Cellular localization and dynamics of Hyaluronan binding protein 1 (HABP1) during mitosis
4.1 INTRODUCTION

Mitosis is a cellular process that facilitates equal partitioning of replicated chromosomes into two identical groups. Before partitioning can occur, the chromosomes must be aligned, so that the separation process occurs in an orderly fashion. The alignment of replicated chromosomes and their separation into two groups constitute the stages of mitosis and are observed in virtually all eukaryotic-cells. These stages are *interphase*, the longest period of the cell cycle during which the DNA replicates, the centrioles divide and protein synthesis takes place. The second phase is the *prophase*, in which the nucleolus fades and the chromatin condenses into chromosomes, with each replicated chromosome comprising of two chromatids. Microtubules of the cytoskeleton disassemble and the building blocks of these microtubules generate the mitotic spindle from the region of the centrioles. *Prometaphase* follows prophase, in which the nuclear envelope breaks down and there is no longer a recognizable nucleus. Some mitotic spindle fibers elongate from the centrioles and attach to kinetochores. Other spindle fibres also elongate, however instead of attaching to chromosomes, they overlap each other at the cell centre. Due to the tension applied by the spindle fibers, all chromosomes align in one plane at the centre of the cell, manifesting the *metaphase*. In *anaphase*, the spindle fibers shorten, the kinetochores separate, and the chromatids are pulled apart and begin moving towards the cell poles. When the daughter chromosomes arrive at the poles and the spindle fibers pulling them apart disappear, it heralds *telophase*. Finally, during *cytokinesis*, the spindle fibers not attached to chromosomes begin breaking down until only the overlapping portion is left, where a contractile ring cleaves the cell into two daughter cells. Microtubules then reorganize into a new cytoskeleton for the return to *interphase*. Though many proteins play a role in mitosis, only few have been studied and well characterized.

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is well documented in living cells. High resolution confocal time-lapse imaging in mitotic cells using the lamin B receptor fused to green fluorescent protein (LBR–GFP), revealed its dispersal into the interconnected Endoplasmic Reticulum (ER) membrane system in prometaphase. LBR–GFP diffused across the cell into the ER network, with little or no fragmentation and vesiculation. However, at the end of mitosis, nuclear membrane reformation coincided with localization of LBR–GFP in the ER elements at contact sites with chromatin. These LBR–GFP-ER membranes then draped the chromatin and generated the nuclear apparatus by compartmentalizing the nuclear material (Ellenberg et al., 1997). Studies on the localization of two inner nuclear membrane proteins (lamin associated polypeptides 1 and 2 [LAP1 and LAP2]) and a nuclear pore membrane protein (gp210) authenticated the dispersal of nuclear proteins into the ER membrane during mitosis. The nuclear membrane lost its identity as a sub-compartment of the ER during mitosis. The nuclear lamins began to reassemble around chromosomes at the end of mitosis, involving reassembly of the nuclear membrane proteins to chromosome surfaces by binding interactions with lamins and chromatin (Yang et al., 1997).

Though the distribution of lamins and other related nuclear proteins during mitosis is studied in detail, the dispersal pattern and their implication for many nuclear proteins are still to be unearthed. One such protein is HABP1, which has been localized in mitochondria (Muta et al., 1997; Seytter et al., 1998), cell-surface (Gupta and Datta, 1991; Eggleton et al., 1995), and the nucleus (Simos and Georgatos, 1994; Matthews and Russell, 1998). However, only a mitochondrial localization signal is well characterized in the precursor form (Dedio et al., 1998). Studies with the tagged mature protein revealed that it mainly localizes in the nucleus (van Leeuwen and O'Hare, 2001). Numerous cellular proteins interact with HABP1, the first one being SF2, with which it was co-purified (Krainer et al., 1991). Though the functional implications of
HABP1 were not known at that moment, it was later shown to inactivate the splicing activity of SF2 and interact with another splicing factor SRp30c (Petersen-Mahrt et al., 1999). Other nuclear proteins interacting with HABP1 include TFIIB (Yu et al., 1995b) and lamin B receptor (Simos and Georgatos, 1994). The interaction of HABP1 with lamin B receptor is presumed to act as a link between the nuclear membrane and the intracellular structures. HABP1 is also reported to interact with several cytoplasmic proteins. Its interaction with α_1B-adrenergic receptor influences the localization and expression of the later (Xu et al., 1999). Its interaction with extracellular plasma proteins Factor XII and kininogen have already been established (Herwald et al., 1996, Joseph et al., 1996). The ability of HABP1 to interact with varied proteins, coupled to its diverse intracellular localization, led to the proposition that HABP1 could be a component of the trafficking pathway connecting the nucleus, mitochondria and cytoplasm and the export pathway to the cell surface (van Leeuwen and O'Hare, 2001).

