Regulation of Involucrin Gene Expression

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The epidermis is a dynamic renewing structure that provides life-sustaining protection from the environment. The major cell type of the epidermis, the epidermal keratinocyte, undergoes a carefully choreographed program of differentiation. Alteration of these events results in a variety of debilitating and life-threatening diseases. Understanding how this process is regulated is an important current goal in biology. In this review, we summarize the literature regarding regulation of involucrin, an important marker gene that serves as a model for understanding the mechanisms that regulate the differentiation process. Current knowledge describing the role of transcription factors and signaling cascades in regulating involucrin gene expression are presented. These studies describe a signaling cascade that includes the novel protein kinase C isoforms, Ras, MEKK1, MEK3, and a p38δ–extracellular signal regulated kinase 1/2 complex. This cascade regulates activator protein one, Sp1, and CCATT/enhancer-binding protein transcription factor DNA binding to two discrete involucrin promoter regions, the distal- and proximal-regulatory regions, to regulate involucrin gene expression.

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Keratinocyte differentiation. Keratinocytes are the major cell type responsible for the structure of the epidermis. They begin as stem cells in the basal epidermal layer (Cotsarelis *et al*, 1989; Li *et al*, 1998; Taylor *et al*, 2000; Alonso and Fuchs, 2003; Gambardella and Barrandon, 2003). As they move to the epidermal surface, the cells cease cell division and undergo morphological changes to form the spinous, granular, transition, and cornified layers. Spinous layer cells are characterized by the presence of extensive intercellular desmosomal connections, whereas granular layer cells are distinguished by the presence of granules that contain the products of keratinocyte differentiation. Continued differentiation of the granular layer cells results in formation of the transition zone which separates the dead from living epidermal layers. It is in this zone that the cellular constituents are extensively enzymatically remodeled. This remodeling ultimately results in the formation of the cornified layer, the covalently cross-linked terminally differentiated corneocytes that form the skin surface (Green, 1980).

Achieving these morphological alterations relies on executing a preset program of differentiation that requires tight regulation of gene expression. The involucrin gene is a model for elucidating the mechanisms that guide gene expression during differentiation (Eckert and Green, 1986; Eckert *et al*, 1997b). Involucrin (hINV) is a 68 kDa precursor of the cornified envelope that was originally described by Rice and Green (1979) and ultimately cloned by Eckert and Green (1986). The protein is rod shaped and includes several reactive glutamine residues that function in the formation of covalent isopeptide bonds (Yaffe *et al*, 1992; Eckert *et al*, 1993; Robinson *et al*, 1997; Lazo and Downing, 1999; Steinert and Marekov, 1999; Kajava, 2000). Involucrin is cross-linked early in cornified envelope formation and forms a scaffold for incorporation of other precursors (Rice and Green, 1979; Eckert *et al*, 1993; Steinert and Marekov, 1997). Involucrin expression initiates in the early spinous layer and is maintained in the granular layer. In the transition zone, involucrin is incorporated, via the action of transglutaminase, as a component of the cornified envelope (Yaffe *et al*, 1992; Eckert *et al*, 1993; Robinson *et al*, 1996).

During the differentiation process, numerous genes are turned on and off at specific stages (Eckert and Welter, 1996; Eckert *et al*, 1997a). The study of involucrin gene expression has identified some key mechanisms whereby intracellular signaling cascades and transcription factors regulate differentiation-dependent gene expression. Moreover, these studies have shed light on the mechanisms whereby a variety of agents, including calcium (Bikle *et al*, 2001; Deucher *et al*, 2002), vitamin A (Poumay *et al*, 1999), protein kinase C (PKC) activators (Welter *et al*, 1995), and antioxidants (Balasubramanian *et al*, 2002), regulate keratinocyte differentiation. This manuscript reviews our understanding regarding the mechanisms that regulate hINV gene expression.

Abbreviations: AP1, activator protein one; C/EBP, CCATT/enhancer-binding protein; DRR, distal-regulatory region; hINV, human involucrin; PKC, protein kinase C; PRR, proximal-regulatory region; URR, upstream-regulatory region

The Involucrin Promoter—The Distal-Regulatory Region (DRR)

Initial transgenic mouse studies revealed that a 6 kb segment of DNA encoding the hINV structural gene and 2.5 kb of DNA upstream of the transcription start site, could drive appropriate hINV gene expression in epidermis (Crish *et al*, 1993) (Fig 1*A*). Expression was observed in all stratified squamous epithelia that were tested, including ectocervix, esophagus, epidermis, footpad, and specific segments of the hair follicle. Expression was not observed in the endocervix, brain, or liver (Crish *et al*, 1993). In addition, expression was differentiation-appropriate (i.e., confined to the suprabasal layers in each tissue). The expression pattern essentially matched the pattern observed in human tissues. In addition, the expressed human involucrin protein was found incorporated as a component of the murine cornified envelope, indicating that it was functioning as a transglutaminase substrate (Crish *et al*, 1993).

