Crystal Structure of CC3 (TIP30)

IMPLICATIONS FOR ITS ROLE AS A TUMOR SUPPRESSOR*[S]

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CC3 (TIP30) is a protein with pro-apoptotic and anti-metastatic properties. The tumor suppressor effect of CC3 has been suggested to result from inhibition of nuclear transport by binding to importin β or by regulating transcription through interaction in a complex with co-activator independent of AF-2 function (CIA) and the c-myc gene. Previous biochemical studies indicated that CC3 has protein kinase activity, and a structural similarity to cAMP-dependent protein kinase catalytic subunit was proposed. By contrast, bioinformatics studies suggested a relationship of CC3 to the short chain dehydrogenase reductase family. To clarify details of the CC3 structural family and ligand binding properties, we have determined the crystal structure of CC3 at 1.7-Å resolution. CC3 has a short chain dehydrogenase reductase fold and binding specificity for NADPH, yet it is unlikely to be normally enzymatically active because it is monomeric. These structural results, in conjunction with data from earlier mutagenesis work on the nucleotide binding motif, suggest that NADPH binding is important for the biological activity of CC3, including interaction with importins and with the CIA/c-myc system. CC3 provides an example of the adaptation of a metabolic enzyme fold to include a regulatory role, as also seen in the case of the NADH-binding co-repressor CtBP.

Apoptosis or programmed cell death plays an opposing role to cell division in the homeostatic regulation of animal cell populations. As well as being of significance in development, apoptosis has an important place in the pathogenesis of some disease states. Mutated and deleted pro-apoptotic genes can give rise to carcinogenesis and tumor growth (1). CC3 (also known as TIP30) is an anti-tumor protein that predisposes cells to apoptosis, thereby giving properties of metastatic suppression (2). CC3 is an evolutionarily conserved gene ubiquitously expressed as a 27-kDa protein in human tissues. CC3 RNA is absent in variant small cell lung carcinoma cell lines that are derived from tumors with highly aggressive metastatic behavior (2). Significantly, ectopic expression of CC3 in variant small cell lung carcinoma cells induces expression of a number of apoptosis-related genes including Bad and Siva (3) and also attenuates metastasis in vivo (2). Moreover, it has been shown that genetically engineered mice deficient in CC3 have a high incidence of liver cancer and that reduced expression of CC3 is also associated with 33% of human hepatocellular carcinomas (4).

A number of studies have been aimed at defining the biochemical properties of CC3, as well as the interactions made with partner proteins that may relate to its pro-apoptotic effects. Recent results have shown that CC3 can bind directly to karyopherins of the importin β family of nuclear transportins in a Ran-GTP-insensitive manner (5). For importin β2, the site of interaction with CC3 involves HEAT domain(s) in the C-terminal third of the protein. Binding of CC3 to importins inhibits the nuclear transport of proteins with either the classical nuclear localization signal or the M9 signal recognized by importin β2. Cells modified to overexpress CC3 have slower rates of nuclear transport and higher sensitivity to death signals. CC3 mutated in the region of a nucleotide binding motif (GXXGXXG, between residues 25 and 31) lacked apoptotic properties and had weakened interactions with importin β2 (5).

In a separate line of research aimed at identifying proteins that bind to the HIV-1 transcripional activator protein tat, TIP30 (tat-interacting protein of 30 kDa) was isolated (6). TIP30 was shown to bind to the N-terminal trans-activation domain of HIV-1 tat, resulting in activation of the HIV-1 promoter. However, in model promoter systems, neither the level of basal transcription nor the level of Gal-VP16- and Gal-SP1-activated transcription was dependent on TIP30, indicating that it is not a general transcription factor. Subsequently, it was realized that CC3 and TIP30 were one and the same protein (7). CC3 (TIP30) has been reported to have Ser/Thr protein kinase activity, to undergo autophosphorylation, and to phosphorylate the heptapeptide repeats of the C-terminal domain of the largest RNA polymerase II subunit in a tat-dependent manner (3). Mutating two of the glycine residues in the putative nucleotide binding motif (GXXGXXG) of CC3 abolished the pro-apoptotic effects of the protein and apparently abolished its protein kinase activity. The biological significance of the CC3-tat interaction is unclear, however, because the

