAN has greater nucleotidase activity than the monomeric form. However, because of the limitations of the enzyme assays used to measure both very low and very high activities, quantitative estimates of the $EC_{50}$ values for the activity data were not made because of the lack of data points that would define the lower and upper plateaus of the putative sigmoidal curve (Fig. 7, filled squares).

The elucidated SCAN dimer interface shown in Fig. 9 is consistent with all of the experimental data and crystallographic analyses performed in this study. It also helps explain earlier data obtained using the E130Y mutant SCAN protein. This E130Y mutant was originally made and characterized because it was observed that this residue is a glutamic acid in all the vertebrate CAN enzymes, although it is a hydrophobic amino acid (Ala, Leu, Phe, or Tyr) in the blood-sucking insect enzymes (9). Thus, one of the purposes of that earlier study was to make the human enzyme more like the insect enzyme in terms of its ability to hydrolyze ADP, because ADP is the agonist for platelet activation and blood clotting. Indeed, the E130Y mutation increased the ADPase activity of SCAN 5-fold, and decreased the amount of Ca$^{2+}$ needed to induce the conformational change by $\sim$2-fold (9). Because this residue is not in the active site, based on the crystal structure (7), it was not clear how this mutation generated its effects. The present study suggests a likely mechanism; this mutation allows dimerization to occur at lower protein and Ca$^{2+}$ concentrations. As described above, replacement of glutamic acid with tyrosine at this position creates a more hydrophobic dimer interface and buries slightly more surface area, resulting in a more energetically favored dimer. The analytical ultracentrifugation data presented here, combined with the prior activity assays on this mutant, suggest that the dimeric form of SCAN has a higher nucleotidase activity than the monomer, consistent with the activity data shown in Fig. 7. Furthermore, the crystal structure of the E130Y mutant revealed that none of the residues implicated in catalysis show any significant changes in the mutant compared with wild-type SCAN, supporting the hypothesis that higher activity is conferred by dimerization rather than significant alterations in the active site. In addition, it is interesting to postulate that the enzymatic differences between the blood-sucking insect and the mammalian members of the CAN family of nucleotidases that are critical for differential biological function might be due, at least in part, to the differential extent of dimerization of the insect and mammalian SCAN proteins. Specifically, the ability of the insect enzymes to efficiently hydrolyze ADP allows an anti-coagulant function, whereas the very poor hydrolysis of ADP observed for the mammalian enzymes rules out an anti-coagulant function in mammals (8). This hypothesis could be tested in the future by mutagenesis of amino acids in the dimer interface of the insect enzymes and measurement of the effects on ADP hydrolysis and dimer formation of the insect enzymes.

To examine whether the dimerization observed for the soluble form also occurs in the membrane-bound form found inside cells, we examined the COS cell-expressed CAN protein by cross-linking and site-directed mutagenesis. This full-length form also forms dimers as demonstrated by cross-linking (Fig. 8), and the wild-type enzyme forms disulfide-linked dimers spontaneously, which are mediated via oxidation of Cys$^{30}$. This cysteine residue occurs just after the single transmembrane helix in the protein and is not present in the soluble form. It is postulated that this disulfide bond, together with putative intermolecular interactions between CAN transmembrane helices, will increase the local concentration of the membrane-anchored form of CAN such that the catalytic domains will be easily within the range required for dimerization to occur. Because the endoplasmic reticulum lumen is an oxidizing environment (22), it seems likely that the full-length CAN membrane-bound dimer will be, at least in part, stabilized by a disulfide bond between Cys$^{30}$ residues. However, the oligomeric state of CAN would also be modulated by the local concentration of Ca$^{2+}$. In fact, analysis of the I170C mutation in the full-length, COS cell-expressed C30S CAN protein revealed that although the protein could be cross-linked by sulfhydryl specific agents in the absence of Ca$^{2+}$, the cross-linking efficiency is substantially increased in the presence of Ca$^{2+}$ (data not shown). This result suggests that the identified dimerization interface of the soluble portion of SCAN is indeed important for the membrane bound form, but it is not the only structural component involved in dimer formation of the membrane-associated form. It seems likely that the trans-membrane helix of each monomer would also be important and would result in a more stable dimer relative to the soluble form.

The rat homologue of CAN exists in both the endoplasmic reticulum and the Golgi (1). Ca$^{2+}$ ion concentrations in the lumen of the endoplasmic reticulum vary from $\sim$100 to 800 $\mu$M (23), whereas the concentrations of calcium in the Golgi are higher, $\sim$1–2 mM (24). Thus, it seems plausible that the activity and oligomeric state of CAN may be modulated by changes in Ca$^{2+}$ concentrations in the endoplasmic reticulum, whereas in the high Ca$^{2+}$ concentrations present in the Golgi, the CAN enzyme may be always highly active. These changes in activity and dimerization associated with Ca$^{2+}$ concentration changes may regulate enzyme activity and its putative function in glycosylation reactions (1). Furthermore, the results presented here on the E130Y mutant raise the possibility of engineering stable dimeric forms of human SCAN for effective therapeutic use in platelet inhibition.
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