Opposite Functions for E2F1 and E2F4 in Human Epidermal Keratinocyte Differentiation*

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Proteins of the retinoblastoma family (pRb, p107, and p130) modulate cell proliferation, a function related to their capacity to control the activity of the E2F transcription factor family. The Rb proteins also control cell differentiation in different tissues. We have recently described their involvement in human epidermal keratinocyte differentiation (Paramio, J. M., Lain, S., Segrelles, C., Lane, E. B., and Jorcano, J. L. (1998) Oncogene 17, 949–957). Here we show that E2F proteins are also involved in this process. We found that E2F1 and E2F4 are expressed differentially during the in vitro differentiation of human epidermal keratinocytes, with the former uniformly present throughout the process, whereas the second is predominantly expressed at the onset of differentiation. This pattern is also observed in human skin by confocal microscopy. Electrophoretic mobility shift assays and immunoprecipitation experiments demonstrated that the complexes formed by E2F1 and E2F4 and Rb family proteins vary throughout in vitro keratinocyte differentiation. In agreement with this observation, several E2F-responsive genes are differentially regulated during this process. To test the functional implications of these observations, we transfected HaCaT keratinocytes with plasmids coding for E2F1 and E2F4. Transfected cells display opposite in vitro differentiation properties. Although E2F1-transfected cells are unable to differentiate, E2F4-transfected cells show an increased differentiation rate compared with nontransfected control cells. Our data demonstrate that the differential and coordinated expression and interaction of E2F and Rb proteins modulate the process of epidermal differentiation and provide clear evidence that members of the E2F family of transcription factors play specific and opposite roles during cell differentiation.

Terminal cell differentiation and proliferation are mutually exclusive processes, as cells committed to differentiation arrest cell division. Molecules that negatively control cell cycle can, therefore, also be involved as positive modulators of differentiation processes. The differentiation program in epidermis (reviewed in Refs. 1 and 2) takes place progressively as committed basal cells move upwards to the epidermal surface and express, in a sequential manner, specific proteins such as keratins, involucrin, filaggrin, and loricrin. The molecular mechanisms governing epidermal differentiation are still largely unknown, in particular with respect to molecules involved in cell cycle control. We have recently reported that the retinoblastoma family proteins (pRb, p107, and p130) are differentially expressed during human keratinocyte differentiation in vivo and in vitro. The forced expression of particular combinations of these proteins triggers specific stages of the differentiation program, demonstrating that these proteins cooperate and play relevant roles throughout this process (3).

The Rb1 family proteins are major regulators of cell proliferation through their ability to suppress entry into S phase (reviewed in Refs. 4 and 5; see also Ref. 3 for their roles in keratinocyte growth control). They perform this key activity by basically controlling the activity of the E2F family of transcription factors, which control gene expression following heterodimerization with the DP family of proteins (reviewed in Refs. 6 and 7). Six members of the E2F family (E2F1 through E2F6) and three DPs (DP1, -2, and -3) have been identified to date. E2F proteins seem to play complex functions. They induce the transcription of genes that regulate S phase entry, and, concomitantly, their overexpression appears to be sufficient to promote transformation of certain immortalized cell lines (8–11). However, Rb-bound E2F may also repress gene transcription (12–14). In addition, deregulated overexpression of E2F1 can trigger apoptosis in several cell types (15–18), and the formation of tumors in E2F1-deficient mice (19, 20) suggests that E2F proteins can also function as tumor-suppressor proteins (see, for a discussion Ref. 21).

The significance of the E2F family complexity is presently unclear. These proteins are divided into three groups based on structural and functional properties. The group formed by E2F1, -2, and -3 is regulated almost exclusively by pRb, whereas p107 and p130 regulate E2F4 and -5. These differences in Rb family protein-E2F binding are not absolute, however, because E2F4 can also bind pRb during late G1 and S phases of the cell cycle (for a detailed review, see Ref. 22). Finally, E2F6 is very different from the rest of the family, because it contains no Rb family protein binding nor transactivation domains and appears to act exclusively as a transcriptional repressor (23–26). E2F proteins also display differences regarding their specific heterodimerization with DP proteins, their expression during cell cycle, development, and differentiation (27–31).

