AP-2α: a regulator of EGF receptor signaling and proliferation in skin epidermis

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P-2 transcription factors have been implicated in epidermal biology, but their functional significance has remained elusive. Using conditional knockout technology, we show that AP-2α is essential for governing the balance between growth and differentiation in epidermis. In vivo, epidermis lacking AP-2α exhibits elevated expression of the epidermal growth factor receptor (EGFR) in the differentiating layers, resulting in hyperproliferation when the receptors are activated. Chromatin immunoprecipitation and promoter activity assays identify EGFR as a direct target gene for AP-2α repression, and, in the absence of AP-2α, this is manifested primarily in excessive EGF-dependent phosphoinositol-3 kinase/Akt activity. Together, our findings unveil a hitherto unrecognized repressive role for AP-2α in governing EGFR gene transcription as cells exit the basal layer and withdraw from the cell cycle. These results provide insights into why elevated AP-2α levels are often associated with terminal differentiation and why tumor cells often display reduced AP-2α and elevated EGFR proteins.

Introduction

Mammalian skin epithelium is a self-renewing tissue that constitutes the barrier between an organism and its environment. To provide the organism with this essential function, epidermis must balance proliferation and differentiation (Niemann and Watt, 2002; Dai and Segre, 2004). Its innermost basal layer adheres to an underlying basement membrane rich in ECM. This layer contains proliferative keratinocytes that are typified by their expression of genes encoding integrins and growth factor receptors, particularly EGF receptor (EGFR; also referred to as ErbB1), as well as the structural keratins 5 and 14 (K5 and K14; Fuchs and Raghavan, 2002; Atit et al., 2003). As basal cells move upward, they repress basally expressed genes and switch to expressing a set of differentiation-associated proteins, including keratins K1 and K10. As keratinocytes continue their trek, they further adjust their transcriptional program to culminate in the production of dead, flattened squames that are sloughed from the skin surface as new cells moving outward replace them.

Epidermal homeostasis is under tight transcriptional regulation (Dai and Segre, 2004). Sequence motifs for the binding of the AP-2 family of transcription factors are found in most epidermal promoters and enhancers irrespective of terminal differentiation status (Leask et al., 1990; Byrne et al., 1994; Wang et al., 1997; Zeng et al., 1997; Maytin et al., 1999; Sinha et al., 2000; Kaufman et al., 2002; Luo et al., 2002; Vernimmen et al., 2003). Of the five known murine AP-2 proteins, four are differentially expressed in the skin. Of these, AP-2α is most highly expressed (Byrne et al., 1994; Panteleyev et al., 2003), making it an attractive candidate transcription factor for regulating epidermal-specific transcription.

Although a role for AP-2 factors in epidermal gene expression seems likely, a clear picture as to how they may be involved has not yet emerged. Do AP-2 family members promote or repress proliferation and/or differentiation? Are these effects dependent on the particular AP-2 family member expressed or the relative differentiation stage of the keratinocytes? Often, studies have led to seemingly opposing conclusions. In cultured keratinocytes, for example, AP-2α seems to repress the promoter activity of the basal cell keratin gene K5 (Byrne et al., 1994), but in vivo, AP-2 factors are expressed throughout the epidermis and K5 is restricted to basal cells. In hyperproliferative skin, AP-2 factors are coexpressed with K5 suprabasally (Panteleyev et al., 2003).
Data on the role of AP-2 proteins in other epithelial cells offer little assistance in resolving these issues, where both active and repressive roles for AP-2 proteins have been described (Johnson, 1996; Maytin et al., 1999; Braganca et al., 2003). In mammary carcinoma cell lines, 5′ regulatory sequences for the growth-promoting TGFα and the ErbB subfamily of EGFR genes seem to be positively regulated by AP-2α (Wang et al., 1997), whereas overexpression of AP-2α appears to be growth and proliferation inhibitory (Zhang et al., 2003). Similarly, in breast cancer tissue, enhanced expression of ErbB2 is a frequent occurrence, and yet diminished AP-2α expression has often been cited as a poor prognostic marker for breast cancer survival (Pellikainen et al., 2004; Friedrichs et al., 2005). These tantalizing but often contrasting results underscore the importance of resolving the possible link between AP-2α and epithelial growth.

A major difficulty in evaluating how AP-2 family members orchestrate transcriptional regulation in skin epidermis stems from the disparate results obtained from functional studies across different vertebrate species. In frog embryos, injection of antisense AP-2α oligonucleotides leads to the loss of epidermal character and the gain of neural gene expression (Luo et al., 2002). Similarly, in breast cancer tissue, enhanced expression of ErbB2 is a frequent occurrence, and yet diminished AP-2α expression has often been cited as a poor prognostic marker for breast cancer survival (Pellikainen et al., 2004; Friedrichs et al., 2005). These tantalizing but often contrasting results underscore the importance of resolving the possible link between AP-2α and epithelial growth.

In this study, we use conditional gene targeting of AP-2α to explore the functional significance of AP-2α in postnatal skin development. We show that AP-2α functions in the epidermis by repressing EGFR gene expression as cells exit the basal layer and commit to terminally differentiate. We show that nuclear AP-2α is present normally in some basal and many suprabasal epidermal cells and that it is essential for governing the EGFR-mediated control of epidermal cell proliferation. Other AP-2 family members do not appear to compensate in the suprabasal differentiating epidermal layers, where EGFR expression fails to switch off in the absence of AP-2α. Upon growth factor signaling and EGFR activation, AP-2α–null epidermis displays hyperproliferation and formation of papilloma-like invaginations accompanied by abnormal suprabasal elevation of activated Akt. Finally, we show that, mechanistically, the EGFR promoter possesses AP-2–binding sites that are occupied by AP-2α and that transcriptionally temper receptor gene expression. In vitro, loss of AP-2α elevates EGFR gene transcription, and regulatory circuitries for the phosphoinositol-3 kinase (PI3K), Akt, and MAPK fail to function properly. These findings have major implications for understanding why reductions in AP-2 expression have been associated with tumorigenesis and cancer.

