Fos and Jun Proteins Are Specifically Expressed During Differentiation of Human Keratinocytes

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Activator protein 1 (AP-1) proteins play key roles in the regulation of cell proliferation and differentiation. In this study we investigated the expression of Fos and Jun proteins in different models of terminal differentiation of human keratinocytes and in skin from psoriasis patients. All Jun and Fos proteins, with the exception of FosB, were efficiently expressed in keratinocytes in monolayer cultures. In contrast, in normal epidermis as well as in organotypic epidermal cultures, the expression pattern of AP-1 proteins was dependent on the differentiation stage. Fos proteins were readily detected in nuclei of keratinocytes of basal and suprabasal layers. JunB and JunD were expressed in all layers of normal epidermis. Interestingly, expression of c-Jun started suprabasally, then disappeared and became detectable again in distinct cells of the outermost granular layer directly at the transition zone to the stratum corneum. In psoriatic epidermis, c-Jun expression was prominent in both hyperproliferating basal and suprabasal keratinocytes, whereas c-Fos expression was unchanged. These data indicate that AP-1 proteins are expressed in a highly specific manner during terminal differentiation of keratinocytes and that the enhanced expression of c-Jun in basal and suprabasal keratinocytes might contribute to the pathogenesis of psoriasis.

Key words: AP-1/hyperproliferation/keratinocytes/skin equivalents
J Invest Dermatol 124:212–220, 2005

Terminal differentiation of epidermal keratinocytes begins when cells migrate out of the basal epidermal layer, stop to proliferate and move outwards to the spinous and granular layers. Keratinocytes thereby undergo defined changes finally giving rise to the mature horny layer of the epidermis composed of tightly connected corneocytes devoid of nuclei that function to protect the body from dehydration and injury (Fuchs and Byrne, 1994).

The Activator protein 1 (AP-1) transcription factor consists of homo- or heterodimers of members of the Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) proteins (Shaulian and Karin, 2001). Loss- and gain-of-function experiments in mice have demonstrated the central role of AP-1 in many biological processes including cell proliferation, differentiation, oncogenic transformation, and apoptosis (Jochum *et al*, 2001). AP-1 proteins play important roles during terminal differentiation of epidermal keratinocytes and therefore the epidermis represents a highly informative and easily accessible *in vivo* system to study AP-1 functions. It is well known that AP-1 regulates many genes encoding critical players of skin homeostasis (Angel *et al*, 2001). These include transglutaminase type I (Liew and Yamanishi, 1992), different cytokeratin gene family members, such as K1 (Lu *et al*, 1994), K5 (Casatorres *et al*, 1994), K14, and K17 (Ma *et al*, 1997) as well as structural epidermal proteins like involucrin (Welter *et al*, 1995), loricrin (DiSepio *et al*, 1995), and profilaggrin (Jang *et al*, 1996). Although in mice inactivation of some AP-1 proteins did not reveal an overt skin phenotype (Angel *et al*, 2001), it is difficult to speculate about AP-1 functions in mouse skin as loss of JunB and Fra-1 results in embryonic lethality (Eferl and Wagner, 2003). Interestingly, the conditional inactivation of c-Jun in the mouse epidermis has demonstrated that c-Jun plays an essential role in regulating eyelid closure, keratinocyte proliferation, and skin tumor development through anti-epidermal growth factor receptor signaling (Li *et al*, 2003; Zenz *et al*, 2003). Expression of individual AP-1 proteins has also been critically implicated in cytokine-mediated mesenchymal–epithelial interactions in the skin (Szabowski *et al*, 2000). Disturbances in tissue homeostasis and an aberrant, altered expression of cytokines are likely to be implicated in human pathological diseases such as psoriasis. Psoriasis is a chronic and inflammatory skin disease, which affects around 3% of the population and is characterized by epidermal abnormalities including keratinocyte hyperproliferation and altered differentiation leading to parakeratosis and desquamation (Stern, 1997). In one study decreased transcription of *c-fos* and *c-jun* has been described in psoriasis (Basset-Seguin *et al*, 1991). In contrast, targeted v-fos expression to mouse keratinocytes efficiently induced epidermal hyperplasia (Greenhalgh *et al*, 1993) indicating that the functions of AP-1 in hyperproliferative skin disorders are still not well understood.

Individual AP-1 proteins are differentially expressed in epidermal layers, although findings on AP-1 expression in

Abbreviations: AP-1, activator protein 1; FCS, fetal calf serum; HDF, human dermal fibroblasts; NHEK, normal human epidermal keratinocytes; PBS, phosphate-buffered saline; SE, skin equivalents

the epidermis from different investigators are somehow discordant. In normal human skin, expression of c-Fos and c-Jun has been reported to be predominantly confined to basal and lower spinous cells (Basset-Seguin *et al*, 1990), whereas in another study c-Fos has been localized to all epidermal layers and c-Jun to granular layers of both authentic and reconstructed skin (Briata *et al*, 1993). In another study, analysis of all AP-1 proteins localized FosB and Fra-2 in lower, c-Jun and c-Fos in upper, Fra-1 in suprabasal and JunB as well as JunD in all layers of human neonatal epidermis (Welter and Eckert, 1995). We assumed that unspecific antibodies against AP-1 proteins are responsible for some of the contradicting results on AP-1 expression in different models of epidermal differentiation. Using *in vitro* translated AP-1 proteins and cells deficient in different AP-1 proteins, we first examined the reactivity of different antibodies with individual AP-1 proteins. These antibodies were subsequently used to analyze AP-1 expression in different models of terminal differentiation of human keratinocytes such as monolayer cultures, *in vitro* reconstructed epidermis as well as normal and psoriatic epidermis. We present novel findings on the specific expression of AP-1 proteins during terminal differentiation of human keratinocytes and also demonstrate high expression of c-Jun in psoriatic epidermis.