In skin, the E2F1 overexpression in epidermis of transgenic mice induces hyperplasia and co-operates with cyclin D1, Haras, and p53 in the development of chemically induced tumors (17, 32). In addition, deregulated E2F expression has been reported during premalignant progression in mouse skin tu-

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1 The abbreviations used are: Rb, retinoblastoma; EMSA, electrophoretic mobility shift assay; wt, wild-type; NaDOC, sodium deoxycholate.
mors (33). However, during two-stage carcinogenesis protocols, the overexpression can act as a tumor suppressor at the promotion stage probably by inducing apoptosis (18). These observations, together with our previous findings regarding the differential expression and function of the Rb family of proteins during epidermal differentiation (3), have led us to study E2F function in keratinocyte differentiation. We found that, both in skin in vivo and during in vitro differentiation of human HaCaT keratinocytes, there is a differential expression pattern for E2F1 and -4. Multiple complexes containing distinct E2F-pocket protein combinations were observed during differentiation. Finally, we found that the transfection of E2F1 or -4 lead to the inhibition or acceleration, respectively, of keratinocyte differentiation in vitro. Collectively, these data demonstrate the involvement of E2F proteins in epidermal differentiation, suggesting that this process is driven by the regulated presence of specific E2F-pocket protein complexes.

MATERIALS AND METHODS

Cell Culture—HaCaT keratinocytes were obtained from Dr. N. Fusenig (DKFZ, Heidelberg, Germany) and were cultured and induced to differentiate as described (3, 34, 35). HA-tagged E2F1 and E2F4 (A-20 polyclonal antibody or D3 monoclonal antibody) or E2F2 (C-20 polyclonal antibody or RH55 monoclonal antibody) or E2F4 (A-20 polyclonal antibody or D3 monoclonal antibody), and counterstained with propidium iodide, essentially as described (3). Specimens were analyzed using a Bio-Rad MRC600 confocal microscope. The specificity of the staining was confirmed by routinely performing the appropriate controls including incubations omitting the primary antibodies and preincubation of the antibodies with five fold excess of the corresponding blocking peptide (Santa Cruz). For immunofluorescence analysis, the expression of the transfected proteins was analyzed by indirect immunofluorescence with the following probes: c-myc (Cia1-EOri fragment expanding exon 3); E2F1 (EOri-Xhol fragment expanding nucleotides 200–1380); DP-1 (Smal-BstXI fragment containing almost the full-length cDNA); E2F-4 (Apol fragment expanding nucleotides 540–930); E2F-5 (EOri fragment expanding nucleotides 597–930); cycE, cycA, p21/mdm2 (full-length cDNAs). A T S rRNA-specific probe was used to normalize the loading.

Western Blotting—Total cell extracts (150 μg) or immunoprecipitates (see below) were separated in SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose filters (Amer sham Pharmacia Biotech). The filters were processed and probed using appropriate primary antibodies. Polyclonal antibodies against E2F2A and DP-1 were purchased from Santa Cruz Biotechnologies; for anti-keratin K10, K14 and involucrin antibodies, see References 3, 34, and 35. Secondary antibodies and detection were as described previously (3, 34, 35).

Immunofluorescence—Fresh cryostat human epidermal sections were stained using antibodies (Santa Cruz Biotechnologies) specific for E2F1 (C-20 polyclonal antibody or RH55 monoclonal antibody) or E2F4 (A-20 polyclonal antibody or D3 monoclonal antibody), and counterstained with propidium iodide, essentially as described (3). Specimens were analyzed using a Bio-Rad MRC600 confocal microscope. The specificity of the staining was confirmed by routinely performing the appropriate controls including incubations omitting the primary antibodies and preincubation of the antibodies with five fold excess of the corresponding blocking peptide (Santa Cruz). For immunofluorescence analysis of transfected cell pools, cultures grown on glass coverslips were methanol-fixed, stained and analyzed in a Zeiss Axiophot microscope, essentially as described previously (3, 34, 35). HA-tagged E2F1/4 and E2F2/4 were detected using the 12CA5 monoclonal antibody (Roche Molecular Biochemicals) against the hemagglutinin tag. Secondary antibodies purchased from Jackson Immunoresearch were used as described previously (3, 34, 35).