Based on these studies, we hypothesized that the DNA sequences required for appropriate expression were localized within the 2500 nucleotide segment upstream of the transcription start site. Characterization of this region, called the upstream-regulatory region (URR), was carried out using cultured keratinocytes and luciferase reporter assays (Welter *et al*, 1995). This study identified, within the URR, specific DNA segments called the distal- and proximal-regulatory regions (DRR, PRR) that are required for optimal promoter activity (Fig 1*A*). Deletion of the DRR (nucleotides −2473/−1953) results in a 50% loss of promoter activity and further deletion of the PRR (−241/−7) results in an additional loss such that the promoter is inactive. An important finding of this study is the presence of activator protein one (AP1) transcription factor binding sites at each location (Fig 1*A*). Subsequent studies reveal that mutation of either AP1 site, AP1-5 in the DRR or AP1-1 in the PRR, results in a substantial loss of transcriptional activity. Moreover, DNA/protein interaction studies confirm that selected AP1 factors, including JunB, JunD, and Fra-1, interact at these sites (Welter *et al*, 1995). In addition, a 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-dependent increase in hINV promoter activity is associated with increased AP1 factor interaction at the AP1-1 and AP1-5

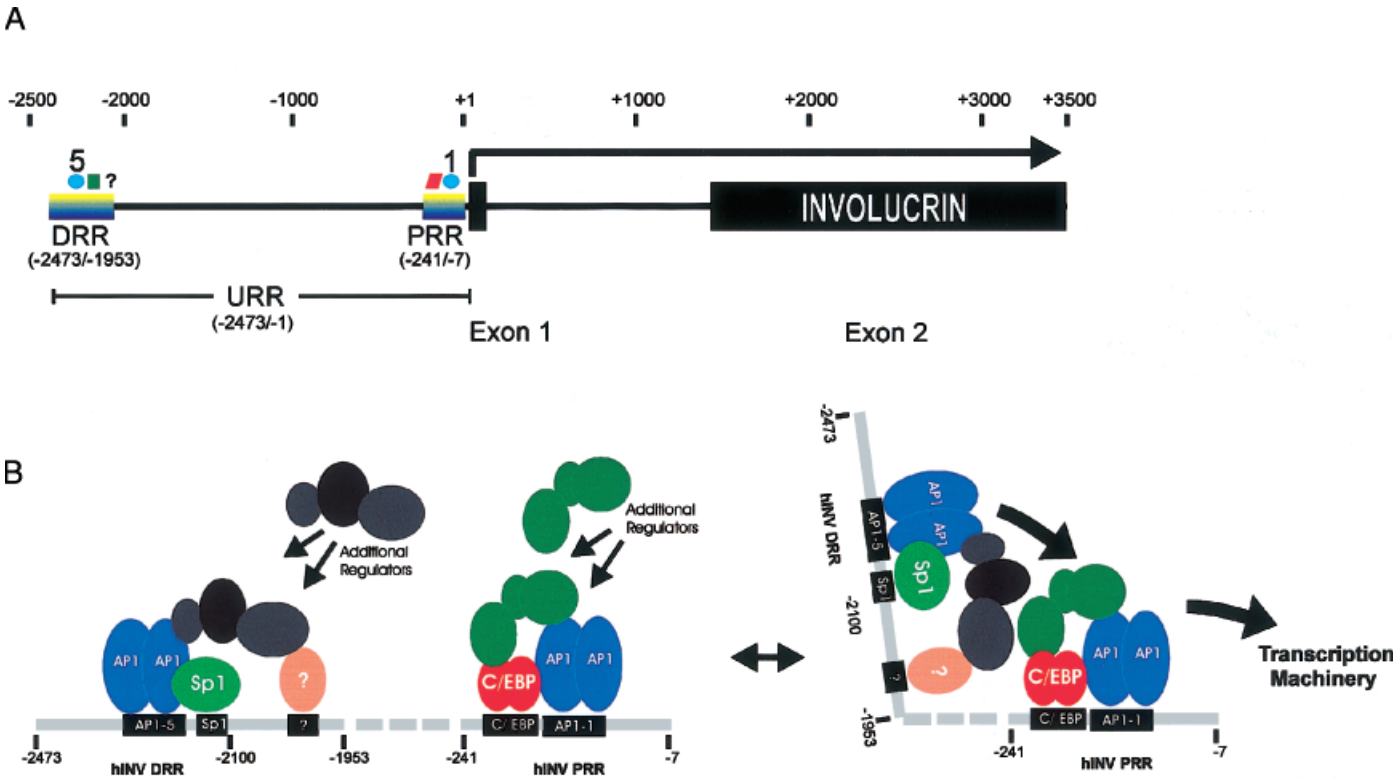

Figure 1
The involucrin gene and regulatory regions. (*A*) The hINV gene regulatory sequences. The entire hINV gene is presented with the transcription start site designated at +1. The sequence is taken from the original reports (Eckert and Green, 1986; Crish et al, 1998). The transcription start site and direction of transcription is indicated by the arrow and the two black rectangles indicate the exons. The entire protein coding sequence is encoded in the second exon (Eckert and Green, 1986). The upstream-regulatory region (URR) includes nucleotides −2473/−1. The distal-regulatory region (DRR) includes nucleotides −2473/−1953 and the proximal-regulatory region (PRR) encompasses nucleotides −241/−7 (Welter *et al*, 1995; Crish *et al*, 1998). The activator protein (AP)1-5 (DRR) and AP1-1 (PRR) sites are indicated as blue spheres, the Sp1 site (DRR) is a green square, and the C/enhancer binding protein (EBP) site (PRR) is a red parallelogram. The immediate early element is indicated by a question mark. (*B*) Cooperation of the DRR and PRR is required for appropriate expression. The hINV gene DRR and PRR are indicated. The DRR encodes an AP1 site (AP1-5) and a Sp1 site that bind AP1 and Sp1 factors, respectively (see text). An additional site, the immediate early element, marked by a question mark, was identified during *in vivo* transgenic mouse gene regulation studies (Crish *et al*, 2002). This element is required for involucrin expression in the immediate suprabasal layers *in vivo*. The PRR includes an AP1 site (AP1-1) and a C/EBP site that bind, respectively, AP1 and C/EBP transcription factors. These complexes also interact with co-activators (Crish and Eckert, unpublished) to form extended transcriptional complexes. We propose that the DRR and PRR, which are separated by 1.7 kb, are brought together to form a larger complex that then interacts with the basal transcriptional apparatus to activate tissue-specific and differentiation-appropriate expression.