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§ The abbreviations used are: HIV, human immunodeficiency virus; CIA, co-activator independent of AF-2 function; SDR, short chain dehydrogenase reductase; AMPCP, adenosine 5′(β,γ-methylene)-triphosphate; PEG, polyethylene glycol; PDB, Protein Data Bank; SRS, Synchrotron Radiation Source; ESRF, European Synchrotron Radiation Facility; r.m.s., root mean square.

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generally accepted mode of action of tat involves interaction with cyclin T and cyclin-dependent kinase 9 (8). Follow-up studies by the same group aimed at establishing the normal cellular role of CC3 have been reported (9). CC3 interacts in a complex formed in response to estrogen, consisting of the estrogen receptor α5, the co-activator C1A, and the promoter and downstream regions of the c-myc gene (9). CC3 overexpression represses estrogen receptor α-mediated c-myc transcription, whereas CC3 deficiency enhances it. Based on these data, it has been proposed that the increased level of c-myc transcription is the mechanism of tumorigenesis associated with low levels of CC3 (9).

The nature of the CC3 protein fold and its consequent implications for ligand binding specificity and likely biochemical properties have, however, been controversial. Bioinformatics studies using hidden Markoff models have indicated a relationship of CC3 to the SDR family, with the closest match to cations for ligand binding specificity and likely biochemical properties of the protein. Such data will be of value in further biochemical characterization and studies of the mechanism of action of CC3 in apoptosis and tumor suppression.

MATERIALS AND METHODS

Cloning Expression and Purification of CC3 (TIP30)—The gene encoding CC3 was obtained from the Sanger Centre (Hinxton, Cambridge, UK). The coding sequence for CC3 was amplified by PCR and cloned into the expression plasmid pET45b, giving rise to a protein with an N-terminal Hisagger tag. The plasmid was transformed into Rosetta-(DE3)pLyS0S for protein expression. The cells were grown overnight in a 60-cm² shake-flask culture supplemented with 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol, with shaking at 37 °C. The culture was then diluted 1:100 into 6 liters of LB (with 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol included) and grown at 37 °C until A₈₀₀ reached 0.7. Isopropyl 1-thio-β-d-galactopyranoside was added to 1 mM, and the induction was carried out at 30 °C overnight. The cells were harvested by centrifugation, and the pellets were resuspended in buffer A (50 mM sodium inorganic phosphate, pH 7.4, 500 mM NaCl, and 0.2% Tween 20). The cells were disrupted by sonication, and the supernatant was clarified by centrifugation and applied to a 5-mL nickel chelating column (Hitrap chelating column; Amersham Biosciences) pre-equilibrated with buffer A. CC3 was eluted with buffer A + 500 mM imidazole by a linear gradient. Fractions containing CC3 were applied to a Superdex S-200 gel filtration column pre-equilibrated in buffer A but without Tween 20. Pure CC3 was concentrated, and buffer was exchanged against 10 mM Tris, pH 8.0. The purification protocol produced, on average, 40 mg of pure CC3. Selenomethionine-labeled CC3 was expressed in the methionine auxotroph strain B834 grown on Selenomet Medium Base with Nutrient Mix media (Arthena Enzyme Systems) and purified using the same protocols as described for the methionine protein. Liquid chromatography-mass spectrometry analysis using a Waters Q-Tof micro mass spectrometer indicated >95% incorporation of selenomethionine. CC3 was shown to be monomeric in solution in the presence of dithiothreitol using dynamic light scattering measured in a Protein Solutions DynaPro.

