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The STIL protein contains intrinsically disordered regions that mediate its protein–protein interactions†

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The STIL protein participates in mitosis and malignant transformation by regulating centrosomal duplication. Using biophysical methods we studied the structure and interactions of STIL. We revealed that its central domain is intrinsically disordered and mediates protein–protein interactions of STIL. The intrinsic disorder may provide STIL with the conformational flexibility required for its multitude binding.

Intrinsically disordered proteins (IDPs) or protein domains (intrinsically disordered regions, IDRs) contain no tertiary structure and rather exist in an ensemble of highly flexible conformations in equilibrium. This lack of structure represents their physiological functional state. Typically, the sequences of IDRs are rich in polar and charged residues, resulting in low overall hydrophobicity and high net charge, followed by the inability to form a stable hydrophobic core.[1] Approximately 30% of the eukaryotic proteins are highly intrinsically disordered or contain a significant proportion of IDRs and other 40% are moderately disordered.[2] More than 70% of signaling proteins are partially disordered and contain IDRs,[2] emphasizing the significance of disordered proteins in signaling pathways. IDRs are rich in phosphorylation sites that regulate protein–protein interactions in signaling processes.[3] Disordered proteins are involved in recognition, signaling and regulation pathways in living cells, in which interactions with many partners are required. The extended surface of the disordered regions combined with their flexibility provides the protein a combination of diversity and specificity that allows it to interact with many different proteins.[1]

The *STIL* gene (*SCL*/*TAL1* interrupting locus) was cloned from a chromosomal aberration associated with T-cell leukemias.[4] It encodes a 150 kDa cytosolic protein containing 1288 residues, which is expressed mainly in proliferating cells. STIL is ubiquitously expressed during embryogenesis and its knockout in mouse causes embryonic lethality.[5] Overexpression of STIL was observed in many types of cancer[6] and STIL was shown to be required for the survival and growth of cancer cells.[7] STIL expression is regulated during the cell cycle and the protein is phosphorylated at mitotic entry and is degraded upon mitotic exit.[8] STIL is a centrosomal protein that is required for centrosomal biogenesis and centriolar duplication.[9–13] Sequence analysis indicates that STIL is a non-globular protein[8] that is predicted to contain an IDR[14] but there is no further information about its structure.

STIL was found to directly interact with several proteins. The interaction of STIL with SUFU, a regulatory protein in the hedgehog signaling pathway of the growing embryonic cell, is mediated by the STIL C-terminal residues 756–1263.[15] STIL also interacts with PIN1, a regulatory protein in mitosis, through residues 567–704 in its central domain. This interaction is mediated by phosphorylation in this STIL domain.[8] STIL also interacts with CENPJ/CPAP, a protein that, like STIL, plays a role in cell division and centrosomal duplication, through residues 231–781 in the central domain of STIL.[12] CHFR, a tumor suppressor that delays mitotic entry in response to mitotic stress, was also suggested to interact with STIL.[9]

Here we present for the first time a structural analysis of the central domain of STIL, showing that this domain is an IDR. Using several disorder prediction servers[16] we found that STIL is predicted to be a partially disordered protein (Fig. S1A, ESI†). Its central part, residues 400–700, is predicted to be disordered by most of the servers. The ~400 N-terminal residues are predicted to form an ordered structure with good agreement between the servers. The disorder predictions of the C-terminal part of the protein, from residue ~720, show discrepancies between the servers, indicating that this part may contain folded and disordered parts. Sequence analysis shows that the STIL IDR is rich in Ser and Pro residues and is slightly more hydrophilic than average, like other IDRs (Table S1, ESI†).

Centrosomal proteins are difficult to express since their over-expression and solubility are very limited.[14] No efficient

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protocol for expressing and purifying STIL at high concentrations that are required for biophysical studies was reported so far. Since full-length STIL is too big to be expressed in bacterial systems, we divided it into fragments. We focused on the central domain of STIL, residues 200–700, which mediates protein–protein interactions of STIL.[8,12] Based on the disorder prediction of STIL, we divided this domain into three fragments: STIL4$_{200–450}$, which was predicted to be partly structured, STIL5$_{450–700}$ and STIL6$_{500—650}$, which represent the fully predicted disordered region as well as its central part (Fig. S1A and S1B, ESI†). Each STIL fragment was purified using a nickel affinity column followed by gel filtration (Fig. S2, ESI†). All three fragments eluted in the GF at lower volumes than expected from their molecular weight, implying disordered features of these fragments. This is because disordered proteins elute earlier than globular proteins with the same molecular weight, due to their extended conformation. Since the STIL fragments tended to aggregate when the HLT tag was cleaved, we used the fused HLT-STIL proteins for further studies.