sites (Welter *et al*, 1995). It is important to note that other AP1 sites, present within the URR, do not function in gene regulation. Thus, only selected AP1 sites within the URR are functional. Additional studies confirm that hINV promoter activity is cell type-specific, as promoter activity and hINV protein expression are observed in normal human keratinocytes but not in cultured fibroblasts (Welter *et al*, 1995).

An analysis of the sequence surrounding the AP1-5 site in the DRR revealed the presence of a canonical Sp1 site, located immediately downstream of the AP1 site and separated from the AP1 site by a single nucleotide (Crish *et al*, 1998). Sp1 transcription factors comprise a family of proteins, including Sp1, Sp2, Sp3, and Sp4. These proteins contain a conserved DNA binding domain composed of three zinc fingers near the C-terminus and serine/threonine- and glutamine-rich domains in the N-terminal region (Apt *et al*, 1996; Suske, 1999). Sp1 factors act by binding to G-rich elements, similar to the G-rich site located adjacent the AP1-5 site in the hINV promoter DRR. Since AP1 and Sp1 factors function as co-regulators in other systems (Wu *et al*, 2003), we suspected that this Sp1 site may be required, with the AP1-5 site, for optimal hINV gene expression. A comparison of the role of these sites reveals that mutation of the AP1-5 site results in a complete loss of promoter activity, whereas mutation of the Sp1 site results in a partial reduction in activity (Banks *et al*, 1998).

Gel mobility supershift studies show that Sp1, but not Sp2, Sp3, or Sp4, bind at the Sp1 site. The selective Sp1 binding is an interesting finding, since Sp1, among the Sp1 family members, is generally considered a transcriptional activator (Suske, 1999) that frequently acts synergistically with other proteins to increase gene expression (Courey and Tjian, 1988; Courey *et al*, 1989; Pascal and Tjian, 1991). We surmised that Sp1 may cooperate with the AP1 factors that bind at the AP1-5 site. Indeed, mutagenesis studies suggest that these sites act together to synergistically activate the promoter (Banks *et al*, 1998). The importance of the close juxtaposition of the AP1-5 and Sp1 sites was confirmed by showing that increasing the distance between these sites results in a reduction in promoter activity (Banks *et al*, 1998).

In addition, Sp1 factors may have a role in regulating cell type-specific involucrin expression. Involucrin is normally not expressed in non-epithelial cells (Rice and Green, 1979). Moreover, hINV promoter activation is not observed in 3T3 cells or HEK-293 (non-epithelial) cells; however, overexpression of Sp1 in 3T3 cells or HEK-293 cells results in an substantial increase in both endogenous hINV and hINV promoter activity (Banks *et al*, 1999). Thus, Sp1 level profoundly influences involucrin expression, suggesting that Sp1 factors may help direct tissue- and cell type-selective expression. Moreover, reducing the effective Sp1 concentration in normal human keratinocytes reduces hINV promoter activity (Banks *et al*, 1999). Thus, Sp1 has an important role as a regulator of hINV gene expression.

Recent transgenic mouse studies (Adhikary and Eckert, unpublished) reveal that unlike the partial loss of expression observed in other tissues, mutation of the Sp1 site results in a complete loss of hINV promoter activity in the corneal epithelium. This suggests that Sp1 plays a more important role in the corneal epithelium than in other surface epithelial tissues, and is consistent with a demonstrated role of Sp1 factors in regulating gene expression in the cornea (Wu *et al*, 1994).

The DRR Region Is Necessary and Sufficient for Normal hINV Expression *In Vivo*

The above studies identify an important role for the DRR region, and specifically the DRR AP1-5 and Sp1 sites in regulating hINV gene expression. The importance of this region was examined in detail using transgenic mice. A promoter deletion series was designed and each construct was tested. These experiments revealed that deletion of the DRR eliminates hINV transgene expression in epidermis and other surface epithelia (Crish *et al*, 1998). In addition, a remarkable finding is that the DRR region, itself, $DRR_{-2473/-1953}$ (Fig 1*A*), when linked to the hINV basal promoter, is sufficient to drive tissue-specific and differentiation-appropriate expression in epidermis (Crish *et al*, 1998).

