Structural basis for the regulatory interactions of proapoptotic Par-4

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Par-4 is a unique proapoptotic protein with the ability to induce apoptosis selectively in cancer cells. The X-ray crystal structure of the C-terminal domain of Par-4 (Par-4CC), which regulates its apoptotic function, was obtained by MAD phasing. Par-4 homodimerizes by forming a parallel coiled-coil structure. The N-terminal half of Par-4CC contains the homodimerization subdomain. This structure includes a nuclear export signal (Par-4NES) sequence, which is masked upon dimerization indicating a potential mechanism for nuclear localization. The heteromeric-interaction models specifically showed that charge interaction is an important factor in the stability of heteromers of the C-terminal leucine zipper subdomain of Par-4 (Par-4LZ). These heteromer models also displayed NES masking capacity and therefore the ability to influence intracellular localization. Cell Death and Differentiation (2017) 24, 1540–1547; doi:10.1038/cdd.2017.76; published online 16 June 2017

Par-4 is a 332 amino-acid proapoptotic protein with tumor suppressor activity. Par-4 had been predicted to be largely disordered.1 Its downregulation or non-functional state has been linked to various cancers.2 In contrast, elevated levels of Par-4 have been observed in neurons affected by various neurodegenerative diseases3–5 and, similarly, in the pathogenesis of HIV encephalitis.6 The most striking feature of Par-4 is that its ectopic expression induces apoptosis selectively in certain cancer cells but not in normal or immortalized cells. Instead, it sensitizes the latter to various apoptotic stimuli.7 Par-4 has been involved in both the extrinsic/death receptor-mediated and the intrinsic/mitochondrial pathways of apoptosis.8 Nevertheless, Par-4 is ubiquitously expressed with few exceptions and evolutionarily conserved in vertebrates,9 indicating that the endogenous Par-4 under normal conditions is inactive.

The N-terminal half of Par-4 contains the SAC domain (residues 137–195), which selectively induces apoptosis in cancer cells. It is sufficient for the activation of the Fas pathway, inhibition of NF-κB activity and apoptotic induction.10 However, the latter two functions require its nuclear translocation and phosphorylation at Thr1558 by protein kinase A (PKA)11 or death-associated protein-like kinase (Dlk/DAPK3).12 The SAC domain includes a nuclear localization sequence (residues 137–153), which is essential for the nuclear translocation of Par-4.10 Between the SAC domain and the C-terminal region of Par-4, a phosphorylation site for Akt1 is located, that is, Ser249. It acts as a general prosurvival switch and sequesters Par-4 to the cytoplasm upon phosphorylation.13

The C-terminal region of Par-4 (Par-4CC, residues 254–332) was predicted to form a coiled coil1 and was shown to sensitize cells to various apoptotic stimuli.14 It was suggested to contain a leucine zipper domain (Par-4LZ, residues 292–332) and a nuclear export sequence (NES).1,8 This Par-4CC region was mainly shown to mediate the interaction with partner proteins such as PKCe,15 WT1,16 Akt1,13 apoptosis antagonizing transcription factor (AATF)/Che-1,17 DAPK3,18 Amida,19 THAP120 and p62.21 Par-4 binds and inhibits the activity of PKCe and thereby NF-κB activity, whereas p62 antagonizes this inhibition by forming a ternary complex.21 Par-4 regulates the processing of the β-amyloid precursor protein, which is blocked by the interaction with AATF.17 Par-4 interacts with DAPK3 and Amida, respectively, whereupon coexpression it initiates their relocation from the nucleus to colocalize at the cytoskeleton causing the cytoskeletal reorganization and apoptosis induction.18,19 Par-4 interaction with WT1 transcriptionally downregulates BCL-2 and thereby induces apoptosis.19 Par-4 interacts with THAP1 (a promyelocytic leukemia nuclear bodies (PML-NBs)-associated protein), which contains a DNA-binding THAP domain.20 In spite of these interactions, some being exclusively nuclear, Par-4CC is not necessary for the direct induction of apoptosis.10 The aforementioned features of Par-4 point to the C-terminal region of Par-4 as a regulatory domain, which interacts with a multitude of proteins forming an obscure signaling network.

