Chapter III

Transformed cells are refractory to alteration in Hyaluronan binding protein 1 (HABP1) level as compared to normal cells.
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Earlier, we reported the involvement of HABP1 in cell signalling including its enhanced phosphorylation in transformed as well as normal cells following PMA stimulation (Rao et al., 1997). In continuation, in Chapter I, we have shown that PMA stimulation of normal fibroblast cells enhances HABP1 synthesis followed by its translocation to the nucleus in a MAP kinase dependent manner. Subsequently, in Chapter II, we demonstrated that alteration of HABP1 levels lead to changes in cellular morphology viz. nuclear condensation, vacuole formation along with growth inhibition in normal fibroblast cells. It was also demonstrated that constitutive overexpression of HABP1 in normal fibroblast cells does not lead to its nuclear translocation but affects the level of MAP kinase and the pre-mRNA splicing factor, SF2 (with which HABP1/p32 is co-purified in HeLa cell nuclear extract).

Cells of tumour origin like HeLa (Human cervical epithelial carcinoma) behave differently in culture as compared to normal cells, due to their disrupted cell cycle regulation. As opposed to their normal counterpart, transformed cells do not arrest at G0 phase. They continue through the cell cycle until they eventually die as a consequence of environmental stress (Thomas, 1996). As their cell cycle regulation is aberrant, the expression of various cell cycle proteins is also altered e.g. MAP kinase stays activated in transformed cells as compared to normal cells (Hulleman and Boonstra, 2000).

Thus, keeping in view that overexpression of HABP1 in normal fibroblasts leads to depletion of MAP kinase and inhibition of cell growth, we were interested in creating similar conditions under a transformed background and follow the consequences. In the following section, altered expression of HABP1 was thus studied in the context of cell growth and morphology in HeLa cells, where MAP kinase is constitutively stimulated.

6.1 RESULTS

6.1.1 Generation of stable transfectants of HeLa cells harbouring HABP1 cDNA in sense and antisense orientation

Transfection of HeLa cells with pCDNA.JM.HABP.M (sense orientation) and pCDNA.JM.PBAH.M (antisense orientation) was carried out as described for F111 cells (Figure 5.2). Stable transfectants obtained after introducing the pCDNA.JM.HABP.M were called H-HABP and those obtained by transfection
Figure 6.1 Confirmation of the presence of HABP1 cDNA in sense and antisense orientation in H-HABP and H-PBAH cells: Genomic DNA was isolated from HeLa, H-HABP and H-PBAH cells and subjected to PCR with S1-MYC and S3-MYC primers. PCR products were resolved on a 0.7% agarose gel and visualised by ethidium bromide staining.
Figure 6.2 Morphological characterisation of H-HABP and H-PBAH cells: HeLa (A), H-HABP (B) and H-PBAH (C) cells were grown on cover slips and fixed with methanol. Fixed cells were double stained with hematoxylin and eosin [A (i), B (i) and C (i)] to visualise the cell morphology. Cells were also stained with hematoxylin alone [A (ii), B (ii) and C (ii)] for visualising the nuclear morphology.
with pCDNA.JM.PBAH.M were called H-PBAH. However, these cells could not be cloned as they cells adhered to each other strongly and single cell colonies were almost impossible to obtain.

Integration of HABP1 cDNA into the host cell genome was confirmed by PCR, using genomic DNA from the neomycin resistant cell pool and the same set of primers as used for characterising the stable transfectants from F111 cells (Figure 5.3) were used. The sizes of the PCR products were confirmed by electrophoresis on a 0.7% agarose gel (Figure 6.1).

6.1.2 Morphological characterisation of H-HABP and H-PBAH cells

HeLa, H-HABP and H-PBAH cells were grown on cover slips, fixed and stained with hematoxylin and eosin for studying the cell morphology and with hematoxylin alone for studying the nuclear morphology (Figure 6.2). Morphology of H-HABP cells appeared to be comparable to that of HeLa cells, except that they were more vacuolated (Figure 6.2B) than HeLa (Figure 6.2A) or H-PBAH (Figure 6.2C) cells. However, the number and size of the vacuoles are less than that in F-HABP07 (F111 cells transfected with HABP). Interestingly, in H-HABP as well as H-PBAH cells, the nuclear size seemed to be partially condensed (Figure 6.2 panels ii) as compared to HeLa cells (Figure 6.2A ii). Therefore, it appears that enhanced HABP1 expression in a transformed background does not have any significant effect on its morphology as compared to the normal (F111) cells (Figure 5.5).

