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Cytoplasmic and nuclear interaction between Rb family proteins and PAI-2: a physiological crosstalk in human corneal and conjunctival epithelial cells

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Abstract

Extracellular plasminogen activator inhibitor type-2 (PAI-2) is a potent inhibitor of urokinase-type plasminogen activator (u-PA) and also acts as a multifunctional protein. However, the biological activity of intracellular PAI-2, as well as its intracellular targets, until now remain an enigma. Here, we show that pRb2/p130 and Rb1/p105, but not p107, interact with PAI-2 in both the cytoplasm and nucleus of normal primary human corneal and conjunctival epithelial cells. We provided the first *in vivo* evidence that a specific fragment of the *PAI-2* promoter is bound simultaneously by pRb2/ p130, PAI-2, E2F5, histone deacetylase 1 (HDAC1), DNA methyltransferase 1 (DNMT1), and histone methyltransferase (SUV39H1), in normal primary human corneal epithelial cells, and by pRb2/p130, PAI-2, E2F5, HDAC1, and DNMT1, in normal primary human conjunctiva epithelial cells. Our results strongly indicate a physiological interaction between pRb family members and PAI-2, suggesting the hypothesis that pRb2/p130 and PAI-2 may cooperate in modulating *PAI-2* gene expression by chromatin remodeling, in normal corneal and conjunctival cells.
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Abbreviations: PAI-2, plasminogen activator inhibitor type-2; u-PA, urokinase-type plasminogen activator; pRb1/105, pRb2/130 and p107, Retinoblastoma family proteins; E2Fs, transcription factor family; DNMT1, DNA methyltransferase 1; HDAC1, histone deacetylase 1; SUV39H1, histone methyltransferase; p300, histone acetyltransferase

Introduction

The plasminogen activator inhibitor type-2 (PAI-2) is a member of the ovalbumin subgroup of serpins (ov-serpins), originally characterized in human placenta and macrophages,[1,2] and now known to be synthesized by a variety of cells, including tumors, after appropriate stimulation.[3–5] Extracellular PAI-2 is a potent inhibitor of urokinase-type plasminogen activator (u-PA) and, although the intracellular targets and the molecular mechanism of PAI-2 remain undefined, different studies have indicated that PAI-2 acts as a multifunctional protein, since, it is involved in the regulation of fibrinolysis, in the regulation of keratinocytes development, in proliferation, in the invasion and metastasis of cancer cells, and in conferring resistance to apoptosis.[6–8] It has been reported that PAI-2 is constitutively expressed in human stratified squamous epithelia and suggested that this inhibitor could be involved in the differentiation or in the antimicrobial defense mechanisms of at least some epithelia.[5] For instance, it has been reported that PAI-2 could be a product of differentiating corneal keratinocytes, since in human corneal epithelium PAI-2 is present exclusively in the most superficial cell layers (i.e. most highly differentiated) and also it appeared to be primarily cytoplasmic rather than secreted.[5,9] On the contrary, it has been reported that in conjunctival epithelium, a tissue adjacent to the cornea with distinct functions, PAI-2 expression was present both in the superficial cells and in lower (including basal) epithelial cell layers.[10] The significance of the different expression pattern of PAI-2 in corneal and conjunctival epithelia is not yet clear, but may suggest a peculiar role of this inhibitor in regulating cell proliferation and cell turnover in the cornea and conjunctiva.

The biological activity of intracellular PAI-2, as well as its intracellular targets, until now remain an enigma. However, it has been recently reported that in Hela and in Jurkat cells PAI-2 expression could result in post-transcriptional recovery of the retinoblastoma protein Rb, and that PAI-2 could inhibit Rb degradation, suggesting an intriguing intranuclear role of PAI-2.[11] Under physiological conditions, the expression of PAI-2 gene is maintained at low to undetectable levels in several cells.[3] However, it has been reported that a wide variety of factors, such as lipopolysaccharide, phorbol myristate acetate, tumor necrosis factor-α, interleukin-1, can modulate PAI-2 expression in different cell types.[3,12] Moreover, a recent

study has suggested that different redox-dependent conformations of PAI-2 may have different targets and or functions.[13] The regulation of *PAI-2* transcription has been found to play an important role in controlling PAI-2 expression and both negative and positive transcription regulatory regions have been identified in the *PAI-2* promoter.[14–16] However, until now the molecular mechanisms controlling the expression levels of PAI-2 remain unknown.