The diverse cellular localization profile and the ability of HABP1 to interact with numerous cellular proteins demanded a study on its distribution pattern during mitosis to explore its participation in the trafficking pathway. In the previous chapter, it has already been shown that the kidney contains more HABP1 as compared to liver. The presence of HABP1 in the basement membrane and epithelial layer of kidney encouraged us to study its localization pattern in cell-lines derived from the kidney. Thus, in this study, a comparative analysis of HABP1 in live and permeabilized CV-1 (a kidney fibroblast cell-line) with COS-1 (transformed derivative of CV-1) was made. Finally, to establish the implication of HABP1 localization in the nucleus, its subcellular dynamics during mitosis was examined and compared with two other proteins; lamin B, a constitutive and p53, a regulatory nuclear protein.
4.2 MATERIAL AND METHODS

4.2.1 Material

Unless otherwise mentioned, all chemicals were procured from Sigma Chemicals Co., (St. Louis, MO, USA). Rabbit anti-lamin B antibody was a kind gift from Dr. Veena Parnaik, CCMB, Hyderabad, while rabbit anti-p53 antibody were purchased from Santa Cruz Biotech. Inc., USA. Media for mammalian cell culture, antibiotics and fetal calf serum were procured from Gibco BRL, Rockville, MD, USA. Plastic-wares for mammalian cell culture were purchased from Corning Costar Corp., Corning, USA. The anti-HABP1 polyclonal antibodies used in the study were raised against rat tissue HABP1.

4.2.2 Cell Culture

Green monkey kidney cells (CV-1) and origin-defective SV40-transformed green monkey kidney cells (COS-1), obtained from National Centre for Cell Science (Pune, India) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. For reasons of simplicity, the DMEM supplemented with all the above components will henceforth be referred to as 'complete medium'. The cultures were maintained in a CO₂ incubator at 37°C in a humidified environment containing 5% CO₂–95% air.

4.2.3 Immunofluorescence studies on live cells

CV-1 and COS-1 cells were cultured overnight on sterile glass cover slips in complete medium. For immunodetection, the cells were washed carefully with DMEM and incubated in DMEM in a CO₂ incubator for 30 min. The cells were then immunoreacted with rabbit anti-HABP1 antibody (1:100 dilution) for 1 h under similar conditions and rinsed thrice with DMEM over a period of 15 min. A final incubation of 1 h with Cy3-conjugated secondary antibody (goat anti-rabbit IgG; 1:250 dilution) was carried out, following which the cells were washed
thrice in DMEM and visualized under a fluorescence microscope. All
dilutions for the antibodies were made in DMEM. To visualize the
nucleus, stain for DNA (Hoechst 33342) was added along with the
secondary antibody and co-incubated for 1 h.

4.2.4 Immunofluorescence studies on permeabilized cells

Indirect immunofluorescence staining was performed on fixed CV-1
and COS-1 cells. The cells were fixed in 2% paraformaldehyde in PBS (pH
7.4), for 15 min at room temperature and washed with 0.1 M glycine for
5 min to quench excess aldehyde. They were permeabilized with 0.1%
Triton X-100 (v/v) for 1 min and the excess detergent was washed off
with PBS. The fixed cells were preincubated in 1% BSA-PBS, then
incubated either with rabbit anti-HABP1 antibody (1:100 dilution) or
alternatively rabbit anti-lamin B antibody (1:100 dilution) or rabbit anti-
p53 antibody (1:100 dilution) for 2 h at room temperature. After the
incubation period, the cells were rinsed thrice over a period of 30 min
and incubated for 1 h with Cy3-conjugated goat anti-rabbit IgG (1:250
dilution). The cells were washed thrice and mounted in PBS-glycerol. The
antibodies were diluted in 1% BSA-PBS. Hoechst was added along with
the secondary antibody and was co-incubated for 1 h. Controls were
carried out with an identical procedure, replacing the primary antibody
with a pre-immune serum.

