The α2 and α5 integrin genes: identification of transcription factors that regulate promoter activity in epidermal keratinocytes

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Abstract We analysed the activity of the proximal promoters of the α2 and α5 integrin genes in human keratinocytes. An AP-1 site, found in the α5 but not the α2 promoter, bound c-Jun/c-Fos dimers and contributed strongly to promoter activity. Both promoters had a CCAAT/enhancer binding protein (C/EBP) binding site: the α5 C/EBP element enhanced activity, while the α2 site was a negative regulatory element. C/EBP overexpression repressed the activity of both promoters, but the effect was independent of occupancy of the identified C/EBP binding sites, suggesting interactions with additional transcription factors. We propose that upregulation of C/EBPs contributes to the inhibition of integrin transcription during keratinocyte terminal differentiation, while AP-1 factors play a role in the selective induction of the α5 gene during wound healing.

Key words: Integrin promoter; CCAAT/enhancer binding protein; AP-1; Differentiation; Epidermis; Keratinocyte

1. Introduction

The key role of cell–extracellular matrix adhesion in cellular differentiation is well illustrated in mammalian epidermis. Adhesion is a negative regulator of keratinocyte differentiation and detachment of keratinocytes from the underlying basement membrane triggers terminal differentiation [1,2]. The keratinocytes with the highest proliferative potential, the stem cells, have higher surface levels of β1 integrins than keratinocytes of lower proliferative potential and elevated integrin expression is required for maintenance of the stem cell compartment [3,4].

Keratinocytes in the basal layer of human epidermis express several integrins constitutively, including α2β1, a collagen receptor, and α3β1 and α6β4, which are laminin receptors [5]. In addition at least two integrins which are expressed either at low levels or not at all in normal epidermis are upregulated when keratinocytes become hyperproliferative, for example during wound healing, in psoriasis or when keratinocytes are placed in culture: these are α5β1, the classical fibronectin receptor, and αvβ6, which may be a receptor for vitronectin, fibronectin and tenascin [6–8]. Normally integrin expression is downregulated during keratinocyte terminal differentiation; however, suprabasal expression is a feature of hyperproliferative epidermis and some keratinocyte tumours [5]. Modelling in transgenic mice has shown that suprabasal integrin expression can contribute directly to the psoriatic phenotype [9].

In order to identify the basis for the regulated expression of the α2β1 and α5β1 integrins in keratinocytes we have analysed the proximal regulatory regions of the α2 and α5 integrin genes. The proximal regulatory regions of a number of integrin α subunit genes have already been isolated and functionally characterised [10–17]. In most cases their respective promoters lack TATA boxes and are dependent on proximal Sp1 binding sites for basal and regulated activity [18–21]. A number of additional transcription factors have been shown to participate in the transcription of the CD11b (PU.1) [22], CD11c (AP-1, CCAAT/enhancer binding protein (C/EBP), PU.1) [23–25], α2 (NF-κB) [26] and α4 (ZEB) [27] integrins. In this report we highlight the importance of C/EBPs in the inhibition of α2 and α5 integrin gene transcription that occurs during keratinocyte terminal differentiation and present evidence that AP-1 factors play a role in the selective upregulation of the α5 gene observed in hyperproliferative epidermis.

2. Materials and methods

2.1. Cell culture

Normal human keratinocytes (strains ka, kc, kq and kz; passage numbers 2–7), derived from neonatal foreskin, were cultured in the presence of a feeder layer of mitomycin C-treated J2-3T3 cells [28] and suspension-induced differentiation was carried out as described previously [29]. For transfection experiments, keratinocytes were grown in keratinocyte serum-free medium (KSFM; Gibco), supplemented with recombinant epidermal growth factor (rEGF), bovine pituitary extract and gentamicin, as recommended by the manufacturer. The human cell lines HepG2 (hepatoma) and U937 (monoblastic leukaemia) were cultured in Dulbecco's modified Eagle's medium (DMEM) and RPMI, respectively, supplemented with 10% FCS, 2 mM glutamine, and 50 mg/ml gentamicin.

2.2. Reporter plasmid construction

The pGL2-derived p173-Luc and p27-Luc plasmids, where luciferase expression is driven by the −173/+23 or the −27/+23 fragments from the α5 integrin gene promoter, were kindly provided by Dr T. Birkenmeier (Washington University). To synthesise the α5 promoter fragment spanning −92/+23, a *Pfu* polymerase-mediated PCR protocol was performed with the oligonucleotides −92/+23 (sense) and +23/−46 (antisense), whose 16 3′ nucleotides are complementary. The PCR protocol included a 5 min denaturation step at 95°C, followed by a 15 min extension step at 72°C and a 35 cycle PCR procedure (1 min at 95°C, 30 s at 50°C, and 1 min at 72°C). An identical approach was used to synthesise the −92/+23 α5 promoter fragment harbouring mutations at the C/EBP binding site (TTTGGCAA mutated to GACTAGTC) or the AP-1 binding site (TGAGTCA mutated to TGCAGCA). In all cases the sense oligonucleotides contained a

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*Bgl*II restriction site at their 5′ ends while the +23/−46 oligonucleotide contained a *Hin*dIII site at its 5′ end. The resulting PCR products were digested with *Bgl*II and *Hin*dIII and the fragments purified and ligated into *Bgl*II/*Hin*dIII-digested pGL3 plasmid (Promega). In the case of the α2 integrin, the promoter region between −92 and +109 was synthesised by PCR using the α2 integrin promoter-based reporter plasmid p900bp-CAT (kindly provided by Dr M. Zutter, Washington University School of Medicine) as template. The primer oligonucleotides −92/−57 and +109/−90 were designed to contain *Bgl*II and *Hin*dIII sites, respectively, and the resulting PCR fragments were inserted into *Bgl*II/*Hin*dIII-digested pGL3. An identical strategy was used to replace the C/EBP binding site TTGCGGAA at −72 by the sequence GACTAGTC, which has been previously demonstrated to prevent C/EBP binding [24]. Insertion of the α2 and α5 regulatory sequences in the pXP2 reporter construct [30] was accomplished by recovery of the −92/+25 (α5) or −92/+109 (α2) fragments from the pGL3-based plasmids after digestion with *Bgl*II and *Hin*dIII, and later insertion of the isolated fragments into *Bam*HI/*Hin*dIII-digested pXP2, to yield the plasmids pA5-92-Luc, pA5-92(CEBPmut)-Luc, pA5-92(AP1mut)-Luc, pA2-92-Luc and pA2-92(CEBPmut)-Luc. All constructs were confirmed by sequencing.