Figure 1. Targeted ablation of AP-2α gene expression in mouse epidermis. K14-Cre:AP-2αlox/lox conditional knockout (cKO) mice were generated and compared with their control wild-type (WT) littermates. (A) PCR genotype analysis. Bands were of the expected sizes. (B) Real-time PCR on P0 epidermal mRNAs. Primers are specific for the five known AP-2 family members. Error bars represent SD. (C) Immunoblot analyses of P0 epidermal extracts probed with antibodies to AP-2α and AP-2γ, the two most abundantly expressed members. (D) Immunofluorescence microscopy. Color coding is for secondary antibodies used in detection. Nuclei are counterstained with DAPI. Note that in WT skin, the basal epidermal layer displayed some cells with strong anti–AP-2α labeling (white arrows) and others with weaker labeling (yellow arrows). In the inner spinous layers, cells were typically strongly labeled. Anti-AP2α labeling was uniformly absent in cKO skin. De, dermis; epi, epidermis; hf, hair follicle. Dotted white lines denote dermo–epidermal boundaries. Solid white lines denote skin surface. Bars, 20 μm.
Results

Conditional ablation of the AP-2α gene in mouse skin epidermis

Recently, mice were genetically engineered so that essential coding sequences of the AP-2α gene were flanked by loxP sites (AP-2αlox/lox; Brewer et al., 2004). To elucidate the role of AP-2α in postnatal skin epidermis, we bred these animals to mice harboring a K14-Cre recombinase transgene, which efficiently expresses Cre throughout epidermis by embryonic day (E) 15.5 (Vasioukhin et al., 1999). The heterozygous K14-Cre:AP-2αlox/+ line was phenotypically indistinguishable from wild type (WT). Offspring generated from matings of K14-Cre:AP-2αlox/+ lines were produced at expected Mendelian ratios of genotypes according to PCR analyses of genomic DNA (Fig. 1 A).

Expression of other AP-2 family members in epidermis is not affected by loss of AP-2α

Microarray analyses revealed the presence of AP-2α, AP-2ε, and AP-2γ mRNAs in E18.5 skin epidermis, whereas AP-2δ is primarily in dermis (unpublished data; for expression analyses, see Williams et al., 1988; Byrne et al., 1994; Panteleyev et al., 2003). We corroborated and extended these results by conducting real-time PCR on mRNAs isolated from WT epidermis that was purified from skin by dispase treatment. Expression of AP-2α and AP-2γ mRNAs was particularly high (Fig. 1 B). Similar analyses on K14-Cre:AP-2αlox/+ showed that the signal for AP-2α’s Mfloxed coding exon was abolished, verifying the efficacy of the targeting event. Importantly, the absence of AP-2α did not appreciably affect the expression of any of the other AP-2 family members, indicating a failure to compensate at the level of gene expression (Fig. 1 B).

Immunoblot analyses confirmed the expression of AP-2α and AP-2γ in WT skin epidermis and verified that the AP-2α targeting event resulted in the loss of AP-2α protein production and also underscored the specificity of the AP-2α antibody (Fig. 1 C). By immunofluorescence, anti-AP2α labeled basal and suprabasal nuclei of WT cells, whereas anti-AP2γ preferentially labeled basal nuclei (Fig. 1 D and not depicted). Consistent with our protein studies, anti-AP2α staining was absent in K14-Cre:AP-2αlox/+ skin, and anti-AP2γ staining was unchanged. Hereafter, we refer to the K14-Cre:AP-2αlox/+ mice as conditional knockout (KO [cKO]).

Morphological changes associated with conditional ablation of AP-2α in epidermis

In agreement with and expected from previous reports of the full KO of AP-2α in mice, the skin surface of newborn cKO pups appeared similar to that of their WT littermates (not depicted; Schorle et al., 1996; Zhang et al., 1996; Talbot et al., 1999). Upon histological inspection, however, cKO skin displayed a thickened interfollicular epidermis (Fig. 2 A). Despite the complete absence of AP-2α, the thickening was not uniform across the postnatal day (P) 0–3 epidermis and began to wane altogether by P6. This aberration had not been noted previously in the straight AP-2α KO animals that died at birth. Whether this is attributable to the relatively mild nature of the defects or to strain-related differences in the mice used for the two studies (Talbot et al., 1999) was not addressed.
Our conditional targeting strategy enabled us to examine, for the first time, the consequences of AP-2α loss to postnatal skin development. As the animals aged, AP-2α cKO adult mice progressively lost their hair in select regions of their coats. The loss of hair was most pronounced on the ventral thoracic surface, where a large area of skin always became bald (Fig. 2 B). Histological analyses revealed that in contrast to the typically thin epidermis of adult WT animals, the AP-2α cKO animals displayed hyperthickened, papilloma-like undulations throughout these expansive areas (Fig. 2 C). As judged by light and electron microscopy, these undulations displayed epidermal rather than hair follicle morphology. In addition to thoracic skin epithelium, dorsal ear epidermis was also markedly affected, typified by both hyperthickening and parakeratosis in cKO skin (Fig. 2 D).