The human *Rb/p105 (RB1)* gene, together with *Rb2/p130 (RB2)* and *p107*, form the retinoblastoma gene family. Each of the Rb family members regulate G_1 progression, are implicated in various forms of differentiation, are regulated in a cell cycle-dependent manner by phosphorylation and exhibit growth suppressive properties in a cell type-dependent manner.[17] Several mechanisms have been proposed to account for transcriptional repression by the Rb proteins.[18] Some of the proposed models stress the importance of chromatin structure in regulating transcriptional activity. Active repression by pRb family members could involve a mechanism by which condensed chromatin structure is enhanced through histone deacetylation and methylation.[19] Recently, we have suggested a functional interaction between pRb2/p130 and chromatin modifying enzymes to control the transcription of *p73* and *ER-*α genes.[20,21]

Here, we show that pRb2/p130 and Rb1/p105, but not p107, interact with PAI-2 in both the cytoplasm and nucleus of normal primary human corneal and conjunctival epithelial cell cultures. Moreover, we reported that, in the same cell lines, pRb2/p130, E2F5, PAI-2 and the chromatin modifying enzymes DNA methyltransferase 1 (DNMT1), histone deacetylase 1 (HDAC1) and histone methyltransferase (SUV39H1) simultaneously bind, *in vivo,* the same region on the *PAI-2* proximal promoter. We also discussed the physiological significance of the pRb family members and PAI-2 interaction and suggested a model of how pRb2/p130 and PAI-2 may cooperate in modulating *PAI-2* gene expression by chromatin remodeling, in normal corneal and conjunctival epithelial cells.

Results and Discussion

Anti-PAI-2 antibody coimmunoprecipitates pRb2/p130 and Rb1/p105, but not p107, in the cytoplasm and nucleus of normal primary human corneal and conjunctival epithelial cell cultures.

Our data reveal for the first time, a cytoplasmic and nuclear interaction between PAI-2 and specific members of pRb family proteins, pRb2/p130 and Rb1/p105, in normal human corneal and conjunctival epithelial cell cultures. In these cells, most of PAI-2 is found to be primarily intracellular rather than secreted, however, the expression pattern of this serpin is different in these epithelia. In the corneal epithelium, PAI-2 is present exclusively in the highly differentiated superficial cell layers, while in the conjunctival epithelium PAI-2 is present not only in the more superficial differentiated cells, but also in the basal, less differentiated, yet more proliferative, epithelial cell layers.[5,10]

This difference in PAI-2 localization may reflect a peculiar role of this inhibitor in regulating cell turnover, and cell proliferation and differentiation, in cornea and conjunctiva epithelia. In this context, it is possible to hypothesize that the distribution of intracellular PAI-2 is controlled through the interaction with specific protein targets involved in the signaling pathways regulating the status of cell proliferation and differentiation. We have previously indicated that pRb2/p130, in association with the transcription factor E2F-4, is the most abundant E2F complex found in differentiated or quiescent cells in G_0.[22–24] High pRb2/p130 expression detected in G_0 *versus* proliferating cells could be due to an increase of protein stability perhaps by the inhibition of the ubiquitination pathway through the interaction with specific intracellular target.

Here, we investigated if pRb2/p130, Rb1/p105, and p107 are targets of intracellular PAI-2. In order to assess whether PAI-2 associates with the retinoblastoma family proteins, PAI-2 was immunoprecipitated from nuclear and cytoplasmic lysates of 12 paired couples of normal primary human corneal and conjunctival epithelial cell cultures. The immunoprecipitates were then analyzed by Western blotting using anti-pRb2/p130, anti-Rb1/p105, anti-p107, and anti-PAI-2 antibodies. We found, for all the cell cultures analyzed, that anti-PAI-2 antibody coimmunoprecitates pRb2/p130 and Rb1/p105 from both nuclear and cytoplasmic fractions. On the contrary, no binding was detected between PAI-2 and p107 (Figure 1a). Interestingly, we observed that most of the pRb2/p130 immunoprecipitated by anti-PAI-2 antibody, from both cornea and conjunctiva cytoplasmic fractions, exhibits a hyperphosphorylated form (Figure 1a, upper band), while in the correspondent nuclear fractions, most of pRb2/p130 exhibits an hypophophorylated form (Figure 1a, lower band). Moreover, as expected, the anti-PAI-2 antibody immunoprecipitated PAI-2 from both nuclear and cytoplasmic fractions of all the cell cultures (Figure 1a). The results were confirmed by performing immunoprecipitations from cytoplasmic and nuclear fractions of normal primary human corneal and conjunctival epithelial cell cultures, and using anti-pRb2/130, anti-Rb1/105 or anti-p107 as immunoprecipitating antibodies. The immunoprecipitates were then analyzed by Western blotting using anti-PAI-2 antibody (Figure 1b).

