Variant-specific patterns and humoral regulation of HP1 proteins in human cells and tissues

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Summary

We have examined the occurrence and distribution of HP1α and HP1β under in vivo, ex vivo and in vitro conditions. Consistent with a non-essential role in heterochromatin maintenance, both proteins are diminished or undetectable in several types of differentiated cells and are universally downregulated during erythropoiesis. Variant-specific patterns are observed in almost all human and mouse tissues examined. Yet, the most instructive example of HP1 plasticity is observed in the lymph nodes, where HP1α and HP1β exhibit regional patterns that are exactly complementary to one another. Furthermore, whereas HP1α shows a dispersed sub-nuclear distribution in the majority of peripheral lymphocytes, it coalesces into large heterochromatic foci upon stimulation with various mitogens and IL-2. The effect of inductive signals on HP1α distribution is reproduced by coculture of immortalized T- and B-cells and can be confirmed using specific markers. These complex patterns reveal an unexpected plasticity in HP1 variant expression and strongly suggest that the sub-nuclear distribution of HP1 proteins is regulated by humoral signals and microenvironmental cues.

Supplementary material available online at
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Introduction

Recent studies have identified a set of chromatin-associated proteins that could presumably 'read' epigenetic signals. As it turns out, these effector molecules are often components of large enzymatic assemblies and possess specialized modules known as bromo-, tudor- or chromodomains (Daniel et al., 2005).

The chromodomain (CD) represents one of the most ubiquitous, yet versatile, structures and is conserved in organisms ranging from mammals to archeobacteria (Ball et al., 1997; Singh et al., 1991). Within eukaryotes, there are more than a dozen chromodomain protein families that include enzymes, remodeling factors and chromatin modulators, such as heterochromatin protein 1 (HP1) (reviewed by Tajul-Arifin et al., 2003).

The HP1 molecule comprises an amino-terminal chromodomain (CD) and a structurally related chromo-shadow domain (CSD), partitioned by a non-conserved 'hinge' region (for reviews, see Singh and Georgatos, 2002; Hediger and Gasser, 2006). HP1 orthologues have been characterized in almost all eukaryotic organisms, from yeast to mammals. Although the structural features of HP1 proteins have only minor variations, the number of individual HP1 genes varies. The *Saccharomyces cerevisiae* genome contains no identifiable HP1 gene; *Neurospora crassa* expresses a single HP1 form (hpo-1); and almost all metazoans and *Schizosaccaromyces pombe* possess multiple variants. For historical reasons, HP1 proteins are referred to as HP1α, HP1β and HP1γ in mammals, as HPL-1 and HPL-2 in *Caenorhabditits elegans*, as HP1a, HP1b and HP1c in flies and as Swi6, Chp1 and Chp2 in fission yeast (reviewed by Hiragami and Festenstein, 2005) (see also Thon and Verhein-Hansen, 2000; Freitag et al., 2004). In mice, there has been a suggestion that there are another two HP1-like proteins (in addition to the three classic variants) that apparently have no homologues in the human transcriptome (Tajul-Arifin et al., 2003). However, this contention has not been confirmed yet at the molecular and/or cellular level. In humans, the three HP1 genes are located on chromosomes 12, 17 and 7, respectively. There is also a number of HP1 pseudogenes scattered throughout the genome (Norwood et al., 2004; Jones et al., 2001).

Although cDNAs encoding the three HP1 variants were isolated more than 15 years ago, information concerning the tissue-specific distribution of these proteins in vivo is fragmentary. Assessing the abundance of the three variants in different microenvironments is of considerable interest, because HP1 molecules have a propensity to homo- and heterodimerize through their CSDs, yielding a range of different oligomers (Brasher et al., 2000; Wang et al., 2000; Nielsen et al., 2001a; Nielsen et al., 2001b). Since the α, β and γ forms appear to have non-redundant functions (Filesi et al., 2002; Cammas et al., 2007), each HP1 oligomer (or oligomer combination) may represent committed machinery utilized in specialized cellular functions.

Clues about this are provided by the interesting dichotomy between HP1α and -β and HP1γ. The former two proteins are predominantly localized in constitutive heterochromatin and are often involved in gene repression, whereas the latter variant shows a pan-nuclear distribution and is implicated in both gene repression and gene activation (Horsley et al., 1996; Nielsen et

