Chapter 9
Phorbol Myristate Acetate upregulates hyaluronan binding protein 1 (HABP) and translocates it to the nucleus. Nuclear translocation is a HAP kinase dependent process.
Hyaluronan (HA), a major extracellular matrix non-sulphated glycosaminoglycan, plays a critical role in the maintenance of the extracellular matrix structure and water balance as well as in cellular signalling (Laurent, 1970). Signalling functions of HA are mediated through its interaction with HA-binding proteins (McCourt, 1999). Several important HA-binding proteins have been reported to be cell surface or intracellular receptors for HA. Any alteration in the interaction of HA with its cellular receptors may lead to perturbation in normal cell growth and differentiation (Toole, 1997). Purification and characterisation of one such HA-binding protein (34 kDa HABP) from rat kidney has been reported by our group (Gupta et al., 1991) and its involvement in cellular transformation process has also been established (Gupta and Datta, 1991). As a continuation, its role in HA mediated cellular signalling has also been demonstrated (Rao et al., 1997). The 34 kDa HABP has been shown to be highly phosphorylated at threonine residues in transformed fibroblasts as compared to normal fibroblasts. Further, enhanced phosphorylation of 34 kDa HABP by HA, PMA (Phorbol 12-myristate 13-acetate) and Calyculin-A has been demonstrated, suggesting its role in cellular signalling (Rao et al., 1997). The cDNA encoding the 34 kDa HABP from human fibroblast has been characterised. The presence of a HA binding motif and subsequently the binding of the bacterially expressed recombinant protein to HA has been confirmed (Deb and Datta, 1996). The gene encoding this protein has been localised on human chromosome 17p12-p13 (Majumdar and Datta, 1998) and has been named as HABP1 by Hugo Gene Nomenclature Committee of GDB (GDB: 9786126). A pseudogene for this protein has also been identified on human chromosome 21 (Hattori et al., 2000).

Sequence analysis of HABP1 revealed its identity with p32, a human protein co-purified with pre-mRNA splicing factor, SF2 (Krainer et al., 1991); gC1qR, the human receptor for globular head of complement component C1q (Ghebrehiwet et al., 1994) and substantial (92%) homology with YL-2, a HIV-type1-Rev binding murine protein (Luo et al., 1994); thereby suggesting its multifunctional nature. Sequence analysis has also supported our earlier report on the role of this protein in cellular signalling, as it has been seen to possess five phosphorylation sites for CKII and one site for extracellular signal regulated kinase (ERK) (Deb and Datta, 1996).

Extracellular signal regulated kinases (ERKs) or Mitogen Activated Protein Kinases (MAP Kinases) are a family of intracellular serine/threonine
kinases that are activated by signals from receptor tyrosine kinases, cytokine receptors and seven spanning heterotrimeric G protein coupled receptors (Marshall, 1994). PMA, a protein kinase C stimulator, is one of the most commonly used extracellular stimulants to study MAP Kinase signal pathway. PMA specifically induces tyrosine phosphorylation of MAP kinases and in turn plays an important role in regulation of cell growth and differentiation (Thomas, 1992). As phosphorylation of HABP1 is stimulated by PMA and it has a MAP kinase phosphorylation site also, we asked the question whether HABP1 is a component of cell signalling pathway leading to cell growth and differentiation. In the following section, we show the effects of PMA on synthesis and nuclear translocation of HABP1 and its possible involvement in MAP Kinase cascade.

4.1 RESULTS

4.1.1 HABP1 synthesis is upregulated following stimulation of HeLa cells with PMA

In order to investigate the role of HABP1 in cell growth and differentiation, initially we tested whether HABP1 level is modulated upon mitogenic stimulation of HeLa cells. To that purpose, HeLa cells were pulse labelled with $^{35}$S-methionine in the absence and presence of PMA, a potent and well-characterised mitogen. Labelled proteins were immunoprecipitated with anti-HABP1 antibody, resolved on a 12.5% SDS-PAGE and analysed by autoradiography as mention in Materials and Methods, Section 3.2.9.5. As seen in Figure 4.1, there was a significant (more than five fold increase) upregulation in the synthesis of HABP1 following PMA treatment. Since PMA mediates its effect via PKC pathway, specificity of the effect of PMA was tested by addition of staurosporine, a protein kinase inhibitor. Treatment with staurosporine inhibited the upregulation of HABP1.