2.3. Transfection

Keratinocytes (2.5–3.5×10^5/35 mm dish) were seeded in KSFM the day before transfection. DNA (2 μg) was diluted in 100 μl of serum- and antibiotic-free DMEM in polystyrene tubes. 10 μl of Superfect reagent (Qiagen) was added and the mixture was vortexed and incubated for 15 min at room temperature. Finally, 600 μl of KSFM was added to the mixture. After washing with phosphate-buffered saline (PBS), keratinocytes were incubated with the DNA–reagent mix for 3 h at 37°C, washed once with PBS and cultured for 24 h in 2 ml of KSFM. In transactivation experiments using the CMV promoter-based C/EBP expression vectors (CMV-0, CMV-CEBPα and CMV-CEBPβ, kindly provided by Dr G.J. Darlington, Texas Children's Hospital, USA), 1.6 μg of luciferase reporter construct was cotransfected with 0.4 μg of the corresponding expression vectors. Transfection of HepG2 cells was carried out using an identical protocol but plating 4×10^5 cells per well. Cells were usually cultured for 24–48 h, washed and processed for luciferase activity measurement. For comparative purposes between cell lines and reporter gene constructs, transfection efficiencies were normalised by cotransfection with the β-galactosidase expression plasmid pCMV-βgal. The activity of each reporter construct was expressed relative to the activity produced by the pCMV-βgal plasmid (relative light units).

2.4. Electrophoretic mobility shift assays (EMSA)

EMSA were performed essentially as described [23–25]. Unlabeled competitor oligonucleotides were added to the nuclear extracts at a 100-fold molar excess. Nuclear extracts were prepared as described previously [31], except that aprotinin, antipain, leupeptin, pepstatin (1 mg/ml) and 10 μM Pefabloc (Boehringer Mannheim) were included as additional protease inhibitors. The α2 and α5 integrin promoter-based oligonucleotides used for EMSA and their relative positions are shown in Fig. 1.

Additional double-stranded oligonucleotides used as competitors and/or probes included AP1CONS (5′-CGCTTGATGAGTCAGCCGGAA-3′), Sp1CONS (5′-ATTCGATCGGGGCGGGGCGAGC-3′), CEBPCONS (5′-TGCAGATTGCGCAATCTGCA-3′) and CEBPCONS-mut (5′-TGCAGAGACTAGTCTCTGCA-3′), which contain the consensus binding sites for AP-1, Sp1, C/EBP, and a mutated C/EBP consensus binding site, respectively. The CD11c promoter-derived oligonucleotides used were Sp1-CD11c (Sp1-70 in [19]) (5′-GCGTACTCTGCCCGCCCCCTCTGACTC-3′); Sp1mut-CD11c (Sp1-70mut in [19]) (5′-GCGTACTCTGCCCGAATTCCTCTGACTC-3′); AP1-CD11c (AP1-60 in 23) (5′-GCCCCCTCTGACTCATGCTGACAA-3′); AP1mut-CD11c (AP1-60mut in [23]) (5′-GCCCCCTCTGCAGCATGCTGACAA-3′); CEBP-CD11c (Box D in [24]) (5′-CCTCGGATCAGTTGCGTACTCTGCC-3′); CEBP-mut-CD11c (Box Dmut4 in [24]) (5′-CCTCGGATCAGGACTAGTCTCTGC-3′).

2.5. Western blotting

Proteins from nuclear extracts (10–15 μg) were subjected to SDS–PAGE under reducing conditions and transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking unoccupied sites with TBS buffer (100 mM NaCl, 10 mM Tris pH 7.4) containing 5% of non-fat dry milk, protein detection was performed using the Amersham ECL protocol according to the manufacturer's instructions. For reprobing, membranes were incubated 2×30 min in stripping buffer (0.2 M glycine, pH 2.2, 0.1% sodium dodecyl sulfate, 1.0% Tween-20) at 50°C, followed by thorough washes with blocking buffer.

3. Results

3.1. A common structure in the proximal regulatory regions of the CD11c, α2 and α5 integrin genes: presence of functional Sp1, AP-1 and C/EBP binding sites

Analysis of the leukocyte integrin gene CD11c has demonstrated the involvement of the AP-1 and C/EBP transcription factor families in its tissue-specific and differentiation-regulated expression [23,24]. To find out whether these factors participate in the transcription of the α2 and α5 integrin genes in keratinocytes we compared the regulatory regions of the CD11c, α2 and α5 integrin genes. Alignment of the three proximal promoter sequences revealed homologous Sp1 binding sites 60–70 nucleotides upstream of the major transcriptional start sites (Fig. 1), two of which (CD11c-Sp1-70 and