Classical signs of a hyperproliferative skin disorder in AP-2α cKO mice

To examine the consequences of AP-2α ablation on epidermal differentiation, we first used immunofluorescence microscopy (Fig. 3). In regions where the AP-2α–null epidermis appeared morphologically normal, differentiation markers were expressed in patterns indistinguishable from WT skin. These included basal keratins K5 and K14, the spinous layer keratins K1 and K10, and granular layer proteins filaggrin and loricrin (Fig. 3 A and not depicted; for review see Fuchs and Raghavan, 2002). In hyperthickened regions, the classical changes in gene expression associated with the hyperproliferative state were observed (Weiss et al., 1984), including sustained expression of keratins K5 and K14 in the suprabasal layers and induction of the outer root sheath keratins K6, K16, and K17 in the suprabasal layers of the epidermis (Fig. 3, A and B; and not depicted). These abnormalities were accompanied by an approximately two- to threefold increase in proliferation in the basal layer as judged by BrdU incorporation (Fig. 3 C) and by labeling with antibodies against the proliferating nuclear antigen Ki67 (not depicted).

The biochemical abnormalities noted in hyperthickened neonatal regions were enhanced in the adult papilloma-like invagination (Fig. 3 D). In addition, the papilloma-like regions displayed some suprabasal proliferating cells as judged by staining for Ki67, which is nuclear in cycling cells (Fig. 3 D, top; arrows). These data are suggestive of a perturbation in the mechanism that normally restricts dividing cells to the basal layer. In contrast, we observed no obvious change in apoptosis, as indicated by either histology or antibody staining against the activated form of caspase 3. The caspase 3–positive cells (Fig. 3 D, bottom; arrows) in catagen-phase hair follicles undergoing cyclic apoptosis provided a nice internal control for these stainings.

Additional changes underlying the hyperproliferative defects in AP-2α conditionally null mice

Because the alterations we observed in differentiation patterns are classical features of hyperproliferation, they could represent a reflection of an abnormality in the proliferative machinery of the epidermis. Alternatively, if these changes were a primary consequence of a loss of AP-2α, it is possible that the alterations in differentiation could perturb the epidermal barrier, which is a process known to result in indirect
Figure 4. Intact epidermal barrier but elevated EGF expression and signaling in hyperproliferative AP-2α-null skin. [A] Barrier function assay. Newborn litters were placed in blue dye to test for the presence of an intact epidermal barrier. Note tail cut for genotyping and umbilicus show areas of blue staining, indicating dye penetration in areas where there is loss of barrier. [B] Semiquantitative RT-PCR of neonatal epidermal mRNAs. Epidermis was separated from the rest of the skin (including hair follicles) by enzymatic treatment with dispase. After mRNA isolation, semiquantitative RT-PCR was conducted using primer sets specific for EGFR (test) and GAPDH (control) mRNAs. All bands were of the expected size and were generated only in the presence of reverse transcriptase (RT). [C] Anti-EGFR and antiphospho-EGFR immunoblot analyses of epidermal proteins isolated from neonatal mice in which the cKO epidermis is hyperproliferative. Anti-tubulin is used as a control. [D] EGFR immunofluorescence of sections of hyperproliferative cKO skins and WT counterparts from P0 mice. Inset shows a high magnification confocal image of the colocalization of phospho-EGFR and E-cadherin at the membrane. Arrows denote suprabasal phospho-EGFR staining. [E] Same as in D, but on adult thoracic skins. Epi, epidermis; de, dermis; hf, hair follicle. Dotted lines denote dermo-epidermal borders. Solid white lines denote skin surface. [F] Same as in C, but on adult epidermis. Normalizations are to anti-tubulin. Bars, 20 μm.

Members of the EGF and insulin growth factor (IGF) families are particularly important in regulating proliferation in the epidermis (for review see Fuchs and Raghavan, 2002). Of these, TGFα and EGFR genes were particularly interesting in that they both harbor AP-2α-binding sites in their promoters. Additionally, constitutive expression of TGFα in transgenic mice results in a transient epidermal hyperproliferation that wanes in the adult and can reemerge after wounding and/or mechanical stress (Vassar and Fuchs, 1991; Dominey et al., 1993), whereas overexpression of constitutively active members of the receptor family can lead to papilloma undulations throughout the skin (Xie et al., 1999). Finally, EGF injection in sheep skin results in concomitant hair loss and epidermal thickening (Moore et al., 1985), whereas EGFR down-regulation has been associated with hair follicle formation (Atit et al., 2003). Collectively, although all prior gene studies have argued for a positive rather than a negative effect of AP-2α on EGF signaling pathway genes (Johnson, 1996; Wang et al., 1997; Vernimmen et al., 2003; Begon et al., 2005), the significant physiological parallels prompted us to focus on the status of genes involved in EGF signaling in our cKO animals.