4.1.2 Kinetics of upregulation of HABP1 by PMA

The immediate effect of PMA stimulation is the upregulation of early response transcription factors viz. AP-1 (FOS-JUN dimer) which occurs within fifteen minutes and is mediated by synthesis of new transcripts (Bakiri et al., 2000). It is believed that these transcription factors are responsible for upregulation of growth responsive genes further downstream. In order to
Figure 4.1: Increased HABP1 Synthesis following PMA treatment: 1 X

10^5 cells were seeded on to each well of a 12-well dish and grown for ~24 h. Cells were then kept in methionine free medium for 3 h. Hundred micro curie of 35S methionine was added to the methionine starved cells along with 40 nM PMA and 2 µg/mL staurosporine as indicated in the figure. Cells were incubated for 2 h, lysed in RIPA buffer and immunoprecipitated with anti-HABP1 antibody. Immunoprecipitated beads were resolved on a 12.5% SDS-PAGE and analysed by autoradiography. The arrow indicates 34 kDa HABP1.
Figure 4.2: Kinetics of upregulation of HABP1 by PMA: Serum starved HeLa (A) and F111 (B) cells were stimulated with 40 nM PMA for the time periods indicated. Total cell lysates were prepared and 50 μg protein was resolved on a 12.5% SDS-PAGE, followed by immunoblotting with anti-HABP1 antibody. A (i) and B (i): immunoblot of lysates from HeLa and F111. A (ii) and B (ii): the CBB stained gel showing the profile of lysates used for blot shown in A (i) and B (i).
understand the place of HABP1 in that hierarchy of events, serum starved HeLa cells were stimulated with PMA for different time periods (2 h, 4 h and 6 h) as described earlier in Materials and Methods (Section 3.2.8.2). Total cell lysates were then prepared and quantitated by immunoblotting with anti-HABP1 antibody (Figure 4.2A). We observed a time dependent increase in the level of HABP1, which reached a maximum at six hours. Similar results were also obtained with F111 cells (Figure 4.2B). It is notable that although both HeLa and F111 cells showed an increase in HABP1 levels following PMA stimulation, the increase was more significant in case of F111 cells.

4.1.3 HABP1 is translocated to the nucleus following PMA stimulation

Following specific stimulation, number of regulatory proteins are translocated to distinct sub-cellular locations to mediate their effect. For example, PKC isoforms are translocated from cytosol to cell membrane and steroid receptors are translocated from cytoplasm to nucleus after ligand binding (Chen et al., 2000). Since HABP1/p32 has been co-purified with splicing factor SF2, we tested whether HABP1 is translocated to the nucleus following PMA stimulation. Cytoplasmic extracts prepared from serum starved HeLa cells after stimulation with PMA for 2 h, 4 h and 6 h were resolved on a 12.5% SDS-PAGE and immunoblotted with anti-HABP1 antibody. As shown in Figure 4.3A1, following PMA treatment, there was a gradual decrease in the cytosolic level of HABP1 with time. Simultaneously, there was an increase in HABP1 levels in the nuclear fraction (Figure 4.3AII). Similar results were also obtained with F111 cells (Figure 4.3B), thus suggesting that following PMA stimulation HABP1 levels increase and then it is translocated to the nucleus.