Western blotting analyses using total lysate displayed that both cornea and conjunctiva cells exhibit similar levels of pRb2/p130, Rb1/p105, p107 and PAI-2 proteins (Figure 1c). The purity of the nuclear and cytoplasmic fractions was validated by using anti-GAPDH and anti-Oct1 antibodies, as cytoplasmic and nuclear markers, respectively (Figure 1d). Moreover, we performed further experiments in order to detect any cross contamination between cytoplasmic and nuclear fractions, by using different and specific cytoplasmic (β-tubulin, α-tubulin and HSP90) and nuclear marker (PCNA, nucleolin and Sp-1) (data not shown).

These data reveal for the first time, a cytoplasmic and nuclear interaction between PAI-2 and specific members of pRb family proteins, pRb2/p130 and Rb1/p105, in normal human corneal and conjunctival epithelial cell cultures.

Previously, Darnell *et al.*,[11] have reported that anti-PAI-2 antibody coimmunoprecipitates Rb1/p105 exclusively from the nuclear fraction of Hela S1a, KJD and activated U937

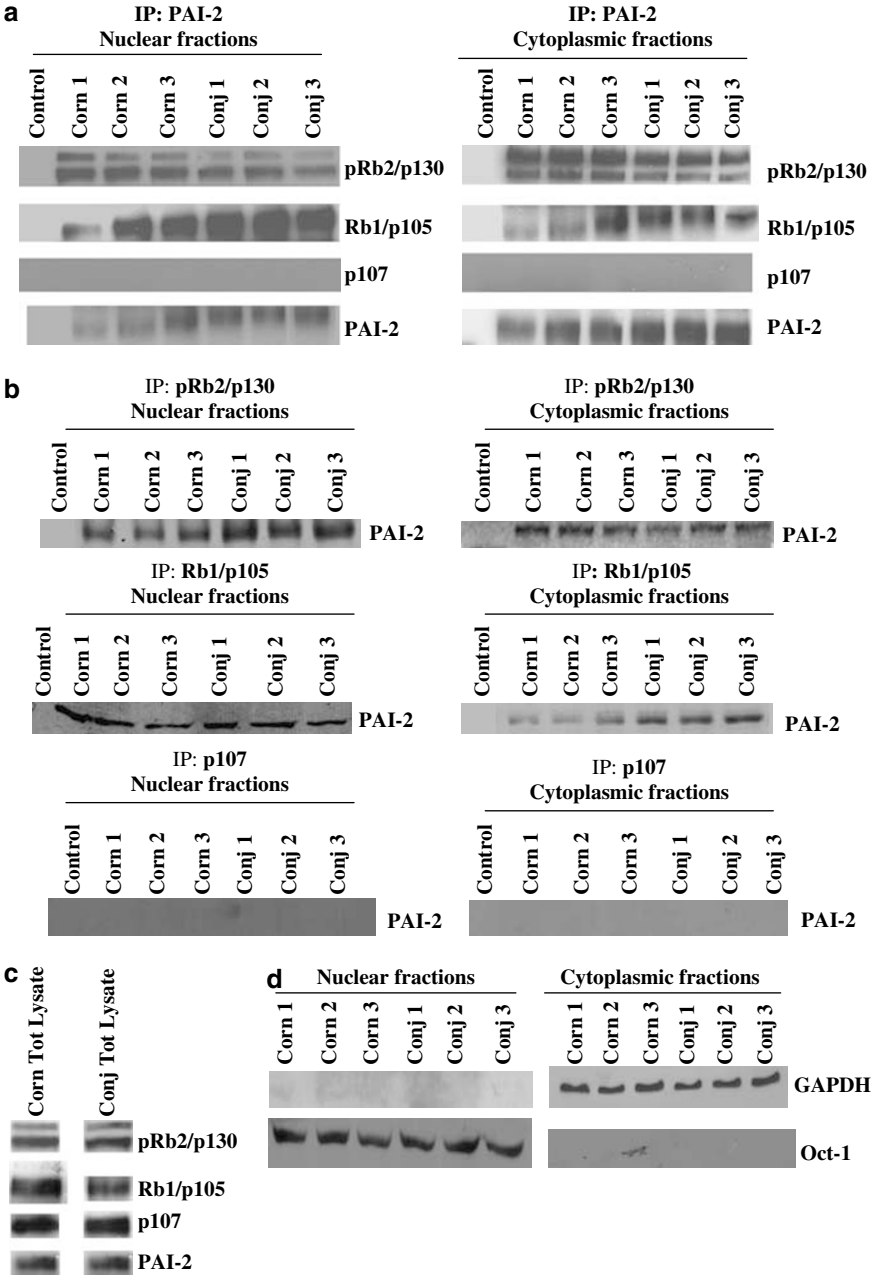

Figure 1 PAI-2 coimmunoprecipitates with pRb2/p130 and Rb1/p105. (**a**) Immunoprecipitation of PAI-2 from nuclear and cytoplasmic fractions from 3 representative cases of paired primary cornea (Corn) and conjunctiva (Conj) (Corn1 and Conj1: donor 1; Corn2 and Conj2: donor 2; Corn3 and Conj3: donor 3) cells by using an anti-PAI-2 antibody, followed by electrophoresis and Western blotting of the immunoprecipitates with anti-pRb2/p130, anti-Rb1/p105, anti-p107 and anti-PAI-2 antibodies. Control (lane 1) represents Western blotting of nuclear or cytoplasmic immunoprecipitates where the anti-PAI-2 antibody was omitted. (**b**) Immunoprecipitations from nuclear and cytoplasmic fractions of primary cornea (Corn) and conjunctiva (Conj) cells by using anti-pRb2/p130, anti-Rb1/p105 or anti-p107 as immunoprecipitating antibodies, followed by Western blotting with anti-PAI-2 antibody. (**c**) Western blotting of equal amounts of total lysates from cornea (Corn Tot Lysate) and conjunctiva (Conj Tot Lysate) cells, with anti-pRb2/p130, anti-Rb1/p105, anti-p107 and anti-PAI-2 antibodies. (**d**) To assess the purity of the nuclear and cytoplasmic fractions, immunoblot analysis was done with anti-GAPDH, as cytoplasmic marker, and anti-Oct1, as nuclear marker

