Structural basis for cpSRP43 chromodomain selectivity and dynamics in Alb3 insertase interaction

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Canonical membrane protein biogenesis requires co-translational delivery of ribosome-associated proteins to the Sec translocase and depends on the signal recognition particle (SRP) and its receptor (SR). In contrast, high-throughput delivery of abundant light-harvesting chlorophyll a,b-binding proteins (LHCPs) in chloroplasts to the Alb3 insertase occurs post-translationally via a soluble transit complex including the cpSRP43/cpSRP54 heterodimer (cpSRP). Here we describe the molecular mechanisms of tethering cpSRP to the Alb3 insertase by specific interaction of cpSRP43 chromodomain 3 with a linear motif in the Alb3 C-terminal tail. Combining NMR spectroscopy, X-ray crystallography and biochemical analyses, we dissect the structural basis for selectivity of chromodomains 2 and 3 for their respective ligands cpSRP54 and Alb3, respectively. Negative cooperativity in ligand binding can be explained by dynamics in the chromodomain interface. Our study provides a model for membrane recruitment of the transit complex and may serve as a prototype for a functional gain by the tandem arrangement of chromodomains.

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Cytosolic membrane protein delivery relies on a signal recognition particle (SRP) machinery found in the cytosol of all prokaryotes and eukaryotes. The SRP core is universally conserved and consists of SRP54 (Ffh in bacteria) and the SRP RNA. In contrast to cytosolic SRPs, SRP in the chloroplasts of higher plants (cpSRP) has lost its RNA and the SRP RNA. In contrast to cytosolic SRPs, SRP in the chloroplasts of higher plants (cpSRP) is universally conserved and consists of SRP54 (Ffh in bacteria) and the SRP RNA. In contrast to cytosolic SRPs, SRP in the chloroplasts of higher plants (cpSRP) has lost its RNA and the SRP RNA. In contrast to cytosolic SRPs, SRP in the chloroplasts of higher plants (cpSRP) has lost its RNA and the SRP RNA.

Post-translational cpSRP-dependent protein transport is a dedicated pathway for members of the light-harvesting chlorophyll $a,b$-binding protein family (LHCPs), the most highly abundant membrane protein in chloroplasts. LHCPs serve as antenna systems in photosynthesis to funnel absorbed light energy to the photoreaction centres. They contain three transmembrane helices (TM1 to TM3) to which chlorophylls and carotenoids bind during insertion into the thylakoid membrane. Synthesized on cytosolic ribosomes, the nuclear-encoded LHCPs are guided to the chloroplast envelope by a cleavable transit peptide and are imported into the chloroplast stroma via interaction with the TOC/TIC import machinery. In the stroma, the LHCPs are transferred from the envelope via the small LTD protein to a soluble transit complex with cpSRP. The transit complex is guided to the thylakoid membrane, where the LHCPs are inserted by the interaction with the membrane-bound SRP receptor cpFtsY and the C terminus of the membrane insertase Alb3 (refs 16,17) (Fig. 1a). Alb3 belongs to the YidC/Oxa1/Alb3 family of membrane insertases, which are responsible for insertion and folding of membrane proteins and their assembly into larger protein complexes. Escherichia coli YidC represents the best characterized member of this family with its C terminus being involved in ribosome interaction.

The C terminus of Alb3 is intrinsically disordered, recruits cpSRP43 to the thylakoid membrane, and participates in cpSRP-dependent post-translational membrane targeting. CpSRP43 is a modular protein with a unique arrangement of three chromodomains (CD1–3) and four ankyrin repeats (Ank1–4) (Fig. 1a,b). Chromodomains and ankyrin repeats are versatile protein–protein interaction modules and allow cpSRP43 to participate in numerous, specific interactions with linear motifs. Chromodomains are typically found in the nucleus where they play a key role in chromatin remodelling and gene expression. The heterochromatin protein 1 and polycomb are key players in chromatin remodelling and gene expression. The chromodomains of these proteins accommodate methylated lysines within an ARKS signature sequence of histone tails in an ‘aromatic cage’. In contrast, cpSRP43 CD2 binds a positively charged arginine-rich motif at the C-terminal tail of cpSRP54 in a twinned and modified cage and thereby recruits cpSRP54 into the transit complex. Ankyrin repeats are typical tandem-arrays of 33 residues that bind linear motifs in different biological contexts and are used for the design of specific binding proteins (DARPins). In cpSRP43, the ankyrin repeats bind a conserved region in the loop between LHCP TM2 and TM3 (the L18 motif) and act as a specific chaperone that prevents LHCP aggregation. Recent studies showed cpSRP43 to exhibit significant inter-domain dynamics, which is reduced on cpSRP54 binding.

We previously showed that the two C-terminal chromodomains CD2 and CD3 of cpSRP43 are important for binding two positively charged motifs in the Alb3 C-terminal tail (A3CT). However, the molecular details of the cpSRP43–A3CT interaction and the selectivity, dynamics and cooperativity with respect to cpSRP54 binding remained unknown. Here we present a detailed structural and biochemical analysis of the cpSRP43 CD2CD3 interaction with A3CT, demonstrating that CD3 binds to a linear motif of A3CT and that LHCP targeting is regulated by a serial connection of ankyrin repeats and chromodomains. Our data provide the structural basis for transit complex tethering to the thylakoid membrane by the Alb3 insertase.