4.2.5 Microscopy and image analysis

Cells cultured on sterile glass cover-slips were either used directly
for live cell imaging or following immunodetection, were observed using
fluorescence microscopy. The fluorescent cells were viewed with an
Axioscope microscope and photographed using the AxioCam camera
system coupled to the AxioVision software (Carl Zeiss, Germany). The
filter system used in this and ensuing studies are in the excitation
wavelength of 320 nm for monodansylcadaverine, 350 nm for Hoechst,
390-480 nm for EGFP, 510-550 nm for Rhodamine and 550 nm for Cy3.
The final images were assembled and processed in MS-PowerPoint.

4.3 RESULTS

4.3.1 Localization profile of HABP1 in live CV-1 and COS-1 cells

The presence of HABP1 in various mammalian tissues has already been documented (Gupta and Datta, 1991), and its sub-cellular localization in some organelles of normal and transfected cells has also been shown (Matthews and Russell, 1998; Dedio et al., 1998; van Leeuwen and O'Hare, 2001). The localization of endogenous HABP1 was studied in live CV-1 and COS-1 cells to ascertain and compare its distribution in such cells. Results indicate prominent cell membrane localization for HABP1 in live CV-1 and COS-1 cells (Figure 4.1) along the margin of the membrane, with distinct Hoechst staining in the nucleus. Another prominent feature of staining along the membrane is discrete dot like structures, resembling podosomes (Marchisio et al., 1984). These structures might assist the cell in adhering to the substratum and have a role in cell migration. The pre-immune sera control for the experiments did not give any staining (data not shown).

![Figure 4.1 Immunodetection of HABP1 in live CV-1 and COS-1 cells. Polyclonal anti-HABP1 antibodies detected HABP1 along the margins on cell membrane, with some discrete dot like structures in CV-1 (A) and COS-1 cells (B). The bar represents 10 µm.](image-url)
4.3.2 Intracellular localization profile of HABP1 in permeabilized CV-1 and COS-1 cells

Having analyzed the distribution pattern of HABP1 in live cells, its intracellular distribution pattern in permeabilized cells was undertaken. Significant level of HABP1 was immunodetected in the nucleus as well as the cytoplasm, with a prominent staining at the ER-Golgi complex in paraformaldehyde fixed and permeabilized CV-1 and COS-1 cells (Figure 4.2 A and B). The staining pattern with anti-HABP1 antibodies was similar in both the cell lines and Hoechst staining for the nucleus was prominent in both. The pre-immune sera control for the experiments did not give any staining. Since a similar intracellular staining pattern was obtained for CV-1 and COS-1, the COS-1 cell line was used as a model in subsequent studies of immunodetection.

![Figure 4.2](image)

**Figure 4.2 Immunodetection of HABP1 in paraformaldehyde fixed, permeabilized CV-1 and COS-1 cells.** HABP1 is detected in the nucleus and cytoplasm, with a prominent staining in the ER-Golgi complex using polyclonal anti-HABP1 antibodies in CV-1 (A) and COS-1 (B) cells. Panel C shows pre-immune serum control in COS-1 cells. The bar in panel A represents 20 μm, while those in panel B and C represent 10 μm.
4.3.3 Dynamics of HABP1 during mitosis

To get an insight into the distribution profile of HABP1, we investigated COS-1 cells through normal mitosis, without the addition of any mitogen or chemical stimulant and analyzed its fate during the process. The normal mitotic stages in COS-1 are depicted in Figure 4.3.