Further transgenic studies revealed additional multiple functions for the DRR. As noted above, the $DRR_{-2473/-1953}$ segment drives near-normal, tissue-specific, differentiation-appropriate expression. This suggests that elements within this region may exist that mediate expression in specific epidermal layers. We examined this by segmenting the DRR into two subfragments, $DRR_{-2473/-2100}$ and $DRR_{-2100/-1953}$, and testing the ability of each to drive expression in transgenic mice. The downstream segment, $DRR_{-2100/-1953}$, does not drive expression in epidermis. The upstream segment, $DRR_{-2473/-2100}$, in contrast, drives expression, but only in the uppermost suprabasal layers (Crish *et al*, 2002). Recombination of these elements, of course, recapitulates the complete differentiation-dependent pattern of expression. These findings demonstrate that the DRR consists of spatially distinct elements, each of which is required to drive differentiation-appropriate expression. This finding is consistent with the modular promoter hypothesis, which states that multiple, spatially distinct DNA segments, containing distinct transcription factor binding sites, assemble a protein regulatory complex that drives appropriate gene expression. The multiprotein complex then interacts with the basal transcription machinery (Hadchouel *et al*, 2003; Ogata *et al*, 2003).

Mutation of the AP1-5 site, in the absence of other mutations in the DRR, results in a complete loss of expression in epidermis, esophagus, and cervix (Crish *et al*, 2002), suggesting that the presence of AP1 factors is necessary for expression. Moreover, mutation of this site in the context of the full-length promoter also results in a complete loss of expression in these tissues, pointing to a physiologic *in vivo* role for this site. Gel mobility shift analysis of extracts taken from mouse epidermis suggests that c-Jun and Fra-1 interact at this site (Crish *et al*, 1998). This AP1 factor binding profile differs slightly from that observed in extracts prepared from human keratinocytes, where JunB, JunD, and Fra-1 are the major interacting proteins (Welter *et al*, 1995), and suggests that expression may be achieved by different AP1 factors in mouse *versus* human epidermis. As will become clear below, in spite of the transgenic studies showing that the DRR is sufficient to

drive differentiation-appropriate expression, this is not the complete story, and the PRR region also plays an important regulatory role.

Other Regulatory Responses Mediated by the DRR

As outlined above, the DRR element, originally identified by Welter *et al* (1995), that is required for hINV expression in transgenic mice (Crish *et al*, 1998), has a broad regulatory role and mediates response to a wide variety of agents. For example, Bikle and coworkers showed that elevation of extracellular calcium resulted in an increase in JunD, Fra-1, and Fra-2 binding to the hINV promoter AP1-5 site and that mutation of this site resulted in a loss of calcium responsiveness (Ng *et al*, 2000). This response was confirmed in the context of calcium and PKC-dependent promoter activation (Deucher *et al*, 2002). Lopez Bayghen *et al* (1996) have also reported calcium-dependent regulation mediated via the DRR. Oxysterols, PPARα activators, cholesterol sulfate, and vitamin D also exert their effects, in part, via this element (Hanley *et al*, 2000, 2001; Komuves *et al*, 2000; Bikle *et al*, 2002). The vitamin D response is interesting, since it acts via a vitamin D response element in conjunction with the DRR AP1-5 site, and the differentiation-dependent increase in gene expression is associated with the exchange of the vitamin D receptor co-activator, DRIP205, for SRC family co-activators, SRC2 and SRC3 (Bikle *et al*, 2003). Thus, selective co-activator use by the vitamin D receptor, may have a role in regulating differentiation. An intriguing finding of all of these studies is the conservation of JunD and Fra-1 as AP1 factors associated with the DRR AP1-5 site following stimulation. Taken together, these studies identify a critical role for these AP1 family transcription factors in the regulation of hINV gene expression.

Characterization of the PRR

The PRR is contained in nucleotides $-241/-7$ and is marked by the presence of the AP1-1 site and a CCAAT/enhancer-binding protein (C/EBP) transcription factor binding site (Welter *et al*, 1995) (Fig 1*A*). As previously noted, mutation of the AP1-1 site, which selectively binds junB, junD, and Fra-1, results in a 50% drop in transcriptional activity (Welter *et al*, 1995). Other investigators have also identified a role for the AP1-1 site. Hudson and coworkers reported that glucocorticoids increase hINV promoter activity and that this increase is inhibited by treatment with all-*trans*-retinoic acid or 9-*cis*-retinoic acid (Monzon *et al*, 1996). This response is mediated via the PRR AP1-1 site. These investigators also noted that TPA-dependent hINV promoter activation via the AP1-1 site is reduced by retinoid co-treatment (Monzon *et al*, 1996).

The C/EBP site also plays an important role in the regulation of hINV gene expression. Mutation of the C/EBP site results in a reduction in basal promoter activity and a reduction in the response of the promoter to treatment of cells with differentiating agents (Agarwal *et al*, 1999). Moreover, individual C/EBP transcription factors differentially regulate promoter activity. In contrast to other C/EBP factors, which are less efficient regulators of promoter activity, C/EBPα binds to the promoter and increases activity. C/EBP-dependent regulation can be complex, since C/EBP factors interact and different combinations can produce different regulatory outcomes (Rosen, 2002; Cassel and Nord, 2003). Indeed, this is the case with hINV, as co-expression of C/EBPβ and C/EBPδ with C/EBPα, suppress C/EBPα-dependent promoter activation. This suggests that the ratio of C/EBP factors present within the cell may help determine the level of hINV gene expression. Likewise, GADD153, a dominant-negative C/EBP transcription factor (Bartlett *et al*, 1992; Fawcett *et al*, 1996), inhibits the C/EBPα-dependent increase in hINV promoter activity (Agarwal *et al*, 1999). Gel mobility shift studies indicate that treatment with the keratinocyte differentiating agent, TPA which increases hINV gene expression, increases C/EBPα loading at the hINV promoter C/EBP binding site. Thus, increased transcriptional activity is associated with increased C/EBPα binding to DNA. Finally, mutation of the C/EBP site results in a loss of TPA-dependent promoter activity, further confirming a role for C/EBP as a positive transcriptional activator (Agarwal *et al*, 1999).