tumoral cells, and also showed that PAI-2 *in vitro* interact with Rb1/p105 and less efficiently with pRb2/p130. Our data, indicating that the anti-PAI-2 antibody coimmunoprecipitates pRb2/p130 and Rb1/p105 with the same efficiency and from both nuclear and cytoplasmic fractions of normal corneal and conjunctival epithelial cell cultures, are apparently in contrast with the results reported from Darnell *et al*.[11] Instead, we strongly believe that these data are not in contrast since they disclose a novel interaction between a cytoplasmic protein, PAI-2, and a family of nuclear proteins, pRb2/p130 and Rb1/p105 in two different complex systems: tumoral and a normal cellular system. It important to point out that in a tumoral

environment several mechanisms are affected and do not work properly. In normal cells, under physiological conditions the interaction between PAI-2 and specific members of pRb family can be required both in the cytoplasm and in the nucleus, while in tumoral cells, an altered environment can limit this interaction to only in the nucleus. In our opinion integrating all these data will be important to gain knowledge about the intracellular functions of PAI-2 as well as to disclose the mechanisms regulating its cellular distribution, both in normal and in cancer cells.

As previously demonstrated, the retinoblastoma family members are nuclear proteins regulated in a cell cycle-dependent manner by phosphorylation and implicated in various forms of differentiation, in the regulation of specific cell cycle genes and in apoptosis.[25,26] Moreover, it has been suggested that this family of proteins exhibit growth suppressive properties in a cell type-dependent manner and could modulate the transcription of certain genes by chromatin remodeling.[27] Our hypothesis is that in the corneal and conjunctival epithelia, the distribution cytoplasm/nucleus of PAI-2 can be controlled by the interaction with pRb2/p130 and Rb1/p105 through multiple pathways. For instance, under specific 'stimuli', and/or at specific times of cell cycle, pRb2/p130 and Rb1/p105 could shuttle PAI-2 between cytoplasm and nucleus, thus controlling the concentration of PAI-2 in these cellular compartments. In addition, PAI-2 may preserve pRb2/p130 and Rb1/p105 from a rapid degradation. In fact, it has been described that PAI-2 expression can inhibit Rb1/p105 degradation in PAI-2 expressing-Jurkat cells with respect to control Jurkat cells.[11]

pRb2/p130, E2F5, HDAC1, DNMT1, SUV39H1 and PAI-2 bind to the PAI-2 proximal promoter region *in vivo*