6.1.3 Growth characteristics of H-HABP and H-PBAH cells

Growth pattern of HeLa, H-HABP and H-PBAH cells was studied using non-radioactive cell proliferation assay kit as described in Materials and Methods (Section 3.2.8.4). As shown in Figure 6.3, the normal growth of H-HABP1 and H-PBAH was not inhibited as was seen in case of F-HABP07 cells. However, overexpression of HABP1 in HeLa cells resulted in a slower growth rate as compared to the normal cells, which was reflected in their enhanced doubling time. The doubling time of HeLa cells is 23 h, while that of H-HABP and H-PBAH cells were 30 h. These observations suggest that in HeLa cells, alteration in the levels of HABP1 expression results in changes in cell morphology and retardation of growth rate. Therefore, our next step was to examine the effects of replenishment of medium on reduced growth rate of H-HABP and H-PBAH cells. The effect of supplementation with fresh growth medium after every 24 h on the
Figure 6.3 Growth kinetics of the stable transfectants: Graph depicting the growth kinetics of HeLa, H-HABP and H-PBAH cells. Doubling time for each cell type is indicated in the inset.
Doubling Time
HeLa — 23 hr
H-HABP — 29 hr
H-PBAH — 30 hr

Cell Number vs. Time (hr)
Figure 6.4 Growth kinetics of H-HABP cells upon periodic replenishment of growth medium: H-HABP (A) and H-PBAH (B) cells were grown with and without changing growth medium every 24 h. Graph depicts the comparison of growth pattern. The arrows on the curves indicate the time points at which the medium was changed.
A. 

- With daily change
- Without daily change

B. 

- With daily change
- Without daily change
growth pattern of H-HABP and H-PBAH cells is shown in Figure 6.4. In this experiment, one set of cells were grown and the growth medium was changed every 24 h and in the other set, cells were grown by media supplementation only after 72 h. In case of both H-HABP (Figure 6.4A) and H-PBAH cells (Figure 6.4B), no significant changes in growth kinetics were observed upon medium supplementation every 24 h. This suggests that the reduced growth rate of H-HABP and H-PBAH cells were not reversed by frequent change of medium and thus, it was not due to the secretion of some inhibitory constituents into the medium.

6.1.4 Immuno-detection of HABP1 in H-HABP and H-PBAH cells

Total cell lysates were prepared from HeLa, H-HABP and H-PBAH cells and equal amounts of the extracts were resolved on a 12.5% SDS-PAGE and immuno-detected with anti HABP1 antibody. Figure 6.5 shows that the level of HABP1 was increased in H-HABP cells as expected, but in case of H-PBAH cells, HABP1 level was not diminished. Here again, it shows that as in the case of F-PBAH, antisense cDNA of HABP1 was not able to suppress the HABP1 synthesis.

6.1.5 Subcellular localisation of HABP1 and its binding molecules in HeLa, H-HABP and H-PBAH cells

HeLa and H-HABP cells were grown on cover slips and fixed with methanol. One set of fixed cells was processed for immunofluorescence with anti HABP1 antibody and the images so obtained were analysed by confocal microscopy (Figure 6.6). Immunofluorescence with anti HABP1 antibody shows higher level of nuclear localization for HABP1 (Figure 6.6B) as opposed to control HeLa cells (Figure 6.6A). This data suggests that the overexpressed HABP1 in transformed cell lines is directed towards the nucleus. We also examined whether enhanced expression of HABP1 results in any change in the level of HA and other HABP1 binding molecules. When HeLa and H-HABP cells were probed using FITC conjugated HABP1, marginally higher levels of HA (or other HABP1 binding molecules) on the cell surface or ECM of H-HABP cells were observed (Figure 6.7). Another interesting feature of these cells was that they existed as aggregates, rather than as individual cells, explaining why they could not be cloned. The occurrence of relatively higher levels of FITC
Figure 6.5 Immuno-detection of HABP1 in stable transfectants of HeLa: Total cell lysates of HeLa, H-HABP and H-PBAH cells from confluent cultures were prepared by boiling cells in Laemmli buffer. Fifty microgram of each lysate was resolved on a 12.5% SDS-PAGE and immunoblotted using anti HABP1 antibody.
Figure 6.6 *Localisation of HABP1 in HeLa and H-HABP cells*: HeLa (A) and H-HABP (B) cells were grown on cover slips and fixed with methanol. Fixed cells were processed for immunofluorescence with anti HABP1 antibody and analysed by confocal microscopy.
Figure 6.7 *Analysis of HABP1 binding activities in HeLa and H-HABP cells*: HeLa (A) and H-HABP (B) cells were grown on cover slips and fixed with methanol. Fixed cells were probed with FITC conjugated HABP1 and analysed by confocal microscopy.
conjugated HABP1 on the edges of H-HABP cells suggest the presence of HABP1 interacting proteins on the extracellular surface.

