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Molecular architecture of the human GINS complex

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Chromosomal DNA replication is strictly regulated through a sequence of steps that involve many macromolecular protein complexes. One of these is the GINS complex, which is required for initiation and elongation phases in eukaryotic DNA replication. The GINS complex consists of four paralogous subunits. At the G1/S transition, GINS is recruited to the origins of replication where it assembles with cell-division cycle protein (Cdc)45 and the minichromosome maintenance mutant (MCM)2–7 to form the Cdc45/Mcm2–7/GINS (CMG) complex, the presumed replicative helicase. We isolated the human GINS complex and have shown that it can bind to DNA. By using single-particle electron microscopy and three-dimensional reconstruction, we obtained a medium-resolution volume of the human GINS complex, which shows a horseshoe shape. Analysis of the protein interactions using mass spectrometry and monoclonal antibody mapping shows the subunit organization within the GINS complex. The structure and DNA-binding data suggest how GINS could interact with DNA and also its possible role in the CMG helicase complex.

Keywords: DNA replication; electron microscopy; mass spectrometry; GINS; CMG helicase complex

EMBO reports (2007) **8**, 678–684. doi:10.1038/sj.embor.7401002

INTRODUCTION

In the last few years, there have been significant findings that have helped to understand the molecular mechanisms of eukaryotic DNA replication; however, the identity of the complex that unwinds DNA has remained elusive. Several lines of evidence provide support for the idea that the minichromosome maintenance mutant (MCM)2–7 hexamer constitutes part of the replicative helicase (Labib *et al*, 2000; Pacek *et al*, 2006). Interestingly, the purified MCM2–7 complex does not show helicase activity *in vitro*, whereas a subcomplex of Mcm4, Mcm6 and Mcm7 presents modest activity with low processivity (Ishimi, 1997). Therefore, it seems that other factors have important roles in the initiation and elongation processes of DNA replication, working as replicative helicase cofactors.

Cell-division cycle protein (Cdc)45 is one of these crucial factors that participates in both initiation and elongation (Zou *et al*, 1997; Aparicio *et al*, 1999). Cdc45 interacts with several DNA replication proteins, including origin recognition complex subunit 2 (Orc2), MCM2–7, Replication Protein A (RPA), DNA polymerases (Saha *et al*, 1998; Zou & Stillman, 2000), synthetic lethality with dpb11-1 (Kamimura *et al*, 2001) and Mcm10 (Christensen & Tye, 2003). In addition, antibodies against Cdc45 disrupt DNA unwinding in a replication assay carried out in cell-free extracts (Pacek & Walter, 2004). Recently, it has been reported that phosphorylation of Mcm4 by the S-phase promoting kinase Cdc7-Dbf4 (Dumb bell former 4) facilitates the formation of a stable Cdc45–MCM complex at the origins of replication (Sheu & Stillman, 2006). The interaction between MCM2–7 and Cdc45 is maintained at the DNA replication forks by means of the four-subunit GINS complex (Gambus *et al*, 2006; Moyer *et al*, 2006).

GINS was first described in yeast as a result of genetic analyses aimed at the discovery of proteins that interact with DNA polymerase B possible subunit 11 (Dpb11) (Takayama *et al*, 2003). The complex is comprised of four conserved proteins—Sld5, Psf1 (Partner of Sld5), Psf2 and Psf3—each distantly related to each other and with no known folding motifs. Three of them were discovered independently by a functional proteomics approach on the basis of induced proteolysis *in vivo* (Kanemaki *et al*, 2003). The GINS complex is essential for initiation of DNA replication and the normal progression of the replisome (Kanemaki *et al*, 2003; Kubota *et al*, 2003; Takayama *et al*, 2003). Previous electron

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Received 12 April 2007; revised 26 April 2007; accepted 27 April 2007; published online 8 June 2007

microscopy images of rotary-shadowed *Xenopus* GINS complex suggested ring-like or C-shaped structures (Kubota *et al*, 2003).