Luciferase Assays—HaCaT cells were cotransfected with 2 μg of pcDNA3 (Invitrogen) to provide Neo resistance and 15 μg of oligonucleotide was added to 7.5 μg of cell extract in 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EGTA, 5% glycerol, and 1 μg of poly(dI-dC) (Amersham Pharmacia Biotech) in a final volume of 20 μl. After 30 min of incubation at 4 °C, DNA-protein complexes were resolved by electrophoresis through 4% polyacrylamide gels in 0.25× TBE at 4 °C at 180 V. Gels were dried and autoradiographed with intensifying screens at −70 °C. The specificity of binding was confirmed by including in the incubation a 20-fold excess of unlabeled wt or mutant oligonucleotides. Sodium deoxycholate (NaDOC) release was performed by adding a 0.1% final concentration of NaDOC to the DNA-protein mixtures prior to incubation.

Immunoprecipitation—Cells were lysed by freeze-thaw cycles and harvested in chilled NET buffer (150 mM NaCl, 0.1% Nonidet P-40, 50 mM Tris-HCl, pH 7.5) containing aprotinin, leupentin, and phenylmethysulfonyl fluoride to inhibit proteases. The crude homogenates were centrifuged (5 min, at 10,000 rpm, 4 °C), and pellets were discarded. Supernatants were precleared by incubation with a mixture of protein G Sepharose beads and mouse IgG (Santa Cruz Biotechnologies) for 2 h at 4 °C and further centrifugation. Total protein (500 μg; quantified using the Bio-Rad protein assay) was incubated in the presence of 2 μl of antibodies against pRB, p107, or p130 (Santa Cruz) for 12 h at 4 °C. Immune complexes were collected by incubation with 30 μl of a mixture of protein A- and protein G-Sepharose beads (Santa Cruz) and centrifugation. Pellets were washed three times with cold NET buffer and stored at −70 °C.

Functional Analysis of E2F Proteins—HaCaT cells were transfected with plasmids (all made in the pcDNA3 backbone) coding for E2F1 (a generous gift from Dr. E. Harlow), E2F4 (a generous gift of Dr. R. Bernards), or the two chimeric constructs E2F1/4 or E2F4/1 (kindly provided by Dr. N. Jones), or Neo empty vector. After selection in G418-containing medium, several clones were pooled and expanded, and the expression of the transfected proteins was analyzed by indirect immunofluorescence and by Western blot (as above). Those cultures positive for expression of the corresponding transfected protein were allowed to differentiate by serum starvation as described (3).

RESULTS

Differential Expression of E2F Proteins during Differentiation of Human Keratinocytes—We recently showed that HaCaT keratinocytes cultured in the absence of serum undergo cell cycle arrest and progressively differentiate, as demonstrated by their stratification and the sequential induction of keratin K10 and involucrin (3). This system also provides the advantage that, because the cells arrest cell proliferation prior to any signs of differentiation at 3–4 days of serum starvation (3, 38), the expression and the possible functional involvement of molecules that are also related to cell cycle progression, such as the Rb family members or the E2F members, can be studied independently for any effect related to cell proliferation (34; see Ref. 3 for a thorough discussion). We also showed that the three Rb family proteins (pRb, p107, and p130) are expressed in a sequential manner during this process, reminiscent of their in vivo localization in skin, and that transfection with particular combinations of these proteins triggers specific stages of keratinocyte differentiation (3). Given the functional relationship between the Rb and E2F families, we studied the expression of E2Fs in the same system. Because E2F proteins are also post-transcriptionally regulated (39, 40), we analyzed their expression in Northern and immunoblotting experiments (Fig. 1, A and B, respectively). By both techniques we observe that E2F1 and DP1 main content throughout the course of the experiment. In contrast, the E2F4 level is high during the early phases of the process, including cell cycle arrest and early differentiation (days 4 and 8), decreasing afterward (20% of the original value as estimated by densitometric analysis). Finally, E2F5 was detectable only at day 0 but not at growth arrest or
Finally, p130 binds E2F4 during late differentiation (days 16 and 20), E2F1 at early differentiation, and E2F4 only in proliferating cells (time 0). p107 binds E2F4 in proliferating cells and also at days 12 and 16. Immunoprecipitates were subsequently probed by Western blotting with antibodies specific for E2F1 or E2F4.

