Interaction between Sp1 and Cell Cycle Regulatory Proteins Is Important in Transactivation of a Differentiation-related Gene

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Abstract

The stratified squamous epithelium is a model system in which to define molecular mechanisms underlying the switch from proliferation to differentiation. This can be achieved through the functional dissection of keratin gene promoters. Having previously established the importance of keratin 4 in maintaining the differentiated phenotype in corneal epithelial cells, we investigated the role of Sp1-mediated transactivation of the keratin 4 promoter given the role of Sp1 in differentiation and cell cycle progression. Sp1 transactivation of the keratin 4 promoter was diminished in cyclin D1-overexpressing cells, which may be mediated through a newly described direct interaction between Sp1 and cyclin D1 and opposed by a complex between Sp1 and pRB.

Introduction

The stratified squamous epithelium comprises proliferating basal cells, differentiating suprabasal and intermediate cells, as well as terminally differentiated superficial squamous cells that line the surface. Ultimately, these superficial cells desquamate, and the process then is continuously renewed. The stratified squamous epithelium is found in many sites in human tissues, including the cornea, esophageal, oropharynx, larynx, skin, and anogenital tract.

Basal cells are subjected to a tightly regulated program of differentiation as they migrate toward the surface accompanied by a series of morphological, biochemical, and genetic changes. Insights into the biochemical and molecular genetic mechanisms that orchestrate the switch from proliferation to early differentiation in this cell type can be achieved through an understanding of the K genes that modulate this process and their transcriptional regulation. In this context, K5 and K14 heterodimerize in proliferating basal cells. The relatively ubiquitous K1 and K10 are linked to early differentiation genes and heterodimerize in suprabasal cells, whereas loricrin, profilaggrin, and transglutaminase are among the late differentiation genes in superficial squamous cells (1, 2). Of significance, the suprabasal K4 and K13 are relatively tissue restricted, with highest expression in esophageal and corneal squamous epithelial cells (3).

We previously generated a model in which K4 is disrupted through homologous recombination in murine embryonic stem cells, resulting in impaired differentiation and basal cell hyperplasia, specifically in esophageal and corneal epithelia of homozygous null mice (4). This formed the basis for studying transcriptional regulatory mechanisms of the human K4 promoter in esophageal and corneal squamous epithelial cells. Previously, we have demonstrated that, in esophageal squamous epithelial cells, the human K4 promoter is transcriptionally regulated by esophageal-specific transcription factors (5). To further elucidate how the regulation of K4 is linked to differentiation, we undertook analysis of its 5′ untranslated regulatory region and promoter in corneal epithelial cells. Given that one of the roles of Sp1 is to modulate cellular differentiation and influence cell cycle progression in cooperation with the retinoblastoma protein (pRB), we focused on putative Sp1 DNA binding motifs in the K4 promoter and the role of Sp1 in regulating the K4 promoter. This was further investigated by comparing Sp1-mediated transactivation of the K4 promoter in parental normal corneal epithelial cells and stably transduced corneal epithelial cells with the cell cycle regulator, cyclin D1, the latter achieved through retroviral transduction. We describe herein that Sp1 transactivation of the K4 promoter in corneal epithelial cells is suppressed by cyclin D1, and that this can be rescued by concurrent ectopic expression of pRB. In particular, we demonstrate that cyclin D1 interacts with Sp1 in vivo, suggesting perhaps a model in which Sp1 may influence differentiation through interactions with either pRB or cyclin D1.

Materials and Methods

Cell Culture and Retroviral Infection. A rabbit normal corneal cell line SIRC (American Type Culture Collection, Rockville, MD) and the cyclin D1-overexpressing SIRC cell line, designated C3D1, were grown in Eagle's minimal essential medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and antibiotics. Additionally, the human esophageal squamous cancer cell line TE-12, the human osteosarcoma cell line U2OS, the human bladder cancer cell line J82, and the lymphoma B-cell line DT40 were grown under standard conditions.

The amphotrophic packaging cell line Phoenix A was grown in supplemented DMEM (Sigma) and transiently transfected with the respective retroviral vector to generate amphotrophic retroviruses. Fresh retroviral supernatant was used for infection of exponentially growing SIRC cells. Several clones were harvested for further processing after puromycin selection. The retroviral expression vectors pBPSTR-D1 and pBabe-lacZ were obtained from S. Reeves (Massachusetts General Hospital, Charlestown, MA). The retroviral vector pBPSTR-D1 is described previously (6) and contains both elements of the tetracycline-regulated system. The retroviral expression vector pBPSTR is based on the vector pBabe, which accounts for comparable infectivity. The puromycin resistance gene under the control of the promoter within the 5′ long terminal repeat is present in both retroviral vectors.