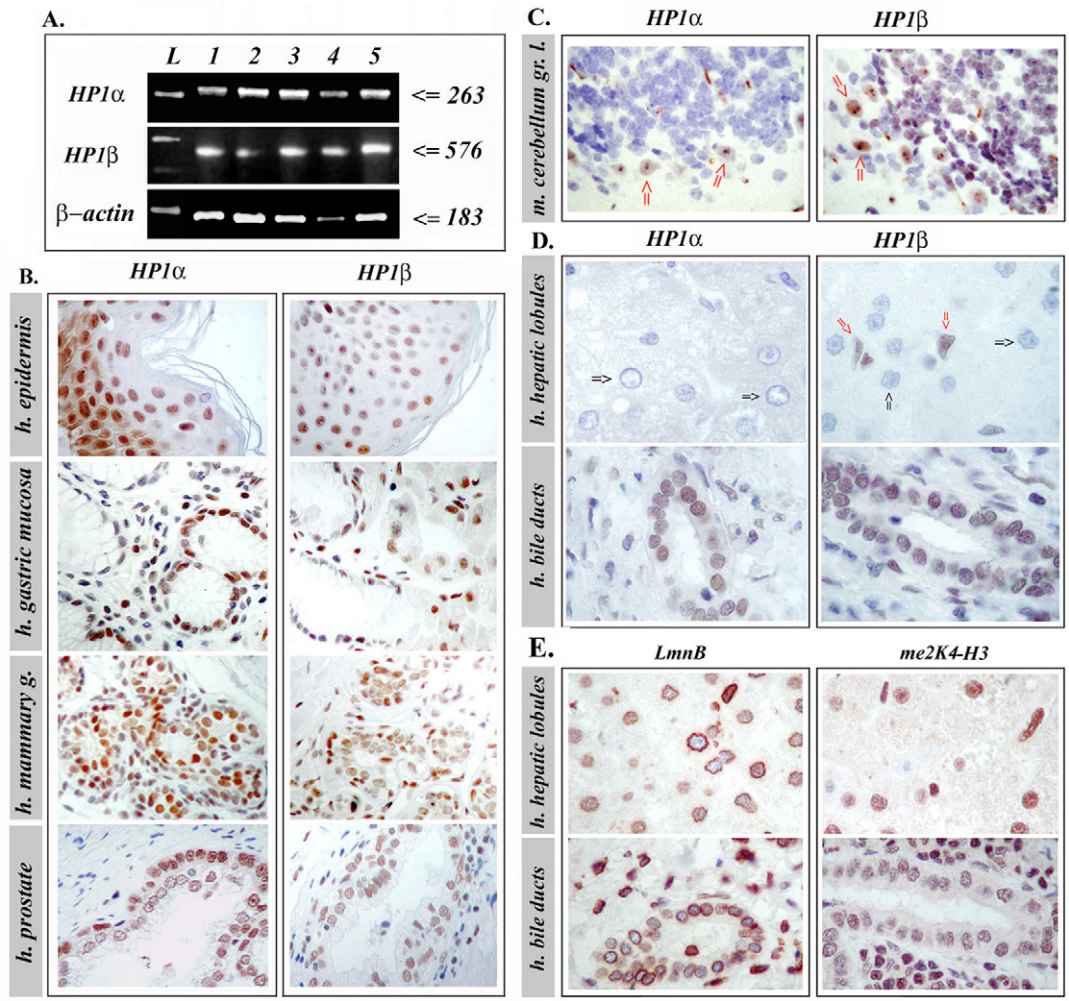

Fig. 1. Expression of HP1α and HP1β in various tissues and cell types. (A) Identification of HP1α and HP1β transcripts in human tissues by RT-PCR. Lanes: 1, endometrium; 2, myometrium; 3, mammary glands; 4, lymph nodes, 5, skin; L, a 1 kb DNA ladder. Sizes of amplified products (in bp) are indicated on the right. Beta-actin amplification was used as a control. For more details see Materials and Methods.
(B,C) Immunocytochemical detection of HP1α and HP1β in (B) human epidermis, gastric mucosa, mammary gland and prostate, and (C) in mouse cerebellar cortex. Red arrows indicate Purkinje cells expressing both proteins. (D) Detection of the HP1α and HP1β proteins in human liver. Red arrows indicate Kupffer cells and black arrows, hepatocytes. (E) Staining of human liver with control (anti-lamin B and anti-me_2K4 histone H3) antibodies. In D and E the two upper panels show hepatic lobules, whereas lower panels show inter-lobular space containing bile ducts.

al., 1999; Hwang and Worman, 2002; Vakoc et al., 2005). So far, a consistent pattern of variant-specific expression has not been reported and the existing literature tacitly assumes that all HP1 proteins are equally abundant in the cells of multi-cellular organisms. However, there are at least two processes where the three HP1 genes are universally downregulated: erythropoiesis (Gilbert et al., 2003; Istomina et al., 2003) and granulopoiesis in humans (Lukasova et al., 2005). There is also an example of selective downregulation that involves the HP1α gene in highly metastatic human breast carcinomas and their derivatives (Kirschmann et al., 2000).

In this work we investigate HP1 expression and distribution in human tissues and various cellular models. The results reveal complex cell-type and tissue-specific patterns for at least two of the three HP1 variants, HP1α and HP1β. Surprisingly, these patterns do not correlate directly with the patterns of classic heterochromatic determinants, such as methylated lysine 9 on histone H3 (me_3K9-H3), but do parallel the proliferation state of the cells and their responsiveness to humoral signals.

Results

Distribution patterns of HP1 variants in human tissues

At the outset of this study we retrieved microarray data from a variety of sources and performed a retrospective evaluation (meta-analysis; see Materials and Methods). Processed data, shown in supplementary material Fig. S1A strongly suggested that the HP1 genes are ubiquitously expressed, a notion that could be experimentally confirmed by examining solid tissues, by RT-PCR. As shown in Fig. 1A, specific products of the HP1α and HP1β genes of the expected length were identified in all samples examined after amplification.

Despite the ubiquitous expression of the HP1 genes at the level of bulk tissues, a closer inspection of the profiling results suggested the existence of fine, variant-specific features. As indicated by the data shown in supplementary

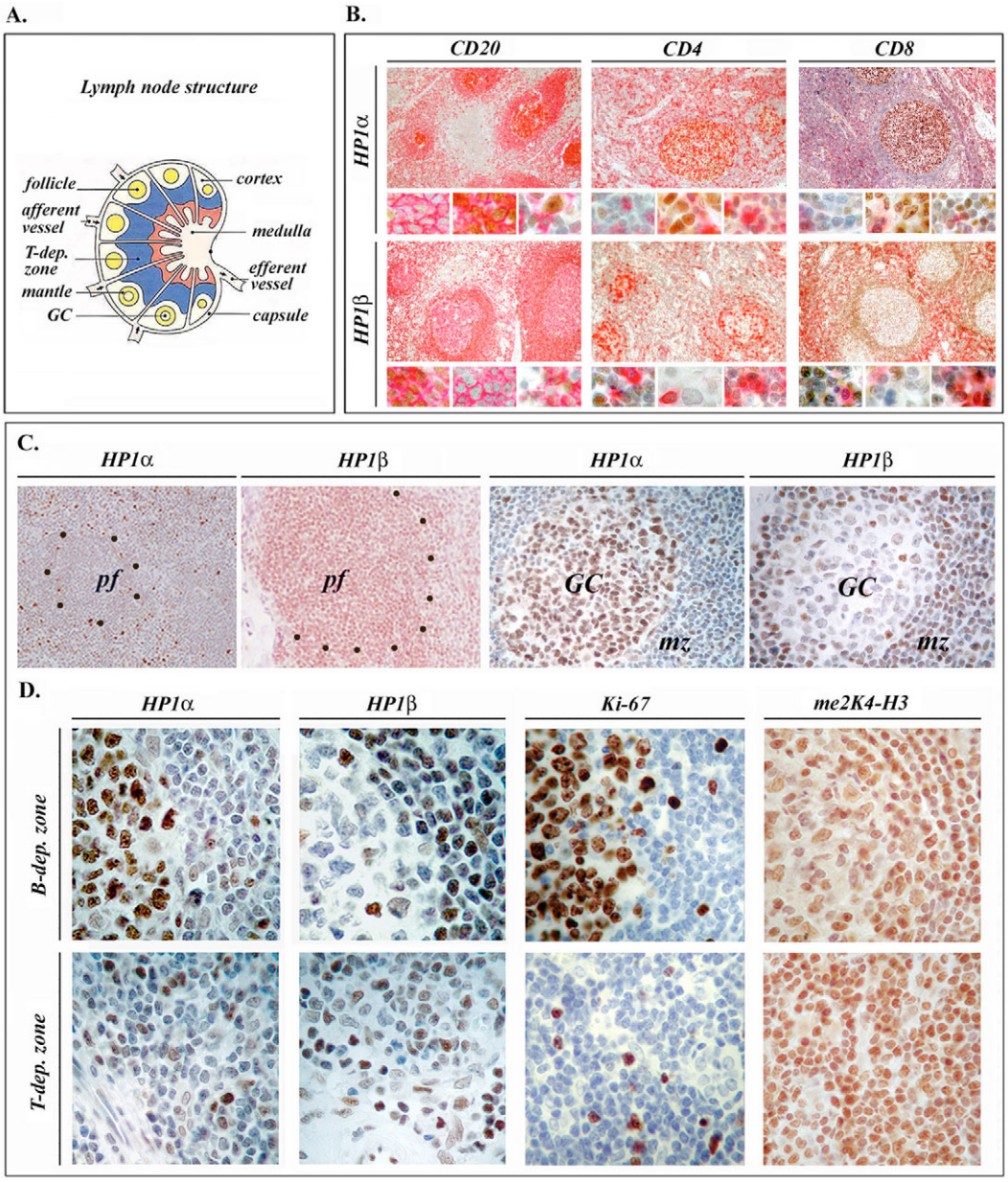

Fig. 2. Distribution of HP1α and HP1β in human lymph nodes. (A) Schematic representation of lymph node structure. (B) Double staining for HP1α or HP1β (brown) and B- (CD20) or T-cell (CD4 and CD8) markers (red). (C) Expression of HP1α and HP1β in primary (pf) and secondary follicles comprising a germinal center (GC) and a mantle zone (mz). The area of primary follicles is indicated by dots. (D) Detection of HP1α, HP1β, Ki-67 and me_2K4-H3 in the B- and T-zone.