Results and Discussion

Assessment of the specificity of anti-AP-1 antibodies Previous reports on AP-1 expression in human epidermis showed that primary antibodies developed in rabbits produced a high background staining (Welter and Eckert, 1995). Therefore, we investigated the specificity of commercially available antibodies against AP-1 proteins (Table S1).

To test antibody cross-reactivity between different subunits, *in vitro* translated AP-1 proteins were analyzed by western blotting. Among the Fos proteins, the rabbit anti-Fra-1 antibody cross-reacted with c-Fos, whereas the other anti-Fos antibodies were detected only the specific subunits (Fig S1*A*). Similarly, among Jun proteins rabbit anti-JunD cross-reacted with c-Jun, whereas both mouse monoclonal antibodies anti-c-Jun and JunB detected only the corresponding Jun proteins (Fig S1*B*). To evaluate whether the respective antibodies also detect the different endogenous AP-1 proteins, serum-starved quiescent human dermal fibroblasts (HDF) were stimulated 3 h with serum and AP-1 reactivity was analyzed by immunostaining. Whereas in resting fibroblasts only Fra-2 and JunD were detected, all Fos and all Jun proteins were induced and accumulated in the nucleus upon serum stimulation (Fig S1*C*, *D*). No nuclear staining and a low cytoplasmic background staining were observed when serum-stimulated fibroblasts were stained with corresponding isotypes or normal rabbit IgG (Fig S1*C*, *D*). Thus, the antibodies used in these experiments detect all human AP-1 proteins by immunostaining and are, with the exception of Fra-1 and JunD AP-1 subunit, specific.

Fos and Jun proteins are expressed in monolayer cultures of human epidermal keratinocytes Since keratinocytes in monolayer cultures exhibit a differentiated phenotype after prolonged confluence (Eckert *et al*, 1997; Chaturvedi *et al*, 1999), we studied differentiation-associated AP-1 expression at different time points by immunostaining and Western blot analysis. c-Fos localized to the nucleus of pre-confluent, as well as in 1 and 2 d post-confluent cells, showed a weak cytoplasmic localization at day 4 and virtually disappeared at day 6 after confluence had been reached (Fig 1*A*). Using western blot analysis, c-Fos was detected in nuclear extracts of pre-confluent as well as 1 and 2 d post-confluent normal human epidermal keratinocytes (NHEK) (Fig 1*B*). The disappearance of c-Fos in later differentiation stages suggests that it is dispensable for the expression of terminal differentiation-associated proteins and the maintenance of differentiation. FosB, which has been shown previously to be unable to bind to the promoter of several epidermal genes, could not be detected by immunostaining at any time point and western blot analysis only detected a very weak signal in proliferating NHEK (Fig 1*A*, *B*). This is in agreement with earlier findings demonstrating only low FosB RNA levels presenting NHEK (Gandarillas and Watt, 1995; Rossi *et al*, 1998). Although only weakly expressed in pre-confluent cultures, both Fra proteins were induced in 1 or 2 d post-confluent cells and were found in nuclear location also at later stages of differentiation (Fig 1*A*). Both Fra proteins were also detectable in nuclear extracts of post-confluent keratinocytes (Fig 1*B*). In contrast to Fos proteins, all Jun proteins were detected in pre-confluent cells as well as at all time points after confluence had been reached (Fig 1*A*). Western blot analysis confirmed that Jun proteins were present at all time points in nuclear extracts of NHEK with higher expression levels in post-confluent than pre-confluent cells (Fig 1*B*). Together these data demonstrate that whereas Jun proteins are continuously expressed at all stages of keratinocyte differentiation, Fos proteins show a differential expression pattern in monolayer cultures. Therefore we assume that in late stages of keratinocyte differentiation, when c-Fos is absent, Fra-1 and/or Fra-2 may replace it as a partner for Jun.

AP-1 proteins are expressed in a differentiation-stage-dependent manner during terminal differentiation of skin equivalents (SE) Keratinocytes co-cultured with fibroblasts from SE mimic the natural architecture of the epidermis in a simplified form and represent a powerful model to study keratinocyte differentiation (Fusenig, 1994). The expression of different AP-1 components was next analyzed during terminal differentiation of keratinocytes in such organotypic cultures.

SE were followed up to 8 d after initiating differentiation by lifting the epidermis to the air–liquid interface (Fig 2*A*). Under these conditions differentiation markers like filaggrin or caspase-14 were expressed as previously described (Eckhart *et al*, 2000; Rendl *et al*, 2002). Although in an earlier study c-Fos was detected in organotypic cultures only after prolonged culture (Basset-Seguin *et al*, 1994), in our study c-Fos was present in the cytoplasm of basal cells already at day 2 after initiating differentiation. Its expression increased and extended to suprabasal cells as differentiation progressed resulting in nuclear localization from day 4 onwards. In the uppermost layers of nucleated cells corresponding to the granular layer of the epidermis, c-Fos expression was