Transient Transfection. Transient transfection of different K4 promoter deletion-luciferase reporter constructs (K4-940, K4-540, K4-163, K4-140, and K4-76; Ref. 5) and cotransfections of expression plasmids pRC/RSV-empty, pRC/CMV-Sp1, pSG5-RB (wild-type pRB), pJ3 RB-592 (mutant pRB), and pJ3 RB-209 (mutant pRB) were carried out using the calcium phosphate precipitation technique (5′→3′, Inc.), as described previously (5, 7, 8). Incubations were performed in triplicate, and results were calculated as the mean ± SE values for luciferase activity. Values were then expressed as fold increase or decrease compared with the control for each set of experiments. Activities were expressed as the mean of at least three independent transfection experiments. Transfection efficiency was controlled by cotransfection with pGreen Lantern-1 (Life Technologies, Inc.) and found not to vary within a
given transfection experiment, indicating that transfection efficiency was unifor-

**Flow Cytometry.** Exponentially growing cells were collected, and the cell pellet fixed overnight in 70% ethanol and then resuspended in a 1-ml solution containing 3.8 mM sodium citrate and 10 μg/ml propidium iodide. After 10 mg/ml RNase treatment at 37°C for 20 min, the samples were analyzed by a fluorescence-activated cell sorter (FACScan; Becton Dickinson).

**Coimmunoprecipitation and Western Blot Analysis.** Lysates from exponentially growing cells were harvested in a buffer [50 mM HEPES (pH 7.4), 0.1% NP40, and 250 mM NaCl] with 1 mM protease and 10 mM phosphatase inhibitors. Total protein (150 μg) was incubated with 1 μg of primary antibody at 4°C overnight, followed by incubation with Protein A/Protein G Plus Agarose (Santa Cruz Biotechnology) for 2 h. Proteins were separated on 6–12% SDS-polyacrylamide gels and transferred to Immobilon membranes (Millipore). Incubation with primary antibodies was performed as indicated (1:3000). The secondary antibody was either peroxidase-conjugated antimouse or antirabbit immunoglobulin (Amersham Corp.; 1:2500). Detection was by chemiluminescence (ECL; Amersham Corp.).

**EMSAs.** Nuclear extracts from SIRC and C3D1 cell lines were prepared, and EMSAs were performed as described previously (5, 7, 8) using probes Sp1-A (5′-AGCTT AACGG GTCCG GG AAG GATGG CTTGC-3′), Sp1-C (5′-AGCTT AGGCT AAGGC TTGAC-3′), and Sp1-consensus (5′-AGCTT ATTCG ATTCG GG CCGG GCCGA GCCC-3′). For competition experiments, the nuclear extract was preincubated with 50-fold excess of unlabelled double-stranded Sp1 consensus oligonucleotide prior to the addition of the α32P-labeled oligonucleotide probe. AP1 consensus oligonucleotide was used as a control. Immune supershift assays were performed using a polyclonal anti-Sp1 (PEP2) or anti-AP1 antibody (D; Santa Cruz Biotechnology). The antibody was preincubated with the nuclear extract at room temperature for 30 min prior to the addition of the α32P-labeled oligonucleotide DNA probe.

**IP-EMSAs.** IPs were done as described. Protein-agarose complexes were then washed three times in gelshift buffer (7, 8) treated with 32 μl of 0.8% deoxycholate to dissociate protein complexes on ice and neutralized with 1% NP40. Ten μl of the supernatant were then used for EMSAs as described above.