material Fig. S1A,B, the steady-state levels of the HP1α mRNA vary significantly, being lower than 40% (on a 0-100 scale) in liver, heart, skin and tongue and higher that 80% in the bone marrow, thymus and neuronal tissues (whole brain and pituitary). Much less variation is seen in mRNA levels of the HP1β and essentially no variation is detected in HP1γ levels.

To assess the abundance and distribution of HP1 proteins at the level of single cells we performed a systematic immunohistochemical survey, examining several non-neuronal human tissues and mouse brain. The results of this study, summarized in supplementary material Fig. S1C, reveal complex patterns of HP1α and HP1β protein expression. As shown in Fig. 1B,C, only minor variations in the abundance and cell-type specificity of HP1α and HP1β were detected in human stomach, mammary gland, prostate, epidermis and in mouse brain. Yet, an exquisite example of cell-type-specific expression was observed in the liver, where hepatocytes were universally negative for both HP1α and HP1β, bile duct epithelia were stained for both HP1 variants and Kupffer cells contained exclusively HP1β (Fig. 1D, upper panels). These variations, as well as the rather restricted expression pattern of HP1α in this organ, are in excellent agreement with the mRNA profiling results summarized in supplementary material Fig.

S1A and readily explain why the levels of HP1α mRNA are lower than that of HP1β mRNA in the bulk sample.

The cell type-specific patterns of HP1α and HP1β were basically the same when mouse hepatic tissue was examined (not shown), but no cell-specific variation was observed when human or mouse liver were stained with antibodies recognizing structural proteins of the cell nucleus (such as lamin B) and widespread chromatin markers (e.g. me_2K4 histone H3; see Fig. 1D, lower panels). This validates the immunohistochemical results presented above and precludes potential artifacts related to specimen preservation.

A striking, regional pattern of HP1 expression was observed in the lymph nodes, which are known to contain histologically and functionally distinct 'domains' (see cartoon in Fig. 2A). In this tissue, anti-HP1α antibodies stained mainly the germinal center cells of secondary follicles, whereas anti-HP1β antibodies decorated exclusively the cells of the primary (i.e. non-stimulated) follicles and the mantle zone, which accommodate primarily naïve B-cells (Fig. 2C,D, supplementary material Fig. S6 and Table 1). The corresponding staining patterns were not as distinct in the T-dependent zone, where the abundance of the two HP1 variants in small lymphocytes was rather variable. In this area, B- and T-blasts stained intensely with anti-HP1α and weakly with anti-HP1β antibodies (Fig. 2D and Table 1).

In line with a region-specific, lineage-independent pattern, HP1 variant expression did not correlate with B-cell (CD20) or T-cell (CD4 and CD8) markers (Fig. 2B). Furthermore, macrophages, as well as follicular and interdigitating dendritic cells, were consistently stained with both antibodies (Table 1). Selective staining of lymphoid and non-lymphoid cells was also detected in sections of human thymus (supplementary material Fig. S2).

The staining pattern of HP1α in the lymph nodes was almost identical to that of Ki-67, a proliferation marker accumulating in the cycling cells of the germinal center and scattered B- or T-blasts residing in the T-zone (Fig. 2D, compare Ki-67 with HP panels). Such a region-specific staining was not observed when anti-histone antibodies were used (Fig. 2D, me2K4-H3), indicating a specific, functional link between expression of HP1α and lymphocyte activation. This is consistent with observations described below and agrees perfectly with results obtained recently by transcriptional profiling of the germinal center reaction (i.e. the transition from naïve B-lymphocytes to centroblasts and centrocytes and, from there, to memory cells). These studies have shown a significant increase in the levels HP1α mRNA upon progression of lymphocyte differentiation (Klein et al., 2003).

Dynamic redistribution HP1 in cultured lymphoid cells

To explore further the differential expression and distribution of HP1 variants in lymphoid cells, we employed two human cell lines representing mature T- and B-lymphocytes (HUT-78 and RPMI-8226, respectively). HUT-78 cells are from mature helper T-cells exhibiting the Sezary phenotype (i.e. a highly convoluted nucleus) (Bunn, Jr and Foss, 1996), whereas RPMI-8226 are from plasma cells (Freund et al., 1993; O'Connell et al., 1995; Gooding et al., 1999).

As would be expected from previous studies (e.g. Kourmouli et al., 2000), in both cell lines the distribution of HP1γ was diffuse, whereas HP1β exhibited the usual 'speckled' pattern (supplementary material Fig. S3 and Fig. 3A, HP1γ/HP1β, monoculture). However, this was not the case with HP1α, which, strongly deviating from stereotype and was localized in a constellation of small 'granules' that were scattered throughout the cell nucleus (supplementary material Fig. S3 and Fig. 3A, HP1α, monoculture).

To find out whether there was 'plasticity' in the distribution of HP1 proteins in these cells we set up a co-culture system allowing exchange of soluble products between the two cell types (Giuliani et al., 2002) and re-assessed the localization of HP1 proteins. Consistent with a dynamic redistribution, the pattern of HP1α (and to a lesser extent the distribution of HP1β) changed dramatically, as the proteins started to coalesce into large heterochromatic blocks (supplementary material Fig. S3 and Fig. 3A, HP1α/HP1β, co-culture). By contrast, the distribution of HP1γ did not change (supplementary material Fig. S3, HP1γ, co-culture) and neither did the pattern of me_3K9-histone H3 (supplementary material Fig. S3, me3K9), a classic heterochromatic marker. The differences in HP1α distribution were more apparent in T- than in B-cells (compare HP1α, co-culture, panels HUT-78 and RPMI-8226), indicating some type of differential responsiveness. We also noticed that the nuclei of HUT-78 cells changed shape upon stimulation, becoming more round and acquiring a smooth contour (Fig. 3A).

Since HUT-78 cells are known to express cytokine receptors, we also cultured this lymphocytic line for several days in the presence and absence of human IL-2. As shown in supplementary material Fig. S3 and Fig. 3A, IL-2 treatment reproduced, at least in part, the effect seen upon co-culturing.