Treatment with thapsigargin, an intracellular calcium mobilizing agent (Thastrup *et al*, 1989, 1990), inhibits the TPA-dependent increase in hINV promoter activity in a C/EBP-dependent manner. This result is surprising, since increased intracellular calcium would be expected to increase hINV gene expression (Deucher *et al*, 2002). But this effect appears to be due to the non-specific ability of thapsigargin to reduce the amount of C/EBP factor bound to the hINV promoter C/EBP binding site (Balasubramanian *et al*, 2000).

In addition, recent transgenic studies (Crish, Gopalakrishnan, and Eckert, unpublished) indicate that mutation of DNA elements within the PRR results in modified expression in transgenic mice. The most striking finding is a loss of continuous expression along the length of the tissue in mice harboring a full-length transgene in which the C/EBP site is mutated.

Involucrin Gene Regulation Model

Based on the results of these studies, we propose a model for regulation of involucrin gene expression via the DRR and PRR elements (Fig 1*B*). In this model, transcription factors load at the AP1-1 and Sp1 sites in the DRR followed by loading of adaptor proteins. Identification of these proteins is presently underway. Binding at three essential sites, the AP1-5 and Sp1 sites in the $DRR_{-2473/-2100}$ segment, and the immediate suprabasal site in the $DRR_{-2100/-1953}$ segment, is necessary for the complete program of differentiation-appropriate transcription. The AP1-5 site functions as an on–off switch, the Sp1 site cooperates with AP1-5 site to synergistically activate expression, and the immediate suprabasal site is required for expression in the immediate suprabasal layers (Crish *et al*, 1998).

Transcription factor loading also occurs at the PRR AP1-1 and C/EBP sites. C/EBPα binds at the C/EBP site (Agarwal *et al*, 1999) and junB, junD, and Fra-1 bind at the

AP1-1 site (Welter *et al*, 1995). This binding is presumably followed by binding of co-regulatory proteins at both the DRR and PRR sites. Since both sites are required for appropriate expression *in vivo*, we speculate that the DNA bends (Tolhuis *et al*, 2002; Ogata *et al*, 2003) to bring the DRR and PRR regions into juxtaposition and that this combined complex then interacts with the basal transcription machinery to drive differentiation-dependent transcription.

Mitogen-Activated Protein Kinases (MAPK)

The above studies define C/EBP, Sp1, and AP1 factors as being important regulators of hINV gene expression. An important question is which intracellular signaling cascades regulate the activity of these factors and, in turn, regulate promoter activity. Initial tests with various pharmacologic inhibitors indicated that the MAPK might regulate involucrin gene expression (Efimova *et al*, 1998).

The MAPK cascades consist of three kinase modules—including a MEK kinase (MEKK), a mitogen-activate protein kinase/extracellular signal regulated kinase (MAPK/ERK kinase, MEK), and a MAPK (Davis, 1995; Robinson and Cobb, 1997). Three MAPK cascades have been extensively studied. These include the mitogen-responsive ERKs, the stress-responsive c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK), and the p38 MAP kinases (Han *et al*, 1996; Jiang *et al*, 1996; Lechner *et al*, 1996; Li *et al*, 1996b; Kumar *et al*, 1997; Cuenda and Dorow, 1998). Activated MAPK phosphorylate a variety of target proteins including transcription factors (Cano and Mahadevan, 1995; Robinson and Cobb, 1997), and cytoplasmic, cytoskeletal, and mitochondrial proteins (Chen *et al*, 2001).

MAPK are characterized by a dual phosphorylation sequence, Thr–X–Tyr (TXY), located in the regulatory loop located between subdomains VII and VIII (where X is Glu, Pro, or Gly) (Hanks and Hunter, 1995). The p38 MAPK possess a Thr–Gly–Try (TGY) motif, the ERK1/2 and ERK5/BMK1 kinases encode a Thr–Glu–Tyr (TEY) motif, and the JNK/SAPK kinases possess a Thr–Pro–Tyr (TPY) motif. The central amino acid in these motifs and the length of the loop influence MAPK substrate specificity and ability to autophosphorylate (Jiang *et al*, 1997). Phosphorylation of the dual phosphorylation sites results in MAPK activation. Although there is a substantial overlap in function among the MAPK classes, it is clear, as outlined below, that each mediates context-dependent functions.

MAPK Regulate hINV Promoter Activity

Efimova *et al* (1998) used pharmacologic agents, dominant-negative and constitutively active kinases, and kinase assays to examine the role of MAPK cascades in regulating hINV promoter activity. Initial studies showed that protein kinase C and Ras activity are required for MAPK activation and hINV promoter response (Efimova *et al*, 1998). For example, treatment with TPA, a known activator of PKC, increases hINV promoter activity, and inhibition of PKC or Ras activity results in a loss of the TPA-dependent activation. In addition, expression of constitutively active Ras triggers downstream events in the MAPK cascade that increase hINV promoter activity. PKC and Ras activation lead to MEKK1 activation. MEKK1, the first kinase in the cascade, appears to be a key integrator of regulatory input. The MEKK1-associated signal is then directed to several MEK, including MEK7 and MEK3, although later studies also suggest a role for MEK6 (Dashti *et al*, 2001a). Ultimately, the signal is channeled to p38 MAPK (Efimova *et al*, 1998).