In budding yeast, chromatin immunoprecipitation studies have shown that GINS is recruited at the paused replication fork together with Mediator of replication checkpoint 1 (Mrc1), Topoisomerase interacting factor 1 (Tof1), polymerases α and ε, Cdc45 and the MCM2–7 complex (Calzada *et al*, 2005). GINS, Cdc45 and the MCM2–7 could form the core of a large complex known as the 'replisome progression complex' (Gambus *et al*, 2006). So far, the physiological role and the biochemical features of the GINS complex are poorly understood. A suggestion comes from the recent purification of a stable high-molecular-weight complex formed by Cdc45/MCM2–7/GINS (CMG) from *Drosophila* that shows helicase activity (Moyer *et al*, 2006). Conversely, the *in vitro* interaction of the human GINS complex with DNA polymerase α-primase seems to stimulate its activity (De Falco *et al*, 2007).

Here, we have characterized the three-dimensional structure and DNA binding of a recombinant human GINS complex. By using single-particle electron microscopy and three-dimensional reconstruction, we have obtained a medium-resolution volume of the human GINS complex showing its horseshoe shape. The arrangement of the subunits in the structure was shown using a combination of mass spectrometry of the intact complex and subcomplexes generated in solution or gas phases, and monoclonal antibody mapping using electron microscopy. The DNA-binding preferences of GINS have been also studied. The three-dimensional structure, in conjunction with DNA-binding experiments, suggests the possible role of GINS in the CMG helicase complex.

RESULTS AND DISCUSSION

Human GINS is a heterotetramer and binds to DNA

The open reading frames of the Sld5, Psf1, Psf2 and Psf3 proteins were cloned in a T7 promoter polycistronic vector. The recombinant protein complex was isolated in three steps by using affinity, anion exchange and gel filtration chromatography (see the Methods and supplementary information online). SDS–polyacrylamide gel electrophoresis of purified recombinant human GINS complex (Fig 1A) showed four bands identified as its subunits by mass spectrometry (data not shown). Analytical ultracentrifugation (supplementary information Fig 1 online) and nano-flow mass spectrometry of the intact complex (Fig 1B) showed that human GINS is a heterotetramer with 1:1:1:1 stoichiometry. The molecular mass of the intact complex, measured by mass spectrometry, showed a mass of 98,373 ± 12.7 Da, which is in close agreement with the theoretical value (98,122.0 Da) of the complex, with a Tobacco etch virus (TEV) cleavage site and a His-tag.

To address the binding of the purified human GINS complex to DNA, different DNA probes resembling several replicative structures were analysed by using electrophoretic mobility shift assays (EMSA; Fig 1C,D). Human GINS showed a clear preference for the probes consisting exclusively of single-stranded DNA (ssDNA) or containing stretches of ssDNA ('ssDNA', '3′ end', '5′ end' and 'bubble') than for a probe consisting of only double-stranded DNA (dsDNA; Fig 1C,D). Remarkably, a supershift was observed with the 'bubble' probe. This could be caused by the loading of more than one human GINS complex on each ssDNA region. These results represent the first experimental evidence that human GINS can associate directly with DNA and indicate its role within the CMG helicase (see below).

Three-dimensional reconstruction of the GINS complex

The human GINS complex was applied to carbon-coated grids and negatively stained with uranyl acetate. Despite the low molecular mass of human GINS complex for electron microscopy analysis, a clean distribution of single particles was observed (Fig 2A,B; for details, see the supplementary information online). The refined volume of the human GINS complex at 33 Å resolution shows a horseshoe shape. The approximate molecule dimensions are 130 × 60 × 80 Å (Fig 2D–F). The complex shows a central hole of 30–35 Å in diameter, which is large enough to accommodate either dsDNA or ssDNA. The upper part of the three-dimensional volume is wide open, whereas the opposite side of the central hole is narrower. Hence, the central hole is arranged in a manner similar to a funnel with an upper diameter of approximately 70 Å and a bottom diameter of approximately 25 Å (Fig 2D,E; supplementary movie online), indicating the possibility of different functions for each side of the complex. Although the human GINS three-dimensional structure forms an open ring, the shape of the volume resembles the structure of proliferating cell nuclear antigen (PCNA)—an essential processivity factor for DNA polymerases (supplementary Fig 3 online). The different human GINS subunits could not be identified in the electron microscopy three-dimensional structure owing to the limited resolution, therefore a combined approach of mass spectrometry and monoclonal Fab labelling was used to show the subunit organization.