Assessment of Nucleotide Binding to CC3 (TIP30)—The assessment of the binding of potential ligands to CC3 was carried out using a parallelized thermal shift assay with Sypro Orange dye as probe (11). Samples of CC3 (1 mg/ml), either unliganded or with 0.5 mM added ligand (ATP, ADP, AMPPCP, NAD, NADP, NADH, or NADPH), were heated in a 96-well plate from 25 °C to 90 °C in 1 °C steps. Experiments were repeated with the addition of 1 mM dithiothreitol. Fluorescence was monitored for each raised degree with a real-time PCR machine (Chromo4 detector; MJ Research).

Crystallization and Data Collection—CC3 at 14 mg/ml was initially screened in crystallization trials with a total of ~4032 droplets (672 standard conditions consisting of Hampton, Wizard, and Emerald kits for 6 different ligand complexes). The crystallization system developed at the Oxford Protein Production Facility was used for the initial screening phase (12). A Cartesian Technologies pipetting robot was used to set up nano-crystallizations consisting of 100 nl of protein and 100 nl of reservoir solution mixed as sitting drops in 96-well Greiner plates. Crystallization plates were placed in a TAP Homebase storage vault maintained at 22 °C and imaged via a Veeco visualization system (12). An initial crystal hit that only yielded very small crystals, even after some optimization, was subjected to an additive screen that indicated that PEG 400 improved crystal size and quality. Further screening with different low molecular weight PEGs revealed that PEG 600 gave dramatically larger crystals of CC3. The optimal conditions for the growth of crystals of the CC3-NADP complex with dimensions of up to 300 × 130 × 130 μm were as follows: 1.8 mM ammonium sulfate, 0.1 M Tris, pH 8.4, and 8% (v/v) PEG 800.

X-ray diffraction data for CC3-NADP crystals were collected to 1.7 Å at station PX14.2, SRS (Daresbury, UK). Multiple wavelength anomalous dispersion data for selenomethionine-labeled CC3-NADP crystals were collected to 1.96-Å resolution at beamline BM14, the ESRF (Grenoble, France). Following a fluorescence scan, the peak wavelength was set as 0.97919 Å. Data were also collected at a remote wavelength (λ = 0.90789 Å).

Indexing and integration of data images were carried out with HKL2000, and data were merged using SCALPACK (13). The statistics for x-ray data collection are given in Table I.

Structure Solution and Refinement—The SHELX program suite was used to evaluate the anomalous signal during the course of data collection from a selenomethionine CC3 crystal. Following data preparation and initial tests, SHELX-L (14) was used to identify selenium sites. The electron density map following SHELXE using just the peak wavelength data was very clear, with “dimples” observable in aromatic rings. Data collection was thus terminated after just two wavelengths. SOLVE/RESOLVE (15) were then used for refinement of selenium positions and phase improvement combined with automated model building. The CC3 structure was refined in CNS (16) using positional and B factor refinement. Initially, the selenomethionine CC3 structure was rebuilt from the RESOLVE model and subjected to a single round of refinement before switching to the 1.7-Å resolution native data for further refinement to give the final statistics shown in Table I.

Structure Alignments and Protein Docking—The refined coordinates of CC3 were aligned with available protein structures in the Protein Data Bank, using the Dali server (17). For preliminary comparisons of CC3 with selected protein structures, SHP was used (18). CC3 was docked with the C-terminal region of importin β2 using PTDock (19). The PDB codes for coordinate sets used are as follows: biliverdin IXβ reductase, PDB code 1HE2 (20); porcine carbonyl reductase, PDB code 1N5D (21); cAMP-dependent protein kinase catalytic subunit, PDB code 1APM; tat, PDB code 1TBC; and importin β2, PDB code 1QBK (22).