Results

Alb3 binds specifically to cpSRP43 CD3. Recruitment of the transit complex to the thylakoid membrane involves the conserved interaction of the A3CT with the two C-terminal chromodomains of cpSRP43 (in the following denoted with CD2 and CD3 only) (Supplementary Fig. 1a,b). To test whether A3CT is able to discriminate between CD2 and CD3, we used nuclear magnetic resonance (NMR) spectroscopy to characterize the interaction with a cpSRP43 construct comprising CD2 and CD3 (CD2CD3) (Fig. 1b). Titration of unlabelled A3CT into $^{15}$N and $^{13}$C labelled CD2CD3 shows NMR chemical shift perturbations (CSPs) for residues located almost exclusively in CD3, while CD2 was only affected at its C-terminal end (residues 313 and 316) (Fig. 1c). These data show that A3CT binds to cpSRP43 CD3 and does not directly compete with cpSRP54 for CD2. To quantify the binding event, we performed isothermal titration calorimetry (ITC, Supplementary Table 1a). CD2CD3 binds A3CT with a dissociation constant of 20.6 mM in contrast to 5.1 mM observed for full-length cpSRP43 (Fig. 1d), indicating a stabilizing effect of the ankyrin repeats on the chromodomains.

In a crystal structure of a cpSRP43–cpSRP54 complex, we previously reported how CD2 binds the C-terminal tail of cpSRP54 harbouring a RRK peptide motif. In a titration of CD2CD3 with the same RRK peptide (RRK$_p$) (Fig. 1b) monitored by NMR, we find that this ligand binds exclusively to CD2 also in presence of CD3, although some CSPs are observed for N-terminal residues in CD3 (Gly316 to Glu318) (Fig. 1e). NMR CSPs for RRK$_p$ binding to CD2 are much stronger than in the NMR titration for A3CT binding to CD3, consistent with the ~10 times higher binding affinity of the cpSRP54 tail in comparison with A3CT as determined by ITC. The data also explain our previous observations that cpSRP43 is able to bind both ligands at the same time and show that CD2 and CD3 provide specific interaction sites for the tails of cpSRP54 and Alb3 despite the high sequence similarity between the two chromodomains and the ligands.

CD3 binds the A3CT motif IV. Having established that A3CT interacts specifically with CD3, we tested whether CD3 discriminates between similar interaction motifs (II and IV) within A3CT. The A3CT harbours three ARKS-like signature sequences that could potentially bind to chromodomains (Supplementary Fig. 1b). From the cpSRP43ACD3–RRKK$_p$ complex it was known that CD2 accommodates the second arginine in the ARKK signature sequence of cpSRP54 in a modified cage. However, the signature is extended to 531-PPTGARRK and all three arginines are read-out by cpSRP43. Motif II of A3CT contains a 375-ARKS sequence flanked by predominantly small and uncharged residues, whereas motif IV is highly positively charged and contains two putative overlapping binding sequences (453-SKRS and 456-SKRK). Therefore, it was a priori not clear, which of the three signature sequences would bind, whether binding is specific or if there is any promiscuity.

Titration of unlabelled CD2CD3 into $^{15}$N,$^{13}$C labelled A3CT showed CSPs most pronounced for the C-terminal region of A3CT harbouring motif IV (Fig. 1f). The CSPs are considerably smaller than for the previous titrations, reflecting the lower affinity of A3CT. Based on these data, we designed an A3CT...
variant comprising only the C-terminal 28 residues including motif IV (A3CT IV peptide, residues 434–461; Supplementary Fig. 2a). Titration of CD3 only into A3CT IV peptide confirmed the specific interaction (Supplementary Fig. 2a). The region including motif II (residues 369–379) did not show any significant CSPs. Titrations of a motif II peptide into double-labelled CD2CD3 or CD3 alone confirmed these results (Supplementary Fig. 2b,c). ITC experiments revealed that A3CT

Figure 1 | NMR and ITC analysis of the cpSRP43–A3CT interaction. (a) Scheme of the cpSRP system in post-translational LHCP targeting. (b) Schematic representation of cpSRP43 and A3CT constructs used in this study. (c) NMR chemical shift perturbations (CSPs) of CD2CD3 on titration with A3CT. Major CSPs are observed only for residues located in CD3. (d) Characterization of the interaction between cpSRP43 and A3CT. ITC titrations are shown for cpSRP43 with A3CT (left) and cpSRP43 with A3CT IVp (middle). $K_D$ values for relevant ITC measurements are listed in a table (right). (e) CSPs of CD2CD3 on titration with RRKRp. Major spectral changes on RRKRp binding occur only for residues located in CD2. (f) CSPs observed in A3CT on titration with unlabelled CD2CD3. Significant CSPs are observed for residues located at the C terminus of A3CT (motif IV and flanking residues). CSPs with a negative value indicate residues that could not be assigned in the bound state.

Including motif II (residues 369–379) did not show any significant CSPs. Titrations of a motif II peptide into double-labelled CD2CD3 or CD3 alone confirmed these results (Supplementary Fig. 2b,c). ITC experiments revealed that A3CT
motif II and IV bind to CD2CD3 with dissociation constants $K_D$ of 150 nM and 15 nM, respectively (Fig. 1d), while the affinity to the complete A3CT corresponds to a $K_D$ of 5 nM. Thus the cpSRP43–A3CT interaction is in a similar range as canonical chromodomain interactions with histone tails, which typically exhibit $K_D$ values of 1–10 nM (ref. 34).