Upon double immunostaining of co-cultured or IL-2-

Table 1. Expression of HP1 proteins in lymph nodes

Cell type	Staining intensity	Percentage
HP1α		
B-dependent zone		
B-blast, centrocytes	+++	>90
Small B	–	>90
Small T	+/–	5-40
Macrophages	–/+	>90
T-dependent zone		
T-blasts	+++	>90
Small T	+/–	50
Small B	–	>90
IDC	–/+	>90
Fibroblasts	–	>90
Endothelial cells	–	>90
HEV	+/–	20
Smooth muscle fibers	–	>90
HPIβ		
B-dependent zone		
B-blast, centrocytes	–	>90
Small B	+++	>90
Small T	+/–	50
Macrophages	+	>90
T-dependent zone		
T-blast	–	>90
Small T	+/–	60
Small B	+++	>90
IDC	+++	>90
Fibroblasts	+	>90
Endothelial cells	+	>90
HEV	–	>90
Smooth muscle fibers	+	>90

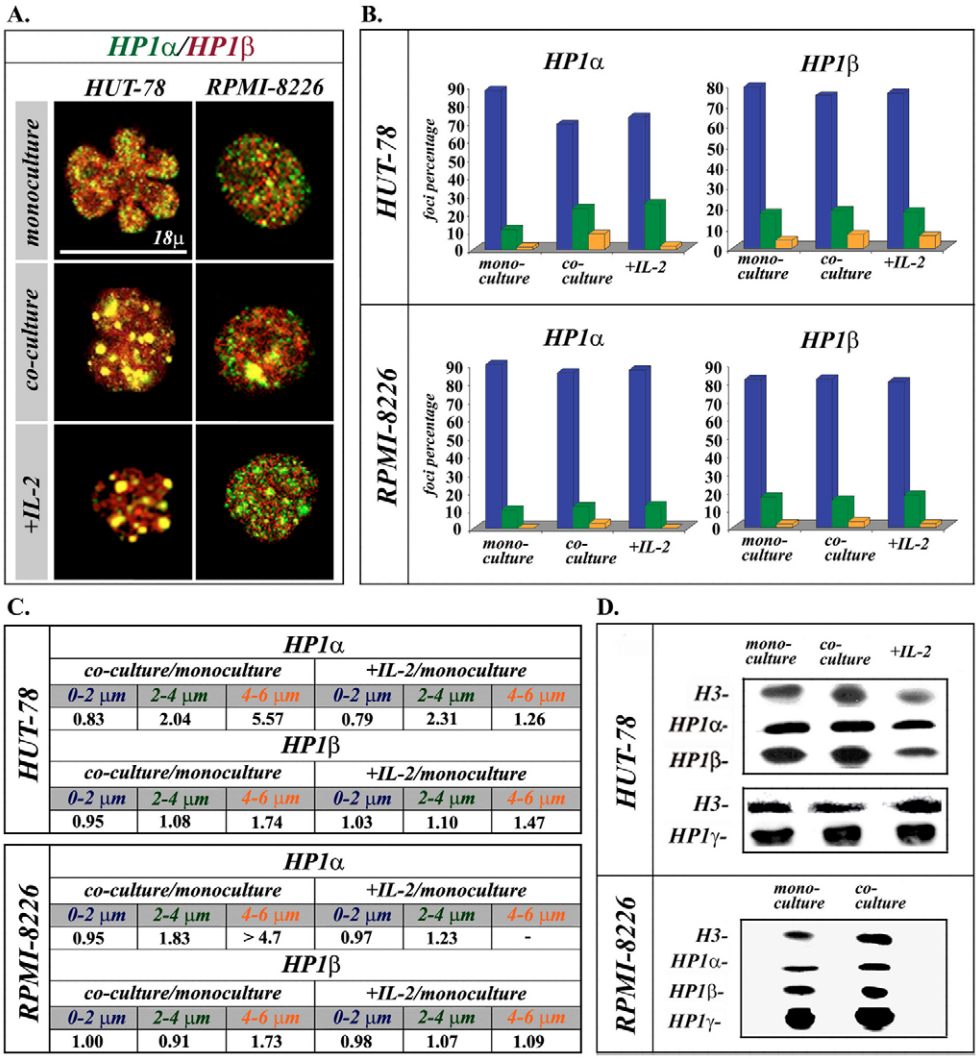

Fig. 3. Redistribution of HP1 in immortalized B- and T-lymphocytes. (A) Monocultures, co-cultures and IL-2-treated cells doubly-stained with anti-HP1α (green) and anti-HP1β (red) antibodies. Coarse HP1 foci that appear upon co-culture or IL-2 treatment are more obvious in the HUT-78 line. Notice the convoluted nuclei in HUT-78 monocultures, a typical feature of Sezary cells. Bar, 18 μm. (B) Morphometric data showing the proportion of HP1α and HP1β foci in the nucleus of HUT-78 and RPMI-8226 cells under different culture conditions (blue bars: 0-2 μm foci; green bars: 2-4 μm foci and orange bars: 4-6 μm foci). Optical sections and 'projections' of 15 different cells were analyzed in each case. (C) Fold-difference in the abundance of the various classes of HP1 particles upon co-culture or IL-2 treatment in relation to controls (monocultures). The results, shown in a tabular form, were derived from the data presented in B. (D) Western blots showing the levels of HP1α, HP1β and histone H3 in nuclear extracts of HUT-78 and RPMI-8226 cells before and after co-culture, or treatment with IL-2.

treated HUT-78 cells, we noticed that large HP1α blocks also contained HP1β (Fig. 3A). Systematic scoring and categorization of the various HP1 particles revealed that HP1α foci in co-cultured cells were significantly bigger (Fig. 3B,C). For instance, HP1α foci measuring 2-4 μm (intermediate category) were twice as abundant in co-cultures than in monocultures of HUT-78 cells, whereas foci with sizes ranging from 4-6 μm (large) showed a 5.6-fold increase. When the T-cell line was treated with IL-2, the relative proportion of large particles did not change significantly, but the intermediate ones were 2.3 times as abundant in treated than in non-treated cells. Under the same conditions, intermediate-sized HP1β foci were as abundant in co-cultures as they were in monocultures, whereas the large foci were slightly increased (a 1.7-fold difference). Interestingly, the average number of HP1 foci per cell did not change upon stimulation, and differences seen at these two particle classes were fully compensated by a relative decrease in the proportion of the small-size particles, i.e. foci measuring <2 μm. In RPMI-8226 cells the results were similar, but the differences seen in HP1α foci were less dramatic.