As noted above, the AP1 transcription factors, Sp1 and C/EBP factors are downstream targets of this activation. Increased p38 MAPK activity results in increased AP1 factor levels, and increased AP1 factor binding to the hINV promoter AP1 sites (Welter *et al*, 1995). JunB, JunD, and Fra-1 are confirmed as regulators that bind to the hINV promoter AP1 sites. Moreover, TAM67, a dominant-negative mutant of c-Jun that inhibits the activity of all AP1 factors (Wu *et al*, 2003), inhibits the p38-dependent promoter activation (Efimova *et al*, 1998). Activation of this MAPK cascade also results in increased C/EBPα and Sp1 binding to DNA (Banks *et al*, 1998; Agarwal *et al*, 1999). The involvement of C/EBP, Sp1, and AP1 factors in regulating differentiation-dependent involucrin gene expression is perhaps not surprising, as these factors have been implicated as differentiation regulators in other systems. These findings suggest a PKC, Ras, MEKK1, MEK3 pathway that activates p38 MAPK. p38 MAPK, in turn, acts to increase binding of selected AP1, Sp1, and C/EBP factors to the hINV promoter to increase promoter activity.

The Role of PKC

Additional studies examined the role of PKC family members in greater detail. The PKC family comprises multiple isoforms that are divided into three subfamilies—the novel, classical, and atypical subtypes (Liu and Heckman, 1998). These kinases have been suggested to have an important role in skin biology and pathobiology (Dlugosz and Yuspa, 1991, 1994; Denning *et al*, 1995, 2002). The observation that TPA, a diacylglycerol analogue, activates hINV gene expression (Welter *et al*, 1995), whereas BIS-IM, a specific inhibitor of PKC, blocks TPA-dependent increase in expression, suggests a role for PKC in regulating hINV gene expression. We therefore sought to assess the role of specific PKC isoforms as regulators of hINV gene expression. Co-expression of individual PKC isoforms with the hINV promoter reveals that the novel PKC isoforms produce a dramatic increase in hINV promoter activity. Endogenous involucrin levels are also increased by overexpression of novel PKC isoforms. Moreover, this activation is not inhibited by Go-6876, an agent that inhibits classical PKC subtypes, but is inhibited by a dominant-negative form of PKCδ (Efimova and Eckert, 2000). Dominant-negative PKCδ expression also inhibits TPA-dependent promoter activation.

A further study assessed the role of PKC as an upstream regulator of MAPK activity. Several striking observations suggest a novel pattern of MAPK regulation by PKCδ and other differentiation activators. Expression of PKCη or PKCδ causes a marked reduction in ERK1/2 activity without changing ERK1/2 protein level (Efimova *et al*, 2002). This

suppression of ERK1/2 activity coincides with a substantial increase in p38 MAPK activity. This inverse regulation—a net increase in the p38 MAPK to ERK activity ratio—is also confirmed for keratinocytes treated with several pharmacological agents that enhance keratinocyte differentiation—including okadaic acid, and TPA (Efimova *et al*, 2003). The shift in p38 MAPK to ERK ratio in favor of p38 coincided with increased hINV promoter activity (Efimova *et al*, 2002). To identify the link between nPKC activation, the shift in the ratio of p38 MAPK to ERK activity, and activation of hINV promoter activity, the effect of nPKC activation on C/EBPα level, was examined.

These studies reveal a substantial increase in the endogenous C/EBPα level in the presence of PKCδ or PKCη, and an inhibition of endogenous C/EBPα levels in the presence of dnPKCδ or dnPKCη (Efimova *et al*, 2002). Consistent with a role for nPKC and C/EBPα in regulating hINV gene expression, co-expression of these regulators prompts an increase in C/EBPα binding to the hINV PRR C/EBP transcription factor binding site and a corresponding increase in hINV promoter activity. Moreover, GADD153, a dominant-negative C/EBP factor, inhibits the increase, and mutation of the C/EBP transcription factor binding site in the hINV PRR results in a loss of this regulation (Agarwal *et al*, 1999). nPKC and C/EBPα also act as partners to increase expression of endogenous hINV. The nPKC-dependent activation of hINV is also inhibited by dnRas, dnMEKK1, and dnMEK3, but is increased by dnERK1 (Efimova *et al*, 2002).