We have determined the X-ray crystal structure of Par-4CC to determine its subdomains. Their regulatory interactions with AATF, Amida, DAPK3 and THAP1 have been analyzed by modeling to discover the structural basis for the formation of stable heteromeric complexes and control of subcellular localization.

Results

Structure of Par-4CC domain confirms homodimerization. The structure of Par-4CC was determined by multiwavelength anomalous dispersion from a selenomethionine-labeled Par-4CC (SeMet-Par-4CC) at 3.0 Å resolution to an Rwork/Rfree of
The asymmetric unit contains seven helical molecules (chains A, B, C, D, E, F and G) forming three homodimeric structures (dimers AB, CD and FG) (Figure 1). A fourth dimer is generated by a crystallographic symmetry (dimer EE'). One of the non-crystallographic homodimers (dimer FG) displayed electron density for their C-terminal halves lacking connectivity, which did not allow completion of the model. Our result confirms the yeast two-Hybrid assay showing the self-association of Par-4 via its Par-4CC region.\textsuperscript{15}

### Table 1: Data collection, phasing and refinement statistics (related to Figure 1)

| Data collection | SeMet-Par-4CC |
|-----------------|--------------|
| **Space group** | P 4\textsubscript{3} 2 2 |
| **Cell dimensions** | 114.99 x 114.99 x 121.81 (Å), α, β, γ (deg.) |
| **Resolution (Å)** | 41.81–3.0 |
| **Wavelength (Å)** | 0.9771, 0.9794, 0.9795, 0.9825 |
| **I/σ(I)** | 15.9 (6.1), 17.4 (5.6), 14.8 (5.1), 24.4 (7.5) |
| **Total observations** | 152 595 (28 283), 152 969 (28 368), 152 789 (28 286), 152 261 (28 257) |
| **Unique reflections** | 16 918 (3002), 16 918 (3002), 16 917 (3002), 16 912 (3002) |
| **Completeness (%)** | 99.7 (100.0), 99.7 (100.0), 99.7 (100.0), 99.7 (100.0) |
| **Redundancy** | 9.0 (9.4), 9.0 (9.4), 9.0 (9.4), 9.0 (9.4) |
| **CC(1/2)** | 0.998 (0.981), 0.997 (0.974), 0.996 (0.968), 0.997 (0.977) |
| **Phasing power** | (see Supplementary Figure S4) |

Phasing (AutoSol in PHENIX)

| Bragg spacing limit (Å) | Total | 6.41 | 4.99 | 4.23 | 3.73 | 3.37 | 3.10 |
|-------------------------|-------|------|------|------|------|------|------|
| **Phasing power (r.m.s. anomalous FH/E)** |       |      |      |      |      |      |      |
| λ<sub>1</sub> | 0.6 | 1.8 | 2.1 | 1.7 | 1.2 | 0.8 | 0.5 | 0.3 |
| λ<sub>2</sub> | 0.6 | 1.3 | 1.8 | 1.7 | 1.4 | 0.9 | 0.6 | 0.4 |
| λ<sub>3</sub> | 0.5 | 0.9 | 1.4 | 1.4 | 1.1 | 0.6 | 0.4 | 0.3 |
| λ<sub>4</sub> | 0.5 | 1.0 | 1.1 | 1.2 | 1.0 | 0.7 | 0.4 | 0.3 |

Mean figure of merit 0.39 (after SOLVE), 0.72 (after RESOLVE)

Phasing (r.m.s. dispersive FH/E)

| λ<sub>1</sub>–λ<sub>2</sub> | 0.1 | 0.3 | 0.3 | 0.3 | 0.2 | 0.2 | 0.1 | 0.1 |
| λ<sub>1</sub>–λ<sub>3</sub> | 0.2 | 0.5 | 0.5 | 0.5 | 0.3 | 0.2 | 0.2 | 0.1 |
| λ<sub>1</sub>–λ<sub>4</sub> | 0.1 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 |
| λ<sub>2</sub>–λ<sub>4</sub> | 0.2 | 0.6 | 0.7 | 0.6 | 0.5 | 0.3 | 0.2 | 0.1 |

