The Forkhead-associated Domain of Ki-67 Antigen Interacts with the Novel Kinesin-like Protein Hklp2*

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The Ki-67 antigen (pKi-67) is widely used as a cell proliferation marker protein. Its actual role in the cell cycle progression, however, is presently unclear. Using a two-hybrid screening in yeast, a novel protein, termed Hklp2 (human kinesin-like protein 2), was identified and shown to interact with the forkhead-associated (FHA) domain of pKi-67. Hklp2 has 1388 amino acids and shows a striking similarity (a 53% identity in amino acids) to Xklp2, a plus-end directed kinesin-like motor found in *Xenopus*. The interaction domain of Hklp2 was mapped to the portion that comprised residues 1017–1237 and that was phosphorylated *in vitro* by incubating with mitotic but not interphasic HeLa cell extracts. That the interaction was striking in the mitotic extract was also verified. In addition, immunofluorescence using specific antibodies revealed an association between pKi-67 and Hklp2 at the periphery of mitotic chromosomes, largely in close proximity to the centromeres. These findings suggest that pKi-67 is involved in the progression of mitosis via its interaction with Hklp2.

The Ki-67 antigen (pKi-67) was originally identified as an antigen for a monoclonal antibody raised against the nuclear extract from a Hodgkin’s lymphoma derived cell line and is characterized as a class of proteins that are localized around mitotic chromosomes (1). Because of this, it is assumed that pKi-67 is involved in mitotic chromosome organization. Moreover, pKi-67 is a convenient cell proliferation marker, because its expression is restricted to growing cells (2). Although the recent identification and characterization of a marsupial counterpart of pKi-67, which is referred to as chmadrin, suggests a role of pKi-67 in the organization of higher order chromatin structure (3), the actual function of the molecule in the cell cycle progression is presently unclear.

To better understand the role of pKi-67 in the cell cycle, a two-hybrid screening from a HeLa cDNA library was carried out using the N-terminal portion of pKi-67 as the bait. This portion is well conserved between human pKi-67 and chmadrin

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB035898.

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The abbreviations used are: FHA, forkhead-associated; GST, glutathione S-transferase.

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 Interaction of the FHA Domain of pKi-67 with Hklp2

Recombinant Proteins—GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech). GST fusions of Ki-FHA (residues 1–99 of pKi-67) and Hklp2-tail (residues 1132–1388) were created by cloning the appropriate inserts into pGEX (Amersham Pharmacia Biotech).
14,000 × g for 10 min at 4°C. The supernatants were used as EB150 extracts. EB150 extracts were incubated with GST or GST-Ki-FHA coupled to glutathione-Sepharose beads (GS-4B; Amersham Pharmacia Biotech) for 1 h at 4°C, after which the beads were washed extensively with EB150. Associated proteins were boiled out in sample buffer and separated in 2–15% gradient gel (Multigel 1 2/5, Duichi Pure Chemicals). Hklp2 was detected by Western blotting using anti-Hklp2 antibodies at 0.5 µg/ml.

RESULTS

Identification of Hklp2 as a Molecule That Interacts with the FHA Domain of pKi-67—To identify cellular proteins that might interact with the FHA domain of pKi-67, a yeast two-hybrid screen of a HeLa cDNA library was carried out using residues 1–99 of human pKi-67 as bait. From the ∼5 × 10⁶ cDNA clones screened, 10 positive clones were obtained. Sequence analyses revealed that these clones could be placed in one of two possible classes. One class (containing five clones), which corresponded to a novel RNA-binding protein, which will be described elsewhere.² Here, we focus on the other class (containing two clones). A full-length sequence was first obtained using the strategy summarized in Fig. 1A. The predicted protein showed striking similarity to Xklp2 (53% identity in amino acids over their entire length) and was therefore termed Hklp2 (human kinesin-like protein 2). Hklp2 and Xklp2 share

Fig. 2. The FHA domain of pKi-67 interacts with Hklp2 (1017–1237) in yeast and in vitro. A, identification of the region of Hklp2 that interacts with the FHA domain of pKi-67 (Ki-FHA) by a yeast two-hybrid system. Schematic representation of Hklp2 and the deletion constructs used. Residue numbers of the N- and C-terminal ends are indicated at the top. Positive interactions (growth on selective medium and activation of the β-galactosidase gene) are represented by +. B, topology diagram of Hklp2 (1017–1237) predicted by the method of Chou and Fasman (13). Helices are shown as boxes and numbered consecutively. Residue numbers at the beginning and the end of the helices are indicated. C, helical wheel plots of residues 1030–1047 in the helix 1 (a), residues 1060–1075 in the helix 1 (b), residues 1094–1107 in the helix 2 (c), and residues 1210–1230 in the helix 7 (d). D, Ki-FHA interacts with His-Hklp2 (1017–1237) in vitro. Purified His-Hklp2 (1017–1237) was incubated in a batch assay with control GST (lane 2) or GST-Ki-FHA (lane 3) coupled to glutathione-Sepharose beads. Bound His-Hklp2(1017–1237) was detected by Western blotting using Penta-His antibody (Qiagen). Lane 1 shows 10% of the amount of input His-Hklp2 (1017–1237).