PKCδ also Functions in Concert with Calcium to Increase hINV Promoter Activity

PKCδ also functions with calcium, an important regulator of keratinocyte differentiation, to enhance promoter activation (Deucher *et al*, 2002). Promoter truncation experiments demonstrate that this response is mediated by DNA elements located within the DRR. Specific mutation of the AP1-5 site eliminates the calcium response, the PKCδ response, and the response when the agents are combined (Deucher *et al*, 2002). Moreover, mutation of the DRR Sp1 site results in a partial loss of response. This study shows that calcium treatment does not alter PKCα or PKCδ level or cause a redistribution of PKCδ to membranes; however, calcium treatment does cause a marked increase in tyrosine phosphorylation of PKCδ. The role of tyrosine phosphorylation of PKCδ is not well understood; however, increased phosphorylation of PKCδ has also been observed in mouse keratinocytes following treatment with increased extracellular calcium (Denning *et al*, 2000). Moreover, this tyrosine phosphorylation may be physiologically important, as tyrosine-phosphorylated PKCδ is also detected in mouse epidermis (Denning *et al*, 2000). But the increase in phosphorylation in this system trails the increase in expression of differentiation-associated genes, suggesting it may not have a role in differentiation. A recent study shows that TPA treatment promotes phosphorylation at PKCδ Tyr_{187} in 3T3 cells; however, mutation of this site does not appear to reduce the ability of PKCδ to phosphorylate substrates (Li *et al*, 1996a). Different phosphorylation sites, including Tyr_{311}, Tyr_{332}, and Tyr_{512}, are selected when COS-7 cells are treated with hydrogen peroxide, with Tyr_{311} being the major phosphorylation site (Konishi *et al*, 2001). *In vitro* studies show that phosphorylation of PKCδ Tyr_{311} increases basal PKCδ activity in the presence of diacylglycerol (Konishi *et al*, 2001). In contrast, the Src family kinase-mediated phosphorylation of PKCδ on Tyr_{64} and Tyr_{565} causes inactivation of PKCδ and promotes a neoplastic phenotype in v-ras-transformed mouse keratinocytes (Joseloff *et al*, 2002). These varying results suggest that additional studies will be necessary to determine whether phosphorylation of PKCδ has a positive effect on involucrin gene expression, and to identify the PKCδ tyrosine residues that are modified.

An interesting finding is that classical PKC isoforms, such as PKCα, inhibit the calcium-dependent activation of the hINV promoter, whereas dominant-negative PKCα enhances the calcium-dependent increase. Thus, PKCα and PKCδ produce opposing effects on hINV promoter activity (i.e., antagonism between classical and novel PKCs). This suggests that the balance between PKCδ and PKCα may influence the extent of keratinocyte differentiation (Deucher *et al*, 2002). This represents one example, wherein members of a single signaling family produce opposing effects on the regulation of hINV gene expression. Another example is the inverse role of ERK1/2 and p38δ in regulating hINV gene expression (Efimova *et al*, 2003). Other studies have also examined the role of PKC in regulating hINV gene expression. Takahashi *et al* (1998) studied the regulation of hINV promoter by PKC in SV40-transformed keratinocytes. In this context, PKCα and PKCη increase hINV promoter activity via effects that require the AP1-1 site within the PRR (Takahashi *et al*, 1998). It is interesting that in the context of this immortalized cell line involucrin expression is increased by PKCα. This suggests that the pattern of PKC-dependent regulation may depend upon cell environment.

An ERK–p38δ Complex Regulates Gene Activation

As noted above, agents that regulate involucrin gene expression consistently increase p38δ MAPK activity and reduce ERK1/2 activity (Efimova *et al*, 2002, 2003). There are multiple mechanisms that might explain such coordinate regulation. It is possible that one signaling pathway acts to inhibit ERK1/2 activity, whereas a second pathway increases p38δ activity. A second possibility is the novel idea that p38δ and ERK1/2 are part of a complex in which the regulatory responses are transferred directly via changes within the complex. Studies designed to distinguish these possibilities reveal that ERK1/2 and p38δ exist in keratinocytes as components of a complex (Efimova *et al*, 2003).

Time course studies show that total p38 activity increases within 4 h after treatment with okadaic acid. This onset of p38 activity is associated with a corresponding reduction in ERK1/2 activity. In each case, this represents an actual change in activity, as the level of individual MAPK is not altered by treatment. Both ERK1 and ERK2 activities are decreased by treatment; however, selective changes are observed in activity of individual p38 MAPK isoforms. Four p38 MAPK isoforms are known to exist, including

p38α, β, δ, and γ (Lee *et al*, 1994; Lechner *et al*, 1996; Goedert *et al*, 1997; Kumar *et al*, 1997). Only p38α, p38β, and p38δ are expressed in keratinocytes (Dashti *et al*, 2001b). Among these isoforms, only p38δ activity is increased following Okada acid treatment. Thus, the major stimulus for activation of hINV promoter activity is provided by a reduction in total ERK1/2 activity with an accompanying increase in activity of the p38δ MAPK isoform (Efimova *et al*, 2003). Additional support for an exclusive role for p38δ is provided by the observation that treatment with SB203580, an agent that inhibits p38α and β activities (Cuenda *et al*, 1997; Kumar *et al*, 1997; Enslen *et al*, 1998), does not stop the increase in p38 activity. Additional studies show that this is likely to be physiologically relevant, as p38δ is expressed in all layers of the epidermis (Efimova *et al*, 2003). Although p38δ appears to be the major regulator of hINV gene activation, under special conditions, in the presence of constitutively active MEK6 or MEK7, a role of p38α has also been identified (Dashti *et al*, 2001a, b).