Taken together, our NMR and ITC experiments show that the cpSRP43–Alb3 complex is based on the specific interaction of CD3 with the C-terminal region of A3CT harbouring motif IV, and neither CD2 nor A3CT motif II significantly contribute to the interaction.

Crystal structure of the CD2CD3-motif IV complex. Having identified the specific interaction between CD3 and A3CT motif IV, we set out to characterize the binding mode by NMR and X-ray structure determination. As the interaction of CD3 with A3CT motif IV is rather weak and attempts to crystallize this minimal complex were not successful, we designed a fusion construct with the C terminus of CD2CD3 (residues 264–373) covalently linked (with a triple GS-linker (GS)3) to A3CT motif IV (A3CT IV, residues 440–461) (Fig. 1b and Supplementary Fig. 3a), to increase the local concentration and to stabilize the interaction. However, as this construct did not result in crystals and motivated by recent success using carrier proteins in crys-

...tallography and NMR (Fig. 2d,e). Both structures are in the crystallographic neighbours emphasizing the high flexibility of isolated chromodomains (Supplementary Fig. 3e). Helix closure corresponds to the ordering of the chromodomain N terminus that folds into strand $\beta_2a$ and integrates the ligand as strand $\beta_1'$ by $\beta$-completion. To characterize changes in the conformation and dynamics of CD3 on ligand binding, we also determined the structure of the unliganded CD3 domain by X-ray crystallography and NMR (Fig. 2d,e). Both structures are in the open conformation characterized by the unstructured first strand $\beta_2a$ due to the absence of its binding partner. In the crystal structure, the CD3 fold is stabilized by helix swapping between crystallographic neighbours emphasizing the high flexibility of isolated chromodomains (Supplementary Fig. 3e). Helix swapping is probably due to crystal packing forces as the NMR structure exhibits a compact monomeric chromodomain fold in solution (Fig. 2e and Supplementary Table 2).

The accommodation of the ligand as strand $\beta_1'$ in CD3 results in the formation of a $\beta$-barrel as described for the CD2 interaction with RRKRp25 (Supplementary Fig. 4b,d). Strand $\beta_1'$ is hereby sandwiched between strand $\beta_2a$ and a short strand $\beta_5$ formed by the loop between strand $\beta_4$ and helix $\alpha_1$. The combined chromodomain–ligand $\beta$–barrel is peculiar in the sense that the parallel strands $\beta_4$ and $\beta_5$ (the latter not formed in CD3) span only half of the barrel and are laterally offset by an inserted helical turn, thus creating a void in the barrel between strands $\beta_4$ and $\beta_1'$, which is filled by the ligand bound to its ‘cage’ (Fig. 2f).

In the classical chromodomain interaction with histone tails, the cage is formed by three aromatic residues that accommodate the methylated lysine of the tail (Fig. 3c, Supplementary Fig. 4c). In the CD2CD3–IV complex ligand binding is modified for the specific recognition of an arginine residue (see below) and the absence of strand $\beta_5$ is due to a twist (usually 25° in $\beta$-sheets) of strand $\beta_1'$ with respect to the neighbouring strands $\beta_2a$ (30°) and the $\beta_5$ region (0°) (Fig. 2f). Therefore, strands $\beta_1'$ and $\beta_2a$ are shorter as observed in the cpSRP43CD3–RRKRp complex25 (Supplementary Fig. 5), which is reflected by the lower binding affinity of motif IV compared with the RRKRp motif (Supplementary Table 1a,b).

Taken together, the CD2CD3–IV complex reveals a unique arrangement of tandem chromodomains that are stabilized by the highly positively charged A3CT IV accommodated in their interface. A3CT IV complements the fold of CD3 by $\beta$-augmentation in a specific manner that is distinct to the CD2–RRKRp interaction.

Ligand recognition by the modified cage in CD3. The most significant difference of the CD2CD3–IV complex in comparison to classical chromodomain–ligand interactions concerns the specific ligand recognition in a ‘modified cage’. Cage modification also occurs in CD2 as seen in the cpSRP43A2CD3–RRKRp complex and is therefore a characteristic of both cpSRP43 chromo-
domains. In the CD2CD3–IV complex, A3CT IV adopts an extended conformation and a positively charged KRKRKRKR sequence (residues 454–460) at its C terminus is threaded into CD3 (Fig. 3a and Supplementary Fig. 6). Insertion of A3CT IV as strand $\beta_1'$ involves Arg548 (0 position, Fig. 3b), which is accommodated in the modified cage, and the flanking four resi-
dues Lys454–Lys457 at positions $-4$ to $-1$, and Lys459 and Arg460 at the +1 and +2 positions. Cage modification reflects the change of ligand from a methylated lysine in context of the ARKS signature sequence for classical chromodomain structures (Fig. 3c,d and Supplementary Fig. 5) to an arginine for CD3 and CD2. While Trp343 forms a $\pi$-cation stacking with the guanidinium group of the arginine (in an orthogonal T-shaped geometry) the two other aromatic residues (tyrosines) of the classical cage are replaced by Glu318 and Asp345, which are involved in salt bridges with the caged arginine. Mutation of Trp343 to alanine drastically reduces the binding affinity ($K_D$ of 74.6 μM) (Supplementary Table 1a). All cage residues are conserved in CD2 and CD3 (Supplementary Fig. 4a). In CD2, the cage accommodates Arg537 (0 position) of the cpSRP54 RRKR motif in an identical manner (Fig. 3b–d and Supplementary Fig. 5b).