| Mean figure of merit | 0.39 (after SOLVE), 0.72 (after RESOLVE) |

Refinement

| Resolution (Å) | 41.81–3.0 |
| No. of reflections | 16 869 |
| R<sub>work</sub>/R<sub>free</sub> | 24.54/29.44 |
| No. of atoms | 3958 |
| Protein | 3913 |
| Potassium ions | 18 |
| Water | 27 |
| Mean B-factors (Å\textsuperscript{2}) | 75.20 |
| Protein | 75.20 |
| Potassium ions | 97.60 |
| Water | 54.90 |
| R.m.s. deviations | 0.005 |
| Bond lengths (Å) | 0.810 |

Abbreviations: r.m.s., root mean square; SeMet-Par-4CC, selenomethionine-labeled Par-4CC

\textsuperscript{a}Data set used during refinement

\textsuperscript{b}Values within parentheses are for highest-resolution shell (3.21–3.0 Å).
Par-4CC homodimerizes forming parallel coiled-coil structure. The structure of Par-4CC includes the C-terminal 79 residues, of which the residues 257–330 form a canonical coiled-coil structure with two right-handed helices running parallel to each other having a left-handed super helical twist. Also, the diffraction images of Par-4CC crystals displayed diffuse scattering characteristic of coiled-coil (Supplementary Figure S1) (see also Supplementary Results, and Supplementary Figure S2) formation of such coiled-coil structures are guided by heptad repeats in their sequence designated (abcdefg)ₘ, with positions a and d being predominantly occupied by hydrophobic amino acids. Analyzing the structure by SOCKET, 22 11 heptad repeats can be assigned to residues 257–330, with only four residues in the eleventh heptad (Figure 2a). The three asparagines of the homodimeric coiled-coil of Par-4CC (Asn278, Asn313 and Asn320 of non-crystallographic dimers), each in position a of their respective heptads, show two rotamer conformations breaking the twofold non-crystallographic symmetry locally (Figure 2b). The structure is in agreement with the observation that the presence of Asn in position a of a heptad favors dimerization with parallel packing of helices.23,24 The Ca–Cβ bonds of the residues at positions a and d of each heptad are oriented parallel and perpendicular, respectively, to the peptide bonds facing each of them in the opposite helix, as shown previously for two-stranded parallel coiled-coils.25 As expected, the structure shows the side chains at positions a and d facing the interhelical space of the coiled coil (Figure 2b). These residues being predominantly (73%) hydrophobic constitute the hydrophobic dimer interface.

The coiled-coil structure of Par-4CC also includes Par-4LZ (Figure 2b). In the LZ motifs, the position d′ of their heptads is predominantly occupied by leucine.25 Such structures are known to dimerize through coiled-coil formation.27 The last six heptads of Par-4CC belong to Par-4LZ featuring four hydrophobic and two polar residues at position a (Ile292, Ile299, Leu306, Asn313, Asn320, Val327), and exclusively leucine residues at position d (Leu295, Leu302, Leu316, Leu323, Leu330), with the exception of the eighth heptad containing a methionine (Met309) (Figure 2b).

N-terminal half of Par-4CC displays stabilizing factors for the homodimer. Coiled-coil structures gain stability via interhelical salt bridges,28 while intrahelical salt bridges can stabilize monomeric α-helices.29,30 The structure of Par-4CC shows both interhelical and intrahelical salt bridges of g–e′, d–e′ and g–c type (Figure 2b). The interactions Arg263-Glu268, Glu270-Arg275 and Glu312-Lys317 belong to the g–e′ type, whereas Glu274-Arg275 and Lys288-Glu289 belong to the d–e′ type (Figure 2b). There are two intrahelical salt bridges of the g–c type, that is, Glu270-Arg273 and Glu277-Arg280 (Figure 2b).