Architecture of the GINS complex

Mass spectrometry of the intact human GINS complex showed the heterotetrameric oligomerization state of the complex (Fig 1B). Interestingly, the Psf2 subunit readily dissociated on activation and tandem mass spectrometry (MS/MS), indicating that Psf2 has fewer intersubunit contacts and is likely to locate at one end of the horseshoe-shaped structure (Fig 1B, inset). Interactions between the subunits in the human GINS heterotetramer were determined by generating subcomplexes using in-solution perturbation and gas-phase dissociation of the resulting complexes (Hernandez *et al*, 2006). After the addition of 42% methanol, two additional charge state series were observed (Fig 3A,B). The measured masses (47,758 and 70,895 Da) indicate that the two series correspond to the Psf2–Sld5 heterodimer and a Psf2–Sld5–Psf1 heterotrimer, respectively. As Psf2 is located at one end and it interacts with Sld5, the Psf1 subunit should be located on the opposite site of the Psf1/Sld5/Psf2 heterotrimer. Thus, a model of the subunit organization in the complex comprises a central core formed by Sld5 and Psf1, and Psf2 and Psf3 are located at the tips of the horseshoe (Fig 3C). This arrangement is in agreement with the network of interactions of the GINS subunits proposed in yeast using genetic and two-hybrid methods (Takayama *et al*, 2003).

On the basis of the interactions observed by mass spectrometry and the restrictions imposed by the subunit organization inside the three-dimensional structure, our model of the human GINS architecture could be confirmed by localizing Psf2 within the complex. Thus, the human GINS complex was incubated with a monoclonal Fab fragment that recognizes Psf2, and the human GINS–Fab complex was purified (supplementary Fig 4A–E online). To obtain the three-dimensional structure of the human GINS–Fab, the purified complex was negatively stained and analysed by using electron microscopy (Fig 4A,B). A total of 2,000 images were selected and processed similarly to the volume representing the