We analyzed the secondary structures of the STIL fragments using CD spectroscopy. The HLT STIL4$_{200–450}$ spectrum exhibited a minimum at 208 nm and a shoulder at 220 nm (Fig. 1A), indicating a combination of α helical structure and a random coil. The CD spectra of HLT STIL5$_{450–700}$ and HLT STIL6$_{500–650}$ exhibited a minimum at 200 nm (Fig. 1B and C), suggesting that these proteins exist mostly in disordered conformations. The CD results were analyzed using the DichroWeb server[17] that calculates the secondary structure contents of CD spectra. HLT STIL4$_{200–450}$ contains 70% α-helical structures and only 25% disordered elements. HLT STIL5$_{450–700}$ contains 60% disordered conformations and HLT STIL6$_{500–650}$ is 80% disordered. The CD results are in good agreement with our disorder prediction (Fig. S1A, ESI†). We used CD spectroscopy to follow the effect of increasing urea concentrations on the secondary structure of HLT STIL4$_{200–450}$, HLT STIL5$_{450–700}$ and HLT STIL6$_{500–650}$. Fig. 1D shows the urea denaturation curves, which suggest that HLT STIL4$_{200–450}$ loses its structure at high urea concentrations while HLT STIL5$_{450–700}$ and HLT STIL6$_{500–650}$ do not undergo any structural change, as typical for IDRs. These results further confirm that HLT STIL5$_{450–700}$ and HLT STIL6$_{500–650}$ are disordered while HLT STIL4$_{200–450}$ is mainly structured.

To test for the functional role of the STIL IDR we studied its interactions with the CHFR protein, previously suggested to interact with STIL.[9] We designed an array of partly overlapping peptides, derived from the STIL-binding domain of CHFR, and screened it for binding the STIL fragments. Several CHFR peptides bound the central domain of STIL (Fig. 2A–C). HLT alone did not bind the peptide array (Fig. 2D).

The STIL-binding CHFR-derived peptides were synthesized and their interaction with STIL quantified using fluorescence anisotropy (Fig. 2E and F). Seven peptides bound HLT STIL4$_{200–450}$ and HLT STIL6$_{500–650}$ with K_d values between 0.37 μM and 4.8 μM (Table S2, ESI†). The concentration of HLT STIL5$_{450–700}$ was too low to perform such experiments. The identification of STIL binding peptides derived from CHFR allowed us to map the precise STIL binding site in CHFR. Fig. 3 shows the CHFR Cys rich domain,[18] which is the STIL binding domain. The STIL binding sites, representing the binding peptides, are located at two major regions in each CHFR monomer and create a putative binding pocket for STIL in the CHFR dimer.

Although STIL plays a fundamental role as a regulator of centrosomal duplication and consequently in embryonic development and cell cycle regulation, there is no information about its biochemical, structural and biophysical properties. Structural and biophysical studies of STIL were impossible so far because of the unavailability of pure recombinant protein required for such studies. Here we described new protocols for expressing and purifying three recombinant fragments of STIL derived from its central domain. The availability of recombinant proteins enables thorough *in vitro*

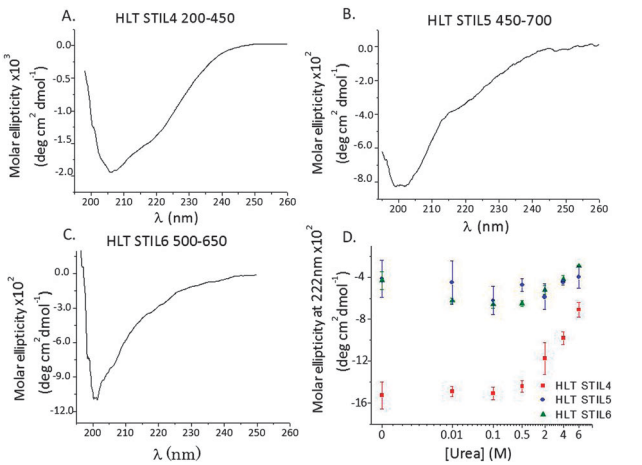

Fig. 1 The secondary structures of STIL fragments: CD spectroscopy. (A) HLT STIL4$_{200–450}$. (B) HLT STIL5$_{450–700}$. (C) HLT STIL6$_{500–650}$. HLT STIL5 and HLT STIL6 are disordered while HLT STIL4 is partly helical. (D) Structural changes of HLT STIL4$_{200–450}$, HLT STIL5$_{450–700}$ and HLT STIL6$_{500–650}$ at increasing urea concentrations. The graph shows the molar ellipticity at 222 nm for each urea concentration for HLT STIL4$_{200–450}$ (red), HLT STIL5$_{450–700}$ (blue) and HLT STIL6$_{500–650}$ (green).