The N-terminal half of the structure with the aforementioned salt bridges displayed significantly more favorable interactions between charged residues than the C-terminal half (Figure 2b). In contrast, Par-4LZ with only one stabilizing salt bridge depended mostly on the hydrophobic interface for stability of the homodimeric coiled-coil structure (Figure 2b). Weakening of side-chain electron density and increasing temperature factors were observed starting around residue 290 (Figure 2b) (Supplementary Table S1). This clearly indicated that the N-terminal half of Par-4CC constitutes the homodimerization subdomain (Par-4HDD).

Masking of the NES by dimerization. Par-4LZ was claimed to contain an NES and Par-4 displayed sensitivity towards selective inhibitors of nuclear export,31 indicating its NES-dependent nuclear export. The protein sequence analysis using NetNES32 and ValidNES33 suggests the residues 295–301 (LKEEIDL) as the possible NES (Par-4NES). The general NES consensus includes hydrophobic residues (shown in bold) that are essential for binding to the hydrophobic pockets of exportin-1/XPO1/CRM1.34 The homodimeric structure of Par-4CC shows the hydrophobic surface formed by Par-4NES being masked by dimerization (Figure 3), suggesting a mechanism to escape CRM1-mediated nuclear export by being inaccessible. It also displays relatively higher temperature factors for Par-4NES (Supplementary Table S1), which is also true for majority of the structures of NES.34

Modeling of Par-4CC regulatory interactions. Docking analysis of Par-4CC using some of its binding partners, that is, AATF, Amida, DAPK3 and THAP1 was performed for parallel helical pairing to understand the regulatory mechanism of Par-4. Only the region of the sequence of the binding partners displaying high probability for the coiled-coil conformation was used for modeling and docking. The predicted coiled-coil regions overlap significantly with the reported binding regions of respective proteins with Par-4.
Three of the four interacting partners, that is, AATF, Amida, and DAPK3, showed interaction in the Par-4LZ region, whereas THAP1 showed the interaction in the Par-4 HDD region with six interhelical salt bridges (Figure 4). This strong interaction with THAP1 could explain the colocalization of Par-4 in the PML-NBs. The model of DAPK3 used for docking did not include the kinase’s LZ region because Par-4 was shown to interact with the LZ deletion mutant of DAPK3.18 The interaction models of Par-4CC with AATF and DAPK3, which are known to interact with each other via their LZ motifs,35 displayed binding with Par-4LZ, respectively. Therefore, these models suggested a competition of AATF and DAPK3 for Par-4LZ. The interaction region of Par-4LZ in the Amida/Par-4LZ model included two leucine residues (Leu295 and Leu316) that were mutated in a leucine zipper triple mutant of Par-4 (Leu295Ala, Leu316Ala, and Leu330Ala), which failed to interact with Amida.19

The interaction models of AATF, Amida, and DAPK3 with Par-4CC covered the complete region of the Par-4 NES, suggesting an NES masking in these heteromers, similar to that in the Par-4CC homodimer (Figure 4). The coiled-coil stability of Par-4LZ was found to depend strongly on pH, which was previously attributed to intermolecular charge repulsion between Asp305 and Glu310′ (g-e′ type) based on mutation analysis.36,37 The region corresponding to this destabilizing g-e′ type charge repulsion of the Par-4CC homodimer in the former three interaction models did not show such repulsion (Figure 4). Moreover, the residues Asp305 and Glu310 of Par-4LZ were stabilized in DAPK3 containing model by forming salt bridges. The region corresponding to the homodimerization and leucine zippers domains are depicted. For clarity only one set of residues involved in salt-bridge formation are shown with their side chains in atom-type representation. Asparagines (blue) occupying the heptad positions a in both the domains, and the residues at every heptad positions d of the leucine zipper domain are labeled. Electron density (2mFo-DFc type) is contoured at 1.0σ. See also Supplementary Table S1
bridges (Figure 4). Overall, the docking analysis suggested charge stabilization as an important factor for specific heteromerization of Par-4CC.