We first performed semiquantitative RT-PCR on mRNAs from purified hyperproliferative AP-2α neonatal cKO and WT epidermis. By this criteria, EGFR mRNA levels were significantly elevated (Fig. 4 B). Immunoblot analysis showed that the increase in EGFR mRNA expression was reflected at the protein level (Fig. 4 C). Immunofluorescence microscopy further revealed sustained expression of EGFR in the suprabasal layers of AP-2α-null epidermis (Fig. 4 D). This difference was evident by P0, making it an early consequence of the AP-2α targeting event. Furthermore, the increase in epidermal thickening was associated with the phosphorylated (i.e., activated) form of the receptor.
Figure 5. EGFR levels are elevated in nonproliferative regions of adult AP-2α–null skin, but EGFR signaling is silent unless externally stimulated. (A) H&E and immunofluorescence of sections of nonproliferative cKO skins and WT counterparts from P6 mice. Antibodies are against EGFR, phosphorylated (active) EGFR, and K6. Note elevated EGFR but not phospho-EGFR or K6 in P6 cKO skin. (B) H&E and anti-EGFR/antiphospho-EGFR immunofluorescence of sections of nonproliferative cKO and WT adult back skins. (C) Immunoblot analyses of epidermal proteins isolated from adult back skin from WT and AP-2α conditionally null mice. Blots were probed with antibodies against the proteins indicated at right. Note elevated EGFR but not phospho-EGFR, which is normally weak in thin adult skin. (D) mRNAs were isolated from WT and cKO adult back skin or neonatal epidermis and subjected to real-time PCR using a primer set specific for TGFα mRNA. Controls shown are for the reaction involving the primer set alone in the absence of added mRNA. Error bars represent SD. (E) Back skins of adult AP-2α–null and control littermates were treated with TPA, a procedure known to elevate the expression of EGFR ligands (Kiguchi et al., 1998). After treatment, skins were processed for H&E and EGFR/phospho-EGFR immunofluorescence microscopy. No papillomas were observed even after 4 mo of treatment. Epidermal thickening was accompanied by EGFR activation, both of which were more robust in the TPA-treated cKO animals. Epi, epidermis; de, dermis; hf, hair follicle. Dotted lines denote dermo–epidermal borders. Bars, 20 μm.

The epidermis of adult mice is typically thin, and its proliferative activity is low. Correspondingly, it was not surprising to see a single layer of anti-EGFR labeling with very little phospho-EGFR labeling in WT adult skin (Fig. 4 E). In contrast, the lesional regions of AP-2α–null epidermis displayed elevated levels of not only total but also autophosphorylated EGFR. The AP-2α null–associated up-regulation in total EGFR was confirmed by immunoblot analyses, as was the elevated levels of activated EGFR in lesional regions of the cKO mice (Fig. 4 F).

EGFR levels are elevated in adult AP-2α–null back skin, which is not hyperproliferative

A priori, the observed elevation in EGFR and/or signaling could be caused either directly by the loss of AP-2α or indirectly by the enhanced proliferation that occurs concomitantly with the absence of AP-2α. To distinguish between these possibilities, we first examined P6 AP-2α–null and WT back skin (Fig. 5 A). Despite the similar morphologies and thickness of WT and AP-2α–null epidermis at this age (Fig. 2 A), EGFR was still elevated in the absence of AP-2α. Interestingly, however, antibodies against the phosphorylated form of EGFR showed no difference between WT and cKO skin, suggesting that the EGFR was not active. Further consistent with the lack of EGFR signaling and hyperproliferation in these regions was the lack of anti-K6 staining in the epidermis.

Similarly, in adult back skin, the AP-2α–null epidermis appeared largely normal (Fig. 5 B). By immunofluorescence analysis, however, the EGFR levels were clearly elevated.
In contrast to hyperproliferative AP-2α cKO thoracic skin, the levels of tyrosine-phosphorylated EGFR remained low. This was confirmed by immunoblot analyses (Fig. 5 C).

Based upon these data, two important findings emerged. First, AP-2α loss resulted in increased EGFR expression. Second, the hyperproliferative phenotype correlated with the activation of these EGFR tyrosine kinases, a process which is typically dependent on ligand. TGFα is both a ligand for EGFR and a potent growth factor of epidermal keratinocytes in vitro (Barrandon and Green, 1987). In nonlesional adult back skin, TGFα mRNAs were extremely low in both WT and cKO mice (Fig. 5 D). In contrast, in neonatal (P2) epidermis, where EGFR activity and proliferation were detected in both WT and cKO mice, TGFα mRNAs were readily detectable (Fig. 5 D). The levels were slightly higher in cKO relative to WT P2 epidermis, and this and/or other EGF signaling molecules could enhance the effects of elevated EGFR in neonatal cKO mice. Altogether, the patterns of TGFα, EGFR, and EGFR activation were consistent with the phenotypic effects observed.

To further explore these parallels, we took advantage of the previous observation that topical application of 12-O-tetradecanoylphorbol-13-acetate (TPA) causes elevation of TGFα, leading to activation of EGFR in adult skin (Kiguchi et al., 1998). If, as we surmise, the epidermal hyperproliferation seen in lesional AP-2α-null skin is at least in part caused by regional activation of elevated EGFRs, nonlesional AP-2α-null back skin might be expected to show elevated thickening upon TPA treatment.

At the doses of TPA administered in this study, TPA treatment had only a modest effect on the back skin of control adult mice. In contrast, these TPA treatments caused hyperthickening of the AP-2α-null back skin accompanied by activation of EGFRs (Fig. 5 E). These morphological aberrations were accompanied by an increase in proliferation and biochemical alterations that are typical of a hyperproliferative state (not depicted). Even after 4 mo of treatment, however, no signs of tumor formation or progression were observed, indicating that AP-2α loss alone was not sufficient to cause skin tumorigenesis.

EGFR is a direct target of AP-2α

To further probe the underlying mechanisms responsible for the up-regulation of EGFR in the absence of AP-2α, we cultured primary keratinocytes from neonatal WT and cKO mouse skins. To evaluate the data, it was first essential to examine the expression of AP-2α and AP-2γ in vitro to see whether the preferential suprabasal expression of AP-2α was recapitulated in culture, as we had observed in epidermis (Fig. 1). To examine this, we induced terminal differentiation by elevating the calcium levels in the medium and then performed immunoblot analyses on protein extracts from low- and high-calcium exposed cells. As shown in Fig. 6 A, within 24 h of the calcium...
shift, the expression of the spinous layer markers K1 and involucrin were elevated, although appreciable involucrin mRNA was present even in the low-calcium state. Within the same time frame, AP-2α was largely unaffected by calcium treatment relative to internal protein loading standards. In contrast, AP-2γ was markedly decreased upon calcium-induced differentiation. Moreover, the levels of AP-2γ were not appreciably changed in the AP-2α–null cells (Fig. 6 A). Together, these data were consistent with our prior in vivo data presented in Fig. 1.