**Results**

**Ectopic Cyclin D1 Overexpression in Normal Corneal Epithelial Cells Causes Cell Cycle Abnormalities.** We used a tetracycline-regulated retroviral vector system, pBPSTR-D1 (6), containing the cyclin D1 cDNA, to infect the normal corneal squamous epithelial cell line termed SIRC. Parallel experiments used a lacZ gene containing retrovirus (pBabe-lacZ) to determine infection efficiency. Infection efficiencies of retroviral supernatants of the two vectors are comparable because pBPSTR is a derivative of pBabe. Infected SIRC cells were puromycin selected, and the clone used for further studies was designated C3D1, although several clones were expanded (e.g., B4D1) without any differences observed in cyclin D1 overexpression and cell cycle kinetics. The levels of endogenous and ectopically expressed cyclin D1 in the parental SIRC and cyclin D1-transduced cell lines were determined using Western blot analysis (Fig. 1A). Ectopic cyclin D1 was induced in asynchronously growing cells in the absence of tetracycline. Of note, the levels of cyclin D1 in the presence of tetracycline were essentially the same observed in the parental cell line (data not shown). Flow cytometry analysis revealed pronounced cell cycle redistribution in C3D1 cells with a 20% increase in cells cycling in S-phase compared with parental SIRC cells (Fig. 1B). Furthermore, C3D1 cells demonstrated an altered morphology and grew in clusters with smaller size and rounded appearance compared with the pleomorphic parental SIRC cells (data not shown).

**Sp1 Transactivation in Corneal Epithelial Cells Is Cell Cycle Dependent.** We next assessed transcriptional regulation of the differentiation-linked human K4 promoter in corneal epithelial cells. Having shown before that multiple positive and negative cis-regulatory elements reside in the K4 promoter (5), here we focused our attention on the role of Sp1 in transcriptional regulation of the K4 promoter. Sp1 is known to regulate differentiation in several cell types (9, 10). Different K4 promoter deletion constructs (K4-940, K4-540, K4-163, K4-140, and K4-76; Ref. 5) were transfected in SIRC cells; an Sp1 expression vector or an empty vector was cotransfected, and luciferase activity was assayed as described (5, 7, 8). Several potential Sp1 binding sites are located throughout the K4 promoter. We found that Sp1 transactivates the K4-940 promoter ~10-fold, which is reduced by 50% in the K4-540, K4-163, and K4-140 deletion constructs and almost abolished in the K4-76 deletion construct. These two reductions in Sp1-mediated transactivation are consistent with two distinct functional Sp1 cis-regulatory elements within the K4 promoter, one within the K4-940 construct and the other one within the K4-140 construct, respectively (Fig. 2A). One putative Sp1 binding site resides at −649 bp and the other one at −125 bp.

To investigate whether cyclin D1 overexpression influences the transcriptional regulation of the differentiation-linked K4 promoter in corneal epithelial cells, the K4-940 construct was transfected in C3D1 cells. Overall, the activity of the K4 promoter was 10-fold lower in C3D1 cells compared with parental SIRC cells. Furthermore, when Sp1 was cotransfected, Sp1-mediated transactivation of the K4 promoter was reduced to 5-fold in C3D1 cells compared with 10-fold in SIRC cells (Fig. 2B).

Because pRB is the major substrate of cyclin D1 and is known to act as a transcriptional activator through Sp1 (11, 12), the role of pRB in influencing the transcriptional regulation of K4 was assessed. Interestingly, cotransfection with wild-type pRB, but not mutated pRB (pRB-592 and pRB-209), resulted in a 10-fold transactivation of the K4 promoter in C3D1 cells (Fig. 2B). Thus, pRB rescues the reduction in Sp1-mediated transactivation of the K4 promoter in cyclin D1-overexpressing cells. It has been described previously that...
pRB regulates the transcription of target genes through cis-acting elements referred to as retinoblastoma control elements, which can bind members of the Sp1 family. In contrast, pRB cotransfection showed only a 5-fold transactivation of the K4 promoter in parental SIRC cells (Fig. 2B). Cotransfection of pRB and Sp1 did not cause any significant additional increase in K4 promoter activity in both cell lines (Fig. 2B).

**Sp1 Binds to cis Regulatory Elements of the Human K4 Promoter.** To examine whether the putative Sp1 cis-regulatory elements indeed bind Sp1, we performed EMSAs with SIRC or C3D1 nuclear extracts. EMSAs were performed with double-stranded oligonucleotide probes containing the putative Sp1 elements within the K4 promoter, residing at positions −649 bp (Sp1-C) and −125 bp (Sp1-A), respectively. As control, a Sp1 consensus oligonucleotide probe was used. One DNA-protein complex was observed with Sp1-A, Sp1-C, and Sp1-consensus. The Sp1-DNA complex was competed specifically by the Sp1-consensus oligonucleotide but not by a nonspecific (AP1-consensus) oligonucleotide (Lanes 3 and 5). The addition of Sp1 polyclonal antibody (PEP2) diminished the Sp1-DNA complex. AP1 monoclonal antibody (D) served as negative control (Lanes 4 and 6).