Discussion

Par-4HDD contains a likely trigger motif for homodimerization. Two-stranded parallel coiled-coil structures were shown to contain a trigger sequence, which represents an autonomous helical folding unit that is indispensable for coiled-coil formation.38 Based on the trigger sequences of different dimeric coiled-coil structures, a trigger motif consensus was derived, that is, xxLExc-hxcxccx, where x, h and c are any residue, hydrophobic residue and charged residue, respectively.38 These consensus sequences occur with little variations. Par-4HDD contains such consensus sequences twice (conserved residues in bold), that is, 258-STLEK-260 and 265-EDKQ-267. They show less disorder than any other part of the Par-4CC structure (Supplementary Table S1). These features of Par-4HDD suggest that this region, which triggers the homodimeric coiled-coil interaction of Par-4, represents the most stable part of the structure. Moreover, the heteromeric-interaction model of THAP1 with Par-4CC shows also for THAP1 such a trigger consensus in the interaction region, that is, HQLEQ-VEKLRKK. This region interacts directly with the second trigger consensus sequence of Par-4HDD (Supplementary Figure S3). In contrast, the Par-4 interacting proteins AATF, Amida and DAPK3 do not contain such a trigger sequence and exhibit dimerization only with Par-4LZ in the interaction models (Figure 4).

The presence of Asn at position a in two heptads of Par-4LZ (Asn313 and Asn320) should have a destabilizing effect besides giving specificity for the dimeric coiled-coil structure formation.24 Par-4HDD also contains such an Asn, that is, Asn278. Therefore, difference in the stability, respectively, apparent disorder, inferred from the structures of Par-4HDD and Par-4LZ (Supplementary Table S1) could be explained at least partially by the difference in the number of destabilizing Asn residues within these subdomains. Furthermore, Par-4HDD contains intrahelical salt bridges that stabilize monomeric α-helices and interhelical salt bridges that stabilize its homodimer (Figure 2b). Interestingly, the majority of these stabilizing salt bridges are located within the trigger sequences of Par-4HDD. The same is true for the trigger sequence in THAP1, which mimics in the heterodimer (Figure 4) the salt-bridge formation of the Par-4HDD homodimer (Figure 2a).

Preferential heterodimerization of Par-4LZ. Par-4LZ was shown to display an environment-dependent structural interconversion between a partially ordered monomer state and a predominantly coiled-coil dimer state.39 Despite the
Regulation of complex formation by Par-4CC. The structure of Par-4CC along with the docking studies suggested a dual function of Par-4CC. One is homodimerization, which is mediated by Par-4\textsubscript{HDD}, and the second is heteromeric oligomerization, which is mainly mediated by Par-4\textsubscript{LZ}. Nevertheless, there is one case in our study where Par-4\textsubscript{HDD} is used for the heteromeric binding interaction with THAP1. This suggests that the THAP1/Par-4 complex is most likely to be heterodimeric, whereas the complexes with the other three proteins, that is, AATF, Amida and DAPK3, might be heterotetrameric assemblies that consist of a Par-4 homodimer formed by Par-4\textsubscript{HDD}, which binds interaction partners via Par-4\textsubscript{LZ}. For example, the interaction of Par-4 and DAPK3 is mediated via Par-4\textsubscript{LZ} and the predicted coiled-coil domain within the interacting region of DAPK3 (Supplementary Table S2). This interaction model is in accordance with the fact that DAPK3 does not need its LZ motif for interaction with Par-4 (Figure 4). In fact, the LZ domain of DAPK3 is needed for its homodimerization and kinase activation.40 Taken together, these considerations suggest that the putative complex of Par-4 and DAPK3 is a tetramer containing two intertwined homodimers (Figure 5). This model for the quaternary structure requires bending of the Par-4 helix. The appearance of high temperature factors after the Par-4\textsubscript{HDD} region starting around residue 290 for molecules A–E and the disorder for molecules F and G point at this region as the most likely area for bending. A closer inspection of the predicted coil-coil region of DAPK3 homodimer (residues 366–393) shows that there are repulsive $g$–$e'$ interaction between Arg382 and Arg387', which are replaced by attractive interactions in the suggested heterotetramer (Figure 4). No structural information is available for the region of DAPK3 adjacent to its terminal LZ. Therefore, there is no clear picture of a possible interaction of DAPK3 with Par-4\textsubscript{HDD} and its preceding segment that connects the Par-4\textsubscript{HDD} region to the Par-4\textsubscript{SAC} domain (Figure 5).