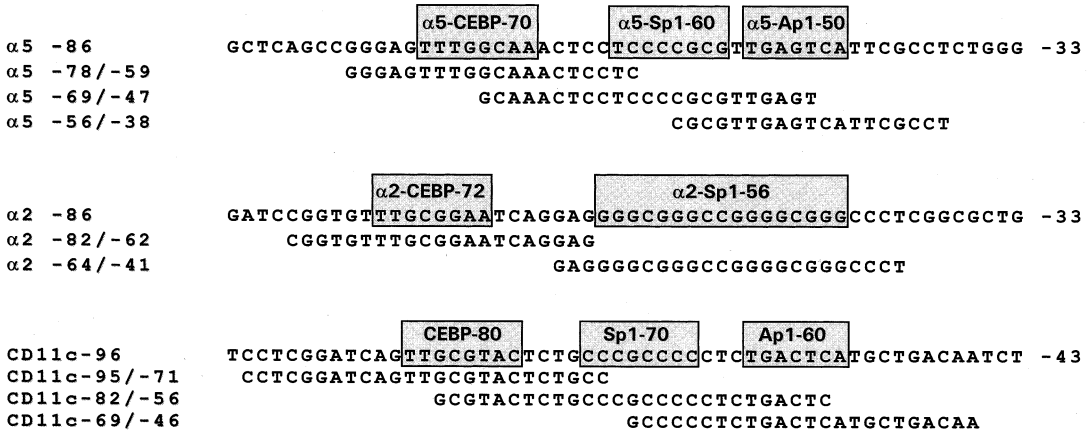

Fig. 1. Alignment of the proximal regulatory regions of the α2, α5 and CD11c integrin genes. Identified and putative *cis*-acting elements are boxed, and their positions are shown relative to their respective major transcriptional start sites. Oligonucleotides used for EMSA are indicated below the respective sequences.

α2-56) are known to be absolutely required for optimal activity of their respective promoters [19–21]. A perfect consensus sequence for AP-1 binding was found at −50 within the α5 promoter (α5-AP1-50), in the same relative location as the AP-1 binding site which controls the differentiation responsiveness of the CD11c promoter [23]. In addition, putative C/EBP binding sites were identified within the α2 (−72) and α5 (−70) promoters, and their location was homologous to that of the previously reported C/EBP binding site in the CD11c promoter [24] (Fig. 1). The putative C/EBP binding site in the α2 promoter very closely resembled the C/EBP binding consensus element (A/GTTGCGT/CAAT/C) [32] (eight identical residues out of 10), while that in the α5 promoter exhibited a lower degree of identity (6/10 nucleotides) (Fig. 1).

^{32}P-labelled oligonucleotides spanning each of the putative *cis*-acting elements were assayed by EMSA. 100-fold molar excess of each corresponding cold oligonucleotide prevented the formation of all the observed complexes, demonstrating the specificity of the assay (Fig. 2 and data not shown). The element at −60 in the α5 promoter (hereafter named α5-Sp1-60) was recognised by Sp1 and Sp1-related factors since the retarded complex was competed by consensus Sp1 binding sequences and by the previously described α2-Sp1-56 and CD11c-Sp1-70 elements [31,34], but not by an oligonucleotide containing a mutated Sp1 site (Fig. 2A). The element at −50 in the α5 promoter (hereafter named α5-AP1-50) was recognised by the AP-1 transcription factor family: formation of the retarded complexes was abrogated in the presence of bona fide AP-1 binding sequences (including the CD11c-60-AP1 element from the CD11c promoter) or anti-c-Jun or anti-c-Fos antisera, but not by a mutated AP-1 site or by anti-JunB (Fig. 2B). The elements at −72 in the α2 promoter and −70 in the α5 promoter (hereafter named α2-CEBP-72 and α5-CEBP-70) were recognised by C/EBP-related factors, as the retarded complexes were inhibited by a consensus C/EBP oligonucleotide or the C/EBP site from the CD11c promoter (CD11c-CEBP-80) [34], but were unaffected in the presence of a mutated C/EBP recognition site or by a mutation of CD11c-CEBP-80 which prevents C/EBP binding (Fig. 2C and data not shown).

We conclude that the proximal promoter regions of the α2, α5 and CD11c integrin genes have a highly conserved structure, with similarly positioned Sp1 and C/EBP binding sites. The α5 and CD11c promoters share, in addition, an AP-1 binding site downstream of the Sp1 element.

3.2. *Recognition of the α2-CEBP-72, α5-AP1-50 and α5-CEBP-70 elements during suspension-induced terminal differentiation*

Suspension-induced terminal differentiation of cultured primary keratinocytes constitutes a very useful model for studying how integrin expression is downregulated during differentiation [29]. After 5 h in suspension, keratinocytes withdraw from the cell cycle and commit to terminal differentiation; at this time surface integrin levels do not decrease but the receptors lose their ligand binding ability [29,33]. After 24 h in suspension transcription of integrin genes is inhibited, integrins are lost from the cell surface and expression of differentiation markers such as involucrin is induced [29,33]. To analyse the functional significance of the Sp1, AP1 and C/EBP integrin promoter elements in keratinocytes, EMSA was performed using nuclear extracts from adherent, proliferating keratinocytes or cells that had been placed in suspension for up to 24 h to induce differentiation.