RESULTS

Co-crystallization Studies with Potential CC3 (TIP30) Ligands—His-tagged CC3 purified by nickel-nitrilotriacetic acid and gel filtration columns was initially used to assess possible ligand specificity. Using a parallelized thermal shift assay, seven potential ligands were screened for binding to CC3. ATP, ADP, AMPPCP, NAD, and NADH at 0.5 mM gave no indication of binding, whereas NADPH gave a significant right shift, and NADP gave an intermediate shift (Fig. 1). The right shifts were observed in either the presence or absence of dithiothreitol. Although efforts were therefore focused on co-crystallization of the putative CC3-NADPH complex, each potential ligand was also tested in our standard set of 672 crystallization conditions. The only CC3 crystals obtained were in the presence of NADPH, again strongly suggesting that it is the preferred ligand for CC3. The original small crystals of CC3 grew significa-
significantly larger in the presence of 8% PEG 600, which was identified from two rounds of additive screening. The crystal volume was increased by a factor of 50-fold by adding PEG 600. The effect of PEG 600 in improving the crystallization was later rationalized by the identification of a PEG 600 molecule binding in the crystal (Figs. 2b and 3a). A PEG 600 molecule binds between two CC3 monomers (and within 5.5 Å of the NADPH site), which potentially may have a rigidifying effect on the protein.

Overall Fold of CC3 and the Site of NADPH Binding—The CC3 map phased by multiple wavelength anomalous dispersion using selenomethionine-labeled protein was of very good quality (Fig. 2a). Refinement of the CC3 structure to 1.7 Å gave an Rwork of 0.167 (Rfree = 0.193) while retaining good stereochemistry (Table I). The CC3 structure is dominated by a dinucleotide (Rossmann) fold largely located in the N-terminal two-thirds of the protein. An N-terminal extension from the Rossmann fold consists of residues 1–18, which (apart from the first four amino acids that are disordered) form an α-helix from residues 5–18 (α1). There is a rather extended loop region between 168 and 206 containing a helix (α7) and two parallel β-strands (β-7 and β-8). There is a cavity in the CC3 structure that is the site of NADPH binding. Adjacent to the nucleotide binding site is a bound PEG 600 molecule that is located in the extended region between two CC3 monomers (and within 5.5 Å of the NADPH site), which potentially may have a rigidifying effect on the protein.

### Table I
Crystallographic data collection, phasing, and refinement statistics for CC3 crystals

| Data collection and scaling | Native | SeMet peak | SeMet remote |
|----------------------------|--------|------------|--------------|
| Beamline                    | SRS PX 14.2 | ESRF BM14 | ESRF BM14 |
| Wavelength (Å)              | 0.9790 | 0.97919 | 0.90789 |
| Degrees collected (°)       | 360 | 360 | 180 |
| Resolution limit (Å)        | 1.7 (1.76–1.7) | 1.96 (2.0–1.96) | 1.96 (2.0–1.96) |
| Space group                 | P3_21 | P3_21 | P3_21 |
| Unit cell a, b, c (Å), α, β, γ (°) | 68.1, 68.1, 119.2, 90.0, 90.0, 120.0 | 68.1, 68.1, 119.1, 90.0, 90.0, 120.0 |
| Asymmetric unit (molecules) | 1 | 1 | 1 |
| Reflections                 | 665,406 | 410,057 | 243,634 |
| Unique reflections          | 25,993 | 43,006 | 43,454 |
| Completeness (%)            | 100.0 (100.0) | 98.3 (95.3) | 97.7 (82.2) |
| I/σI                        | 33.0 (6.5) | 35.9 (13.6) | 25.3 (8.7) |
| Rmerge                     | 0.086 (0.348) | 0.063 (0.143) | 0.064 (0.151) |

**Phasing**

| Se sites found by SOLVE | 2 |
| Mean FOM after SOLVE at 2.0 Å | 0.55 |
| Mean FOM after RESOLVE DM at 2.0 Å | 0.76 |
| RESOLVE auto-build to 2.0 Å: residues built | 205 (82%) |

**Refinement**

| Resolution range (Å) | 30–1.7 |
| R factor (Rwork/Rfree) | 0.165/0.190 |
| No. of atoms | 2258 |
| Mean B factor (Å²) | 20.8 |
| r.m.s. bonds (Å) | 0.007 |
| r.m.s. angles (°) | 1.3 |

* Rmerge = Σ|I–〈I〉|/ΣI,

* R factor = Σ|Fo–Fc|/ΣFo.