The quantitative data presented above corroborated the qualitative observations presented in Fig. 3A and further suggested that the 'lumpy' staining pattern of co-cultured cells probably does not reflect an increase in HP1α levels, but rather a redistribution of pre-existing protein. This could be independently confirmed by western blotting, which showed that the relative amounts of HP1α in HUT-78 monocultures, HUT-78 co-culture and HUT-78 cells incubated with IL-2 did not differ (Fig. 3D). Therefore, it can be inferred that extrinsic

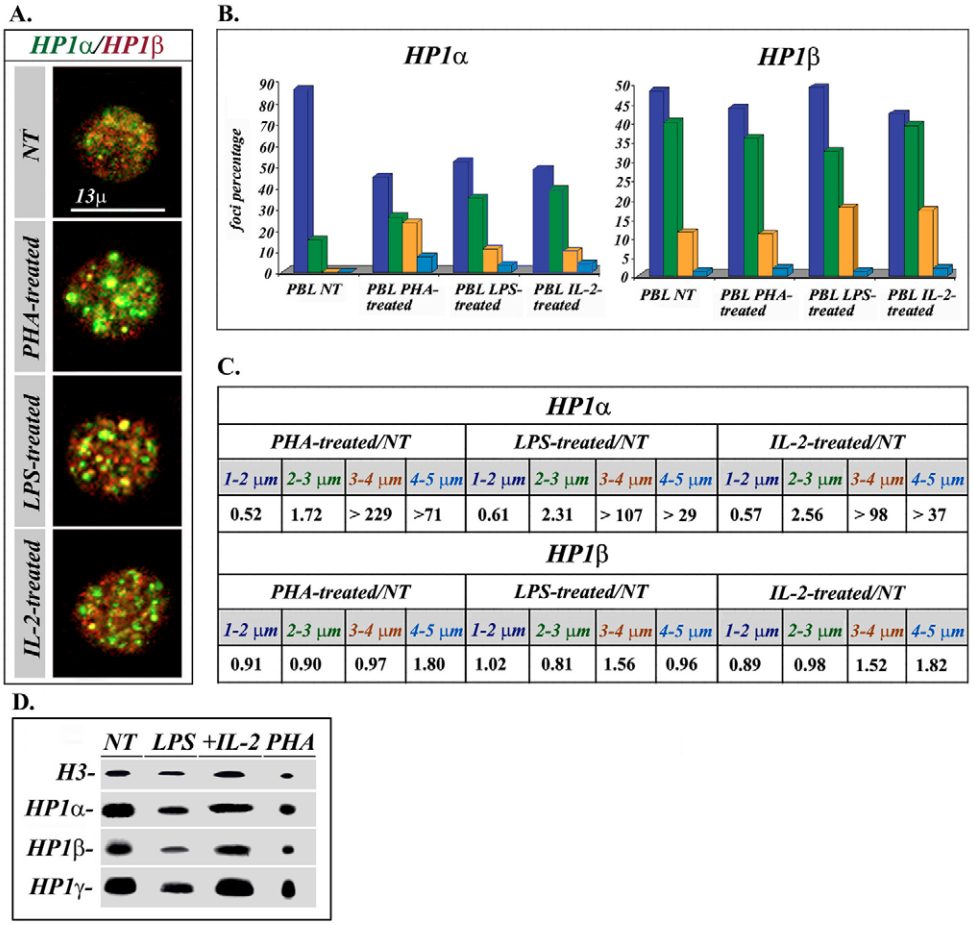

Fig. 4. Redistribution of HP1 in peripheral blood lymphocytes (PBLs). (A) Double staining with anti-HP1α (green) and anti-HP1β (red) antibodies. The panel shows non-treated (NT), phytohemagglutinin-treated (PHA), lipopolysaccharide-treated (LPS) and IL-2-treated (+IL-2) human PBLs. Bar, 13 μm. Note the difference in the size of the nucleus. (B) Morphometric data showing the percentage of HP1α and HP1β foci in the nucleus of non-treated and treated cells, as indicated (dark blue bars: 1-2 μm foci; green bars: 2-3 μm foci, orange bars: 3-4 μm foci, and light blue bars: 4-5 μm foci). Optical sections and 'projections' of 15 different cells were analyzed in each case. (C) Fold-difference in the abundance of the various classes of HP1 particles upon treatment in relation to controls. The results, shown in a tabular form, were derived from the data presented in B. (D) Western blots of PBLs before and after treatment with LPS or IL-2. The blots were probed for HP1α, HP1β and histone H3, as indicated.

signals affect primarily the distribution and not the expression levels of this protein.

Next, we examined peripheral blood lymphocytes (PBLs) ex vivo. As could be observed in supplementary material Fig. S4 and Fig. 4A (NT panels; compare HP1α and HP1β), the large majority of PBLs contained small HP1β foci and exhibited a completely dispersed HP1α and HP1γ pattern. Nonetheless, upon closer inspection PBLs that were intensely stained with anti-HP1α antibodies and contained large heterochromatic blocks were occasionally seen (supplementary material Fig. S5A). Based on what is currently known, these figures may represent rarely occurring activated lymphocytes circulating in the blood stream (supplementary material Fig. S5B).

After mitogenic stimulation with phytohemagglutinin (PHA) or lipopolysaccharide (LPS) antigen, the HP1α pattern changed dramatically and became visibly 'speckled' (Fig. 4A and supplementary material Fig. S4, PHA/LPS, HP1α). A similar pattern was observed when PBLs were cultivated in the presence of human IL-2 (Fig. 4A and supplementary material Fig. S4, +IL-2, HP1α), in accordance with the experiments described previously. In contrast to this, no major changes were observed in the staining profile of HP1β, HP1γ and me_3K9-histone H3 between non-treated and mitogen-activated lymphocytes (supplementary material Fig. S4, HP1β, HP1γ, me3K9). Similarly to HUT-78 cells, HP1α blocks in activated PBLs also contained HP1β (Fig. 4A, +IL-2).

As shown in Fig. 4B,C, HP1α foci measuring 3-5 μm (intermediate class) were increased up to 229-fold in stimulated cells, whereas HP1β foci of the same category were increased only 1.8-fold, strongly suggesting a variant-specific effect. Moreover, a more precise assessment of the data shown in Fig. 4B,C further suggested that the effect of different humoral factors on HP1 distribution was selective. For example, the largest HP1α particles (4-5 μm) were more abundant in PHA-treated than in LPS- or IL-2-treated cells, and approximately the same was observed with 3-4 μm particles. Conversely, 2-3 μm particles were substantially more abundant in IL-2- and LPS-treated cells than in PHA-treated cultures. No such differences were detected when HP1β foci were compared. Therefore, HP1α-specifc differences, as fine as they might be, are indicative of the fact that each activator affects the distribution patterns of HP1 proteins, and thereby the epigenetic status of different lymphoid sub-classes, in distinct ways.

As in the case of HUT-78 cells, the average number of HP1α/β foci in human PBLs was not significantly altered upon stimulation and the differences seen in bigger particle classes were fully compensated for by a relative decrease in the proportion of the smaller-size particles, i.e. foci measuring <3 μm. This further suggested that the 'lumpy' staining pattern of co-cultured cells did not reflect an increase in HP1α levels, but rather a redistribution of pre-existing protein, an interpretation that could be directly confirmed by western blotting (Fig. 4D).