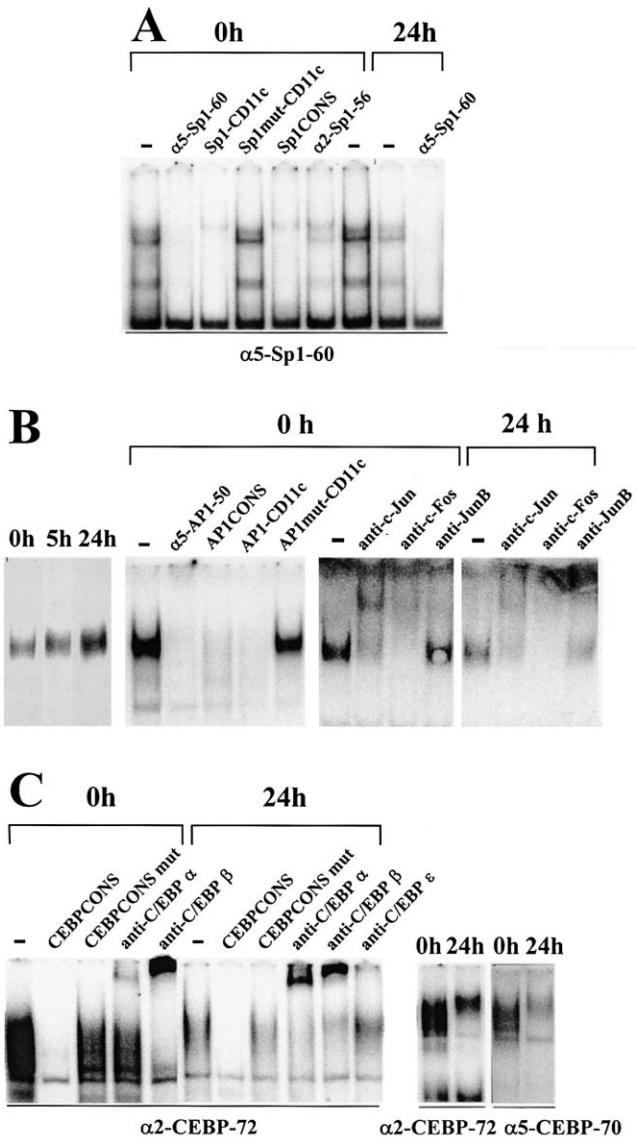

Fig. 2. Specificity and characterisation of EMSA complexes formed on the α5-Sp1-60, α5-AP1-50, α5-CEBP-70 and α2-CEBP-72 elements. Nuclear extracts from primary keratinocytes, either adherent (0 h) or after 5 or 24 h of suspension-induced differentiation, were tested for their capacity to recognise the α5-Sp1-60 (A), α5-AP1-50 (B), α5-CEBP-70 and α2-CEBP-72 (C) elements. The specificity of the interactions was determined in the presence of either a 100-fold molar excess of the indicated competitor oligonucleotides or antisera against members of the C/EBP family (C/EBPα, β and ε) or AP-1 family (c-Fos, c-Jun and JunB).

As shown in Fig. 2A the α5 Sp1 site still gave rise to retarded complexes after 24 h in suspension. The α5-AP1-50 element also gave rise to retarded complexes with nuclear extracts from both adherent (0 h in suspension) and differentiating (5 or 24 h in suspension) keratinocytes. The formation of the retarded complexes on α5-AP1-50 was inhibited by AP-1 consensus sequences or by antisera against c-Jun and c-Fos. We conclude that the α5-AP1-50 element is mainly recognised by c-Jun/c-Fos dimers in keratinocytes and that its occupancy is not altered during suspension-induced differentiation.

EMSA analysis of the α2-CEBP-72 and α5-CEBP-70 elements using keratinocyte nuclear extracts revealed formation of specific retarded complexes which were competed by consensus C/EBP binding oligonucleotides (Fig. 2C), demonstrating that C/EBP-related factors bound both elements. Moreover, comparison of the EMSA pattern produced by nuclear extracts from adherent and differentiating cells showed a change in the mobility of the retarded complexes, a shift which was more obvious in the case of the α2-CEBP-72 site (Fig. 2C). With nuclear extracts from adherent keratinocytes an antiserum against C/EBPβ abrogated the formation of complexes on the α2-CEBP-72 and α5-CEBP-70 elements, while C/EBPα-specific antiserum had no effect (Fig. 2C and data not shown); this indicates that most complexes in adherent keratinocytes were composed of C/EBPβ dimers. In contrast, the retarded bands obtained with nuclear extracts of differentiating keratinocytes were greatly affected by the presence of C/EBPα-specific antiserum, the antiserum to CEBPβ having a somewhat lower effect (Fig. 2C). Antisera against C/EBPδ and C/EBPε had no effect on complex formation in either adherent or suspended keratinocytes (Fig. 2C and data not shown).

We conclude that both α2-CEBP-72 and α5-CEBP-70 are recognised by members of the C/EBP transcription factor family in keratinocytes and that the factors that bind to them are altered during terminal differentiation. Only C/EBPβ was found in retarded complexes from adherent keratinocytes while both C/EBPα and C/EBPβ could be observed in the complexes from differentiating cells. The complexes in differentiating cells could potentially be C/EBPα dimers or C/EBPα-CEBPβ heterodimers.

3.3. Role of the α2-CEBP-72, α5-AP1-50 and α5-CEBP-70 elements in integrin promoter activity

Since expression of the α2 and α5 integrins in keratinocytes is differentiation- and, in the case of α5, activation-dependent we analysed the activity of their corresponding promoters in cultured primary human keratinocytes. α2 and α5 promoter-based plasmids were constructed that included the regions −92/+23 of the α5 promoter or −92/+109 of the α2 promoter in front of a luciferase reporter gene (plasmids A2-92-Luc and A5-92-Luc; Fig. 3A). Transient transfection of the A2-92-Luc and A5-92-Luc reporter constructs showed that both promoters were active in primary human keratinocytes, with activities ranging between 10 and 250 times higher than that of the promoterless plasmid pXP2 (Fig. 3B). The activity of A2-92-Luc was usually 2–5 times higher than that of A5-92-Luc (Fig. 3B). Therefore, the α2 and α5 proximal promoters had transcriptional activity in keratinocytes.

To determine the role of the α2-CEBP-72, α5-AP1-50 and α5-CEBP-70 sequence elements, each one was disrupted by introducing mutations known to prevent recognition by either AP-1 or C/EBP factors. The resulting promoter constructs A2-92(CEBPmut)-Luc, A5-92(AP1mut)-Luc and A5-92(CEBPmut)-Luc were analysed by transient transfection in primary keratinocytes (Fig. 3A,B). Disruption of the α5-AP1-50 element had a profound effect on the activity of the α5 promoter, as it lowered its activity to 15–20% of the wild-type