**FIG. 1.** Thermal shift curves for CC3 in the presence or absence of potential ligands. The first derivative of the fluorescence change (in arbitrary units) is plotted on the y axis against temperature on the x axis. Curves are color-coded for the ligands as indicated on the right.
residues 168 and 206 and shared between two CC3 monomers, related by a crystallographic 2-fold axis (Figs. 2b and 3a). The contacts of CC3 with the PEG 600 include residues Val169, Leu171, Gln175, Leu183, Val184, Lys186, Phe187, Phe188, Trp195, His199, and Ser200.

Electron density for NADPH was clearly identified in an extended conformation in the initial selenomethionine multiple wavelength anomalous dispersion-phased map (Fig. 2a). Refinement at 1.7-Å resolution confirmed that NADPH was well defined with holes visible for each of the aromatic rings in the 2Fo − Fc electron density map, allowing a detailed description of the stereochemistry of the nucleotide interactions with CC3.

For the ADP moiety of NADPH, the adenine ring is sandwiched between a cluster of hydrophobic residues (van der Waals contacts with Phe72, Leu92, Val107, and Tyr111) and the side chain of Arg52. Arg52 lies parallel with the plane of the purine ring, making contacts along part of its length (Fig. 4a). The specificity for the 2′-phosphate of NADPH is generated by salt bridge formation with Arg52 and Arg53 as well as via an H-bond to Ser27. The ribose ring oxygen forms an H-bond to the main chain of Gly93. The 3′-hydroxyl H-bonds with the main chain nitrogen of Gly93, as well as with the side chain of Ser27. The NADPH pyrophosphate group has the expected close approach to the tight turn (β-1 to α-2) containing the signature nucleotide binding motif GXXGXXG. The contacts to the cofactor are via main chain hydrogen bonds from residues 29 and 30 with the phosphate groups. The helix dipole of α-2 giving some positive charge at the N-terminal end of the helix may also contribute to the binding of the pyrophosphate group.

The nicotinamide ring of NADPH forms main chain H-bond interactions involving the amide group O to the NH group of Leu170 as well as via a water molecule to the carbonyl oxygen of Leu170 (Fig. 4a). The nicotinamide ring of NADPH is in a syn-conformation and forms an internal H-bond with the nicotinamide pyridine nitrogen in a direction approximately normal to the ring. In contrast, the opposite face of the nicotinamide ring is in a much more hydrophobic environment in a pocket lined by Leu130, Pro167, and Leu170. In the crystal, a CC3 dimer is present across a crystallographic 2-fold axis, which leads to an additional interaction with the NADPH pyrophosphate group via Arg178 from the opposing subunit.

CC3 Is Related to the SDR Superfamily Rather Than to cAMP-dependent Protein Kinase—DALI searches (17) revealed that the protein structures most closely related to CC3 are members of the SDR family (23). The highest match was with biliverdin IXβ reductase (20), whereas carbonyl reductase (21, 24) (Fig. 3, b and c) and UDP-galactose epimerase (25) are among the next most closely related structures. More detailed
analyses comparing CC3 with additional SDR homologues in different ligand complexes were carried out with SHP. Biliverdin IXβ reductase and CC3 have 158 equivalent residues with an r.m.s. deviation in α-carbons of 1.8 Å. In contrast, murine cAMP-dependent protein kinase catalytic subunit (26) (closely related to the C. elegans orthologue used in published amino acid sequence alignments) was not within the list of significant matches in DALI. Attempts at overlapping CC3 and cAMP-dependent protein kinase catalytic subunit with SHP indicated that the folds of these two proteins are largely unrelated. A structure-based sequence alignment of CC3 with other SDR proteins is shown in supplemental Fig. 1A. For comparison, an alignment of CC3 and cAMP-dependent protein kinase catalytic subunit sequences, similar to that described previously (3), is in supplemental Fig. 1B.