In order to confirm the translocation of HABP1 following PMA stimulation, HeLa (Figure 4.4 A) and F111 (Figure 4.4 B) cells were grown on coverslips, serum starved and stimulated with PMA for 2 h and 6 h. Methanol fixed cells were then probed with anti-HABP1 antibody and analysed by confocal microscopy. As shown in Figure 4.4, indirect immunofluorescence analysis of individual cells showed that PMA stimulation not only led to an increase in HABP1 levels but also resulted in its nuclear translocation. It is distinct that in cells stimulated for 2 h with PMA, there is an increase in HABP1 level in the cytosol (Figure 4.4 Aiii and Bii), which shifts to the nucleus at 6 h of PMA.
Figure 4.3A: **HABP1 is translocated into the nucleus, following PMA stimulation in HeLa cells**: (I) Cytoplasmic extracts of serum starved HeLa cells stimulated with 40 nM PMA for the indicated time periods were prepared, 50 µg of the extracts were resolved on a 12.5% SDS-PAGE and immunoblotted with anti-HABP1 antibody. (II) Serum starved HeLa cells were stimulated with 40 nM PMA for 6 h. Unstimulated and stimulated cells were fractionated into nuclear and cytoplasmic fractions and 80 µg of each was used for 12.5% SDS-PAGE, followed by immunoblotting with anti-HABP1 antibody (II a). CBB stained gel showing the profile of the extracts used for blotting is represented in II b.
Figure 4.3B: HABP1 is translocated into the nucleus, following PMA stimulation in F111 cells: (I) Cytoplasmic extracts of serum starved F111 cells stimulated with 40 nM PMA for the indicated time periods were prepared, 50 µg of the extracts were resolved on a 12.5% SDS-PAGE and immunoblotted with anti-HABP1 antibody. (II) Serum starved F111 cells were stimulated with 40 nM PMA for 6 h. Unstimulated and stimulated cells were fractionated into nuclear and cytoplasmic fractions and 60 µg of the each was used for 12.5% SDS-PAGE, followed by immunoblotting with anti-HABP1 antibody (II a). CBB stained gel showing the profile of the extracts used for blotting is represented in II b.
Figure 4.4A: *Indirect immunofluorescence analysis showing translocation of HABP1, following PMA stimulation in HeLa cells:*

HeLa cells were grown on cover slips, serum starved and stimulated with 40 nM PMA for 0 h (ii), 2 h (iii) and 6 h (iv). After fixation with methanol, the coverslips were processed for immunofluorescence with anti-HABP1 antibody. Analysis was done using confocal microscopy. Pre-immune serum was used as a control as shown in A (i).
Figure 4.4B: *Indirect immunofluorescence analysis showing translocation of HABP1, following PMA stimulation in F111 cells:*

F111 cells were grown on cover slips, serum starved and stimulated with 40 nM PMA for 0 h (ii), 2 h (iii) and 6 h (iv). After fixation with methanol, the coverslips were processed for immunofluorescence with anti-HABP1 antibody. Analysis was done using confocal microscopy. Pre-immune serum was used as a control as shown in B (i).
4.1.4 Nuclear translocation of HABP1 in PMA stimulated cells is MAP Kinase dependent

As mention in the Review of Literature (Section 1.4.3), HABP1 has a substrate phosphorylation site for MAP kinase. MAP kinase is also known to be stimulated by PMA (Thomas, 1992). To check the possible role of MAP kinase in HABP1 activity, the effect of a synthetic inhibitor for MAP kinase, PD098059 (Alessi et al., 1995) was tested upon the translocation of HABP1, following PMA stimulation. HeLa cells were treated with PD098059 along with PMA for 6 h and processed for immunofluorescence as mentioned in the previous section. As seen in Figure 4.5 C, cells treated with PD098059 alone show only cytoplasmic staining, while cells treated with PMA showed nuclear translocation of HABP1 (Figure 4.5 B). When cells were treated with both PMA and PD098059, HABP1 was primarily localised in the cytoplasm as evident by immunofluorescence (Figure 4.5 D). This observation shows that PD098059 inhibits translocation but not the upregulation of HABP1 by PMA. Therefore, it is concluded that following PMA stimulation, HABP1 expression is upregulated that is independent of MAP kinase but its subsequent translocation to the nucleus is MAP kinase dependent.