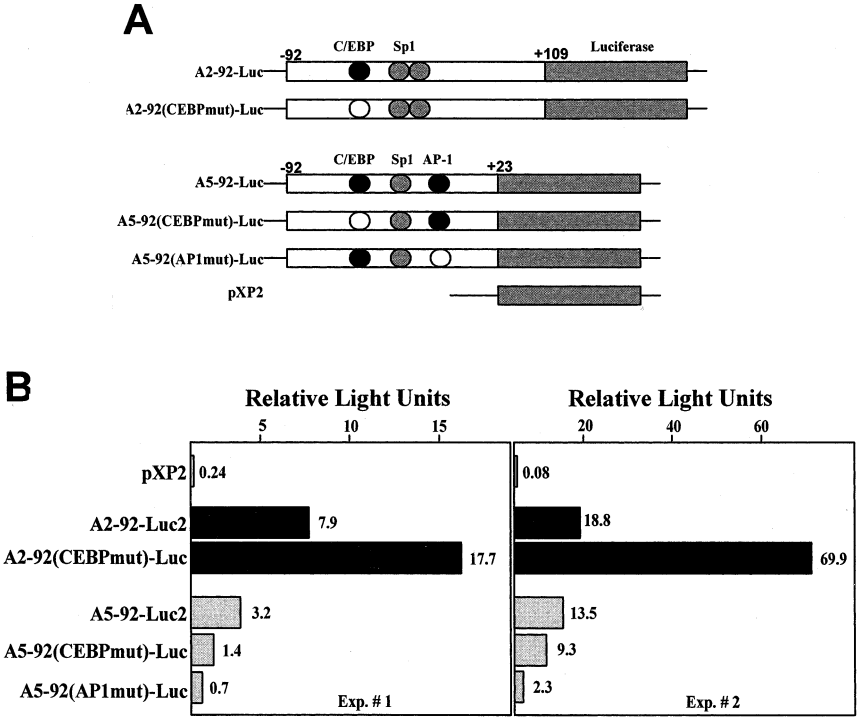

Fig. 3. Activity of the α2 and α5 integrin gene promoter regions in primary human keratinocytes. A: Schematic representation of the wild-type and mutated α2 and α5 integrin promoter constructs. Wild-type *cis*-acting elements are indicated by filled dots, while mutated elements are depicted as empty circles. B: Primary human keratinocytes were transfected with the indicated reporter constructs and luciferase activity determined after 24–30 h. In all experiments, each plasmid was transfected at least five times, with at least two distinct DNA preparations, and transfections were performed in the presence of pCMV-βgal plasmid to normalise the transfection efficiency. Two independent experiments are shown.

values (Fig. 3B), indicating that AP-1 transcription factors positively contribute to transcription of the α5 integrin gene. Mutation of the α5-CEBP-70 site lowered the activity of the α5 promoter between 30 and 60%, with the activity of A5-92(CEBPmut)-Luc being on average 68% of that of A5-92-Luc (Fig. 3B), suggesting that C/EBP factors also contribute positively to transcription of the α5 gene.

Disruption of the α2-CEBP-72 C/EBP binding site caused an increase in the activity of the α2 promoter, which was 2.6-fold on average and in some experiments was as high as 13-fold (Fig. 3B). The C/EBP binding site therefore acted as a negative regulatory element within the α2 promoter. A similar increase was observed after transfection of the mutated α2 promoter in Jurkat lymphoblastic cells and in bovine aortic endothelial cells (data not shown), indicating that the α2-CEBP-72 element decreases the activity of the α2 promoter in distinct cell lineages. Therefore, the C/EBP binding sites within the α2 and α5 proximal regulatory regions mediate opposite transcriptional activities on the respective promoters.

3.4. *Influence of C/EBPα and C/EBPβ overexpression on α2 and α5 integrin promoter activity*

Having demonstrated that the C/EBP binding sites contributed to the activity of the α2 and α5 integrin proximal promoters we investigated the effects of elevated expression of C/EBP factors on promoter activity. Each promoter construct was co-transfected with expression vectors for C/EBPα and C/EBPβ into adherent primary human keratinocytes. Expression of either C/EBPα or C/EBPβ caused a dramatic decrease in the activity of the α2 proximal promoter, reducing activity more than eight-fold (Fig. 4A). The same effect was observed with the A2-92(CEBPmut)-Luc construct, showing that the repressor effect of C/EBP factors was independent of occupancy of the α2-CEBP-72 element. Analysis of the α5 promoter constructs revealed a similar decrease on cotransfection with C/EBPα or C/EBPβ (Fig. 4A). In both integrin promoters, C/EBPα was a more effective repressor than C/EBPβ (Fig. 4A).

To examine how general the transcriptional repression by C/EBP factors was we also transfected the hepatoma cell line HepG2, where C/EBP-dependent transactivation can be readily demonstrated [34,35]. In contrast to their activity in human keratinocytes, the basal activity of the A2-92-Luc or A5-92-Luc constructs was almost undetectable, with luciferase activity values very close to those produced by the promoterless pXP2 plasmid (Fig. 4B). Cotransfection of either C/EBP expression vector led to a marked increase in the activity of both integrin promoters, C/EBPα-mediated transactivation usually being five times higher than that mediated by C/EBPβ (Fig. 4B). C/EBPα and C/EBPβ transactivated the A2-92-Luc construct 60- and 15-fold, respectively (Fig. 4B).

Both C/EBP factors significantly transactivated the α5 promoter constructs in HepG2 cells, albeit to a lower extent than the α2 constructs. C/EBPα cotransfection produced a 20-fold increase in the activity of the proximal α5 promoter, while C/EBPβ augmented the activity three-fold (Fig. 4B). Mutation of α5-CEBP-70 or α2-CEBP-72 decreased C/EBPα- or C/EBPβ-mediated transactivation by 30–40% (Fig. 4B), suggesting the presence of additional elements mediating the C/EBP transcriptional effect. The C/EBP-mediated transactivation of A5-92(AP1mut)-Luc was usually higher than of the wild type or A5-92(CEPmut)-Luc construct, indicating that occupancy of the AP-1 binding site contributes to the effect of C/EBP factors on the α5 promoter.