The expression level of PAI-2 could be mainly controlled by repressor and/or activator mechanisms modulating the basal transcription. It has been described that 5′-flanking region of *PAI-2* contains negative and positive transcription regulatory regions, which could be controlled in a cell type specific manner.[14] For instance, this regulation could occur through the interaction of specific DNA binding factors with the regulatory regions. Here, we explored the in *vivo* promoter occupancy of *PAI-2* in normal primary human corneal and conjunctival epithelial cell cultures. Our investigation was focused in a specific *PAI-2* promoter fragment, between the residues −2062 and −1643, which defines a negative regulatory element that represses *PAI-2* promoter activity in a cell type independent manner,[15] and contains possible putative E2F binding sites (Figure 2a). This information and the results indicating that anti-PAI-2 antibody coimmunoprecitated pRb2/p130 and Rb1/p105 from both nuclear and cytoplasmic fractions of normal corneal and conjunctival epithelial cells (Figure 1a), suggested the intriguing hypothesis that the pRb family proteins may be recruited on the *PAI-2* promoter via E2F factors binding and that this interaction could have a physiological significance in controlling *PAI-2* transcription, perhaps by chromatin remodeling. In attempt to verify our hypothesis, we performed crosslink chromatin immunoprecipitation (XChIP) experiments using anti-pRb2/p130, anti-pRb1/p105, anti-E2F4, anti-E2F5, anti-E2F1, anti-HDAC1, anti-SUV39H1, anti-p300, anti-DNMT1 or

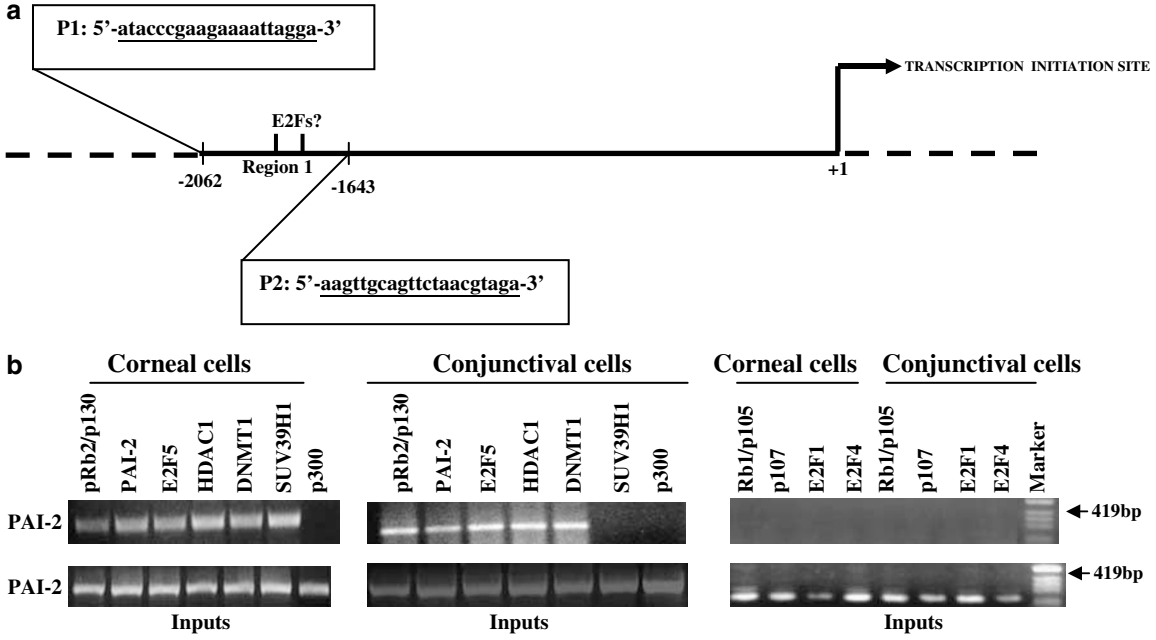

Figure 2 pRb2/p130, PAI-2, E2F5, HDAC1, DNMT1 and SUV39H1 bind to *PAI-2* promoter *in vivo*. (**a**) Schematic representation of region 1 on the *PAI-2* promoter recognized by P1/P2 primers (GenBank accession no. M22469). (**b**) Representative results from XChIP analyses in human primary corneal and conjunctival cells. Formaldehyde crosslinked chromatin was immunoprecipitated using the antibodies indicated on the top of each panel. The presence of PAI-2 promoter region in the immunoprecipitates was tested by PCR using specific primers (P1/P2) spanning the region 1 of *PAI-2* promoter. 1% of total chromatin (inputs) was used as a positive control in PCR reactions

anti- PAI-2 as immunoprecipitating antibodies. As shown in Figure 2b, the XChIPs experiments clearly indicate that pRb2/p130, E2F5, HDAC1, DNMT1, and PAI-2 *in vivo* bind to, simultaneously, a specific fragment of *PAI-2* promoter, in both normal primary human corneal and conjunctival epithelial cell. Moreover, only in the cornea cells, does SUV39H1 bind to the same *PAI-2* promoter fragment, while in both cornea and conjunctiva cells we were not able to detect pRb1/p105, p107, E2F4, E2F1 and p300.