Fig. 2. Sp1 transcriptionally regulates the K4 promoter in SIRC and C3D1 cells. A, SIRC corneal cells were transfected with 2 µg of K4 promoter deletion-luciferase reporter constructs (K4-940, K4-540, K4-163, K4-140, and K4-76) and cotransfected with 1 µg of pRc/RSV-empty or pRc/CMV-Sp1 vectors. B, effects of Sp1 and/or pRB on K4 promoter activity in SIRC and C3D1 cells. SIRC and C3D1 cells were transfected with the K4-940 promoter-luciferase construct and cotransfected with 1 µg of pRc/RSV-empty, pRc/CMV-Sp1, pSG5-RB (wild-type pRB), pJ3 RB-592 (mutant pRB), or pJ3 RB-209 (mutant pRB). In both panels, luciferase activity is expressed as fold increase relative to the empty vector-cotransfection (means; bars, SE) and is calculated from three independent transfections. C, EMSAs were performed with SIRC nuclear extracts using probes Sp1-A, Sp1-C, and Sp1-consensus. D, equal amounts of SIRC and C3D1 nuclear extracts were compared using probe Sp1-C (Lanes 1 and 2). The Sp1-DNA complex was competed specifically by the Sp1-consensus oligonucleotide but not by a nonspecific (AP1-consensus) oligonucleotide (Lanes 3 and 5). The addition of Sp1 polyclonal antibody (PEP2) diminished the Sp1-DNA complex. AP1 monoclonal antibody (D) served as negative control (Lanes 4 and 6).
Comparison of Sp1-DNA complexes in both cell lines indicated a decrease in C3D1 nuclear extracts compared with SIRC nuclear extracts, thereby suggesting diminished DNA binding by Sp1 in cyclin D1-overexpressing cells (Fig. 2D). These results indicate that cyclin D1 overexpression influences the transcriptional regulation of the K4 promoter in corneal epithelial cells by interfering with the function of Sp1 as a transcriptional activator.

**Differential Expression of pRB and Sp1 in Cyclin D1-overexpressing Corneal Epithelial Cells.** To clarify differences in Sp1-DNA complex formation in C3D1 cells versus SIRC cells, we determined Sp1 and pRB protein levels in these cell lines. Western blot analysis of pRB levels, using a pRB antibody that equally recognizes hypo- as well as hyperphosphorylated pRB, revealed a shift toward the hyperphosphorylated form of pRB in C31D1 cells (Fig. 3A), consistent with cyclin D1 overexpression. Western blot analysis of Sp1 revealed no appreciable differences between C3D1 and SIRC cells (Fig. 3B).

Sp1 Interacts with pRB and Cyclin D1 in Corneal Squamous Epithelial Cells. We next asked whether Sp1 physically interacts with cell cycle regulatory proteins, i.e., pRB and cyclin D1, leading to this differential transcriptional regulation of K4. Initially, C3D1 and SIRC cells were immunoprecipitated with either a pRB antibody, a cyclin D1 antibody, or as a control an AP1 antibody. The immunoprecipitates were resolved by SDS-electrophoresis and immunoblotted with an anti-Sp1 antibody (data not shown). Sp1 immunoprecipitates prepared in parallel were immunoblotted with either anti-pRB or anti-cyclin D1 antibodies, respectively (Fig. 4A and B). We found that Sp1 physically interacted with either pRB or cyclin D1. Whereas no differences in levels between the two cell lines could be determined with the recently described Sp1-pRB interaction (Ref. 12; Fig. 4A), a more prominent Sp1-cyclin D1 interaction was detected in C3D1 cells compared with SIRC cells (Fig. 4B). However, in C3D1 cells, Sp1 interacts predominantly with hyperphosphorylated pRB, whereas in SIRC cells an interaction with both forms of pRB could be observed (Fig. 4A). As a negative control, J82 cells, where pRB is truncated, and DT40 cells, where cyclin D1 is deleted, were immunoprecipitated with Sp1 antibody and immunoblotted with either anti-pRB antibody (Fig. 4A) or anti-cyclin D1 antibody (Fig. 4B), respectively.