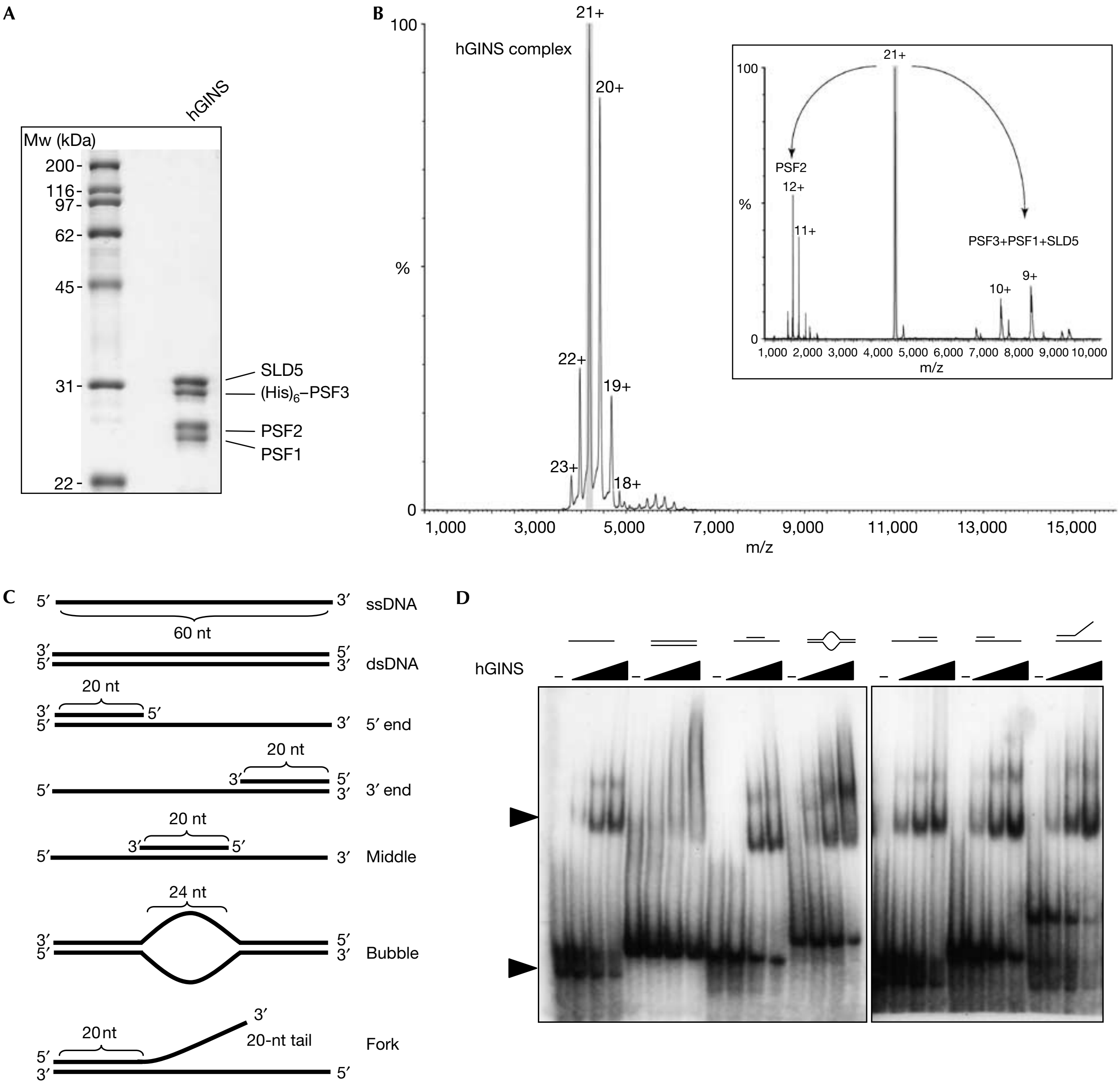


Fig 1 | The human GINS complex is a heterotetramer that binds to DNA. (**A**) SDS–polyacrylamide gel electrophoresis of the purified recombinant human GINS (hGINS) complex shows four bands and their identity as human GINS subunits was confirmed by using mass spectrometry. (**B**) Nanoelectrospray mass spectrometric analysis of the intact human GINS complex shows a well-resolved charge state series (labelled 23+ to 18+), which is consistent with the presence of the four subunits in stoichiometric amounts. Inset: gas-phase acceleration of the isolation at approximately 4.700 *m/z* (21+ charge state) and tandem mass spectrometry clearly showed the dissociation of the subunit Psf2 from the intact complex. (**C**) Scheme of the DNA probes forming different structures used for EMSA. (**D**) EMSA of the human GINS complex. Increasing amounts of the human GINS complex were used, whereas the probe concentration was constant. EMSA, electrophoretic mobility shift assays.

human GINS complex alone (without Fab). The resultant three-dimensional volume (Fig 4C,D) resembles the human GINS structure and shows an additional mass on one tip of the horseshoe-shaped human GINS structure, which corresponds to the size and shape of a Fab molecule (Fig 4E,F). This result confirms the localization of Psf2 at one end of the structure and, combined with the mass spectrometry data, supports the proposed model of the organization of human GINS subunits within the complex.

Possible roles of the GINS complex in the replication fork

A certain parallel could be drawn between the structures of human GINS and PCNA (Krishna *et al*, 1994). Indeed, it has been proposed recently that GINS binds to and enhances the activity of DNA polymerase α-primase (De Falco *et al*, 2007). However, we believe that the structural similarities are not sufficient to indicate that GINS, as PCNA, has the characteristics of a DNA processivity factor. First, the dimensions of PCNA (90 × 40 × 90 Å) are smaller