Conclusions

Rb family and PAI-2 interaction: a physiological crosstalk?

The results presented in this study provide the first *in vivo* evidence that a specific fragment of *PAI-2* promoter is bound simultaneously by pRb2/ p130, PAI-2, E2F5, HDAC1, DNMT1 and SUV39H1, in normal primary human corneal epithelial cells, and by pRb2/ p130, PAI-2, E2F5, HDAC1, and DNMT1, in normal primary human conjunctival epithelial cells (Figure 2). An explanation of these protein/DNA interactions could be that pRb2/p130, PAI-2 and the chromatin modified enzymes may form a multiprotein complex, which by altering the local chromatin structure can create an environment that affect the binding and /or the function of specific DNA binding factors, which modulate the levels of *PAI-2* basal transcription. Moreover, the specificity of the recruited enzymes may be a key element in determining a specific pattern of local chromatin remodeling that may dictate different 'transcription modulation environments', closely correlated to *PAI-2* expression in cornea and conjunctiva cells. In our context, SUV39H1 may be one of these key enzymes, since we found that only in the cornea cells SUV39H1 was able to immunoprecipitate the *PAI-2* promoter fragment analyzed. Moreover, the presence of SUV39H1 binding only in the *PAI-2* promoter fragment immunoprecipitated from cornea cells, as well as the absence of p300, E2F4 and E2F1 binding in both cornea and conjunctiva cells, may stem from unrelated differences between these two cell types. For example, the expression levels of p300, SUV39H1, E2F4 and E2F1 may differ between normal corneal and conjunctival cells. It is also possible that p300, SUV39H1, E2F4 and E2F1 are sequestered by other proteins and are unavailable to bind the particular multiprotein complex that we found to be associated with *PAI-2* promoter in corneal or conjunctival cells. A third possibility is that the aforementioned complexes contain additional components that are not probed for here and that may influence the recruitment of p300, SUV39H1, E2F4 and E2F1. Actually, we are considering these and other possibility. For instance, we have data showing that the protein expression levels of p300, SUV39H1, E2F4 and E2F1 are similar in the normal primary human corneal and conjunctival cell cultures investigated as well as the protein expression levels of pRb family members (data not shown). In addition, we are performing functional studies in order to test our hypothesis that the binding of pRb2/p130, PAI-2, E2F5, HDAC1, SUV39H1 and DNMT1 on the *PAI-2* promoter may be directly correlated with a specific transcriptional environment. Here, we reported the results from multiplex semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) showing that the level of PAI-2 mRNA is higher in conjunctival cells than in corneal cells (Figure 3), suggesting that these cell cultures exhibit a specific *PAI-2* gene expression pattern.

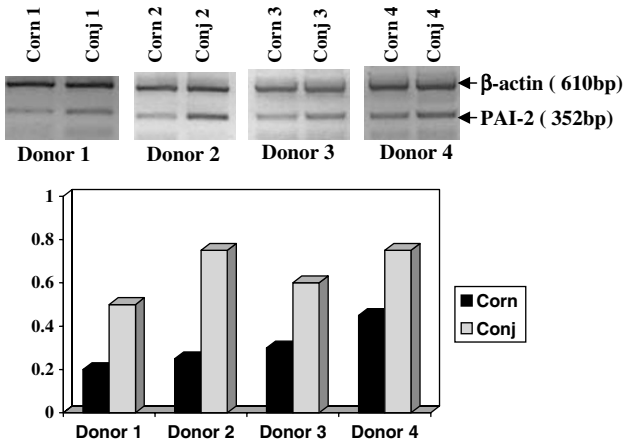

Figure 3 Steady-state of PAI-2 mRNA levels in four representative cases of paired primary cornea (Corn) and conjunctiva (Conj) normal cells. (**a**) Multiplex RT-PCR was performed using total cellular RNA from Corn and Conj cells. Each RT-PCR reaction contained 1/100 of cDNA. 0.3 : 2.0 was the primer ratio for β-actin and PAI-2 used to amplify both products logarithmically and in relatively similar amounts. The upper band and lower band in each line represent PCR products of β-actin and PAI-2- gene, respectively. (**b**) The relative PAI-2 expression levels in Corn and Conj are presented graphically. The values were calculated as the density of the product of PAI-2 gene divided by that of the β-actin from the same cDNA. The panels in the upper of figure, showing the results from the multiplex RT-PCR, are representative of four separate experiments