To further confirm these Sp1 protein-protein interactions, IP-EMSA were performed essentially as described (14). C3D1 and SIRC cells were immunoprecipitated with pRB or cyclin D1 antibodies, complexes were dissociated, and then EMSAs were performed with radiolabeled Sp1-C probe, the region of the K4 promoter where differences in Sp1-mediated transactivation were most apparent. This IP-EMSA revealed a specific Sp1-DNA complex in both cell lines, released from either pRB (Fig. 4C, Lanes 5 and 6) or cyclin D1 immunoprecipitates (Fig. 4C, Lanes 9 and 10). However, a more prominent DNA-protein complex could be observed in immunoprecipitates from C3D1 cells. Addition of Sp1-specific antibody, but not AP1 antibody, attenuated this immunoprecipitated Sp1 complex (Fig. 4C). Sp1 immunoprecipitates (Fig. 4C, Lanes 1 and 2) and AP1 immunoprecipitates (Fig. 4C, Lanes 15 and 16) served as positive or negative controls, respectively. In aggregate, our findings are consistent with an interaction between Sp1 and cyclin D1 that leads to differential regulation of a differentiation-linked K4 promoter.

**Discussion**

The equilibrium between proliferation and differentiation is regulated extensively by a complex network of signals, both extracellular and intracellular. This is illustrated by the rapid turnover of cells in the stratified squamous epithelium at many sites. The switch from proliferation to differentiation is in part governed by the differential expression of cytokeratins in basal and suprabasal cells (1, 2). K4 is an excellent model in which to study early differentiation in the corneal epithelium (4, 5). We now describe that Sp1 transcriptionally regulates the K4 promoter in corneal epithelial cells, which is mediated through binding of Sp1 to two independent Sp1 cis-acting elements. However, these functional and biochemical effects were partially abrogated in cyclin D1-overexpressing cells, which was restored by ectopic expression of pRB.

Indeed, a natural question emanating from these findings was whether cyclin D1 and Sp1 interacted with each other apart from the recently described Sp1-pRB interaction (12). Our data are consistent with the notion that cyclin D1 and Sp1 do interact. It is tempting to speculate that the Sp1-pRB complex may mediate transactivation of genes, the protein products of which in turn favor growth suppression and/or commit the cell to early differentiation. The role of Sp1 in differentiation or growth suppression is further evidenced by its interaction with Smad proteins to regulate the p21/Cip1/WAF1 promoter (15). Nonetheless, the functional effects of pRb-Sp1 may be opposed by the Sp1-cyclin D1 complex, a consequence of which is to retard or partially disrupt the commitment to differentiation. Certainly, such a phenomenon would be evident in an exaggerated form in some tumor types, for example squamous cell cancers, which frequently overexpress cyclin D1. It should be emphasized that we were able to detect the Sp1-cyclin D1 interaction in normal corneal epithelial cells, indicating that this is not the result of cyclin D1 overexpression in the C3D1 cells. However, the Sp1-cyclin D1 interaction is more dramatic in C3D1 cells with apparent functional consequences of diminished Sp1-mediated transactivation of the K4 promoter. Mapping of the domains involved in the Sp1-cyclin D1 interaction will be of interest.

Our data do not preclude the possibility that the cyclin D1-Sp1 complex may be part of a larger complex that regulates transcription, and indeed, such a candidate may be TFIID because members of this family have been demonstrated recently to interact individually with cyclin D1, Sp1, or pRB (16–18). It is also conceivable that cyclin D1 phosphorylates Sp1 because phosphorylation of Sp1 has been shown to be important in cell cycle regulation of its own transcriptional activity (19). Additionally, the cyclin D1-Sp1 complex may also contain pRb, a possibility not excluded by our experiments. Whether cyclin D1-Sp1 complex formation is dependent upon cdk4/ cdk6, as with the phosphorylation of pRB, or independent of cdk activity as is the case with the cyclin D1-estrogen receptor interaction (20), requires further investigation. Whereas the ability of Sp1 to
influence the equilibrium between proliferation and differentiation is mediated in part through its interactions with cell cycle regulatory proteins, cyclin D1 in turn may promote the tendency to proliferation through interaction with proteins such as estrogen receptor in breast epithelial cells and Sp1 in corneal epithelial cells and possibly, in different cell types.

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