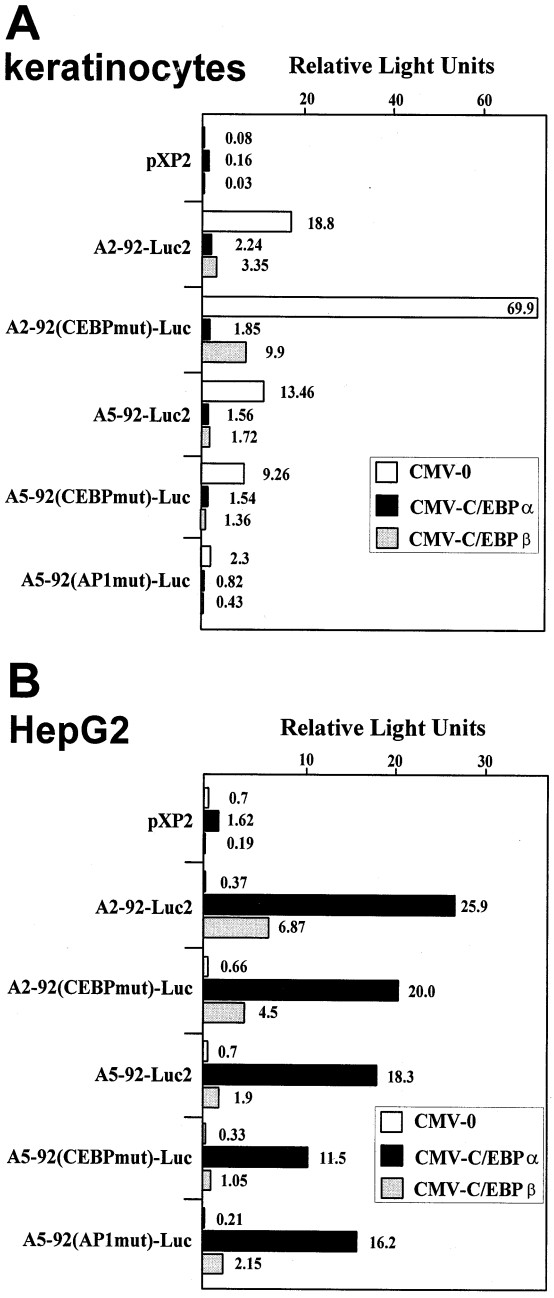

Fig. 4. Contribution of the α5-AP1-50, α5-CEBP-70 and α2-CEBP-72 elements to the activity of the proximal α5 and α2 integrin promoters. Primary human keratinocytes (A) or HepG2 cells (B) were transfected with the indicated reporter constructs in the presence of either an empty vector (CMV-0), or expression vectors for C/EBPα (CMV-C/EBPα) or C/EBPβ (CMV-C/EBPβ), and luciferase activity was determined after 24–30 h. In all experiments each plasmid was transfected at least three times, with two distinct DNA preparations, and transfections were performed in the presence of pCMV-βgal plasmid to normalise the transfection efficiency.

In summary, transactivation experiments demonstrated that C/EBPα and C/EBPβ differentially affected the activity of the α2 and α5 promoters in distinct cell lineages, repressing in human keratinocytes and activating in hepatoma cells. The repressive effects of C/EBP factors in keratinocytes were not mediated by the identified C/EBP binding sites.

3.5. Detection of C/EBPα and C/EBPβ in nuclear extracts of differentiating keratinocytes

The presence of C/EBP factors in the nuclei of cultured primary human keratinocytes and their effects on the proximal regulatory regions of the α2 and α5 integrin genes prompted us to analyse their expression during suspension-induced terminal differentiation, using U937 cells as a positive control. In agreement with the EMSA results (Fig. 2C), Western blotting of nuclear proteins showed that C/EBPβ was highly expressed in the nuclei of adherent keratinocytes and its expression was maintained during suspension-induced differentiation (Fig. 5). C/EBPα was not detected in the nucleus of adherent cells but was present in cells that were undergoing terminal differentiation after 24 h in suspension (Fig. 5). Both the 42 and 30 kDa isoforms of C/EBPα were found in the nuclei of differentiating keratinocytes (Fig. 5). Therefore, expression of C/EBPα was specifically induced during terminal differentiation, in agreement with the detection of C/EBPα-containing EMSA complexes exclusively in differentiated keratinocytes.

4. Discussion

Our results suggest possible mechanisms by which α2 and α5 integrin gene expression is downregulated during epidermal terminal differentiation and expression of the α5 integrin gene is upregulated in hyperproliferative epidermis. The α5 proximal promoter has an AP-1 site that is absent from the α2 promoter and this site contributed positively to α5 promoter activity. We propose that upregulation of AP-1 expression in response to growth factors [36] would contribute to activation of α5 when keratinocytes are stimulated to proliferate, for example in culture or during wound healing. Indeed, serum addition is capable of increasing the activity of the α5 promoter in keratinocytes, and preliminary experiments indicate that EGF also increases α5 integrin promoter activity (data not shown). Conversely, C/EBPα and C/EBPβ negatively regulate the activity of the α2 and α5 integrin promoters in keratinocytes and expression of these transcription factors is upregulated during terminal differentiation in the epidermis [37–39].

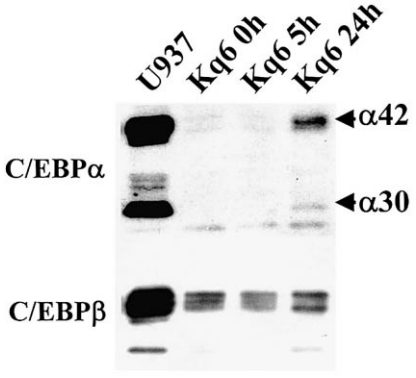

Fig. 5. Expression of C/EBPα and C/EBPβ during suspension-induced differentiation of primary human keratinocytes. Nuclear extracts from myeloid U937 cells and keratinocytes (kq, passage 6) placed in suspension for 0 (kq6 0h), 5 (kq6 5h) or 24 h (kq6 24h) to induce terminal differentiation, were subjected to SDS–PAGE, transferred to Immobilon membranes and incubated with antisera against C/EBPα or C/EBPβ (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The positions of the C/EBPα isoforms (α42 and α30) are indicated.