Taken together, our data suggest the intriguing hypothesis that in normal human corneal and conjunctival epithelial cells, the binding of pRb2/p130-PAI-2 complexes on a specific region of *PAI-2* promoter may modulate the *PAI-2* basal transcription by inducing local changes in chromatin structure, maybe by altering the activity of transcription regulators bound nearby (Figure 4). Under specific 'stimuli', and/or at specific times of cell cycle, the interaction of PAI-2 with pRb2/p130 and Rb1/p105 could permit the shuttle of PAI-2 between cytoplasm and nucleus, then controlling the concentration of PAI-2 in these cellular compartments. Then, the transcription of *PAI-2* gene may be controlled by a feedback trigger loop from a specific PAI-2 concentration in the nucleus, which governs the binding of specific pRb2/p130-PAI-2-chromatin modifying enzymes complexes on the *PAI-2* promoter. Finally, the interaction with E2F5 could be the primary mechanism by which pRb2/p130 is recruited to the chromatin regardless of the cell cycle stages.

Materials and Methods

Tissue procurement and cell culture

A total of 12 couples of paired normal human cornea and conjunctiva biopsies were obtained from the Delaware Valley Lions Eye Bank, from patients undergoing routine cataract surgery after informed consent according to the statutes of the Institutional Review Board of the University