Subcellular Distribution of Hklp2 in Relation with Mitotic Spindles, Chromosomes, and pKi-67—As an approach to understanding the biological significance of the interaction between the FHA domain of pKi-67 and Hklp2, the subcellular localization of Hklp2 was examined using polyclonal antibodies raised against its C-terminal portion (residues 1132–1338). The antibodies specifically recognized a 160-kDa protein from the total HeLa extract by immunoblotting (Fig. 3A). In the interphase, Hklp2 was detected on centrosomes as described for Xklp2 (data not shown). From prometaphase to metaphase, Hklp2 was detected primarily on the mitotic spindles emanating from the spindle pole regions (Fig. 3B). Hklp2 was not uniformly located over the entire length of the spindles. A certain population of Hklp2 in metaphase was localized in the equatorial region, distinct from the microtubules (Fig. 3B, panels d and e). It is likely that the population is integrated in, or closely associated with, the chromatin structure (Fig. 3B, panels d–f). When the mitotic spindles were disrupted by nocodazole before fixing the cells, the chromatin-integrating population of Hklp2 was detected in the form of dots on mitotic chromosomes (Fig. 3C). These dots were not co-localized with but were close to the centromeres (Fig. 3C). In anaphase, Hklp2 was also detected at the equatorial region of the spindle in a punctate pattern (Fig. 3B, panels g and h), raising the possibility that Hklp2 may be involved in the sliding of antiparallel...
microtubules in anaphase B. Considering the fact that pKi-67 is localized around the mitotic chromosomes (9), pKi-67 and Hklp2 could, in theory, be co-localized at the surface of the chromosomes where mitotic spindles are pointed to. In fact, it is likely that a certain population of Hklp2 is colocalized with pKi-67 on mitotic chromosomes (Fig. 3D, yellow).

**Hklp2 Interacts with pKi-67 Preferentially in Mitosis**—A previously published report (7) concluded that the FHA domain interacts preferentially with a phosphorylated peptide. We examined the issue of whether this was also the case for the FHA domain of pKi-67. We first asked whether the partner protein, Hklp2, was modified differently through the cell cycle. When the electrophoretic mobilities of Hklp2 derived from differently synchronized HeLa cells were compared, the mitotic Hklp2 showed a slower mobility than the interphase Hklp2 (Fig. 4A). Whether this was due to mitosis-specific phosphorylation was not determined in this study.

We next examined the issue of whether Hklp2 is phosphorylated within the interaction domain to pKi-67 (residues 1017–1238). When histidine-tagged Hklp2 (1017–1238) was purified and incubated with differently synchronized HeLa extracts in the presence of $[^{32}P]ATP$, the protein was labeled only when the mitotic HeLa extract was used, suggesting that Hklp2 is phosphorylated within this region in the mitotic phase. The possible presence of other phosphorylation sites in the Hklp2 sequence, however, cannot be excluded.
A novel kinesin-like protein, referred to as Hklp2, has been identified and shown to interact with the FHA domain of pKi-67 (Ki-FHA). The interaction occurred efficiently in the mitotic phase, during which Hklp2 was likely to be phosphorylated. These findings suggest that the pKi-67 antigen might play a crucial role in the progression of mitosis, thus providing a clue to our understanding of the strict correlation between the expression of pKi-67 and cell proliferation. Further analysis of the mode of interaction between Ki-FHA and Hklp2, including an investigation of whether phosphorylation itself is essential, will obviously be required for a more complete understanding of the biological significance of the interaction. Because the phosphoproteins that are recognized by the FHA domain have, thus far, been specified only in limited cases (5, 6), these data will be also helpful in exploring the general mechanism of phosphorylation-dependent protein recognition mediated by the FHA domain.

Given the mitotic localization of pKi-67, it is possible that it co-operates with Hklp2 on the surface of mitotic chromosomes to allow mitosis to proceed. Given the fact that Hklp2 is a plus end-directed motor by analogy to XKlp2 (8), Hklp2 could generate “polar ejection force” (14) by acting along chromosome arms via an interaction with pKi-67. However, this notion is apparently inconsistent, given the observed localization of Hklp2 (Fig. 3C), in which a certain population of this molecule is associated with chromosomes in a punctate pattern but are not distributed throughout the entire chromosome arms. It is possible that we overlooked detecting the localization of Hklp2 on the chromosome arms because of its low levels in that area. Alternatively, our protocol (nocodazole treatment and methanol fixation) might disrupt the actual localization. In any case, it would be intriguing to examine the issue of whether the working point of Hklp2 exists on the mitotic chromosome arms.

In an early study, we identified a molecule, termed chmadrin, from a marsupial cell line (3). Chmadrin shows certain similarities to pKi-67 in its primary structure and cellular localization and thus is considered to be a marsupial counterpart of pKi-67. The C-terminal portion of chmadrin has a novel domain that is characterized by repetitive occurrences of LR (leucine and arginine) pairs with irregular spacing. The domain, called the LR domain, gave rise to severe heterochromatin formation when overexpressed (3) and, as a result, can be implicated in the organization of higher-order chromatin structure. The C-terminal portion of human pKi-67 also possesses the LR domain and, in fact, has the activity to drive heterochromatin formation in a manner similar to that of chmadrin. Thus, pKi-67 appears to play a role in chromatin condensation in its C-terminal region (LR domain). If pKi-67 serves as an adapter between chromosomes and the mitotic apparatus in its N-terminal region (FHA domain), this protein may be a multifunctional protein that acts throughout the cell cycle.

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