The position and spacing of the Sp1, AP-1 and C/EBP binding sites within the α5 proximal promoter were identical to the CD11c integrin promoter where a composite element (CESpAP) that includes all three sites mediates the C/EBP transcriptional effect [24]. Although an AP-1 site was lacking in the α2 promoter the Sp1 and C/EBP sites were conserved, as previously reported [20,21]. The almost identical arrangement of *cis*-acting elements within the α2, α5 and CD11c proximal promoters points to the existence of shared mechanisms for coupling integrin transcription to the state of cellular differentiation/activation in distinct cellular backgrounds.

In normal human epidermis both C/EBPα and C/EBPβ are induced during terminal differentiation [37–39]. C/EBPα was also induced during terminal differentiation of cultured keratinocytes in suspension, but functional C/EBPβ was constitutively expressed, showing that C/EBPβ expression is compatible with keratinocyte proliferation. The expression of C/EBPβ can be induced by numerous factors, including growth factors (EGF) and hormones (insulin) [40,41] that are present in keratinocyte culture medium. Overexpression of C/EBP factors can contribute to keratinocyte growth arrest and differentiation; this could be through their capacity to enhance p21 expression and function [42–44], but also through their ability to downregulate integrin expression [1,4].

The proximal α2 and α5 integrin promoters exhibited some cell type-specific activity, as shown by the functional analysis in distinct cell types (HepG2 and keratinocytes). Both regulatory regions were active in keratinocytes while their activity was virtually undetectable in hepatoma cells. Overexpression of either C/EBPα or C/EBPβ transactivated the promoters in HepG2 cells, but repressed promoter activity in keratinocytes. The repressive effect of overexpressing C/EBPα or C/EBPβ in keratinocytes did not depend on the C/EBP sites in either promoter. The influence of C/EBP factors on the integrin promoters resembles the cell type-dependent activity that C/EBPβ has on the upstream regulatory region (URR) of human papillomavirus type 18 [45,46]. C/EBPβ transactivates the URR in HepG2 cells, while it represses its activity in epithelial (HeLa) cells in a DNA binding-independent manner through its interactions with the YY-1 transcription factor. Since YY-1 is an initiator binding protein [47] and integrin promoters exhibit initiator-like sequences at their major transcriptional start sites [10,13], it is tempting to speculate that a similar mechanism could also exist in keratinocytes.

Given the broad range of transcription factors known to physically interact with C/EBP factors (Sp1, AML1, c-Myb, NF-κB, c-Jun or E47 [48–53]), C/EBP-mediated repression of the integrin promoters in keratinocytes might also be exerted by interfering with the functional activity of transcription factors whose binding sites are adjacent to those of C/EBP. The presence of the AP-1 site in the α5 promoter might thus explain why although mutation of the C/EBP site in the α2 promoter led to a large increase in promoter activity, this was not the case when the same site in the α5 promoter was mutated.

Previous studies have suggested a link between adhesion to extracellular matrix and the expression of the distinct C/EBP proteins. In adipocytes, adhesion to different extracellular surfaces affects the pattern of C/EBP factor expression [54], and a C/EBPβ binding site is located within the extracellular matrix-

responsive enhancer in the bovine β-casein gene [55]. When keratinocytes become committed to differentiate the integrins on the cell surface lose ligand binding activity [33] and it is possible that the subsequent detachment of keratinocytes from the underlying basement membrane is the stimulus for induction of C/EBPα and C/EBPβ in vivo; this in turn would lead to downregulation of integrin expression. It will be of interest to test this hypothesis and also to analyse whether C/EBP expression is downregulated in hyperproliferative epidermis, where suprabasal integrin expression is observed [5,9].