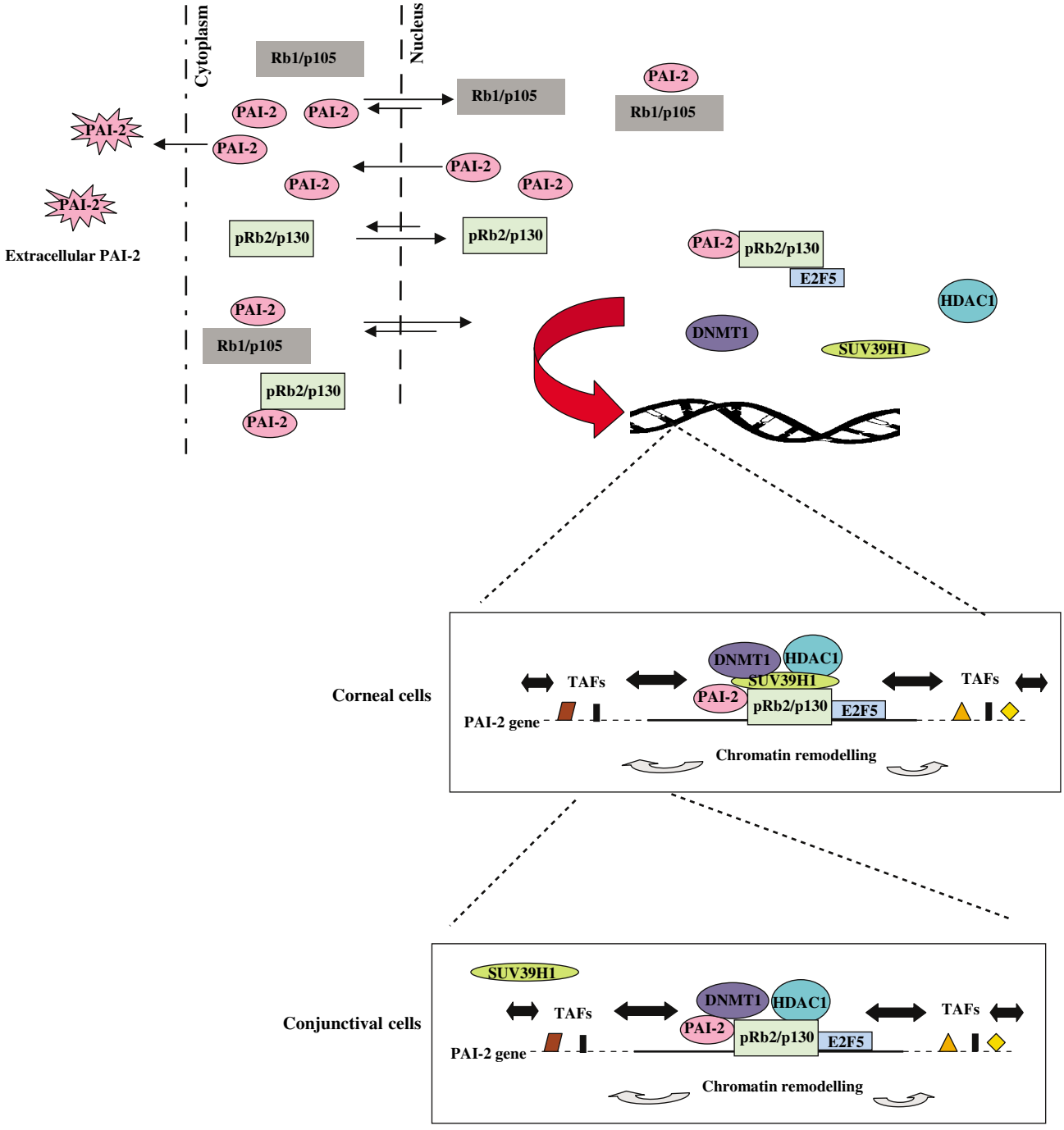

Figure 4 Rb family and PAI-2 interaction: a physiological crosstalk? In normal human corneal and conjunctival cells, the interaction of PAI-2 with pRb2/p130 and Rb1/p105 could permit the shuttle of PAI-2 between the cytoplasm and the nucleus, thus controlling the concentration of PAI-2 in these cellular compartments. The binding of pRb2/p130-PAI-2-chromatin modified enzymes complexes on a specific region of the *PAI-2* promoter may modulate the *PAI-2* basal transcription by inducing local changes in chromatin structure and altering the activity of transcription regulators (e.g., TAFs) bound nearby. Moreover, the specificity of the recruited enzymes may be a key element in determining a specific pattern of local chromatin remodeling that may dictate different 'transcription modulation environments', closely correlated to *PAI-2* expression in corneal and conjunctival cells. (See further explanation in the text)

of Pennsylvania. Primary cornea and conjunctiva cell cultures were initiated from the biopsies as previously described by Williams *et al.*,[5] and Risse-Marsh *et al.*[6] Cells between the first and sixth passages were used for the experiments.

Multiplex RT-PCR

Total RNA was extracted from paired normal human primary cornea and conjunctiva cells by using the RNeasy kit (Qiagen). Before further use, the

total RNA was treated with DNase I, amplification grade (1 U DNase/1 mg total RNA; Life Technologies). Reverse transcription-polymerase chain reaction (RT-PCR) was performed by using the Reverse Transcription System (Promega). Multiplex RT-PCR was carried out using 1/100 of cDNA and the following primers for each reaction: PAI-2: 5′-tgacaaactcaacaagtgga-3′ (forward), 5′- tgcataagataaccaactgc-3′ (reverse); β-actin: 5′-tgacgggctcacccacactgtgccca-3′ (forward), 5′-ctagaag catttgcggtggacgatgg-3′ (reverse). 0.3 : 2.0 was the primer ratio for β-actin and PAI-2, respectively. The amplified fragments were detected by 1.5% (w/w) agarose gel electrophoresis. Each band was quantified and the specific gene expression level was determined semi-quantitatively by calculating the ratio of densitometric value from the PAI-2 band in relation to the internal standard represented by β-actin.

Western blot and chromatin immunoprecipitation assay

Cytoplasmic and nuclear proteins were extracted from 12 couples of paired normal human corneal and conjunctival epithelial cells by using the PARIS kit (Ambion). Efficient cytoplasmic and nuclear fractionation was confirmed by Western blotting analysis using anti-GAPDH antibody for cytoplasmic fraction and anti-Oct-1 antibody for nuclear fraction. Immunoprecipitations experiments were performed from cytoplasmic and nuclear fractions and using PAI-2 as immunoprecipitating antibody (N-18, Santa Cruz Biotechnology, CA). The presence of pRb2/p130, Rb1/p105 and p107 in both PAI-2 nuclear and cytoplasmic precipitates was assessed with anti-pRb2/p130, anti-Rb1/p105 and p107 antibodies (211.6, C-15 and C-18, respectively, Santa Cruz Biotechnology, CA) by Western blotting.

XChIP

XChIPs were performed as previously described by Macaluso *et al*.[21] Cornea and conjunctiva cells were crosslinked by adding formaldehyde (1% final concentration) directly to culture medium, and incubated at 37°C. Immunoprecipitations were carried out using 3–4 μg of antibodies against pRb2/p130, Rb1/p105, p107, E2F1, E2F4, E2F5, DNMT1, p300, PAI-2 (Santa Cruz Biotechnology), HDAC1 or SUV39H1 (Upstate Biotechnology). As negative controls, no-antibody immunoprecipitations and immunoprecipitations with an irrelevant antibody were performed. The crosslink was reversed by incubating samples at 65°C overnight, and DNA extracted with phenol : chloroform and ethanol precipitation. Primers spanning a specific region of *PAI-2* promoter were used in PCR reactions (P1: 5′-atacccgaagaaaattagga-3′, P2: 5′-aagttgcagttctaacgtaga-3′; GenBank accession no. M22469). Total chromatin (1%) (Input) was used as positive control. As a negative control, we performed PCR amplification of XChIP samples with primers annealing to the *β-actin* promoter because the transcription of *β-actin* gene is not thought to be under the control of either the E2F or pRb family.

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