However, CD2 supplies a second cage (residues Phe267, Tyr269 and His304) for specific read-out of Arg536 (position -1) that is not conserved in CD3. Here the corresponding residues Leu317, Tyr319 and Asn356 do not form a cage for Lys457 (position $-1$ in A3CT IV) (Fig. 3a,d). Instead, Tyr319 is involved in stabilizing the interface with CD2 (see below).

Taken together, CD2 and CD3 interact with their charged ligands by using a modified cage and the recognition of flanking residues distinct from canonical chromodomains. While in CD2,
Figure 2 | Structural characterization of the cpSRP43–A3CT complex. (a) Crystal structure of cpSRP43 CD2CD3 (blue) with bound A3CT IV (salmon) as part of the fusion construct with thioredoxin (not shown). (b) The electrostatic surface potential of CD2CD3 is shown with positive charges in blue and negative charges in red (contoured at ± 5 kT). (c) ConSURF analysis showing the degree of conservation mapped on the surface of the CD2CD3 structure. Highly conserved residues are represented in dark red, partially conserved residues in magenta and less conserved residues in cyan. (d) Solution structure of cpSRP43 CD3. A superposition of the 10 lowest energy structures is shown. (e) Close-up view of CD3 bound to A3CT IV highlighting the gap between strands β4 and β1' induced by a helical turn (green) and forming the cage for substrate recognition. The increased β-sheet twist in the β-barrel, resulting in the loss of strand β5 is indicated (50°).

Flanking residues determine selectivity. From the cpSRP43ACD3–RRKRp complex it was known that CD2 recognizes the extended 531-PPGTARRKR sequence of cpSRP54 and that the residues flanking the arginine (0) and especially the arginine at the last position (+2) are important for binding25. Our biochemical and structural data for the CD2CD3–IV complex show that also here the extended 454-KRSKRKR sequence of Alb3 is specifically recognized. To understand how the two chromodomains discriminate between similar linear motifs from cpSRP54 and Alb3, we mutated residues from the chromodomains to alanine still fits into place. In general, for sterical reasons chromodomains need an alanine at the −2 position of the ligand, which holds true for the cpSRP54 tail binding to CD2 and for histone tails binding to canonical chromodomains38 (Fig. 3b). This restriction is, however, not valid for the CD3–A3CT IV interaction due to the ‘super-twist’ of strand β1', which creates extra space for a serine residue. The importance of the −2 position for binding specificity is underlined by the recent finding that cpSRP54 tails of green algae of the chlorophyte division have a valine at this position, which specifically inhibits binding to cpSRP43 (ref. 39).

The introduction of alanine mutations at the −7 to −4 positions all show detrimental effects according to their distance to the 0 position, even though Arg451 (−7) to Ser453 (−5) are not visible in the X-ray structure. Interestingly, the corresponding region in the cpSRP54 tail (S30-APP) forms a tight turn when bound to CD2, which would clash with Asp358 of CD3 (alanine in CD2) (Supplementary Fig. 5a,b). The double mutation of Lys459 and Arg460 (+1 and +2) results in a fourfold reduction of binding affinity (K_D of 20 μM). Their binding modes differ slightly in the two complexes present in the crystallographic asymmetric unit. Either the side chain of Lys459 is involved in a salt bridge with CD3 Glu352 or the guanidinium group of Arg460 is positioned on the negative dipole of the CD2 C-terminal z1
helix and stacks on the side chain of CD2 Tyr313 (Fig. 4a). These interactions highlight the importance of the preceding CD2 for the CD3–A3CT IV interaction as seen from the ITC data (Supplementary Table 1a). Intriguingly, the same binding mode utilizing the helix dipole is observed also for RRKRp Arg539 at position +2 in its interaction with the Ank4 C-terminal helix25 (Fig. 3d). In contrast, the canonical chromodomain interaction with histone tails does not involve the +2 position, which is therefore characteristic for cpSRP43 chromodomain interactions.

Taken together, the specific interaction of CD2CD3 with A3CT IV is dominated by the flanking regions with a high-positive charge density and the formation of salt bridges. The serine in position –1/C0 adds sterical control for A3CT IV binding. Importantly, binding of linear motifs to the cpSRP43 chromodomains involves the interface with the adjacent domain and does not solely depend on the individual chromodomains. While Ank4 contributes to binding of the cpSRP54 tail to CD2, CD2 is necessary for Alb3 binding to CD3.

A3CT binding to CD3 shapes the CD2–CD3 interface. The biochemical and structural analysis of A3CT IV binding to CD3 showed that the interface with CD2 is an important determinant for the interaction. As A3CT IV complements the chromodomain, the ligand can be seen as part of CD3. Closer inspection shows that the interface with CD2 (330 Å²) is built-up by strand b2a of CD3 and by A3CT IV. Residues interacting within CD3 strand b2a include Leu317, Glu318 and Tyr319, which create a hydrophobic core within the CD2 interface surrounded by polar interactions. Tyr319 forms a hub of the interface and is triangulated by contacts with Val309 and Asp312 in helix a1, as well as with Glu274 from strand b2b (Fig. 4a). A3CT IV residues Arg455 to Lys457 complete the framing of Tyr319, with the two