Analysis of HABP1 during mitosis showed a gradual increase from interphase to telophase, as illustrated in figure 4.5. The intracellular protein was immunodetected with Cy3-conjugated secondary antibody (Column-1), while Hoechst stained the DNA (Column-2). The images of the cells are presented in mitotic order, beginning with a cell in interphase and ending with a cell in telophase, with the intermediate stages in between. For ease in localization, the superimposed images of protein and DNA are...
shown in Column-3. Figure 4.5A shows cells in interphase, with the detection of nucleus through Hoechst staining. Intracellular HABP1 localizes to the nucleus, ER-Golgi complex and as scattered cytoplasmic dots. In prometaphase, cells containing condensed chromatin appeared much brighter with HABP1 being dispersed throughout the cell (Figure 4.5B). The perception was reinforced when images for protein and DNA staining were overlaid (Figures 4.5A,B Column-3). In metaphase, HABP1 is dispersed throughout the cell but excluded from the metaphase plate (Figure 4.5C). Throughout anaphase, the staining was intense, yet diffused and extended into the space between separating chromosomes and was excluded from the volume occupied by DNA (Figure 4.5D). The intensity of staining persisted through telophase, where HABP1 was expressed as a ring around the DNA, but gradually decreased as the cell entered cytokinesis (Figure 4.5E) and the nucleus started to form.

4.3.4 Dispersion of lamin B during mitosis

The dispersion of nuclear proteins during mitosis is already reported (Ellenberg et al., 1997; Yang et al., 1997). As the distribution pattern of lamin B during mitosis is well established, it was chosen as a control for our comparative study with HABP1. Cy3-conjugated secondary antibodies immunodetected lamin B (Figure 4.6, Column-1), while DNA was stained with Hoechst (Figure 4.6, Column-2). The images are in mitotic order, starting from interphase to telophase, with the intermediate stages in between. Lamin B localized as bright dots in the nucleus during interphase (Figure 4.6A) and dispersed throughout the cell during prometaphase (Figure 4.6B). An overlay of the two images validated the observation (Figures 4.6A,B; Column-3). A similar profile remains in metaphase (Figure 4.6C), with elimination of staining at the metaphase plate. Throughout anaphase (Figure 4.6D) and telophase (Figure 4.6E), lamin B staining was diffused and stretches into the space between separating chromosomes, forming a halo around the region occupied by DNA. The intensity of staining gradually decreases as cytokinesis is approached and the formation of nuclear envelope starts. It
is during this phase that lamin B would get relocalized to the nucleus.

**Figure 4.4 Dynamics of HABP1 during mitosis in COS-1 cells.** Intracellular HABP1 localizes to the nucleus, ER-Golgi complex and the cytoplasm during interphase (A). In prometaphase, a dispersed staining is observed (B), while in metaphase, HABP1 fills the cell and surrounds the chromosome at the metaphase plate (C). In anaphase (D), staining is present between the chromosomes as they move to opposite poles of the cell and the staining persists through telophase (E). The bar represents 10 μm.
Figure 4.5 Lamin B dispersion during mitosis in COS-1 cells. Lamin B is localized in the interphase nucleus as distinct dots (A), but disperses in the cytoplasm during the prometaphase stage (B). In metaphase, lamin B fills the cell, barring chromosomes at the equatorial plate (C) and in anaphase (D) and telophase (E), stains between the chromosomes, as they separate. The staining intensity however appears to decrease from metaphase to telophase through anaphase. The bar represents 10 μm.
4.3.5 Analysis of DNA and Protein staining during stages of mitosis for HABP1 and lamin B

Analysis of mitotic stages revealed an increase in the amount of both HABP1 and lamin B during mitosis. To validate this observation, a statistical analysis was carried out to compare the intensities of DNA (detected with Hoechst) or proteins (detected with Cy3-conjugated secondary antibody) with respect to the cell size during mitotic stages. A ratio between Hoechst intensity (calculated as arbitrary units) and the cell size was plotted against the stages of mitosis (Figure 4.6A). A similar pattern was followed for Cy3 intensity, in order to analyze the distribution pattern of the protein (Figure 4.6B). The data presented is an average of 15-20 independent observations for each stage.