Next, we conducted immunoblot studies in which we could examine EGFR levels under conditions where we could more rigorously control for microenvironment and cell numbers. As shown in Fig. 6 B, EGFR levels were elevated in the AP-2α–null keratinocytes relative to their WT counterpart. Under enriched culture conditions, both cell populations displayed phosphorylated EGFR (not depicted). These changes in AP-2α–null keratinocytes were also reflected at the mRNA level (Fig. 6 B). Real-time PCR showed a fourfold increase in EGFR mRNAs in KO versus WT cells. TGFα mRNA levels were only modestly elevated in the AP-2α–null state (not depicted).

If AP-2 acts to repress EGFR gene transcription, it should bind to the endogenous EGFR promoter. Previous studies have shown that recombinant AP-2α can bind to an AP-2 consensus motif in the human EGFR promoter (Johnson, 1996; Oyama et al., 2002). In the mouse EGFR promoter, multiple AP-2 consensus binding sites are situated within a kilobase upstream from the transcription initiation site (Fig. 6 C, ovals). Two of these sites are conserved across mammalian species (Fig. 6 C, ovals highlighted in red). To evaluate whether endogenous AP-2α binds directly to one or more of these sites in epidermal cells, we conducted chromatin immunoprecipitation (ChIP) assays using the nonspecific anti–AP-2α antibody. Anti–AP-2α antibodies specifically immunoprecipitated chromatin–protein complexes that contained an ~500-bp DNA fragment encompassing the conserved putative AP-2α–binding sites (Fig. 6 C). In contrast, ChIP analysis of WT skin did not display PCR bands with primers corresponding to either the EGFR promoter or downstream regions that did not contain AP-2–binding motifs (not depicted). These data provided the first ChIP data illustrating the binding of AP-2α to an endogenous gene that contains AP-2 consensus binding motifs.

To test whether EGFR gene transcription is affected by the loss of AP-2α, we transfected Ca²⁺–grown keratinocytes with a luciferase reporter gene driven by a 1.1-kb EGFR promoter fragment harboring the AP-2α–binding sites. By 48 h, luciferase activity was markedly elevated in AP-2α–null versus WT cells (Fig. 6 D). Point mutations in the AP-2α motifs raised EGFR promoter activity levels in WT but did not affect AP-2α–null cells. Finally, we infected WT and KO keratinocytes with a retroviral AP-2α expression vector and repeated the EGFR reporter assay. Exogenous expression of AP-2α resulted in a potent repression of luciferase activity driven by the WT EGFR promoter but not its mutant counterpart (Fig. 6 D). These data underscore the AP-2–specific nature of the manipulations and the repressive effects on EGFR transcription. These findings further suggest that AP-2α functions in suppressing EGFR gene expression as epidermal cells commit to terminally differentiate.

Abnormal EGFR signaling circuits in AP-2α–null skin

To evaluate how downstream signaling events may be affected when EGFR levels are elevated, we first examined the proliferative potential of primary AP-2α–null and WT epidermal keratinocytes. Both populations grew equally well when plated at high density on a fibroblast feeder layer and in rich growth media (unpublished data). However, when subjected to more stringent conditions such as sparse plating with no feeder layer for long term growth (>5 d) or low serum/growth factor–supplemented medium, only KO keratinocyte cultures grew well (representative examples in Fig. 7 A). Moreover, the growth advantage of the KO keratinocytes was largely eliminated by the addition of AG1478, an EGFR-specific protein kinase inhibitor, to the culture medium, suggesting that increased EGFR signaling might be responsible for the enhanced proliferative potential observed in KO keratinocytes.

EGFR stimulation typically leads to downstream activation of the MAPK family members Erk1/2, but it can also lead to activation of Akt kinases. Both are required for epidermal growth and differentiation (Schlessinger, 2000; Sibilia et al., 2000). To determine whether the loss of AP-2α and elevated EGFR signaling results in an elevated activation of MAPK (Erk1/2) and/or Akt pathways, we stimulated 24-h serum-starved cells with EGF. Within a minute after stimulation, the two populations activated Erk1/2 with similar kinetics (Fig. 7 B). Relative to total Erk1/2, the phosphorylated (i.e., activated) Erk1/2 signals were always higher in the KO keratinocytes. This was visualized over a range of EGF concentrations (Fig. 7 C). Although AP-2α–null keratinocytes activated more Erk1/2 in response to EGFR ligands, the most significant difference observed was in EGFR-mediated Akt activation. Again, the first signs of activation appeared within a minute after exposure to EGF. However, relative to equivalent levels of total Akt, phosphorylated (i.e., activated) Akt was nearly an order of magnitude higher in KO cells across a range of EGF concentrations.

Remarkably, as little as 0.5 ng/ml EGF was sufficient to elicit these differences in Erk1/2 and Akt activation. Curiously, the differential effects on Akt appeared to be specific for EGFR signaling, whereas the differential activation of Erk1/2 was also seen with IGF-1, another potent stimulator of keratinocyte growth and survival (Fig. 7 D). The effects were obliterated by treatment with the PI3K–specific inhibitor LY294002, indicating that Akt activation in EGF-treated keratinocytes was mediated through PI3K activation (Fig. 7 E).