### Table 1 | Data collection and refinement statistics (molecular replacement).

|                          | Trx–CD2CD3–IV | CD3 |
|--------------------------|--------------|-----|
| **Data collection**      |              |     |
| Space group              | P 2_1 2_1    | 1 4 3 2 |
| Cell dimensions          |              |     |
| a, b, c (Å)              | 79.6, 163.7, 37.4 | 103.9, 103.9, 103.9 |
| x, y, z (Å)              | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| Resolution (Å)           | 45-2.8 (2.9-2.8) | 42.4-2.75 (2.9-2.75) |
| Rmerge (%)               | 11.3 (44.3) | 3.7 (34.2) |
| l/i                        | 6.7 (19.9) | 23.7 (37.7) |
| Completeness (%)          | 99.9 (99.8) | 100.0 (100.0) |
| Redundancy                | 5.5 (5.4) | 48.8 (52.2) |
| **Refinement**           |              |     |
| Resolution (Å)           | 45-2.8 | 42.4-2.75 |
| No. of unique reflections | 12,743 | 2,708 |
| Rwork/Rfree (%)           | 25.0/29.2 | 24.7/28.0 |
| No. of atoms              |              |     |
| Protein                   | 3,490 | 416 |
| Ligand/ion                | 14 | 1 |
| Water                     | 41 | 16 |
| Average B-factor (Å²)     | 71.6 | 109.6 |
| Ligand/ion                | – | – |
| Water                     | – | – |
| r.m.s. deviations         |              |     |
| Bond lengths (Å)          | 0.003 | 0.004 |
| Bond angles (°)           | 0.73 | 0.78 |

CD2, chromodomain; Trx, thioredoxin. Statistics for the highest resolution shell are shown in parentheses.

**Figure 3 | Ligand recognition by chromodomains.** (a) Close-up view of the backbone interactions of CD3 with A3CT IV. Hydrogen bonds are indicated by dashed lines. (b) Sequence alignment of chromodomain ligands. The 0 position binding to the cage is depicted in red. (c) Specific read-out of the 0 position in different chromodomains. Left: A3CT IV in CD3, middle: RRKRp in CD2, right: H3K9me3 in HP1. (d) Schematic representations of the respective chromodomain-ligand interactions.
positively charged residues involved in salt bridges with CD2 Asp273 and Asp312, respectively. Analysis of CD2CD3 by small-angle X-ray scattering (SAXS) confirms that the domain arrangement observed between CD2 and CD3 in the crystal structure is stabilized by ligand binding (Supplementary Fig. 7 and Supplementary Table 3). These data also correlate also with previous SAXS data on cpSRP43, which indicated a reduced flexibility of CD2 with respect to the ankyrin repeats when the cpSRP54 tail was present.25

Structural basis for negative cooperativity. Previous pull-down and ITC experiments showed that cpSRP43 can bind both the cpSRP54 and Alb3 tails at the same time and that the two binding events are linked with negative cooperativity.23 Binding of A3CT to cpSRP43 loaded with RRKRp leads to a five time lower affinity to cpSRP43 loaded with RRKRp, which according to cpSRP43ACD3–RRKRp complex (Supplementary Fig. 9). In complex with RRKRp, improvements in NMR lineshapes indicate stabilization of Gly316, which according to cpSRP43ACD3–RRKRp25 corresponds to extension of helix z1 by one turn (Fig. 4c). However, in complex with A3CT IV the NMR signal for Gly316 shifts back, and secondary chemical shifts (Supplementary Fig. 9) confirm formation of strand β2a as observed in CD2CD3–IV. When the NMR titration experiment is performed in opposite order and RRKRp is titrated into the pre-assembled CD2CD3–IV complex (Supplementary Fig. 8b), CSPs in CD2 helix z1 are much lower than in the previous experiment. In this case, the CD3 strand β2a is already formed due to A3CT binding and residues 316 to 318 are not available for helix z1 extension.

In summary, the conformational changes in the interaction network between the tandem chromodomains provide the structural basis for negative cooperativity observed for the binding of cpSRP54 to CD2 and of Alb3 to CD3.

Discussion

Post-translational targeting of LHCPs to the thylakoid membrane relies on the formation of the transit complex and is a specific route designed for high-throughput delivery of an abundant membrane protein. Along this route, cpSRP43 serves as the hub for multiple interactions. As central component of the transit complex it recruits cpSRP54, it serves as specific chaperone for LHCPs, and provides the docking site for the Alb3 insertase at the
thylakoid membrane. The modular assembly of cpSRP43 by three chromodomains and four central ankyrin repeats is the basis for simultaneous recognition of linear motifs provided by these diverse binding partners. Previously, we deciphered the structural basis for interactions between the ankyrin repeats and the L18 peptide of the LHCPs and between CD2 and the C-terminal tail of cpSRP54 (ref. 25).