**Immunoprecipitation and Western Blotting**

In vitro transcriptional activators during differentiation, we transfected HaCaT cells with a reporter plasmid coding for luciferase under the control of three E2F consensus sites (pGL3xwtE2F-Luc (37)). The clones obtained were pooled and allowed to differentiate, and the luciferase activity was measured at different times and related to that obtained in proliferating cells (day 0). The results (Fig. 3B) indicate that E2F transcriptional activity decreases by day 4, when the cells are arrested at the differentiation onset. This activity increases progressively during the

**Transcriptional Activity of E2F during Keratinocyte Differentiation**—E2F proteins have a dual function in transcription. When they bind promoters in complexes lacking Rb, they act as transcriptional activators, whereas, if they recruit Rb proteins to the bound promoters, they act as repressors. Some of the genes modulated by E2F, such as p21<sup>waf1</sup>, c-myc (42), and cyclins A (43–45) and E (45–48), modulate S phase entry. We studied the expression pattern of these genes during HaCaT cell differentiation. We found (Fig. 3A) that cyclin E and A expression is completely repressed in differentiating cells, in agreement with the complete cell cycle arrest displayed by these cells after 3 days in the absence of serum (see Refs. 3 and 38 and references therein). In addition to their role during cell cycle progression, c-myc and p21<sup>waf1</sup> are positive modulators of keratinocyte differentiation (49, 50). We found that c-myc is highly induced at days 4 and 8, when E2F4 is also maximally expressed and uncomplexed with Rb proteins (Fig. 1). Finally, p21<sup>waf1</sup>-immortalized cells with a reporter plasmid coding for luciferase under the control of three E2F consensus sites (pGL3xwtE2F-Luc (37)). The clones obtained were pooled and allowed to differentiate, and the luciferase activity was measured at different times and related to that obtained in proliferating cells (day 0). The results (Fig. 3B) indicate that E2F transcriptional activity decreases by day 4, when the cells are arrested at the differentiation onset. This activity increases progressively during the

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**Fig. 1. Expression of E2F members during in vitro differentiation of human HaCaT keratinocytes.** Differentiation was induced by serum starvation as described previously (3), and at the stated times (days) total RNA or protein extracts were obtained and analyzed by Northern (A) or Western (B) blot with the indicated specific probes or specific antibodies (see “Materials and Methods”). Keratin K10 was used to test the induction of differentiation, and 7 S and keratin K14 were used to normalize the loading for Northern and Western analysis, respectively. C, specificity of the E2F-DNA complexes during differentiation binding analyzed by EMSA. The complexes formed using a consensus E2F-specific oligonucleotide (see “Materials and Methods”) and cell extracts from day 4 of differentiation are displaced by a 20-fold excess of the same unlabeled oligonucleotide (not shown) but not by a point-mutated form (not shown). D, EMSA analysis using extracts from HaCaT cells at the stated days after serum starvation reveals the formation of multiple E2F-DNA complexes. E, pretreatment with 0.1% NaDOC leads to the formation of a single band, corresponding to free E2F-DNA complexes, indicating that the bands observed throughout differentiation are due to the presence of other proteins in the complexes. F, interaction of Rb family proteins with E2F during differentiation. Protein extracts (500 µg), obtained at the indicated days (0–20) after induction of differentiation, were immunoprecipitated with antibodies against the indicated Rb family proteins (lppT column). Immunoprecipitates were subsequently probed by Western blotting with antibodies specific for E2F1 or E2F4 (wB column). Note that pRb binds E2F1 at early differentiation, and E2F4 only in proliferating cells (time 0). p107 binds E2F4 in proliferating cells and also at days 12 and 16. Finally, p130 binds E2F4 during late differentiation (days 16 and 20).
differentiation process, reaching the level observed in proliferating cells by day 20. As a control, when a reporter plasmid without E2F sites was used, we did not observe changes in transcriptional activity (not shown), thus excluding that the observed variations with pGL3xwtE2F plasmid can be due to the activation of the basal promoter during differentiation.