To examine if the 'speckled' phenotype of HP1α occurs preferentially in cycling cells, we doubly stained resting and stimulated lymphocytes with anti-HP1α and anti-Ki-67

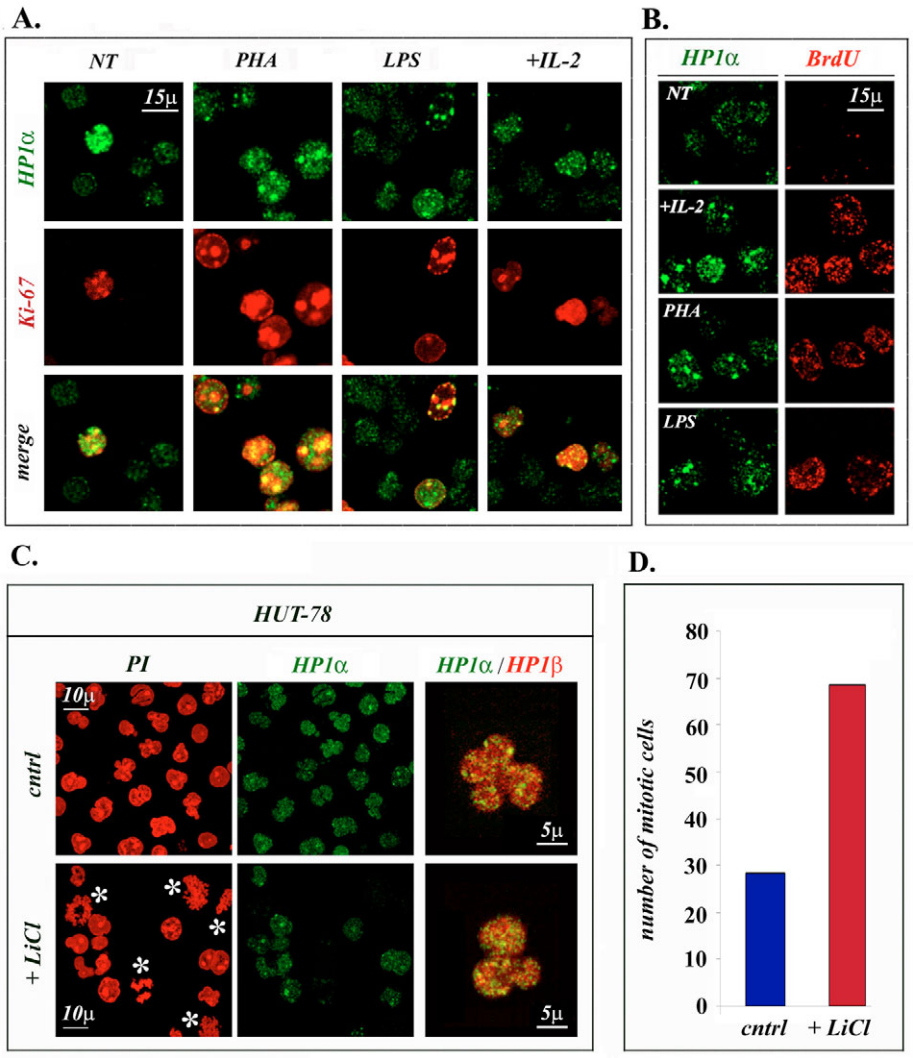

Fig. 5. Correlation of HP1α redistribution with lymphocyte activation. (A) Co-staining of non-treated (NT) and stimulated (PHA, LPS) PBLs with anti-HP1α (green) and anti-Ki-67 (red) antibodies. Note that cells with coarse HP1 foci are scarce in the control but increase substantially after treatment. As expected, the number of the intensely stained figures is rather small in the LPS sample since B cells constitute a very small percentage (25-28%) of total blood lymphocytes. Bar, 15 μm. (B) Staining of non-treated and stimulated PBLs with anti-HP1α (green) antibodies and BrdU (red). The induced cells have entered the cell cycle and incorporated BrdU. Bar, 15 μm. (C) HUT-78 monocultures and LiCl-treated cells stained with the indicated antibodies. Asterisks denote mitotic cells. Notice that addition of LiCl affects only the rate of cell division and not the distribution pattern of HP1α. Bars, 10 μm and 5 μm as indicated. (D) Graphic representation of the mitotic index of HUT-78 cells prior to and after incubation with LiCl. 1,000 cells were analyzed in each case.

antibodies. As seen in Fig. 5A, a close correlation could be established between the HP1α pattern and the cycling state of the cells. The same was observed when we examined mitogen-stimulated and IL-2-induced lymphocytes, utilizing BrdU as a probe. Fig. 5B clearly shows that cells that had incorporated BrdU (stimulated figures) invariably contained large HP1α blocks. No such connection could be established when PBLs (before or after stimulation) were probed with anti-histone modification and anti-nuclear envelope protein antibodies (not shown), suggesting that HP1α distribution represents a specific marker of cycling lymphocytes.

Since lymphocyte activation via antigen receptors involves two simultaneous processes, i.e. clonal expansion and functional differentiation, we wanted to check if the mere induction of cell proliferation would lead to HP1α redistibution. To test this, we employed LiCl, an inducer of Wnt signaling (Lucas and Salinas, 1997) that is known to enhance lymphocyte proliferation (Hart, 1979; Bray et al., 1981; Kucharz et al., 1988; Gauwerky and Golde, 1982). For such purposes, we resorted to a cycling T-cell line (HUT-78 cells), since LiCl is known to enhance cell proliferation only in mitogen or antigen-stimulated primary lymphocytes and not in resting cells.

As seen in Fig. 5C, no changes were observed in HP1α distribution in the presence of LiCl. To verify that LiCl had acted as expected, increasing the proliferation rate of HUT-78 cells, we counted mitotic figures. Fig. 5C,D documents a 2.4-fold increase of the mitotic index in the treated cells and show that, at least in T-lymphocytes, incorporation of HP1α in large heterochromatin blocks is linked primarily to functional differentiation and not to cell proliferation per se.

Downregulation of HP1α and HP1β during erythropoiesis

Finally, to examine whether HP1 expression correlates with differentiation state, we employed the MEL (murine) and K562 (human) cell systems, two erythroleukemic lines that recapitulate the developmental changes occurring during the transition from an early erythroblast to a normoblast. Induction of MEL and K562 cells by DMSO or HMBA is known to induce globin synthesis and production of erythroid-specific markers (for reviews, see Marks and Rifkind, 1978; Koeffler and Golde, 1980).