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References

[1] Adams, J.C. and Watt, F.M. (1989) Nature 340, 307–309.
[2] Levy, L., Broad, S., Diekmann, D., Evans, R.D. and Watt, F.M. (2000) Mol. Biol. Cell 11, 453–466.
[3] Jones, P.H., Harper, S. and Watt, F.M. (1995) Cell 80, 83–93.
[4] Zhu, A.J., Haase, I. and Watt, F.M. (1999) Proc. Natl. Acad. Sci. USA 96, 6728–6733.
[5] Watt, F.M. and Hertle, M.D. (1994) in: The Keratinocyte Handbook (Leigh, I.M., Lane, E.B. and Watt, F.M., Eds.), pp. 153–164, Cambridge University Press, Cambridge.
[6] Clark, R.A.F. (1990) J. Invest. Dermatol. 94, 128S–134S.
[7] Pellegrini, G., De Luca, M., Orecchia, G., Balzac, F., Cremona, O., Savoia, P., Cancedda, R. and Marchisio, P.C. (1992) J. Clin. Invest. 89, 1783–1795.
[8] Haapasalmi, K., Zhang, K., Tonnensen, M., Olerud, J., Sheppard, D., Salo, T., Kramer, R., Clark, R.A., Uitto, V.J. and Larjava, H. (1996) J. Invest. Dermatol. 106, 42–48.
[9] Carroll, J.M., Romero, M.R. and Watt, F.M. (1995) Cell 83, 957–968.
[10] Birkenmeier, T.M., McQuillan, J.J., Boedeker, E.D., Argraves, W.S., Ruoslahti, E. and Dean, D.C. (1991) J. Biol. Chem. 266, 20544–20549.
[11] Donahue, J.P., Sugg, N. and Hawiger, J. (1994) Biochim. Biophys. Acta 1219, 228–232.
[12] Lin, C.S., Chen, Y., Huynh, T. and Kramer, R. (1997) DNA Cell Biol. 16, 929–937.
[13] López-Cabrera, M., Nueda, A., Vara, A., Garcia-Aguilar, J., Tugores, A. and Corbi, A.L. (1993) J. Biol. Chem. 268, 1187–1193.
[14] Obata, H., Hayashi, K., Nishida, W., Momiyama, T., Uchida, A., Ochi, T. and Sobue, K. (1997) J. Biol. Chem. 272, 26643–26651.
[15] Ziober, B.L. and Kramer, R.H. (1996) J. Biol. Chem. 271, 22915–22922.
[16] Zutter, M.M., Santoro, S.A., Painter, A.S., Tsung, Y.L. and Gafford, A. (1994) J. Biol. Chem. 269, 463–469.
[17] Kim, L.T. and Yamada, K.M. (1997) Proc. Soc. Exp. Biol. Med. 214, 123–131.
[18] Chen, H.M., Pahl, H.L., Scheibe, R.J., Zhang, D.E. and Tenen, D.G. (1993) J. Biol. Chem. 268, 8230–8239.
[19] López-Rodriguez, C., Chen, H., Tenen, D.G. and Corbi, A.L. (1995) Eur. J. Immunol. 25, 3496–3503.
[20] Ye, J., Xu, R.H., Taylor-Papadimitriou, J. and Pitha, P.M. (1996) Mol. Cell. Biol. 16, 6178–6189.
[21] Zutter, M.M., Ryan, E.E. and Painter, A.D. (1997) Blood 90, 678–689.
[22] Pahl, H.L., Scheibe, R.J., Zhang, D.E., Chen, H.M., Galson, D.L., Maki, R.A. and Tenen, D.G. (1993) J. Biol. Chem. 268, 5014–5020.
[23] López-Rodriguez, C., Kluin-Nelemans, H.C. and Corbi, A.L. (1996) J. Immunol. 156, 3780–3787.
[24] López-Rodriguez, C., Botella, L. and Corbi, A.L. (1997) J. Biol. Chem. 272, 29120–29126.
[25] López-Rodriguez, C. and Corbi, A.L. (1997) Eur. J. Immunol. 27, 1843–1847.
[26] Xu, J., Zutter, M.M., Santoro, S.A. and Clark, R.A.F. (1998) J. Cell Biol. 140, 709–719.
[27] Postigo, A.A., Sheppard, A.M., Mucenski, M.L. and Dean, D.C. (1997) EMBO J. 16, 3924–3934.
[28] Watt, F.M. (1998) in: Cell Biology, A Laboratory Handbook (Celis, J.E., Ed.), Vol. 1, pp. 113–118, Cambridge University Press, Cambridge.
[29] Hotchin, N.A. and Watt, F.M. (1992) J. Biol. Chem. 267, 14852–14858.
[30] Nordeen, S.K. (1988) BioTechniques 6, 454–457.
[31] Schreiber, E., Matthias, P., Muller, M.M. and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419.
[32] Osada, S., Yamamoto, H., Nishihara, T. and Imagawa, M. (1996) J. Biol. Chem. 271, 3891–3896.
[33] Adams, J.C. and Watt, F.M. (1990) Cell 63, 425–435.
[34] Trautwein, C., Caelles, C., van der Geer, P., Hunter, T., Karin, M. and Chojkier, M. (1993) Nature 364, 544–547.
[35] Umek, R.M., Friedman, A.D. and McKnight, S.L. (1991) Science 251, 288–292.
[36] Su, B. and Karin, M. (1996) Curr. Opin. Immunol. 8, 402–411.
[37] Maytin, E.V. and Habener, J.F. (1998) J. Invest. Dermatol. 110, 238–246.
[38] Oh, H.S. and Smart, R.C. (1998) J. Invest. Dermatol. 110, 939–945.
[39] Swart, G.W., van Groningen, J.J., van Ruissen, F., Bergers, M. and Schalkwijk, J. (1997) Biol. Chem. 378, 373–379.
[40] Lekstrom-Himes, J. and Xanthopoulos, K.G. (1998) J. Biol. Chem. 273, 28545–28548.
[41] MacDougald, O.A., Cornelius, P., Liu, R. and Lane, M.D. (1995) J. Biol. Chem. 270, 647–654.
[42] Chinery, R., Brockman, J.A., Peeler, M.O., Shyr, Y., Beauchamp, R.D. and Coffey, R.J. (1997) Nature Med. 3, 1233–1241.
[43] Timchenko, N.A., Wilde, M., Nakanishi, M., Smith, J.R. and Darlington, G.J. (1996) Genes Dev. 10, 804–815.
[44] Zhu, S., Oh, H-S., Shim, M., Sterneck, E., Johnson, P.F. and Smart, R.C. (1999) Mol. Cell. Biol. 19, 7181–7190.
[45] Bauknecht, T., See, R.H. and Shi, Y. (1996) J. Virol. 70, 7695–7705.
[46] Bauknecht, T. and Shi, Y. (1998) J. Virol. 72, 2113–2124.
[47] Seto, E., Shi, Y. and Shenk, T. (1991) Nature 354, 241–245.
[48] Lee, Y.H., Williams, S.C., Baer, M., Sterneck, E., Gonzalez, F.J. and Johnson, P.F. (1997) Mol. Cell. Biol. 17, 2038–2047.
[49] Zhang, D.E., Hetherington, C.J., Meyers, S., Rhoades, K.L., Larson, C.J., Chen, H.M., Hiebert, S.W. and Tenen, D.G. (1996) Mol. Cell. Biol. 16, 1231–1240.
[50] Mink, S., Kerber, U. and Klempnauer, K.-H. (1996) Mol. Cell. Biol. 16, 1316–1325.
[51] Ray, A., Hannink, M. and Ray, B.K. (1995) J. Biol. Chem. 270, 7365–7374.
[52] Hsu, W., Kerppola, T.K., Chen, P.-L., Curran, T. and Chen-Kiang, S. (1994) Mol. Cell. Biol. 14, 268–276.
[53] Lu, M., Seufert, J. and Habener, J.F. (1997) J. Biol. Chem. 272, 28349–28359.
[54] Rana, B., Mischoulon, D., Xie, Y., Bucher, N.L. and Farmer, S.R. (1994) Mol. Cell. Biol. 14, 5858–5869.
[55] Myers, C.A., Schmidhauser, C., Mellentin-Michelotti, J., Fragoso, G., Roskelley, C.D., Casperson, G., Mossi, R., Pujuguet, P., Hager, G. and Bissell, M.J. (1998) Mol. Cell. Biol. 18, 2184–2195.