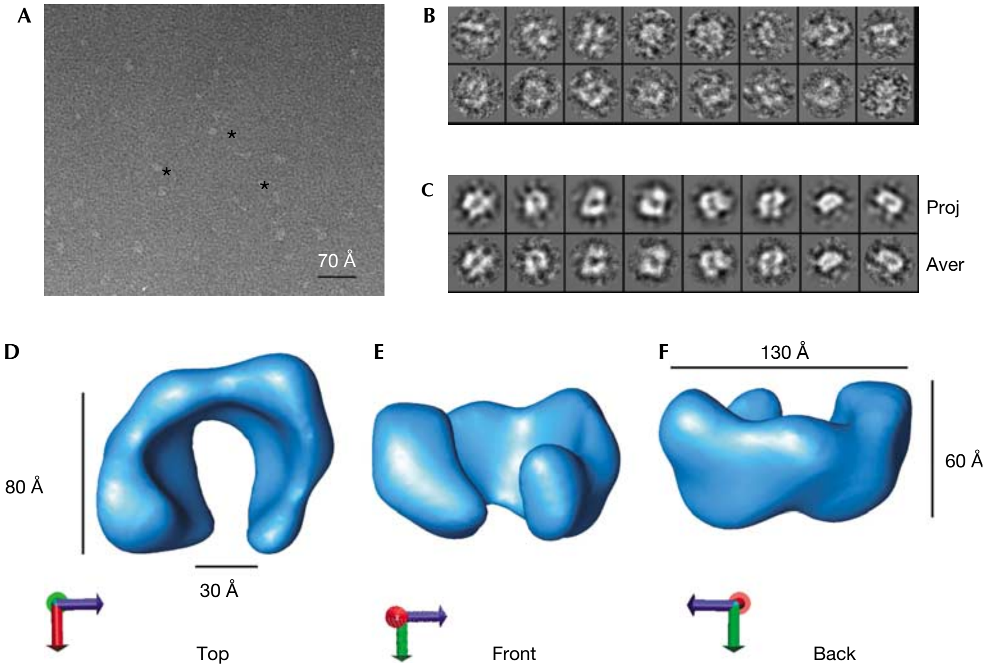


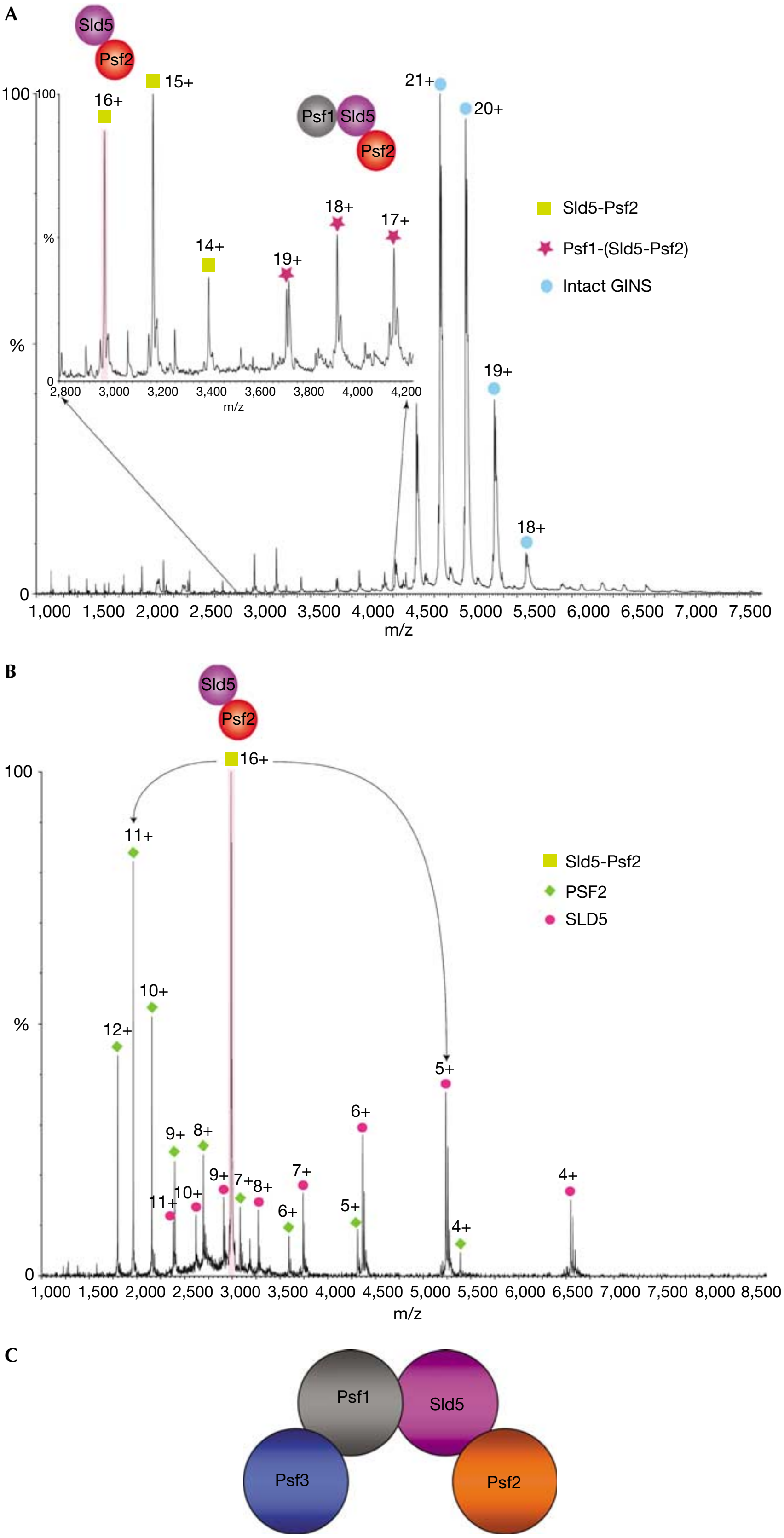
Fig 2 | Electron microscopy and three-dimensional structure of the human GINS complex. (**A**) Representative area of the human GINS micrographs. Some images of human GINS single molecules are indicated by asterisks. (**B**) Gallery of single particles showing some representative views. (**C**) A collection of selected projections of the final volume (Proj) and three-dimensional averages of the images within the corresponding class (Aver). (**D–F**) Different views of the reconstructed volume from human GINS.