Par-4 is known to show different localizations in different cell types,10 but the regulatory mechanism of its nuclear import/export is not described. Masking of Par-4\textsubscript{NES} was observed for the homodimERIC structure of Par-4\textsubscript{CC} as well as the protein.
interaction models (Figures 3 and 4). The nuclear export of Par-4 by exportin-1 requires the Par-4NES being accessible. A masking of NES by oligomerization to regulate nuclear localization was already suggested for p53.41 Similarly, the masking of Par-4NES could also be such a regulatory mechanism to retain Par-4 in the nucleus to induce apoptosis by inhibiting NF-κB activity. Par-4-mediated recruitment of DAPK3 to the actin cytoskeleton in the cytoplasm is known,18 and the NES of Par-4 was suggested to assist in the relocation of the Par-4/DAPK3 complex.2 However, the formation of the complex between Par-4NES and DAPK3 (Figure 4) would mask Par-4NES and block the translocation of the Par-4/DAPK3 complex to the cytoplasm. Therefore, it is more likely that the interaction of Par-4 with DAPK3 occurs in the cytoplasm.

In summary, there appears to be two very different modes of interaction of Par-4 with signaling proteins: (i) a dimerization via the HDD subdomain, which may appear in two different subtypes by binding of trigger motifs, (ii) a hetero-oligomerization by Par-4NES binding and blocking of Par-4NES.

Materials and Methods

Production of SeMet-labeled Par-4CC. Details regarding Par-4CC plasmid construct and protein expression have been reported earlier,42 except for changes mentioned below. DYT medium of 750 ml with 200 μg/ml ampicillin was inoculated with a preculture to get an initial OD600 of 0.2 and allowed to grow in a shaker at 37°C and 110 r.p.m. until it reached an OD600 of 0.8–1.2. The culture was centrifuged at 4000 r.p.m. for 15 min at 4°C. The cell pellet was rinsed with 5–10 ml of SeMet minimal medium,43 resuspended gently in 20 ml of SeMet minimal medium and inoculated into 750 ml of SeMet minimal medium with 200 μg/ml ampicillin and 100 μg/ml l-SeMet. The culture was immediately induced with 1 mM isopropyl-/D-thiogalactopyranoside and continued for 4 h before it was harvested and stored at −20°C.

The SeMet-labeled Par-4CC (SeMet-Par-4CC) was purified as reported for native protein,42 and the protein sample was analyzed for labeling by mass spectroscopy. The SeMet incorporation was confirmed to be complete. The m/z values of the native and the SeMet-Par-4CC proteins were 12 258.401 and 12 402.916 Da, respectively, with a mass difference of 144.5 Da. This was in reasonable agreement with the theoretical value of 140.69 Da for replacement of the three methionines.

Crystallization of SeMet-Par-4CC. Crystallization of the purified SeMet-Par-4CC was performed with our reported crystallization condition:42 crystals were obtained using the condition 42% PEG 4000, 0.1 M HEPES pH 7.0, −2.0°C. The nucleation was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.41 Similarly, the localization was already suggested for p53.

Data collection and processing. Multilayer wavelength anomalous dispersion ( MAD ) data were collected on beamline ID23-1 at the European Synchrotron Radiation Facility ( ESRF ) ( Grenoble, France ). Data measurements were performed at −173°C. MAD data were collected at wavelengths of 0.9794 Å ( peak ), 0.9795 Å ( inflection point ), 0.977 Å ( high-energy remote ) and 0.9825 Å ( low-energy remote ), respectively.

Crystallographic data were processed in space group P43212 till 3.0 Å using CNS. We acknowledge the European Synchrotron Radiation Facility for provision of synchrotron radiation facilities and we thank the local staff for assistance in using beamline ID23-1. The room temperature diffraction measurements were performed on beamline P11, DESY, we are also thankful to the local contact for providing assistance.

Author contributions

UJE, JK and UKTS isolated and crystallized the protein. UKTS generated and analyzed the interaction models. UKTS and JL performed crystallographic experiments, data analysis and completion of manuscript.

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