6.1.6 MAP Kinase and SF2 levels in stable transfectants
Since we have shown earlier that HABP1 is translocated in a MAP Kinase dependent pathway and is also co-localised with the pre mRNA splicing factor SF2 in HeLa cells, we also estimated the level of these two regulatory proteins in H-HABP and H-PBAH cells. HeLa, H-HABP and H-PBAH cell pellets were lysed and fractionated into cytosolic and nuclear fractions as described in Materials and Methods. Those fractions were resolved on a 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane. To compare the expressions of HABP1, MAP kinase and SF2, the blot was sequentially probed with anti-HABP1 antibody (Figure 6.8A), anti-MAP kinase antibody (Figure 6.8B) and anti SF2 antibody (Figure 6.8C). In agreement with our immunofluorescence data, as shown in Figure 6.8A, HABP1 content in the cytosolic extract of normal, H-HABP and H-PBAH cells were comparable, whereas a significantly increased level of HABP1 was detected in the nuclear extract of both H-HABP and H-PBAH cells. Interestingly, when the blot was probed with anti MAP kinase antibody, only cytosolic localization of MAP kinase was observed. The level of MAP kinase was also enhanced in H-HABP and H-PBAH cells as compared to HeLa cells. Since the antibody against MAP kinase was against the inactive non-phosphorylated form, it did not detect the nuclear localised activity. Similarly, the expression of nuclear localised SF2 was highest in H-HABP cells followed by H-PBAH and HeLa cells. It is thus inferred that increased expression of HABP1 in HeLa cells results in an increased level of MAP kinase and SF2.

6.2 DISCUSSION
The main objective of this study was to check the effect of changes on the constitutive level of HABP1 on growth regulation of HeLa cells, as a prototype of transformed cells. Interestingly, our observations suggest that unlike normal fibroblasts cells (F111), transformed cells (HeLa) are refractile to changes in HABP1 level.
Figure 6.8 MAP kinase and SF2 levels in H-HABP and H-PBAH: HeLa, H-HABP and H-PBAH cells were fractionated into cytosolic and nuclear fractions. Equal amounts (50 µg) of each extract was resolved on a 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane. The blot was sequentially probed with anti-HABP1 antibody (A), anti-MAP kinase antibody (B) and finally with anti-SF2 antibody (C).
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After confirming the genomic integration of HABP1 cDNA in the stable transfectants of HeLa (Figure 6.1), these cells were characterised for their growth and morphological behaviour. Morphologically, H-PBAH cells appeared similar to HeLa cells, whereas in case of H-HABP cells, a slight change was observed (Figure 6.2). H-HABP cells appeared to be vacuolated like F-HABP07 cells (as shown in Figure 5.5). However, in case of H-HABP cells, the number of vacuoles were not as much as those in F-HABP07 cells. This could be due to lesser cytoplasmic volume in HeLa cells. However, the size of nucleus in both H-HABP and H-PBAH were slightly smaller than that of HeLa cells.

Another interesting observation is that in case of H-HABP as well as H-PBAH cells, growth regulation is not greatly affected (Figure 6.3). Both these cell lines showed reduced growth rate but did not show any growth arrest. Frequent medium supplementation did not have any effect on the growth rate of those cells (Figure 6.4). Thereby, suggesting that the enhanced level of HABP1 only partially hampers the growth regulation in HeLa cells.

We subsequently checked for HABP1 levels in total cell lysates of HeLa, H-HABP and H-PBAH cells. As we observed in F-HABP cells, in H-HABP cells too, the HABP1 level was elevated. However, in H-PBAH cells, there is no lowering of HABP1 levels, which was again in conformity with F-PBAH cells. Therefore, it can be suggested that abrogation of HABP1 expression by antisense strategy is not effective. The reason for this insufficiency could be attributed to two possibilities. Firstly, if the antisense RNA binds to a fraction of the existing pool of HABP1 mRNAs, it may only partially inactivate it, which might lead to a stabilisation of the remaining ones. These stable mRNAs would then be translated more efficiently to maintain the threshold level of HABP1. Secondly, a pseudogene for this protein has already been reported on human chromosome 21 (Hattori et al., 2000). HeLa being a human cell line, possesses this pseudogene. If the pseudogene was capable of synthesising a non-coding transcript, it would act as sink for squelching out the anti sense RNA.