than the human GINS complex (see previous section), according to the number of components and their molecular weight. Second, the PCNA structure is a closed ring, whereas human GINS is an open ring. Third, although the internal diameter of the central hole has similar dimensions of around 30–35 Å in both, the PCNA internal channel does not show an internal funnel-like shape similar to that observed in human GINS (Fig 2D–E; supplementary Fig 3 online). Furthermore, the EMSA assays (Fig 1C,D) indicate that human GINS does not show preferential binding to dsDNA, which is the molecule bound by PCNA during DNA replication (Johnson & O'Donnell, 2005). Finally, PCNA possibly does not change its overall conformation on DNA binding. This might not be the case for human GINS and a conformational change induced by DNA binding could occur. The structure suggests that DNA binding might promote a more compact complex to embrace the nucleic acid.

An attractive idea is that the biochemical function of GINS resides within the recently described CMG complex consisting of Cdc45, GINS and the MCM2–7 hexamer. All the components of the CMG are present at DNA unwinding sites (Calzada *et al*, 2005; Gambus *et al*, 2006), and a purified CMG complex from *Drosophila* shows ATP-dependent helicase activity (Moyer *et al*, 2006). The association of the MCM2–7 hexamer with these two cofactors seems to stimulate DNA unwinding and strand displacement activities, which have been predicted and experimentally sought for the MCM2–7 hexamer for a long time (Aparicio *et al*, 2006). The need for essential activators of the helicase activity represents a change in the model about the mode of action of eukaryotic replicative helicases and could help to explain the delay between the assembly of the MCM2–7 complexes on the chromatin during late telophase/early G1 and the initiation of DNA replication several hours later (Mendez & Stillman, 2000).

Previous models on the eukaryotic replicative helicase function, based on steric exclusion (Lee & Hurwitz, 2001; Kaplan *et al*, 2003) or rotary pumps (Laskey & Madine, 2003; Mendez & Stillman, 2003), were focused on the MCM2–7 complex as the unique assembly responsible for the unwinding and strand displacement activities.

On the basis of the described association of GINS with the MCM2–7 complex and Cdc45 to form a molecular machine that unwinds DNA (Moyer *et al*, 2006), and on our observation that purified human GINS shows preferential binding for DNA structures containing ssDNA, it is tempting to speculate about the possible role of GINS after its association with the other components of the CMG complex. We foresee two main possibilities (Fig 5). In both cases, the MCM2–7 complex would work as an engine to unwind the dsDNA coupled to ATP hydrolysis and GINS as a crucial structural element required for the successful separation of the two DNA strands. In the first model (Fig 5A), MCM2–7 pumps dsDNA through its inner channel by helical rotation, destabilizing the double helix. Hence, the GINS complex would function as a strand displacement blade, or 'ploughshare' (Takahashi *et al*, 2005), located where unwound DNA exits from the MCM hexamer, preventing re-annealing and providing room for the activity of the polymerases. In the second model (Fig 5B), GINS would be located in front of the MCM2–7 complex and would have a more active role in DNA unwinding. The main difference is that, in this case, only one strand of DNA goes through the MCM2–7 inner channel. This model would share more structural features with the recently proposed mode of action of the MCM4–6–7 helicase (Kaplan *et al*, 2003) and the viral E1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Intact GINS
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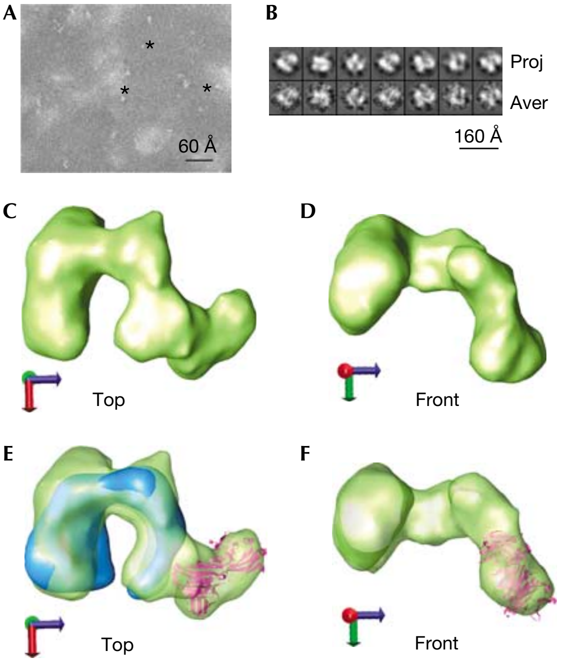