We now complete the structural portfolio of cpSRP43 interactions by showing that cpSRP43 CD3 specifically interacts with a linear motif in the C-terminal tail of Alb3. We delineate the importance of the domain interfaces in cpSRP43 for recruitment of the transit complex to the thylakoid membrane (Fig. 5a). CD3 recognizes the positively charged motif IV with a central arginine being accommodated in a modified cage with respect to classical chromodomains. The same principle applies to the cpSRP43–cpSRP54 interaction, which is therefore the key-feature of the cpSRP43 chromodomains. Selectivity for CD2 and CD3 is achieved by adaptation of the flanking residues in the interacting linear motifs. CpSRP54 binding to CD2 involves two consecutive arginine residues in the C-terminal tail that are accommodated in a twinned cage and two consecutive proline residues that form a tight β-turn. Alb3 binding to CD3 is weaker due to distortion of the β-completion and removal of the second cage. Specificity arises as the prolines of the cpSRP54 tail do not fit in CD3 and a serine in the Alb3 tail cannot be accommodated in CD2. In addition, discrimination between motifs II and IV in Alb3 is supported by the read-out of positive charges in the flanking regions of motif IV. Therefore, although the recognized linear motifs are highly similar, each of them contains unique features for distinct recognition. Likewise, preceding domains of cpSRP43 contribute to the ligand read-out in CD2 and CD3: the Ank4 repeat to cpSRP54 and CD2 to Alb3 recognition, respectively. The mode of interface arrangements is also similar and includes the negative dipole of the terminal α helices of the preceding modules that bind respective arginines of the ligands. CpSRP43 contains a third chromodomain at its N terminus (CD1), which shows high sequence and structure conservation. However, CD1 has so far not been implicated in interactions with components of LHCP biogenesis. In contrast to CD2 and CD3, the modified cage of CD2 and CD3 is not present in CD1 and a preceding folded domain is missing, which apparently prevents cpSRP54 and Alb3 binding and a ligand, if any, remains to be identified. Interestingly, although A3CT IV (Kd 15 μM) contributes most to the overall affinity of A3CT (Kd 5 μM) for cpSRP43, additional low affinity interactions involving A3CT-II increase the avidity of the interaction. Previously, fluorescence complementation assays with protoproducts indicated a stronger contribution of motif II (than motif IV) to the interaction with cpSRP43 and an additional binding site in transmembrane domain five of Alb3 (TMD5). A recent study in planta described that an Alb3 truncation (including motif IV) results only in a slight reduction in LHCP accumulation compared with wild-type plants when grown under low light conditions. Our structural and biochemical study focused on the interaction between cpSRP43 CD2CD3 and the Alb3 tail, but additional binding sites in full-length Alb3 may contribute to the interaction in vivo. The presence of multiple linear motifs in the long Alb3 tail suggests that fly-casting contributes to the interaction with cpSRP43.

Our data provide the structural basis for cpSRP43 tandem chromodomain interaction with their substrates. This interaction differs from the interactions of chromo shadow domains with their binding partners. There, recognition of a central PXVXL pentapeptide requires the interface of a symmetrical chromo shadow domain dimer. However, like for the cpSRP43 chromodomain interaction with cpSRP54 and Alb3, the recognition of flanking residues is also important for the interaction of chromo shadow domains with their substrates and determines specificity.

The C-terminal tails of cpSRP54 and Alb3 can bind simultaneously to cpSRP43 (ref. 23). The two binding events show negative cooperativity as binding of the first ligand lowers the affinity of the second ligand by a factor of five. Our NMR data indicate that C-terminal residues of CD2 are involved in this crosstalk and the crystal structure of CD2CD3–IV reveals Gly316 as a hinge point between the two chromodomains. When only the cpSRP54 tail is bound to CD2CD3, the helix propensity increases for residues beyond Gly316; however, when the Alb3 tail binds to CD3, residues 316–319 undergo a conformational change to a β-strand conformation and constitute the CD2–CD3 interface. Negative cooperativity might be important for membrane docking of cpSRP to Alb3 and for the handing over of LHCP to Alb3. The lower affinity observed in the Alb3 interaction might reflect the necessity of a transient membrane tether, which can be released when LHCP has been handed over to Alb3 for insertion. The negative cooperativity between the CD2 and CD3 interactions indicates inter-domain communication, which could allow for sensing the presence or absence of LHCP. However, further studies are needed to clarify the biological implications of this gain-of-function.

Alb3 belongs to the YidC/Oxa1/Alb3 family of membrane insertases, which are responsible for insertion and folding of membrane proteins and their assembly into larger protein complexes. YidC and Oxa1, members of the insertase family, have been implicated in co-translational membrane protein
biogenesis. YidC recruits ribosomes to the plasma membrane in bacteria, while in mitochondria, which lack SRP, Oxa1-mediated ribosome docking to the inner membrane is required for efficient membrane insertion of proteins involved in oxidative phosphorylation. Oxa1 and YidC utilize their positively charged C-terminal extensions for ribosome binding4–6. The C-terminal tails of YidC in most Gram-negative bacteria are short and contain less positive charges. Fusion of an extended C-terminal tail of YidC from marine bacteria like Rhodopillarella baltica and Oceaniculis alexandrill to E. coli YidC highly improved ribosome binding1. Both Alb3 and Oxa1 have a long C-terminal tail with pronounced clusters of positively charged residues8,8. While the details of the Oxa1-ribosome interactions are not yet resolved, Alb3 utilizes the tail to specifically interact with CD3 of the negatively charged cpSRP43. Overall, the interaction of Alb3 with cpSRP43 and Oxa1 or YidC with ribosomes appears mechanistically similar, and the evolutionary role of cpSRP43 might be to adapt the cpSRP system to post-translational targeting (Fig. 5b). The tandem array of cpSRP43 chromodomains allows interacting with both Alb3 and cpSRP45 to efficiently deliver its LHC5 cargo to the membrane insertase and to regulate the targeting process by the SRP machinery.

Methods
Cloning. The different A3CT constructs encoding the amino acid sequence 363–462 were cloned into pET21d via Ncol/Xhol restriction sites. Single and double point mutations in A3CT were generated in pET21d using the QuikChange system (Stratagene). The cpSRP43 CD2CD3 deletion construct encoding amino acids 264–373 and the cpSRP43 CD3 deletion construct encoding amino acids 316–373 were cloned into pETRlx1a57 with a cleavable tobacco etch virus (TEV) site via Ncol/Xhol restriction sites. Single point mutations in cpSRP43 were generated in the pET24a vector using the QuikChange system (Stratagene). The cpSRP43 CD3 fusion construct, A3CT IV encoding residues 440–462 was covalently linked with a (GS)2, to cpSRP43 CD2CD3 encoding residues 264–373. CD2CD3–IV was then cloned with a G5 linker into pETRlx1a via Ncol/Xhol restriction sites.