When we studied the localization of HABP1 in normal HeLa and transfected cells, we observed that HABP1 is mainly localised in the nucleus of H-HABP cells (as opposed to its cytoplasmic localization in F-HABP cells). Therefore, this data is in corroboration with the observation by Krainer et al (1991), that HABP1 is co-purified along with pre-mRNA splicing factor SF2 in HeLa cells.
In case of staining with FITC conjugated HABP1, there is no distinct difference between HeLa and H-HABP cells (Figure 6.7). However, the staining appears to be higher on the edges of the cells in case of H-HABP, as opposed to the intense cytosolic staining obtained in case of F-HABP07 cells (Figure 5.10). Therefore, in H-HABP cells, not only the localization of HABP1 is different, but the localization of HABP1 interacting proteins was also different.

Having compared the localization and levels of HABP1 in total cell extracts of HeLa, H-HABP and H-PBAH cells, we checked levels of SF2 and MAP kinase in those cell lines after separating them into nuclear and cytosolic fractions. As opposed to F111 cells, we observe HABP1 only in the nuclear fractions of HeLa, H-HABP and H-PBAH cells (Figure 6.8A), consistent with our observation with the immunofluorescence analysis. The levels of MAP kinase as well as SF2 appeared to be slightly higher in case of H-HABP as compared to H-PBAH cells, although both H-HABP and H-PBAH cells showed higher levels of SF2 as compared to the control (HeLa, Figure 6.8C). However, the MAP kinase level was comparable in both H-PBAH and HeLa cells (Figure 6.8B).

Overexpression of HABP1 in HeLa cells (H-HABP) shows its nuclear translocation. This localization is similar to that obtained in PMA stimulated cells (Chapter I). This could be attributed to the elevated MAP Kinase levels in H-HABP cells. Another noteworthy feature is the lesser vacuolation in these cells as opposed to F111 cells; possibly due to the differential localization of HABP1 in those two cells. The nuclear translocation of HABP1 in PMA stimulated F111 cells and in H-HABP cells needs a clarification in context of the previous reports. Even though there is only one mitochondrial localisation signal in the full-length HABP1, it is cleaved off during the formation of the mature protein that is purified from HeLa nuclear extracts along with SF2 (Krainer et al., 1990). Further, HABP1 is also capable of interacting with the serine-arginine motif of the nuclear envelope associated protein Lamin B Receptor and translocates it to the nuclear envelope (Simos and Georgatos, 1994; Nikolakaki et al., 1996). Its nuclear localization has also been reported in cells infected with HIV-Type 1 (Yu et al., 1995 a&b; Tange et al., 1996), Adenovirus (Matthews and Russell, 1998), Epstein-Barr Virus (Scoc et al., 2000) and Rubella Virus (Beatch and Hobman, 2000). It is evident from its crystal structure, HABP1 can sequester Ca\(^{2+}\) and it is believed that the Ca\(^{2+}\) sequestration is responsible for its shuttling between the nucleus and mitochondria (Jiang et al., 1999). In case of cells infected with Rubella Virus, it has been shown that this protein is trapped by the viral protein.
and transported to the nucleus in a Ca\textsuperscript{2+} dependent manner, which eventually may lead to apoptosis (Beatch and Hobman, 2000). Apart from its transportation to the nucleus, in HIV- type 1 infected cells, HABP1/p32 interacts with splicing factors and other transcription factors (Luo et al., 1994; Yu et al., 1995 a&b; Tange et al., 1996), leading to an inhibition in the splicing/transcription machinery. This could be correlated with our observation that SF2 level is increased in H-HABP cells, a possible cause for growth retardation.

In this study, therefore we demonstrate that altered levels of HABP1 impart different effects in transformed and normal cells. Since it is not allowed to remain in the cytosol, increase in HABP1 level may not affect the phosphorylation or the protein synthesis machinery, thereby not inhibiting the growth regulation of these cells. Whereas, in case of F-HABP07 cells, it remains in the cytosol and may interfere with the phosphorylation and/or protein synthesis machinery, which is reflected in the growth inhibition. Our observation on the nuclear translocation of HABP1 on PMA stimulation (Chapter I) and its nuclear localisation in H-HABP cells further support the hypothesis that it is the cell type, not the motifs & domains that determines the localisation of HABP1, which eventually decides its function.