To test the physiological relevance of these findings, we examined the status of activated Akt in our mice. In nonlesional regions of AP-2α–null skin where EGFRs were elevated but not activated, phospho-Akt levels remained low and comparable with WT skin (Fig. 7 F and not depicted). However, in either TPA-treated or thoracic cKO skin, activated Akt was markedly elevated. Even in WT skin, TPA treatment resulted in mildly up-regulated levels of activated Akt, which is consistent with the modest epidermal thickening we noted previously (Fig. 5 D). Based upon these criteria, the mechanisms uncovered in our in vitro analyses appeared to be operative in vivo.
Discussion

The AP-2α and EGFR connection and its relevance to growth control in the epidermis

AP-2-binding sites have been found in a myriad of genes differentially expressed in both basal and suprabasal compartments of mammalian epidermis (Hilger-Eversheim et al., 2000; for review see Fuchs and Raghavan, 2002). However, to date, their functional significance has remained elusive. In this study, we have shown that AP-2α plays a role in the switch between epidermal cell proliferation and differentiation, and we have provided the first experimental evidence to integrate this transcription factor into a key signaling pathway in the skin. The finding is especially important given that some studies have highlighted AP-2α as a tumor suppressor (Ropponen et al., 2001; Pellikainen et al., 2004; Friedrichs et al., 2005), whereas others have suggested a growth-promoting effect of the protein (Bosher et al., 1996; Vernimmen et al., 2003). Our study clarifies how AP-2α governs epidermal proliferation and provides important new insights into why inverse correlations are often found between AP-2α and EGFR family members in human epithelial cancers.

Our studies place AP-2α in the EGFR signal transduction cascade. Using in vivo and in vitro approaches, we found that EGFR gene expression was elevated upon genetic ablation of AP-2α or by mutation of the AP-2–binding sites in the EGFR promoter. Correspondingly, EGFR gene expression was down-regulated when AP-2α was added back to the KO cells through retroviral transgene expression. Although AP-2α is likely to have many other target genes in epidermis, several of the phenotypic abnormalities in the AP-2α conditionally null mice can be explained by EGFR misregulation.

EGFRs have long been known to play an important role in regulating the development of the epidermis and its appendages (Luetke et al., 1994; Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). In mammals and birds, overexpression or injection of EGF can arrest epidermal appendage development and promote epidermal thickening, and, conversely, activation of EGFRs is typically diminished in areas of follicle formation (Moore et al., 1985; Kashiwagi et al., 1997; Atit et al., 2003). Postnatally, EGFRs are predominantly expressed in the basal epidermal layer and down-regulated as cells commit to terminally differentiate (King et al., 1990).

Once activated, EGFR has the ability to activate Ras–MAPK signaling, which is often linked to proliferation, as well as PI3K–Akt signaling, which is more typically associated with cell survival (Rodeck et al., 1997; Schlessinger, 2000). Relevant to the data presented here, tumorigenesis can arise from superactivated Ras–MAPK signaling in transgenic mouse skin but only in conjunction with EGFR signaling to activate PI3K–Akt (Sibilia et al., 2000). A key feature of EGFR signaling is that its activation is dependent on ligand stimulation. Made and secreted by salivary glands, EGF can enter the bloodstream and reach epidermal tissue, whereas TGFα is the major autocrine growth factor of the epidermis. TGFα is down-regulated postnatally but can be up-regulated upon injury or TPA treatment (Kiguchi et al., 1998). That postnatal skin is limiting for EGFR ligands is graphically illustrated by the papilloma-like undulations that develop in skin of mice engineered to overexpress an EGFR relative (ErbB2) that lacks the requirement for ligand stimulation (Xie et al., 1999; Kiguchi et al., 2000).

When placed in the context of these prior studies, we can account for many of the features of the seemingly complex phenotype of our AP-2α cKO mice. The hyperproliferation that occurs in cKO newborn epidermis despite elevated EGFR levels suggests that the ligand pool for these receptors must be quite large at this age. The levels appear to be saturating for WT but not cKO EGFR levels, and this is reflected by the observed enhancement of phosphorylation and activation of the EGFRs in newborn cKO skin. Soon after birth, hyperproliferation and phosphorylation of EGFRs returns to normal in cKO despite sustained elevated EGFR levels in cKO skin. After embryogenesis, the ligand pool is known to be attenuated along with other growth-promoting signals, and our results indicate that these pool levels have dipped below saturation even for WT EGFRs. Finally, the markedly hyperproliferative epidermis and phosphorylation of EGFRs in lesional but not nonlesional regions of adult cKO thoracic and ear skin and TPA-treated cKO back skin is reflective of the elevated ligand pools that are known to arise upon mechanical irritation and TPA treatment. Additionally, when animals lick their wounds, the EGF-rich saliva could further contribute to the robust proliferative response in the ventral chin area.

An additional point worthy of mention is that the AP-2α-null papilloma-like undulations were not as severe as the spontaneous skin tumors that arise from chemical or transgenic mutations in epidermally expressed Ha-Ras. We surmise that the underlying reason for this may be rooted in the normal feedback regulatory loops that are operative in dampening the deleterious effects of overly active EGFR signaling. Indeed, for sustained epidermal hyperproliferation and tumorigenesis to occur on a background of Ha-Ras mutations, sustained EGFR signaling must still be permitted to activate and sustain elevated PI3K–Akt signaling (Sibilia et al., 2000).