Unusually for an SDR-like protein, CC3 does not form a dimer involving the packing of two pairs of helices (including one containing the YXXXX active site motif) related by a 2-fold axis. SDR dimer formation allows correct orientation of the active site residues and thus is normally required for enzymatic activity (21, 24). In CC3, the equivalent pair of helices (α-5 and α-6) contain rather bulky, mainly charged side chains (e.g. Arg109, Lys114, and Glu117 (α-5) and Gln145, Glu149, and Lys153 (α-6)) compared with other SDRs, which presumably precludes the usual dimer formation.

Having established the relationship of the three-dimensional
structure of CC3 to the SDR superfamily, it was then possible to assess into which subcategory of SDRs it belonged (23). The length of the polypeptide chain of CC3 is consistent with it being a member of the classical SDR category, however, in relation to sequence motifs it is somewhat between the classical and extended classes. Thus motifs that include the nucleotide recognition sequence G\textsubscript{X}XXG\textsubscript{X}XG (rather than G\textsubscript{XXX}G\textsubscript{X}XG), a glutamic acid 3 residues beyond the lysine in the Y\textsubscript{XXX}K motif, are typical of the extended group. However, the serine positioned -12 residues upstream of the conserved tyrosine is more characteristic of the classical SDR category (supplemental Fig. 1A).

An SDR-like Conserved Motif in CC3 (TIP30)—Although the closest matched SDR to CC3 is biliverdin IX\(\beta\) reductase, the latter enzyme is a member of a subset of SDRs that do not have the commonly found Tyr-Lys-Ser motif. Because the next closest SDR to CC3 in terms of three-dimensional structure was carbonyl reductase (24), which does contain the conserved 3-residue motif, it was used for detailed comparisons of this region of the two proteins.

Overlap of CC3 and carbonyl reductase reveals that of 242 residues of carbonyl reductase, 148 \textsuperscript{148}-carbons are structurally and topologically equivalent with an r.m.s. deviation of 1.5 Å (Fig. 3c). The lysine and serine residues of the Tyr-Lys-Ser conserved motif of the active site of carbonyl reductase and the equivalent region of CC3 form similar interactions with NADPH (Fig. 4b). However, the Tyr\textsuperscript{143} of the triad is displaced in CC3 compared with the position of the equivalent tyrosine in carbonyl reductase. Thus, although active site residues are present in CC3, they may not be correctly orientated for reductase activity. The reason for this is likely to be the monomeric state of CC3 because SDR activity requires dimer formation via an interface involving the juxtaposition of pairs of helices to correctly orientate the catalytic Y\textsubscript{XXX}K motif. Carbonyl reductase has an insertion containing two \textsuperscript{21,24} helices that mimic the required subunit interface and thus is active as a monomer (21, 24). CC3 lacks this insertion but nevertheless could become active if it formed a heterodimeric complex with a further protein interacting with the active site helix to allow correct orientation of Tyr\textsuperscript{143} for catalytic activity.

Assessment of Protein-Protein Interactions Formed by CC3 (TIP30)—As noted previously, CC3 does not form the usual dimer found in SDRs involving the packing of pairs of helices across a subunit interface. However, a CC3 dimer in the crystal is present across a 2-fold axis, which involves a disulfide bridge via Cys\textsuperscript{172} residues, as well as a PEG 600 molecule, shared between two CC3 molecules. If the stability of this dimer is dependent on disulfide bridge formation, then it is unlikely to

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**Fig. 4. The CC3 conserved SDR-like motif.**

(a) Stereodiagram shows the CC3 NADPH binding site. The blue ribbons and coils represent the protein backbones. The protein main chain and side chains are drawn as thin sticks and colored cyan. The NADPH and water molecules are shown as thick orange sticks and red spheres, respectively. The yellow broken bonds are hydrogen bonds between CC3 and NADPH. The backbone from a symmetry-related CC3 is shown as red ribbons and coils. 

(b) Stereodiagram comparing the conserved SDR-like motif in CC3 with the equivalent region of carbonyl reductase. The protein backbones are shown as ribbons and coils with CC3 colored blue and carbonyl reductase colored cyan. The protein side chains and NADPH are drawn as orange and gray ball and sticks for CC3 and carbonyl reductase, respectively. The red coil is the backbone of a symmetry-related molecule of CC3.
be physiologically significant under normal intracellular redox conditions. However, if a natural ligand mimics the binding of PEG 600, then formation of such a dimer may occur under cellular conditions.