4.2 Discussion

In this study, we report the enhanced synthesis and nuclear translocation of HABP1 in both normal fibroblast (F111) and transformed cells (HeLa) upon treatment with PMA. Increased synthesis as well as translocation is time dependent. The cytoplasm localised HABP1 was enhanced after PMA treatment during first 2 hours following which it was translocated to the nucleus during the next 4 h (Figure 4.3 & 4.4). Further, the PMA dependent translocation of HABP1 was significantly inhibited by treatment with PD098059, a known inhibitor for
Figure 4.5: Nuclear translocation of HABP1 is MAP kinase dependent: HeLa cells were grown on coverslips, serum starved and treated with 40 nM PMA for 0 h (A) and 6 h (B); 50 μM of an inhibitor for MAP kinase, PD098059 for 6 h (C) and with both PMA and PD098059 for 6 h (D). Cells were fixed, processed for immunofluorescence with anti-HABP1 antibody and analysed by confocal microscopy. Figures A & B are the same as figures 4.4 A (ii) and 4.4 A (iv).
MAP kinase (Alessi et al., 1995) (Figure 4.5), thereby suggesting that activated MAP kinase probably phosphorylates HABP1 and triggers its translocation to the nucleus.

The precise detail of the nuclear translocation of HABP1 upon PMA stimulation remains to be elucidated. However, there are evidences to speculate that the PMA induced nuclear translocation of HABP1 is dependent on MAP kinase activation. We have reported earlier that in vivo phosphorylation of HABP1 is significantly enhanced in normal as well as transformed cells by HA and PMA (Rao et al., 1997). Phosphorylation of HABP1 by stimulators occurs at threonine and not at the tyrosine residue (Rao et al., 1997). Further sequence analysis of the cDNA encoding HABP1 revealed the presence of a proline directed \((^{60}\text{PELTSTP}^{166})\) substrate phosphorylation site for protein kinases like extracellular signal regulated kinase, ERK (Deb and Datta, 1996). It has also been well established that ser/thr–proline motif is sufficient for phosphorylation by ERK (Marshall, 1994). The presence of an N-terminal proline residue at least one amino acid distant from the phosphorylation site in the motif increases the efficiency of substrate recognition. This substantiates our observation that bacterial recombinant HABP1 is phosphorylated by MAP kinase and both HABP1 and MAP kinase are co-localised in MAP kinase activated transformed cells (unpublished data), suggesting that HABP1 may act as an endogenous substrate for MAP kinase.

Rapid and transient activation of tyrosine phosphorylation of cellular proteins by HA was reported earlier (Ohita et al., 1997; Ranganathan et al., 1995), but it has been recently established that HA activates MAP kinase via Ras-signalling pathway (Serbulea et al., 1999). It is important to mention in this context that we have demonstrated that HA hexa-saccharide is equally competent in interacting with HABP1 as the HA polymer (Deb et al., 2000). This finding has contributed significantly in expanding our understanding of the role of HABP1 in cellular signalling, as HA fragments, and not the HA polymer, induce the activation transcriptional regulator, NF-κβ (Noble et al., 1996).

The other agents that activate MAP kinase are growth factors, cytokines and tumour promoters like PMA (Marshall, 1994). PMA, the activator of PKC leads to alterations in gene expression, thereby regulating the transcription factors that bind to cognate regulating cis-elements. Apart from acting as a tumour promoter, PMA is also a potent stimulator for oxidative burst or the
generation of reactive oxygen species (ROS). Stimulation of cells with PMA results in a burst of ROS, which has a significant effect on the fragmentation of glycosaminoglycans (Moseley et al., 1997), among which, HA is the most susceptible to fragmentation.

From observations on the PMA induced translocation of HABP1, we suggest the possibility that PMA stimulated cells on one hand generate ROS leading to the production of low molecular HA fragments and on the other hand activate PKC mediated MAP kinase cascade. These processes probably bring about the phosphorylation and translocation of HABP1 to the nucleus. Therefore, we would like to propose here that MAP kinase activation may initiate the phosphorylation and nuclear translocation of HABP1 which ultimately allows this protein to interact with nuclear proteins like SF2, as reported earlier (Krainer et al., 1991, Peterson-Mahrt et al., 1999).