Undifferentiated or partially differentiated cells exhibited the characteristic heterochromatic patterns observed in other cell types (Kourmouli et al., 2000), whereas normoblast-like

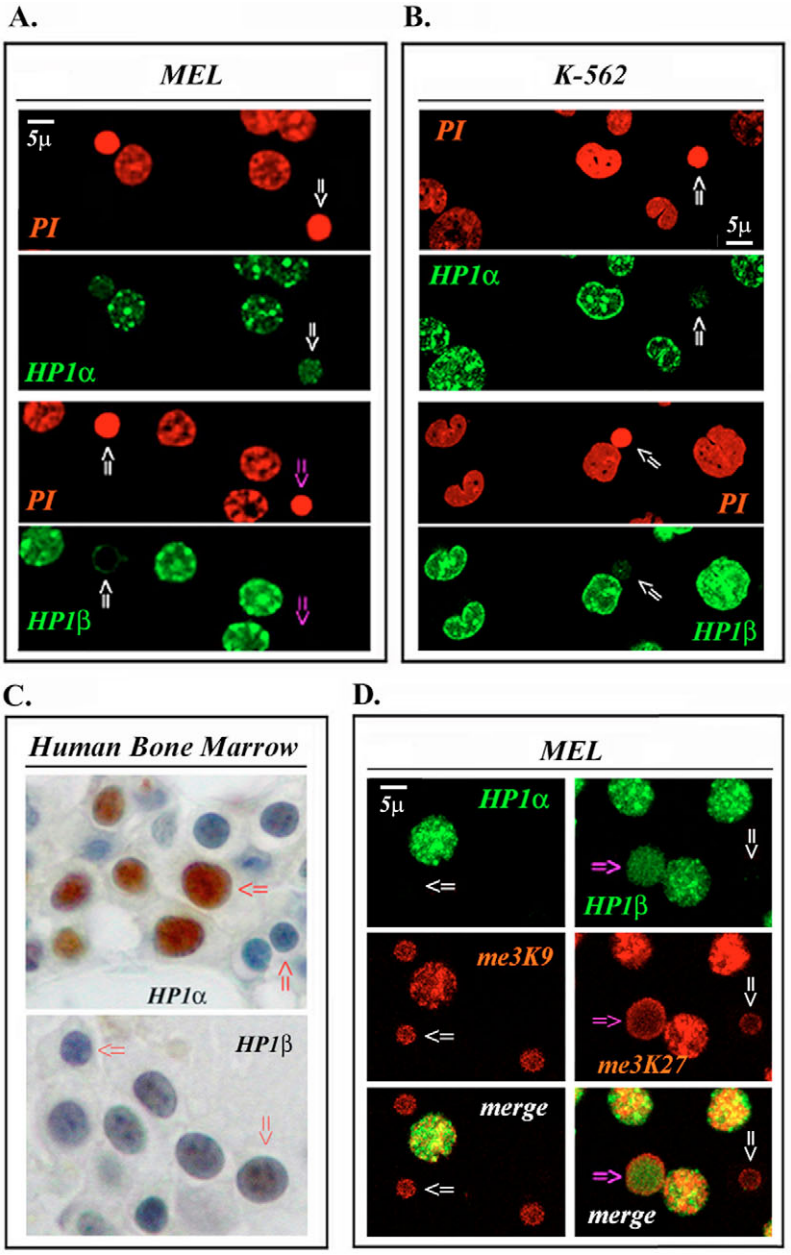

Fig. 6. Downregulation of HP1α and HP1β during mammalian erythropoiesis. (A) Staining of HMBA-treated mouse erythroleukemia cells (MEL) with anti-HP1α or anti-HP1β antibodies (green) and propidium iodide (red). Arrows indicate differentiated, normoblast-like figures possessing condensed nuclei. Cells with bigger nuclei are undifferentiated early erythroblasts that have not responded to HMBA treatment (for more details see text). Bar, 5 μm. (B) The same experiment as in A using human erythroleukemic cells (K-562). Bar, 5 μm. (C) Immunohistochemical detection of HP1α and HP1β in human bone marrow sections. The images show erythropoietic islands containing early and late erythroblasts (red arrows) stained with the corresponding antibodies and Hematoxylin. (D) Double staining of undifferentiated and partially differentiated mouse eythroleukemia cells (as in A) with anti-HP1β (green) and anti-histone modification (red) antibodies. Note that the anti-me_3K9 and anti-me_3K27-H3 antibodies readily penetrate the condensed nuclei of the normoblasts, indicating access to intranuclear antigens. Bar, 5 μm.

figures were faintly stained, or not stained at all by anti-HP1α and anti-HP1β antibodies (Fig. 6A,B). This was surprising, because previous observations have claimed that, unlike avian erythrocytes that are devoid of HP1 proteins, mouse embryonic erythrocytes are abundant in HP1 (Gilbert et al., 2003). Since the mouse erythrocytes examined by Gilbert and coworkers corresponded to *early* erythroblasts that did not contain condensed nuclei, whereas the MEL normoblasts were further differentiated and contained pycnotic nuclei, we reasoned that the lack of HP1α and HP1β could be explained either by specific downregulation of the corresponding genes at late stages of erythropoiesis, or by inaccessibility of the antibodies to areas containing compacted chromatin.

Since the HMBA-treated MEL cultures contained only 20-30% of fully differentiated erythrocytes this question could not be answered by RT-PCR or western blotting. Thus, to distinguish between these two possibilities we used in situ methods. Co-staining with antibodies to me_3K9- or me_3K27-histone H3 and HP1β showed persistent histone fluorescence in normoblast nuclei (Fig. 6D), ruling out inaccessibility to antibodies. Also, consistent with earlier observations (Dialynas et al., 2006), me_3K9-H3 and HP1β largely colocalized in undifferentiated MEL cells, but this overlap was restricted to large heterochromatic blocks. As expected the localization pattern of me_3K27-H3 did not coincide with that of HP1β.

To confirm these observations in an in vivo system, we also examined human bone marrow specimens. In these samples, erythoblastic islets containing early and late erythroblasts could be easily identified by Hematoxylin staining, whereas HP1-expressing cells could be detected by specific antibodies. Results depicted in Fig. 6C show that HP1α was abundantly present in early erythroblasts, but remained undetectable in normoblasts, in line with the data presented previously. However, HP1β was under the threshold of detection in both

early and late erythroid precursors, indicating very low expression levels under in vivo conditions.

Discussion

The transcriptional activity of eukaryotic genes is controlled by specific regulatory factors that include small RNAs and chromatin-associated proteins (reviewed by Elgin and Grewal, 2003). A variety of stimuli (either intrinsic or extrinsic) are known to modulate the levels and properties of such regulatory elements, thus inducing a cascade of interactions that are transmitted downstream and eventually target various effectors to distinct sites of the genome. The exact role of HP1 in these processes is not well defined. Considering HP1 proteins merely as 'repressors', or constitutive elements of heterochromatin, is a rather simplistic view and does not explain findings such as an involvement of HP1 in gene activation (Vakoc et al., 2005), or its absence in terminally differentiated cells (Gilbert et al., 2003) (this report). In fact, the non-redundant functions of the three HP1 variants (Filesi et al., 2002), their ability to oligomerize (Ye et al., 1997; Brasher et al., 2000; Nielsen et al., 2001a; Nielsen et al., 2001b; Wang et al., 2000) and their multiple post-translational modifications (Huang et al., 1998; Minc et al., 1999) confer versatility and endow them with a potential to serve as 'reporters' of different chromatin states.