In order to evaluate potential sites of interactions of CC3 with partner proteins, docking studies were carried out using FTDOCK. For HIV tat-CC3 interactions, the highest scoring solutions did not include the proposed interaction with the CC3 N-terminal helix extension (10). For importin β2 (22), the reported region of interaction with CC3 (the C-terminal region of residues 590–890) (5) was used for docking. The highest-scoring solution docked CC3 on to a region of importin β2 that showed no steric clashes with the N-terminal region of importin β2 including the bound Ran-GTP (Fig. 5). The parts of importin β2 that interacted with CC3 include the following: residues 877–880 and 840–841 (with residues 187–189 of CC3); residues 791–795 and 759 (with residues 95–98 of CC3); and residues 830–834 (with residues 141–142 of CC3). Based on analysis of the importin β2 structure and the conserved functions within the importin family, the putative docking site for CC3, as also judged from the importin β-SREBP-2 complex (27), is adjacent to and would sterically clash with the likely position of cargo binding in importin β2. Interestingly, this position of CC3 is located toward the C terminus of importin β2, and the avoidance of a clash with Ran-GTP is consistent with the binding of CC3 being Ran-GTP-independent. Such an interaction can be interpreted as being consistent with CC3 acting as an antagonist for other proteins binding to importin βs, one of the previously proposed mechanisms of CC3 inhibition of nuclear transport (5). Such preliminary results should be interpreted with some caution because there are likely to be conformational changes on binding of cargo proteins to importins; nevertheless, some functional properties of CC3 can apparently be explained. Significantly, the helices that would normally form the SDR dimer interface, including α-6 containing the catalytic YXXXK motif, are not occluded in CC3 when docked onto importin β2, despite the N-terminal region of α-6 being in contact. The main interaction surfaces for α-5 and α-6 are thus available for forming a further protein-protein interaction.

DISCUSSION

Prior to this report, the nature of the protein fold and ligand binding properties for CC3 were uncertain because there was conflicting evidence indicating a relationship to either SDR or cAMP-dependent protein kinase catalytic subunit folds. Determination of the crystal structure of CC3 has definitively established that it belongs to the SDR superfamily. Of the two different proteins used to predict folds for CC3, UDP-galactose epimerase has a lower sequence identity of 17.5%, yet it is of the correct fold, whereas cAMP-dependent protein kinase catalytic subunit, although of higher sequence identity (22%), is not closely related (see supplemental Fig. 1, A and B). The bioinformatics and modeling work correctly predicted the CC3 fold despite being based on the least experimental data (10). It has not been possible to compare in detail our crystal structure of CC3 with the homology model, because coordinates of the latter are not available. However, we note some differences. For the 2'-phosphate, the predicted side chain contacts were with Arg282 and Lys34. In fact, Arg280 and Arg327 (as well as Ser27) bind the 2'-phosphate, whereas Lys34 points in the opposite direction, with its NH2 group ~15 Å from the phosphate group.

The origin of the previously reported protein kinase activity associated with CC3 is not clear (3). It may be that there were differing levels of protein kinase, deriving from the baculovirus expression system, present in wild-type and putative kinase-negative mutant CC3 preparations. However, differing results on the ability of bacterially expressed CC3 to autophosphorylate, showing either the presence (3) or absence of activity (5), appear to directly contradict one another. An alternative way of rationalizing putative kinase and reductase activities for CC3 is a dual function involving the ability to bind ATP and NADPH for the different reactions. Whereas such a dual specificity appears unlikely, it may be noted that the SDR biliverdin IXα reductase (28), in addition to catalyzing a reductase reaction, has been reported to have protein kinase activity and be capable of autophosphorylation. Based on our current report, we find no evidence for ATP binding to CC3 in thermal shift and co-crystallization studies. In contrast, CC3 specificity for NADPH binding is observed, and thus the latter is most likely the physiologically relevant ligand. It is thus clear that the effect of mutating CC3 within the nucleotide binding motif (G285/G31A), thereby abolishing its effects on apoptosis and reducing repression of c-myc gene transcription (9), can be reinterpreted as resulting from a decrease in NADPH rather than ATP binding. This would argue that NADPH is an essential part of the biological functioning of CC3. A consequence of the loss of cofactor binding resulting from the mutations in CC3 is the ablation of interactions with partner macromolecules, including importin βs (5) as well as c-myc/CIA (9).