Fig 4 | Localization of the Psf2 in the human GINS–Fab complex. **(A)** Electron microscopic field of a negatively stained sample of purified human GINS–Fab complex. Some particles are indicated with asterisks. **(B)** Panel containing projections (Proj) and their corresponding class averages (Aver) obtained after refinement. **(C)** Top and **(D)** front views of the three-dimensional structure of human GINS–Fab complex and **(E,F)** the fitting of human GINS and atomic structure of Fab (2HFF.pdb) coloured in blue and magenta, respectively.

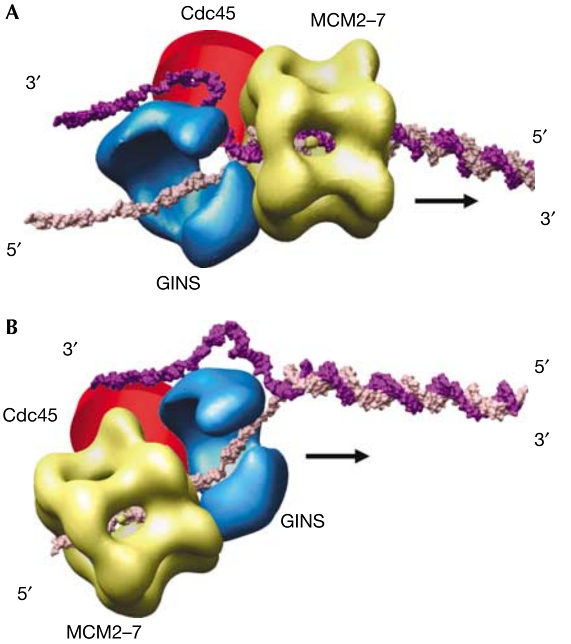

Fig 5 | Hypothetical models of the function of Cdc45/Mcm2–7/GINS complex function (see Discussion for details). **(A)** The MCM2–7 complex (yellow) interacts with double-stranded DNA (pink and magenta), and GINS (blue) could act as a plough preventing the separated strands from the unwound DNA from re-associating. **(B)** In this model the MCM2–7 interacts with single-stranded DNA, and the GINS complex keeps the single strand available to other replication factors. Cdc45, cell-division cycle 45; MCM2–7, minichromosome maintenance mutant 2–7.

helicase (Enemark & Joshua-Tor, 2006). The two hypothetical models in Fig 5 represent two alternatives of cooperation between a motor engine formed by MCM2–7 and a 'strand displacement unit' provided by GINS, but other variations could also be envisioned. So far, no structural information on Cdc45 is available and its position between GINS and MCM2–7 is speculative. However, it is worth noting that an immunoprecipitation with anti-Cdc45 was the original method to isolate the CMG complex (Moyer *et al*, 2006).

Our study is a first step to unravel the architecture of human GINS, and further structural work regarding the association of GINS with DNA and other components of the CMG will be crucial to understand fully the molecular mechanisms involved in DNA unwinding during eukaryotic DNA replication.

METHODS

Full protocols are available in the supplementary information online.

Human GINS expression and purification. The complementary DNAs of the human GINS subunits were cloned in a polycistronic vector and expressed in *Escherichia coli* Rosetta (DE3) cells (Novagen, Madison, WI, USA). Transformed cells were grown in lactose broth medium supplemented with ampicillin (at 100 μg/ml) and chloramphenicol (at 34 μg/ml). The cells were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at 16 °C. The recombinant human GINS complex was isolated using nickel affinity, anion exchange and gel filtration chromatographic steps. Fractions containing human GINS after the gel filtration were pooled, concentrated and stored at −80 °C in small aliquots.