Figure 4.6 Statistical analysis of intensity ratio of DNA and protein to the cell size during mitotic cycle. (A) The ratio of Hoechst intensity and cell size during stages of mitosis is graphically represented for HABP1 and lamin B. The differential ratio for Hoechst staining during mitosis shows an overlapping wavy pattern for both HABP1 and lamin B. (B) The dispersal of protein during mitosis was measured as a ratio of Cy3 intensity to the cell size and plotted as a line graph against the stages of mitosis. The data shows that the intensity of lamin B increases from interphase to anaphase, but decreases in telophase, while the intensity of HABP1 shows a gradual increase across the stages. The x-axis represents the mitotic stages, while the y-axis shows the ratio of intensity (in arbitrary units) to the cell-size.

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4.3.6 Distribution of p53 during mitosis

The intracellular distribution of p53 during interphase is confined to the nucleus, where it shows a diffused pattern, as immunodetected through with Cy3-conjugated antibodies (Figure 4.7A, Column-1). The staining for DNA and the merged images substantiate the observation (Figures 4.7A, Column-2 and 3). This distribution pattern is different from that of lamin B, another nuclear protein, which is localized as distinct dots in the nucleus during interphase (Figure 4.7A). During mitosis, p53 is typically localized as discrete spots. In prometaphase, it is seen as spots between the condensing chromosomes (Figure 4.7B). In metaphase (Figure 4.7C), when the chromosomes are confined to the equatorial plate, p53 spots are localized above the chromosomes, and seem to be associated with the centriole pair. The dots separate out with the sister chromatids and move towards the pole during anaphase and telophase (Figure 4.7D, E). The amount of p53 increases, once it re-enters the nucleus following mitosis. Thus, the distribution pattern of p53 during interphase and stages of mitosis is different from HABP1 and lamin B, another nuclear protein.

4.4 DISCUSSION

The localization profile of cellular proteins throws some light on their functional implications. Using indirect immunofluorescence, the distribution of HABP1 was analyzed in live and permeabilized CV-1 and COS-1 cells in the present study. Apart from this, the dynamics of HABP1 during mitosis in COS-1 cells has been studied to determine its dispersal mechanism following nuclear envelope disassembly.

Results from this study indicate that HABP1 is localized mainly in the cell membrane of live cells as discrete dots resembling podosomes (Marchisio et al., 1984). These dots might represent focal adhesion points, assisting the cells in migration and adhesion. The role of HABP1 in cell adhesion has already been established (Gupta and Datta, 1991) and the present data supports earlier observations from our laboratory.
**Figure 4.7 Distribution of p53 during mitosis in COS-1 cells.** p53 shows a diffused pattern throughout the nucleus during interphase (A), but organizes as discrete dots in the condensing chromosomes from prometaphase (B) to telophase (E) through metaphase (C) and anaphase (D). The discrete dots of p53 for each mitotic stage are marked with arrows. The dots separate out and move towards the poles, resembling the movement of centrioles during mitosis. The bar represents 10 µm.
In permeabilized cells however, HABP1 shows diverse intracellular localization pattern, with visible staining in the nucleus, ER-Golgi complex and the cytoplasm. Though a significant level of HABP1 is present in the nuclear compartment, it is not an exclusively nuclear protein. The detection of the protein in the ER-Golgi complex coincides with the fact that it is a glycosylated protein, which has to traverse the Golgi for post-translational modifications. Similar intracellular pattern of distributions was observed in the untransformed CV1 cells and its transformed counterpart, COS-1 cells.