Follow-up biochemical and structural studies should further define the role that NADPH plays in CC3 binding to importins and CIA/c-myc. Preliminary docking studies to investigate possible interaction regions of CC3 with importin β2 indicate a site that could antagonize the binding of cargo proteins to importins, although ultimately crystal structures of importin β-CC3 complexes will be needed to test such models. Additionally, it

FIG. 5. Ribbon diagram showing the putative interaction site between CC3 and importin β2 (PDB code 1QBK) (22) as derived from FTDOCK. Importin β2 and Ran-GTP (RAN) are colored red and cyan, respectively. CC3 is shown in blue, with the two helices (α-5 and α-6) that correspond to the dimerization interface in SDRs colored gray. The area colored green in importin β2 shows the corresponding region for the binding of SREBP-2 in the importin β-SREBP-2 complex (27).
CC3 Crystal Structure

would be of interest to establish whether CC3 can be active as a reductase. Comparison with carbonyl reductase shows that CC3 has an apparently viable active site, with the typical SDR conserved 3-residue motif in place, albeit with the tyrosine displaced. However, because CC3 lacks the SDR dimer interface four-helix bundle, which borders the key XXXXY motif, it is likely to be inactive. However, it is possible that if CC3 interacts with a further protein via the usual SDR dimer helix interface, then a reductase activity could result. In order for enzyme turnover to occur, a substrate for CC3 is required. Although a CC3 substrate has not been identified, there is nevertheless a second ligand binding site on the protein occupied by PEG 600 in the crystal structure that is also close to NADP. It is plausible that an extended chain molecule such as a lipid could occupy the PEG 600 site and that enzyme turnover would result in the oxidation of NADPH, leading to the release of NADP. Because NADPH binding appears necessary for CC3 function, then enzyme turnover could provide a mechanism for the termination of the binding event by disrupting the interaction of CC3 with partner proteins. Site-directed mutagenesis of the conserved Tyr-Lys-Ser motif and residues of the outer surface of α-5 and α-6, to reduce the bulk of side chains in order to promote dimerization and hence potential enzyme activity for CC3, will be of value in assessing whether a reductase reaction plays a role in CC3 function. An enzyme turnover mechanism has been proposed as a release step as part of the regulatory properties for the transcriptional co-repressor CtBP (29). CtBP is related to the NAD-dependent 2-hydroxy acid dehydrogenase and shows a 100-fold tighter binding of NADH compared with NAD. CtBP is enzymatically active (30), and although the activity does not appear to be essential for co-repressor function, it may be involved in the release step. Based on these properties, it has been proposed that CtBP acts as a redox sensor linked to transcription (29). NADH, via its interaction with CtBP, is an example of the emerging role of cofactors normally associated with intermediary metabolism that act as regulators of transcription (31). Clearly, it is of interest to investigate possible redox sensing properties of CC3 with regard to its transcriptional regulatory role in the c-myc/CIA system. CC3 provides an example of the adaptation of the SDR fold from purely a metabolic enzymatic function to a role that includes protein binding and regulatory properties. The negative transcriptional regulator NmrA has been shown to be a member of the SDR superfamily. Although NmrA can bind NAD, it is monomeric and lacks two of the key residues from the conserved SDR motif and is thus enzymatically inactive (32).

The work reported here has clarified the nature of the structural fold for CC3 and also points to the key role of NADPH binding in CC3 function. It is hoped that additional studies to characterize the properties of CC3, a molecule that has an important role in the suppression of cancer, will be stimulated by the structural data presented here.

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