Electrophoretic mobility shift assays. Different DNA structures were obtained by hybridization of the ^{32}P-labelled 60-mer oligonucleotide. Protein–DNA binding reactions were carried out by incubating recombinant GINS complex (1–10 pmol) with 150 fmol of each probe in buffer B (50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.5 mM EDTA, 10% glycerol and 1 mM dithiothreitol) at 25 °C for 30 min; protein was always added last. After incubation, the mixtures were resolved by in 5% polyacrylamide–TBE non-denaturing gel electrophoresis. Gels were dried and exposed to autoradiography.

Electron microscopy. For negative staining, a few microlitres of purified human GINS complex and its anti-Psf2-Fab bound complex were diluted to an appoximate concentration of 0.1 and 0.2 mg/ml, respectively. Samples were applied to glow-discharged

◀ **Fig 3** | Identification of human GINS subcomplexes by mass spectrometry. Spectrum from **(A)** mass spectrometry and **(B)** tandem mass spectrometry analysis of the human GINS complex after disruption of the complex using 42% methanol. Two charge state series were observed in the *m/z* region 2,800–4,250 **(A**, inset). The measured masses (47,758 Da, light green squares; 70,895 Da, magenta stars) indicate that the two series correspond to the Psf2 and Sld5 dimer, and the heterotrimer composed of Psf1, Psf2 and Sld5. **(B)** The dissociation products Psf2 (green diamonds) and Sld5 (pink circles) of the 16+ charge state (highlighted in red) of the Psf2, Sld5 dimer confirm its composition. **(C)** Interactions of the GINS subunits.

carbon-coated copper–rhodium grids, negatively stained with 2% uranyl acetate (w/v) and observed in a JEOL 1230 electron microscope at an accelerating voltage of 100 kV. The human GINS complex images were recorded under a low-dose condition at a nominal magnification of × 60,000, and images of a human GINS–Fab complex were taken at × 25,000. Good micrographs were digitized in a Dimage Scan Multi Pro scanner (Minolta, Osaka, Japan) at 2,400 d.p.i. and averaged to a final 3.56 Å/pixel at the specimen for human GINS complex and 4.2 Å/pixel at the specimen for human GINS–Fab complex.

Mass spectrometry. Mass spectra collected for the intact protein complexes were recorded on a QSTAR XL mass spectrometer (MDS Sciex, Concord, Canada) modified for high-mass detection (Sobott *et al*, 2002). The human GINS complex (1 μg/μl) was exchanged into 300 mM ammonium acetate (pH 7.5) by using microbiospin-6 columns (Bio-Rad Laboratories, Hercules, CA, USA), and 2 μl aliquots were introduced by gold-coated nanoflow capillaries prepared in-house. The conditions within the mass spectrometer were adjusted to preserve noncovalent interactions (Hernandez *et al*, 2006). The mass spectrometer was operated at a capillary voltage of 1,200 V and a declustering potential of 40 V. An MS/MS spectrum of the intact human GINS complex was obtained by MS/MS of an isolation at 4,685 *m/z* with collision energy of 100 V. The intact human GINS complex was disrupted through the stepwise addition of methanol up to 42% (v/v) and MS/MS of the resulting subcomplex was carried out at collision energy of 80 V.

Accession code. The structure of this complex has been deposited at the European Bioinformatics Institute, with the unique accession code EMD-1355.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

Note added in the proof. Following the submission of this paper, the crystallographic structure of a truncated mutant of the human GINS complex has been published (Kamada K *et al* (2007) *Nat Struct Mol Biol* **14**: 388–396). It is interesting to note that although the intersubunit interactions are similar in both studies, the overall conformation described by Kamada *et al* is different from our structure.

ACKNOWLEDGEMENTS

We thank O. LLorca and J.M. Valpuesta for the use of the electron microscopy and for helpful comments, and J. Prieto for advice with the analytical ultracentrifugue. J.B. and J.C. thank the Ministerio de Educación y Ciencia for a Ramón y Cajal contract and a pre-doctoral fellowship. Funding was obtained through MEC grants BFU2005-02403, GEN2003-20642-C09-02 to G.M., and European Union 3D-Repertoire LSHG-CT-2005-512028 to G.M., M.Z. and C.V.R., and MEC BFU2004-04886 and Fundación Caja Madrid to J.M.

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