An analysis of HABP1 during mitosis reveals a concomitant increase in the amount of intracellular HABP1 in proliferating cells during mitosis. During the mitotic stages, HABP1 completely filled the cytoplasm and surrounded the chromosomes during their separation from metaphase to telophase through anaphase. Interestingly, this dispersion pattern during mitosis is highly similar to that of nuclear proteins (Chaly et al., 1984; Ellenberg et al., 1997; Yang et al., 1997). Nuclear envelope markers (Yang et al., 1997) and Golgi membranes (Zaal et al., 1999) are reported to be completely dispersed throughout the ER during mitosis. The similarity in HABP1 distribution during mitosis could be ascribed to the fact that it is localized in the nucleus, ER and the Golgi. The localization of the protein in Golgi might allow it to disperse and reform through the intermediary ER, exploiting the constitutive recycling pathway. Lamin B and HABP1 disperse in a similar fashion during mitosis, inspite of their differential location in interphase. This could emphasize the fact that both of them are constitutive proteins, and most such proteins (e.g. actin, tubulin and lamins) demonstrate a similar distribution pattern. A constitutive protein is required to be present at an optimal level in the cell. During mitosis, an increase in the amount of HABP1 could be to maintain the optimal quantity in daughter cells upon division. One can hypothesize that the Golgi might serve as a reservoir for HABP1 during interphase, for the protein might be necessary to meet
the requirement during cell division. The presence of HABP1 as discrete dots in the cell membrane of live cells has already been shown, which are presumed to be focal adhesion points. As a cell divides, the progeny cells would require an augmented level of HABP1 for adhering to the substratum. This explains the enhanced level of HABP1 associated with mitosis. Analysis of another nuclear protein p53, reveals a characteristic dot like distribution during mitosis, resembling that of centrin, a constituent of the centriole (Ciciarello et al., 2001). The regulatory nature of p53 is well established; therefore its localization follows a definitive pattern.

Most interestingly however, the distribution pattern and retention of HABP1 during different stages of mitosis resembles that of HA, one of its prime ligands (Evanko and Wight, 1999). Earlier studies have given evidence for the association of HA with chromatin (Furukawa and Terayama, 1977, 1979; Kan, 1990). Combined with the distribution profile of HA during mitosis, it has been postulated that HA might be involved in chromosome condensation in some cells, possibly through interactions with lamins (Evanko and Wight, 1999). The ability of HA to regulate cell-cell and cell-substrate adhesion (Toole, 1982; Lee et al., 1993) combined with its water retention ability might have some functional role during mitosis. Studies in hamster oocyte reported the presence of HA in the rough ER (Kan, 1990) and the diffuse pattern of HA during mitosis might actually be the distribution of rough ER during the process. Though the exact correlation between similar localization patterns of HA and HABP1 during mitosis has yet not been established, these may presumably be involved in chromosomal organization in cells, perhaps through interactions with histones, lamins or lamin binding proteins. Numerous HA binding proteins have been characterized and one such protein, Cdc37 has been identified in chick, which apart from binding HA is a homologue of Cdc37, an essential cell cycle regulatory protein in yeast and Drosophila (Grammatikakis et al., 1995). Yeast
Cdc37 has also been classified as a chaperone with a role in numerous kinase-regulated signaling pathways (Kimura et al., 1997). The presence of HABP1 in the nucleus and its dispersion during mitosis might unearth another HA binding protein, which has a role in cell division and trafficking pathway in the cell. The role of HABP1 in cell-adhesion is well established and combined with HA, it might facilitate the process of adhesion and de-adhesion during mitosis. It is also presumed that the intense staining of mitotic COS-1 cells for HABP1 might be an effect of mitogenic stimulation and these observations indicate that HABP1 may have functional implications inside the cell during mitosis.

Therefore, from this study, it can be concluded that:

1. **A detectable level of HABP1 is present in the cell membrane of live COS-1 and CV-1 cells.**

2. **Though already reported in the nucleus, cell membrane and mitochondria, the intracellular distribution of HABP1 also extends to ER and Golgi complex.**

3. **The increase in the level of intracellular HABP1 during mitosis could be to meet and maintain its optimal level required by the daughter cells, arising upon division.**

4. **The distribution pattern of HABP1 during mitosis is similar to constitutive proteins, as evident from its profile, which is similar to that of lamin B.**

5. **The similarity in the distribution patterns of HABP1 and HA during mitosis might extrapolate their role in cell adhesion and de-adhesion.**

As evident from the diverse sub-cellular distribution, HABP1 now appears to be a molecule with diverse cellular as well as sub-cellular functions. Clearly, these studies open a new direction for elucidating the exact role of HABP1 in cell division.