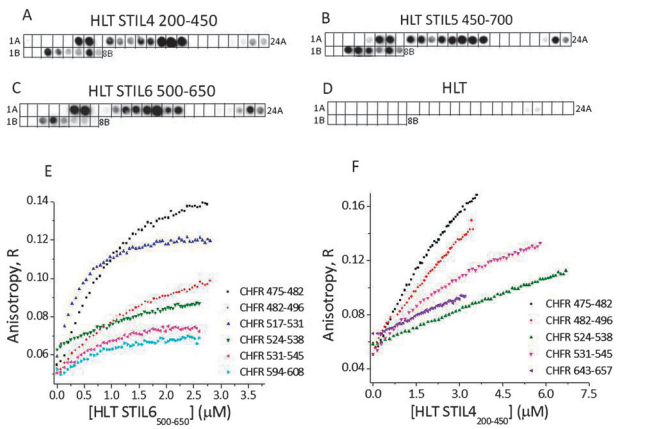

Fig. 2 The STIL fragments bind CHFR derived peptides. (A–D) Screening of HLT STIL4$_{200–450}$, HLT STIL5$_{450–700}$ and HLT STIL6$_{500–650}$ for binding to an array of CHFR derived peptides: (A) HLT STIL4$_{200–450}$, (B) HLT STIL5$_{450–700}$, (C) HLT STIL6$_{500–650}$ and (D) HLT. Each black spot indicates binding between the STIL fragment and the corresponding peptide. (E and F) Fluorescence anisotropy results: (E) titration of HLT STIL6$_{500–650}$ into fluorescein labeled CHFR-derived peptides: CHFR 475–782 (black), CHFR 482–496 (red), CHFR 517–531 (blue), CHFR 524–538 (green), CHFR 531–545 (pink), CHFR 594–608 (cyan). (F) Titrations of HLT STIL4 200–450 into fluorescein labeled CHFR-derived peptides: CHFR 475–782 (black), CHFR 482–496 (red), CHFR 524–538 (green), CHFR 531–545 (pink), CHFR 643–657 (purple).

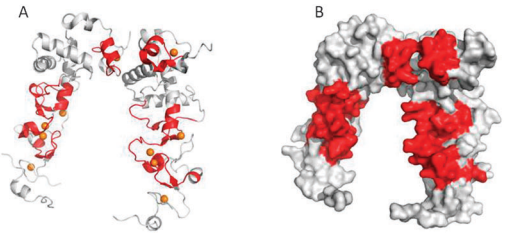

Fig. 3 The STIL binding site in CHFR shown on the structure of the CHFR cys rich domain dimer (residues 407–663, PDB ID: 2xoc). The peptides that bound STIL in the fluorescence anisotropy experiments are colored red (A) ribbon diagram and (B) space filling model.

research into the structure and interactions of STIL using quantitative biophysical techniques.

Our results show that the central domain of STIL is mainly disordered, as was shown by a combination of experimental and computational techniques. Residues 400–700 represent the IDR of STIL protein. The intrinsic disorder of STIL may be required for its involvement in regulating cell proliferation and may enable the protein to interact with different partner proteins at different locations. Many signaling and regulating proteins contain IDRs that take a significant part in their mechanism of action.[19] For example: p21 residues 1–164;[20] P53 1–61;[21] ASPP2 residues 693–918;[22] ARTS 248–274;[23] Axin1 residues 295–500[24] and APC residues 800–2843 are all IDRs.[25] Several interactions of STIL with its target proteins are mediated through the disordered central domain (200–700).[8,12] The central part of STIL is phosphorylated during mitosis and this phosphorylation is required for its interaction with the PIN1 protein.[8] There are seven putative S/T phosphorylation sites in the *murine* STIL, all located between residues 567–704, which are involved in the interaction with PIN1.[8] Disordered regions are common in phosphorylation sites in signaling proteins and take part in the interaction of the phosphorylated domain.[26] We showed here that the phosphorylation site in STIL, residues 567–704, is an IDR.

The fragments that represent the IDR of STIL ($STIL5_{450–700}$ and $STIL6_{500–650}$) are overlapping, making $STIL6_{500–650}$ the central part of $STIL5_{450–700}$. Both fragments bound the peptide array with the same pattern (Fig. 2A–C). This indicates that residues 500–650 are the CHFR peptide-binding sites within STIL 450–700. All three STIL fragments bound approximately the same CHFR peptides, suggesting that $STIL4_{200–450}$, $STIL5_{450–700}$ and $STIL6_{500–650}$ all bind CHFR at the same site. This indicates that STIL 200–700, the full domain, mediates the interaction with CHFR. The binding affinity of the CHFR peptides to the STIL IDR is tighter than the affinity to the structured part of this domain (Fig. 2E and F, Table S1, ESI†). This emphasizes again the important role of the IDR in the STIL interactions.

The IDR of STIL mediates its interactions with proteins that participate in different pathways in the cell. Many scaffold proteins contain IDRs that provide the flexibility, specificity, binding reversibility and multitude interactions they need.[2] We propose that STIL may act as a scaffold protein using its IDR for mediating the interactions. Further studies are required for characterizing STIL interaction network. Our purification protocol described herein paves the way for such studies.

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