Our data shed additional insight onto this signaling circuitry by demonstrating that the EGFR response to Akt activation is different than that of IGF receptor in epidermal cells and that in situations in which EGFR signaling is hyperactive, Akt activation can be very high and suprabasal proliferation can occur. Although Akt activation is typically associated with cell survival, several examples that link PI3K–Akt activation to proliferation may be relevant to the findings we report. Sustained PI3K–Akt activation also occurs in liver tumors, where it posttranslationally silences the C/EBPα transcriptional repressor that controls hepatocyte proliferation (Wang et al., 2004). Intriguingly, C/EBPα has also been implicated in epidermal differentiation and is repressed in some skin cancers (Shim et al., 2005). Although beyond the scope of this study, such a mechanism could explain why AP-2α null–mediated hyperactivation of the EGFR–PI3K–Akt pathway leads to an increase in proliferation and epidermal thickening in vivo.

AP-2 and transcriptional regulation in skin

After 15 yr since the original implication of AP-2 in transcriptional regulation in the epidermis (Leask et al., 1990; Snape
Figure 7. **In vitro and in vivo superactivation of the PI3K–Akt pathway in an EGF-dependent and AP-2α null-dependent fashion.** (A) Primary WT and KO keratinocytes were cultured ± the EGFR-specific protein kinase inhibitor AG1478 without the addition of fibroblast feeder cells after the original plating. Growth curves represents three independent experiments performed in duplicate (see Separation of epidermal...transfections). Error bars represent SD for these experiments. (B) Keratinocytes were serum-starved for 24 h and at t = 0, 50 ng/ml EGF was added. At the times indicated, cell extracts were prepared, proteins were resolved by SDS-PAGE, and immunoblot analyses were conducted with the antibodies indicated at right. The antibodies against the active forms of Erk1/2 and Akt (p-Erk and p-Akt, respectively) are phosphospecific antibodies and do not recognize the inactive states of the kinases. β-tubulin antibodies are used as a loading control, along with ponseau red staining of the blots (not depicted). Note EGF-dependent superactivation of Akt and Erk1/2 in AP-2α-null keratinocytes. (C) Same experiment as in B, except 10× increments of EGF concentrations (0–50 ng/ml) were used, and extracts were harvested at t = 2 min. (D) Same experiment as in B, but insulin growth factor 1 (IGF1) was added at 50 ng/ml. Note that in contrast to EGF, IGF1 did not generate enhanced activated Akt in KO relative to WT cells. (E) Same experiment as in B, but the PI3K-specific inhibitor LY294002 was added at 20 μM. Note that the activation of Akt, but not Erk1/2, is dependent on PI3K activation. (F) Antiphospho-Akt staining of frozen sections (8 μm) from (top) lesional KO and WT thoracic skins and (bottom) TPA-treated KO and WT back skins. Note the superactivation of Akt in KO skin regions that are lesional and that correlate with elevated EGFR ligand expression. De, dermis; epi, epidermis; hf, hair follicle. Dotted lines denote dermo–epidermal borders. Bars, 20 μm.
et al., 1991), the functional importance of these proteins in epidermis is now emerging. Recent knockdown experiments underscore a role for Xenopus laevis AP-2α in embryonic skin development (Luo et al., 2002), and our studies now reveal an essential role for mammalian AP-2α in orchestrating the balance between epidermal proliferation and differentiation. This process can be largely explained by AP-2α’s repressive effects on EGFR gene transcription, but given the repertoire of epidermally expressed genes with AP-2-binding sites, there are likely to be many additional key genes controlled by AP-2α. Why have these other target genes not surfaced in our analyses?

The answer seems, at least in part, to reside in functional redundancy among AP-2 family members, four of which are expressed in the epidermis. This is substantiated by the fact that both in vivo and in vitro, the consequences of AP-2α ablation appeared to be more dramatic in the differentiating cells where AP-2γ expression is down-regulated. Additionally, AP-2α appears to be more highly expressed in those basal cells that are not actively cycling, leading us to speculate that AP-2’s role may be most critical at the juncture between proliferation and differentiation in the epidermis. Evaluating the degree of AP-2 functional redundancy in the epidermis must await functional studies on the other members of the AP-2 family that are expressed in skin.

Cell type and differentiation-specific cofactors are likely to further impact the complexities of when and how AP-2 proteins act in the epidermis. Studies in other systems have already indicated that interactive partners for AP-2 proteins can influence whether AP-2 proteins act as transcriptional repressors or activators (Pfisterer et al., 2002). Differences in AP-2 recognition motifs are also likely to influence target gene specificity, and the differential expression of AP-2 family members and possibly putative AP-2 cofactors could further magnify differences in the spatial and temporal behavior of putative AP-2 target genes within a tissue. Such differences are also likely to contribute to our understanding of why the loss of AP-2B results in massive apoptosis in the kidney (Hilger-Eversheim et al., 2000) and why the loss of AP-2α in neural crest impaired craniofacial development and pigmentation (Brewer et al., 2004). These issues are also likely to underlie the seemingly opposing findings that the addition of recombinant AP-2α to a nuclear extract from a human squamous cell carcinoma line in vitro led to an increase in EGFR transcription (Johnson, 1996), whereas AP-2α in mouse epidermis and in cultured epidermal keratinocytes had an inhibitory effect on EGFR promoter activity (our study). The finding that AP-2 proteins positively regulate cell proliferation in some cells and inhibit growth in others may help in the future to explain the seemingly disparate results obtained concerning the roles for AP-2 family members in human cancers (Bosher et al., 1996; Ropponen et al., 2001; Vernimmen et al., 2003; Pellikainen et al., 2004; Friedrichs et al., 2005).