**Forced Expression of E2F Proteins Severely Affects Keratinocyte Differentiation**—To test the involvement of E2F proteins in keratinocyte differentiation, we generated cell clones overexpressing these proteins. In addition, and given the involvement of the Rb family proteins in this process, we also used two hybrid molecules, E2F1/4 and E2F4/1, composed of the DNA binding domains of E2F1 or E2F4, respectively, but with the Rb family protein binding region interchanged (51). Fig. 4 shows schematically the features of these molecules. The clones derived from each transfection (E2F1, E2F4, E2F1/4, E2F4/1, or empty vector) were pooled, and the expression of the transfected genes was confirmed by Western blotting in asynchronous cultures (Fig. 4B). In the course of these experiments, we observed that the constructs containing the E2F1 backbone displayed decreased colony forming efficiency, suggesting that they hinder cell growth (Fig. 4C). This may be due to the reported ability of E2F1 to promote apoptosis under overexpression conditions (data not shown; see also Refs. 15–20) and may explain why E2F1 expression levels are increased only 2- to 3-fold in the pooled clones as compared with the control cells, whereas E2F4 expression levels are increased about 10-fold. Finally, we observed by immunofluorescence that transfected and endogenous E2F1 and E2F4 displayed a similar cellular localization, although, as expected, staining was brighter in transfected cells (Fig. 4D).

Pooled clones were used in serum starvation differentiation experiments (Fig. 5). Neo-transfected cells differentiate as the non-transfected HaCaT cells (3), and by day 16 after serum removal the presence of stratification domes (Fig. 5A, arrows), in which there is overt expression of differentiation markers such as keratin K10 and involucrin (data not shown), is evident. In contrast, we did not observe either stratification or expression of differentiation markers in E2F1 transfecants, even after 20 days in the absence of serum (Fig. 5B, and results not shown), suggesting that this molecule can hinder epidermal
differentiation. Conversely, E2F4-overexpressing cells displayed accelerated and massive stratification as compared with Neo cells, suggestive of increased differentiation (see an example corresponding to day 12 in Fig. 5E). However, cells expressing E2F4/1 displayed a higher number of stratified domes than did Neo-transfected cells (Fig. 5D, corresponding to day 12), although lower than the number and size observed in E2F4-overexpressing cells (compare Figs. 5D and 5E). Finally, when E2F1/4-transfected cells were induced to differentiate, massive cell death was observed very early after serum removal (Fig. 5C, day 4), with some cells displaying clear signs of apoptosis (arrowheads in Fig. 5C).

To further confirm the effects of the overexpression of the different E2F constructs on HaCaT differentiation, pooled clones from Neo, E2F1, E2F4, and E2F4/1 transfections were allowed to differentiate by serum starvation during 8 days. At this time, protein extracts were obtained, and the expression of keratin K10 and involucrin was analyzed by Western blotting. The results (Fig. 5F) clearly confirmed the morphological observations. The expression of involucrin and keratin K10 was higher in E2F4/1 than in Neo cells. The highest expression of both differentiation markers was detected in E2F4 cells, whereas no expression of such markers was detected in E2F1 cells.

**DISCUSSION**

The function of E2F proteins in the regulation of cell proliferation is presently well established (6, 7). In this article, we have studied their involvement in cell differentiation, and we have explored the possibility that different E2F proteins have distinct functions. We found that E2F1 and -4 are differentially expressed during HaCaT epidermal keratinocyte differen-
tion (Fig. 1, A and B). In contrast, E2F5 is not detected during this process. In addition, distinct DNA-binding complexes containing specific E2F-Rb combinations were detected along epidermal differentiation (Fig. 1, C–F).