Protein production and purification. A3CT wild-type and A3CT mutants with a C-terminal His6-tag were produced in E. coli BL21 (DE3) cells. Protein production was induced with 1 mM DTT. N-terminally His6-tagged cpSRP43 wild-type and cpSRP43 mutants were produced in E. coli Rosetta2 (DE3) pLYS3 cells. Cells were grown at 37 °C to a cell density of 2.5 ml−1. Protein production was induced by 0.4 mM IPTG. After 12 h at 18 °C, the cells were harvested and stored at −80 °C. Protein production was performed as described above. N-terminally His6-tagged Trx–CD2CD3–IV was produced in E. coli Rosetta2 (DE3) pLYS3 cells in auto-induction medium. His6–Trx–CD2CD3–IV was purified as described above. N-terminally His6-tagged Trx–CD3–IV with a cleavable TEV site were produced in E. coli Rosetta2 pLYS3 cells. Cells were grown at 37 °C to a cell density of 0.6–0.8 OD per ml. Protein production was induced by 0.2 mM IPTG. After 12 h at 18°C, the cells were harvested and stored at −80 °C. His6–glutathione-S-transferase (GST)–A3CT IV peptide was produced in E. coli BL21 (DE3) cells. Cells were grown at 37 °C and protein production was induced by 0.4 mM IPTG at an A600 of 0.8–1.0. After 3 h at 37 °C, the cells were harvested and stored at −80°C. Purification of proteins fused to Trx and GST was performed as described in a previous work19 with the following adaptation for A3CT IV peptide: after TEV cleavage overnight, the sample was reloaded on a HisTrap column and the A3CT IV peptide was purified by 0.8–1.0. After induction, the proteins were expressed for 16 h at 18 °C. The different A3CT constructs encoding the amino acid sequence 600 of 0.8–1.0. After 3 h at 37 °C, the proteins were purified for M9 medium supplemented with [15N]HCl and [13C]-glucose as the sole sources of nitrogen and carbon. After protein loading, after induction of AfuG75R43, the proteins were purified as described previously19. For NMR, the proteins were purified as described previously23. For size-exclusion chromatography, the samples were loaded on a 26/60 Superdex75 column equilibrated in NMR buffer (20 mM Na-phosphate (pH 6.5), 150 mM NaCl).

Protein production and purification for NMR. N-terminally His6-tagged Trx–CD2CD3 and Trx–CD3 were produced in E. coli Rosetta2 pLYS cells, and N-terminally His6-tagged Trx–A3CT and His6–GST-tagged A3CT IV peptide were produced in E. coli BL21 cells. The proteins were produced in M9 medium supplemented with [15N]HCl and [13C]-glucose as the sole sources of nitrogen and carbon. After protein loading, after induction of AfuG75R43, the proteins were purified as described previously23. For size-exclusion chromatography, the samples were loaded on a 26/60 Superdex75 column equilibrated in NMR buffer (20 mM Na-phosphate (pH 6.5), 150 mM NaCl).

Isothermal titration calorimetry. ITC experiments were performed in a buffer containing 20 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM MgCl2, and 0.25 mM TCEP (tris(2-carboxyethyl)phosphine). All protein samples and peptides were dialysed over night in this buffer and extensively degassed prior to titration. ITC experiments were performed on a VP-ITC microcalorimeter (MicroCal, Northampton, MA) at 20 °C with 307 r.p.m. stirring. Titrations consisted of 23 injections of 12 µl aliquots of the titrant into the solution of the cell. Typical protein concentrations in the cell were 0.01–0.1 mM and 0.1–1.0 mM in the syringe. Data were analysed with Origin 7.0 software. The ITC measurements were performed in duplicate or triplicate. Peptides were purchased from PSL (Peptide Speciality Laboratories, Technology Park, Heidelberg), purified by HPLC and analysed by mass spectrometry.

Structure determination and refinement. The construct His6–Trx–CD2CD3–IV was crystallized in the orthorhombic space group P2_12_1 with two molecules in the asymmetric unit. The cell parameters are a = 79.6 Å, b = 163.7 Å, c = 37.4 Å, and the solvent content is 42%. The structure was solved by molecular replacement as implemented in PHASER26 using Trx (PDB code 3DXB) and one ensemble of the solution structure of cpSRP43 CD3 as a search model. The structure was reﬁned using Phenix.refine50 and iterative model building in COOT52 and was validated with MOLPROBITY53 as implemented in PHENIX51. Ramachandran statistics for the final model show 97% of residues in most favoured regions and 3% of residues in additionally allowed regions. All structural figures were prepared with PyMOL24. Electrostatic surface potentials were calculated with APBS integrated in PyMOL25. Sequence alignments were performed with ClustalX25 and protein sequence conservation were performed using the ConsURF server56.