Materials and methods

Generation of cKO mice

AP-2α floxed mice [AP-2αlox/lox] and K14-Cre transgenic mice were generated as described previously (Vasioukhin et al., 1999; Brewer et al., 2004). Genotyping was conducted by PCR of tail skin DNAs.

Histology, immunofluorescence, and in situ hybridization

For light or fluorescence microscopy, tissues were embedded in optimal cutting temperature compound and frozen on dry ice. For semi-thin sections and transmission EM, tissues were fixed and processed as previously described (Vasioukhin et al., 2001). For indirect immunofluorescence, 10-μm sections were permeabilized with 0.1% Triton X-100 and washed. For mouse monoclonal antibodies, we used the MOM kit (Vector Laboratories); for antibodies from other species, we used 2.5% normal donkey serum, 2.5% normal goat serum, 1% BSA, 2% gelatin, 0.1% Triton X-100 as block, and antibody diluent.

Primary antibodies used were as follows: (1) mouse: AP-2α (1:1.5; T. Williams) and phospho-EGFR (1:200; Upstate Biotechnology); (2) rabbit: K6 (1:200; Fuchs laboratories), K1 (1:250; Fuchs laboratory), K67 (1:500; Novoceastra laboratories), active caspase 3 (1:500; R&D Systems), K17 (1:1,000; a gift from P. Coulombe, The Johns Hopkins University School of Medicine, Baltimore, MD), EGFR (1:100; Upstate Biotechnology), and phospho-Akt (1:250, Cell Signaling); (3) guinea pig: K5 (1:200; Fuchs laboratory); (4) rat: BrdU (1:150, AbCam) and β4 integrin (1:250, BD Biosciences). For phospho-EGFR staining, sections were fixed in cold 100% methanol, primary antibody incubations were performed as above, and the signal was amplified using the ABC kit (Vector Laboratories) and visualized using the tyramide signal amplification Plus Fluorescence detection kit (PerkinElmer). In situ hybridizations of K5 transcripts were performed as described previously (Byrne et al., 1994). Probe synthesis was performed according to the manufacturer’s instructions (Roche).

Image acquisition and manipulation

The histology, immunofluorescent, and in situ hybridization images were taken by a mot plus microscope (Axioskop 2; Carl Zeiss Micromaging, Inc.). The objectives used were 20× NA 0.5 plan Neofluar 40×/0.17 and 40× NA 1.3 oil plan Neofluor ∼0.17 (Carl Zeiss Micromaging, Inc.). The images were taken at room temperature in antifade as an imaging medium for immunofluorescent images and 80% glycerol for hematoxylin and eosiin (H&E) images. The fluorochromes used were FITC, Texas red/rhodamine red X, and DAPI. A slider camera [SPOT RT; Diagnostic Instruments] and MetaMorph 6 (Molecular Devices) software were used to acquire the pictures. Adobe Photoshop 6.0 software was used for contrast and brightness adjustment. The immunoblot and PCR gel images were acquired with an Alphaimager (Innotech), and Quantity One software (Bio-Rad Laboratories) was used for contrast and brightness adjustment.

Separation of epidermis, keratinocyte isolation, culture, and transfections

Enzymatic separation of epidermis from skins and primary newborn mouse epidermal cell cultures were performed as described previously (Blanpain et al., 2004). To induce keratinocyte differentiation in culture, the calcium concentration of the media was raised from 0.05 to 1.5 mM for 24 h, after which protein and RNA were extracted.

For growth comparisons, 2.5 × 10⁴ of freshly isolated cells were plated with feeder layers in 24-well dishes. For stringent growth conditions, serum was adjusted to 2%, and/or growth factor supplements and the addition of fibroblast feeder cells were omitted. For growth comparisons, with the presence of AG1478, 200 nM of the inhibitor (Sigma-Aldrich) was added to the serum daily after plating. Keratinocyte numbers were determined with a couler counter.

Nucleotides −1,180 to −29 (transcription initiation site = 0) of the mouse EGFR promoter were cloned upstream of the firefly luciferase gene in the pGL2 basic plasmid (Promega). Cells were transfected into 12-well dishes and grown to 30–40% confluency before Fugene 6 (Roche) reagent-assisted transfections of 2 ng cytomegalo virus–Renilla luciferase DNA (control) and 100 ng of either WT or mutant EGFR promoter firefly luciferase constructs [pEGFRpro-Luc or pmutEGFRpro-Luc] or empty vector [p-Luc; Promega]. 48 h after transfection, luciferase assays were performed as described previously (Sinha et al., 2000; Kaufman et al., 2002). Transfection efficiency was 2–3%. A retroviral vector containing the full-length mouse AP-2α cDNA was engineered and infected for AP-2α rescue studies (70% efficiency).

TPA treatment

Mice were anesthetized, and their backs were shaved. Each mouse received 2.5 μg TPA (dissolved in acetone) on the right half of the back twice per week for 4 mo. As a control, the left half was treated with acetone.

ChIP

In vivo ChIP was performed as described previously (Jamora et al., 2003). The presence of AP-2α sites was confirmed by iVista analysis of 5′ upstream
sequences as defined by the ECR Browser and Ensemble software (Euro-
pean Bioinformatics Institute and Sanger Institute). AP-2 sites were chosen for
ChIP analysis based on the conservation and alignment between mouse and
at least one other species, including human, canine, and rat, and clus-
tering of sites when applicable. As a control, PCR was also performed
using primers that recognize other sites within the same promoter or down-
stream portions of the same gene to demonstrate the specificity of the
pull-down.

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