Since little is known about the expression mode and the distribution patterns of HP1 variants in mammalian organs, we performed a systemic screening of HP1α and HP1β in a large number of human and mouse tissues, primary cells and in vitro differentiation models. HP1γ was not pursued in these studies because initial observations showed that it is more or less ubiquitously and universally expressed in all systems studied. Our results reveal a variety of cell type- and tissue-specific patterns, in line with the idea of 'distinct HP1 repertoires' that may match in part the repertoires of epigenetic modifications in histones and non-histone proteins (Singh and Georgatos, 2002). The plasticity of HP1 proteins is demonstrated best by studying the distribution and expression of HP1α in circulating lymphocytes and lymphoid organs. Immunohistochemical observations and morphometric data show clearly that this protein is highly expressed in proliferating, Ki-67-expressing cells populating the germinal centers and the T-dependent zones of the lymph nodes and are much less abundant in resting (G0) lymphocytes. However, apart from transcriptional regulation, which may affect primarily the abundance of HP1 proteins, their sub-nuclear distribution is also regulated at a post-transcriptional level, at least in PLBs. Thus, incorporation of HP1α into heterochromatic 'blocks' is induced when PBLs or lymphocytic lines are stimulated by mitogens and purified IL-2, without an apparent increase in the expressions levels.

Finally, it is interesting to note that HP1α and HP1β are undetectable in a number of differentiated cells, including cells of the liver parenchyma, sub-sets of neurons, fibroblasts and some epithelial cells. Moreover, downregulation is observed in two classic cellular models (MEL and K562 cells) that have been extensively used to study erythopoiesis in vitro. The paucity of HP1 expression is fully confirmed by examining erythropoietic islands in human bone marrow specimens, substantiating these data in vivo. Our findings are consistent with previously published data showing that the terminally differentiated erythrocytes of non-mammalian vertebrates lack HP1 proteins. However, they do not comply with the observation that mouse embryonic erythrocytes possess rather high levels of HP1α (a phenotype exhibited only by partially differentiated cells, as shown in this study). The downregulation of HP1 proteins in these systems may not be as paradoxical as it seems at a first glance, because, once cells become terminally differentiated, the need for a dynamic molecule, such as HP1, might become redundant. In such cases, chromatin states that will be permanently maintained as cells exit the cell cycle and enter G0 are likely effected by other proteins, such as MENT (Istomina et al., 2003) and specialized forms of linker histones (Gilbert et al., 2003).

Materials and Methods

RT-PCR

Total RNA was isolated from cells and tissues using the Trizol method. The extracted RNAs were used as a template for first strand cDNA synthesis (Invitrogen kit). The cDNA products were amplified using previously described primers (Lessard et al., 1998). Beta-actin amplification was utilized as a control. The primers were designed to produce a 186 bp aplicon of the mouse β-actin mRNA sequence (accession no. NM_007393; forward: 617-636; reverse 842-819). PCR amplification was performed at 94°C for 4 minutes once, then 35 cycles at 94°C for 30 seconds, 52°C for 2 minutes and 72°C for 2 minutes, and one cycle at 72°C for 5 minutes.

'Meta-analysis'

Profiling data from 15 microarray studies were retrieved from Entrez GEO-Profiles (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=geo). Of these, observations from three large-scale studies (Su et al., 2004; Ge et al., 2005; Martens et al., 2006), which had used the GPL96 platform (Affimetrix Gene Chip Human Genome U133, array set HG-U133A) and the same set of primers (HP1α: 212126-at; HP1β: 201518-at; HP1γ: 200037-at), were retrospectively analyzed.

Cell culture

HUT-78, RPMI-8226 and Jurkat cells were cultured in RPMI-1640; MEL and HeLa cells were cultivated in DMEM; and K-562 cells were cultured in RPMI-1640 GLUTAMAXI^{TM} (Invitrogen). Differentiation was induced as previously described (Tsiftsoglou et al., 2003). Briefly, the appropriate inducer was added to the medium when MEL, K-562 and Jurkat cultures were at the exponential phase. All lines were cultivated in the presence of the inducer for 4 days.

Isolation of peripheral blood lymphocytes and proliferation assays

Mononuclear cells were isolated from fresh human blood by Histopaque gradient centrifugation. Briefly, whole blood was layered on Histopaque-1077 and centrifuged for 30 minutes at 400 ***g***. The opaque phase was collected and washed extensively with PBS. Cells were resuspended in RPMI-1640 complete medium and incubated overnight at 37°C in a humidified chamber. The non-adherent cells were collected, washed three times with PBS and cultured with the appropriate mitogen, i.e. phytohemagglutinin (PHA; 5 μg/ml; 3 days), lipopolysaccharide (LPS; 25 μg/ml; 3 days) or interleukin 2 (IL-2; 40 ng/ml; 7 days).

Induction of Wnt signaling in HUT-78 cells

To raise the levels of β-catenin (through inhibition of GSK-3β), the cells were cultured in the presence of 20 mM LiCl for 48 hours [for background see Lucas and Salinas (Lucas and Salinas, 1997)].

Immunohistochemistry

Normal tissue blocks were selected from the archives of the Laboratory of Pathology, University of Ioannina. The blocks were sectioned at a thickness of 4 μm, dried for 16 hours at 56°C, de-waxed and finally rehydrated in a graded ethanol series. Antigenic retrieval was achieved by heat treatment in a microwave oven (12 minutes, 700 W) using a commercial unmasking buffer (DAKO). The samples were incubated with the relevant antibodies and immunodetection was accomplished with the UltraVision LP kit employing DAB substrate. For double labeling, the alkaline phosphatase method was used as indicated (Biogenex).

Microscopy

Cells were collected by mild centrifugation, washed three times with phosphate-buffered saline (PBS) and allowed to adhere to Alcian Blue-treated coverslips. The samples were fixed with 1-4% formaldehyde in PBS, permeabilized with 0.2% Triton X-100 and blocked with 0.5% fish skin gelatin. DNA staining (propidium iodide) and probing with the relevant primary and secondary antibodies (see Table 1) was performed according to the method of Maison et al. (Maison et al., 1993). BrdU labeling was done as specified by manufacturer (Roche). Samples were visualized in a Leica SP confocal microscope.

Morphometric analysis

For morphometric analysis we followed a standard routine. First, individual optical sections (0.3-0.4 μm) were viewed in an enlarged format to examine as closely as possible the location of HP1 foci relative to peripheral, perinucleolar and interstitial heterochromatin. Stacks of such sections were then combined (in a 'projection' mode) and the number of HP1 foci, as well as the fluorescence intensity per unit of nuclear surface, was systematically measured using ImageJ software, allowing a comparison among differently stained specimens. To account for confluence and ploidy differences, these experiments were repeated multiple times (at least 15), using cell aliquots that were thawed, cultured and analyzed independently.

Nuclear extract preparation and western blot analysis

Cells were collected by mild centrifugation and washed three times with PBS containing 2 mM $MgCl_2$ and 1 mM PMSF. The pellet was resuspended in isotonic buffer (150 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and supplemented with other protease inhibitors). After centrifugation for 10 minutes, the resulting pellet was vortexed and resuspended in an 8 M urea buffer containing 20 mM Tris-HCl pH 8, 10 mM EDTA, 10 mM EGTA, 1 mM DTT and 1 mM PMSF and then sonicated for 20 seconds. The preparation was ultra-centrifuged (40 min, 18°C) and the supernatant collected. For immunoblotting, whole cell lysates or nuclear extracts were analyzed in 13.5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and processed for electrochemiluminescence (ECL).

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