Protein crystallography and data collection. Crystals of His6–Trx–CD2CD3–IV were grown in an in-house automated crystallization platform at 18 °C in sitting drops containing 0.2 µl of His6–Trx–CD2CD3–IV (17 mg ml−1) and 0.2 µl of a reservoir solution consisting of 20% (v/v) PEG 3500 and 0.2 M Ca(OAc)2. Crystals grew as thin and fragile plates after 5 days. The crystals were cryoprotected in mother liquor containing 20% (v/v) glycerol and flash-cooled in liquid nitrogen. Data were collected at the European Synchrotron Radiation Facility (ESRF, Grenoble) on beamline ID29 at 0.992 Å and 100 K. Data were integrated and scaled with XDS58 and AIMLESS from the CCP4 package49. Crystals of cpSRP43 CD3 were grown at 4 °C in sitting drops containing 0.2 µl of cpSRP43 CD3 complexed with A3CT IV (12 mg ml−1) and 0.2 µl of a reservoir solution consisting of 25% PEG 3500, 0.1 M Hepes pH 7.5 and 0.2 M MgCl2. Crystals were grown only after the CD3 domain grew as little needles after 21 days. The crystals were cryoprotected in mother liquor containing 35% PEG and 20% glycerol, flash-frozen in liquid nitrogen and measured at the ESRF on beamline ID29 at 1.033 Å and 100 K. Data were integrated and scaled with XDS58 and AIMLESS from the CCP4 package58.

NMR spectroscopy. Spectra were acquired at 298 K (all experiments, except backbone assignment experiments of A3CT motif IV and its titrations with CD3, which were carried out at 278 K) on Bruker Avance III NMR spectrometers equipped with cryogenic triple resonance gradient probe heads at magnetic field strengths corresponding to proton Larmor frequencies of 600 and 800 MHz. All spectra were processed with NMRPipe28 and analysed using Sparky28 and CARA (http://cara.mrzn.ch). For backbone and side chain assignments HNCACB, CBCA(CO)NH and HCCH-TOCSY spectra were recorded. Distance restraints were calculated from a solution structure of cpSRP43 CD3 as a search model. The structure was reﬁned using Phenix.refine50 and iterative model building in COOT52 and was validated with MOLPROBITY53 as implemented in PHENIX51. Ramachandran statistics for the final model show 96% of residues in most favoured regions and 4% of residues in additionally allowed regions. All structural figures were prepared with PyMOL24. Electrostatic surface potentials were calculated with APBS integrated in PyMOL25. Sequence alignments were performed with ClustalX25 and protein sequence conservation were performed using the ConsURF server56.

NATURE COMMUNICATIONS | DOI: 10.1038/ncomms9875 | www.nature.com/naturecommunications

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by according to the study by Wishart and Sykes60. NMR CSPs of CD3, CD2CD3, A3CT and motif II/IV on titration with each other and RRRK were observed by titrating their signal intensity until saturation was reached. A series of 1H, 13C HSOQC spectra were recorded for 15N-labelled CD3, CD2CD3, A3CT or motif IV with various amounts of binding partner. The following stoichiometries were used for diverse interactions (with the 15N-labelled partner being first): CD2CD3:A3CT (1.0, 1.1, 1.3, 1.5, 1.9; 1.0, 1.1, 1.3, 1.5, 1.9; 1.0, 1.1, 1.3, 1.5, 1.9; 1.0, 1.1, 1.3, 1.5, 1.9; 1.0, 1.1, 1.3, 1.5, 1.9). Automated assignments of NOEs and completeness of the NOE cross-peaks were manually inspected. Distance restraints from the CYANA calculation and Automatically assigned NOEs and completeness of the NOE cross-peaks were assignment and torsion angle dynamics, using the software CYANA 3.0 (ref. 62). Structural quality of the final ensemble of 10 structures with lowest g of all samples were extracted by the Guinier approximation with the 70 in Supplementary Table 3. Small-angle X-ray scattering. The constructs cpSRP43 CD2CD3 (residues 264–373) and cpSRP43 CD3–IV were produced and purified as described above. About 50 μl of CD3 (5.10, 20 mg ml−1) and CD2CD3 with 7 × molar excess of A3CT IV (2.5, 5.10 mg ml−1), as well as buffer and buffer with the same amount of A3CT IV were measured at 298 K on a Rigaku BioSAXS100 using a Pilatus detector. Six frames with 900-s exposure time per frame were recorded for each sample used in the titrations were in the same buffer to prevent CSPs based on buffer or pH effects. Affinities from NMR data could not be obtained in most cases as interactions were in the intermediate exchange regime. Interacting residues were identified by manually tracing peaks in the NMR spectra and confirmed by recording and analysing backbone assignment experiments for the bound form. Atom-specific chemical shift weighting was done according to Mulder et al.61. The NMR structure of CD3 was calculated by automated NOE cross-peaks assignment and torsion angle dynamics, using the software CYANA 3.0 (ref. 62). Distance restraints from the CYANA calculation and TALOS + derived38 were used in a water refinement calculation64 using ARIA 1.2 (ref. 65). Structural quality of the final ensemble of 10 structures with lowest energy was validated using the iCING web server66. Ramachandran statistics for the final NMR structure of CD3 show 90.9% of residues in most favoured regions and 9.1% in additionally allowed regions. The structural statistics are shown in Supplementary Table 2. References 1. Cross, B. C., Sinning, I., Luirink, J. & High, S. Delivering proteins for export according to the study by Wishart and Sykes60. NATURE COMMUNICATIONS | DOI: 10.1038/ncomms9875 10 NATURE COMMUNICATIONS | DOI: 10.1038/ncomms9875 2015 Macmillan Publishers Limited. All rights reserved.
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