This in vitro expression pattern was concordant with the expression of E2F proteins in human skin sections (Fig. 2) and suggests that these proteins indeed play a functional role in epidermal differentiation. The observed expression pattern in human epidermis is different from that described in murine epidermis, where basal cells express E2F2 and -4, and suprabasal cells express E2F5, whereas there is no expression of E2F1 (30). This absence of E2F1 may help to explain why no skin abnormalities have been reported in E2F1-deficient mice (19, 20). Surprisingly, the epidermis of E2F5-deficient mice also appears histologically normal (52), although the aberrant induction, as a consequence of E2F5 absence, of other E2F species that may act as compensatory factors has not been discarded in these mice. Nonetheless, this discrepancy in the E2F expression pattern in murine and human skin is not entirely surprising, because it has also been described for pRb (see Ref. 3 for a discussion). Differential E2F expression and complexation with the Rb family of proteins has also been found during differentiation in hematopoietic, neuronal, and muscle cells (29, 31, 53–55). How-

FIG. 5. Appearance of differentiating pooled clones from transfections with the indicated E2F plasmids. A, Neo cells after 16 days in the absence of serum, showing characteristic stratification domes (arrows). B, E2F1 cells show no signs of stratification after 20 days without serum. C, E2F1/4 induces massive cell death immediately after serum removal (shown at day 4), with signs of apoptosis (arrowheads). D, E2F4/1 cells displaying increased numbers of stratified domes at 12 days after serum starvation. E, E2F4 cells displaying areas of massive differentiation at 12 days of differentiation. F, Western blots against keratin K10 and involucrin using protein extracts from the stated pooled cell clones after 8 days in absence of serum demonstrating the increased expression of both differentiation markers in E2F4 and E2F4/1 cells compared with Neo cells, and the absence of expression in E2F1 cells. The same blots were probed against β-tubulin no normalize the protein loading.

FIG. 6. Model explaining the role of E2F4 (A, B) and E2F1 (C, D) at early (A, C) or late differentiation (B, D) times. Crossed-out arrows indicate that the corresponding Rb and E2F proteins, although present, cannot form complexes. Crossed-out Rb family molecules are not present during early (A) or late (B) differentiation. Plus or minus signs indicate active or inhibited gene transcription, respectively. See text for further details.
ever, the discrepancies among these data, and also with ours, in epidermal cells, support the existence of cell type-specific roles of E2F during differentiation.

The use of an E2F-responsive reporter demonstrated a fall in the E2F transcriptional activity after serum removal (and, consequently, proliferation inhibition) followed by a progressive recovery during differentiation. These data seem to be in disagreement with the observed expression pattern of E2F species (Fig. 1). A possible explanation for this apparent discrepancy would implicate that the reporter plasmid is more sensitive to E2F1 than to E2F4. Therefore, the decay in luciferase activity observed at early stages would indicate the binding of pRb to E2F1, whereas during terminal differentiation (days 16 and 20), the recovery would be due to the uncomplexed E2F1 promoted by the absence of pRb expression at these time points (3). In addition, we have studied the expression of several E2F-regulated genes. We found that cyclin E and A genes are silenced during this process (Fig. 3A), indicating that, although free E2F4 (early stages) and E2F1 (late stages) are found during differentiation as demonstrated in Fig. 1F, these molecules cannot overcome the silencing of cyclin A and E genes. On the other hand, c-myc and p21\textsuperscript{waf}\textsubscript{1} in agreement with their reported function as positive modulators of keratinocyte differentiation (49, 50) are expressed during in vitro HaCaT differentiation. These two genes appear to be expressed under the control of E2F among several other elements (41, 42). The fact that the maximal expression of c-myc is coincident with that of uncomplexed E2F4 at days 4 and 8 (Fig. 1), suggests that, as during cell cycle progression (42), E2F4 can be down-regulated upon differentiation. These two genes appear to be expressed under the control of E2F among several other elements (41, 42).

The identification of the postulated E2F-responsive genes, as well as the characterization of skin abnormalities in animals lacking the appropriate E2F species expressed in mouse epidermis, should provide further support for these conclusions.

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