An important and unique finding is the presence, in keratinocytes, of an ERK1/2–p38δ complex (Efimova *et al*, 2003). The complex is constitutively present in both untreated and treated keratinocytes. Significant ERK1/2 activity, but little p38δ activity, is observed in untreated cells; however, treatment with okadaic acid results in a reduction in ERK1/2 activity and an increase in p38δ activity within the complex (Efimova *et al*, 2003). The level of the kinases is not altered by okadaic acid treatment. This finding alters some common assumptions regarding regulation by MAPK. Previously, the MAPK cascades have been envisioned as linear regulatory pathways in which the MAPK (ERK, p38, etc.) are independent signaling proteins (Cobb, 1999). But our studies suggest that there is a convergence at the level of MAPK and that incoming signals in keratinocytes impinge on a MAPK signaling complex that may include multiple MAPK. The composition of this complex is an important topic that is presently under investigation.

Regarding the downstream changes observed following activation of this complex, both C/EBP and AP1 factor levels increase. The increase is not inhibited by the p38α/p38β inhibitor, SB203580, confirming that it is likely to be mediated by p38δ. There is also a substantial increase in the level of c-Jun, JunB, JunD, Fra-1, Fra-2, C/EBPα, and C/EBPβ. The time course of increase for each of these transcription factors parallels the increase in p38δ activity and the decline in ERK1/2 activity, suggesting that transcription factor levels are controlled by the changing activity of the ERK1/2–p38δ complex.

The increased transcription factor level is associated with increased binding to JunB, Fra-1, and JunD, and C/EBP factors to the hINV promoter PRR AP1-1 and C/EBP sites, respectively. The altered expression of these factors ultimately leads to increased hINV transcription—both the endogenous gene and the promoter. Moreover, the increased transcription is inhibited by the dominant-negative form of MEK3, the kinase immediately upstream of p38δ in the MAPK signaling cascade (Efimova *et al*, 1998).

A Signal Transduction Model

Taken together, these findings suggest that the novel PKC enhance hINV gene expression by activation of a pathway that includes novel PKC isoforms, Ras, MEKK1, and MEK3. MEK3, in turn, regulates the activity of a p38δ–ERK1/2 complex such that p38δ activity is increased and ERK1/2 activity is reduced. This alteration in MAPK activity is correlated in time with an increase in transcription factor binding to DNA and activation of hINV promoter activity (Fig 2). An interesting and important feature of this regulation is the convergence of the signal at the level of the MAPK. The MAPK cascades have been viewed as functioning as distinct entities at the MAPK level, without direct cross-talk. Our model, in contrast, indicates that the signal actually converges onto a complex that includes multiple MAPK. Another important finding is that the ERK1/2–p38δ complex is constitutively present in cells and that incoming stimuli alter the relative activity of the MAPK isoforms within the

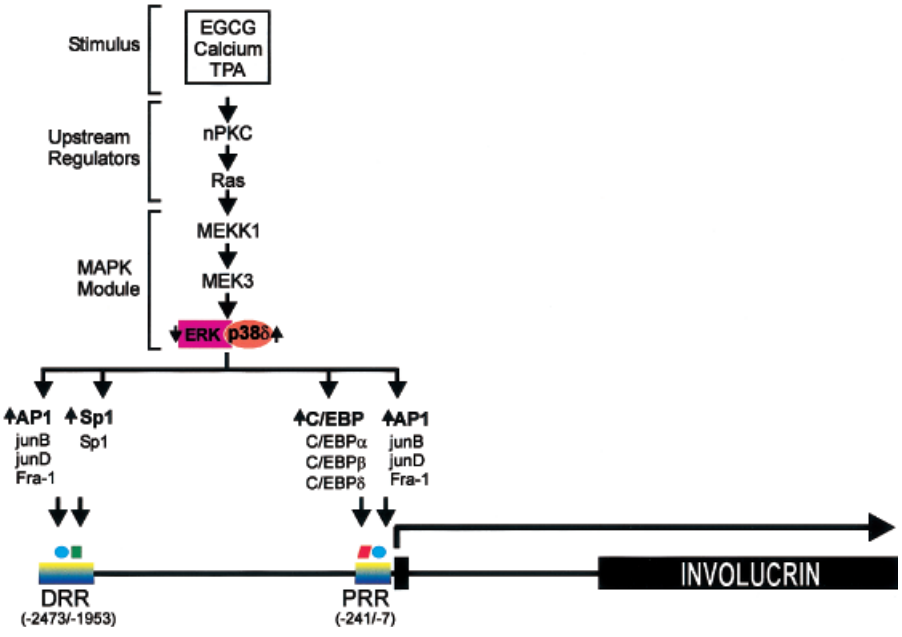

Figure 2
Mitogen-activated protein kinase (MAPK) regulation of involucrin gene expression. The stimulus activates the indicated cascade which ultimately alters the activity of MAPK encoded in a signaling complex. This complex exists in resting and stimulated cells. The net effect of the stimulus is to increase p38δ activity (small upward arrow) and reduce extracellular signal regulated kinase (ERK)1/2 activity (small downward arrow) without changing the level of these kinases. This shift in activity results in an increase in the level (upward small arrows) of the indicated transcription factors which interact with the appropriate sites to increase hINV promoter to increase transcriptional activity. The activator protein one (AP1)-5 (distal-regulatory region (DRR)) and AP1-1 (proximal-regulatory region (PRR)) sites are indicated as blue spheres, the Sp1 site (DRR) is a green square, and the C/enhancer binding protein (EBP) site (PRR) is a red parallelogram. The position of the involucrin exons and start of transcription, etc. are as outlined in Fig 1.

complex, but not their association (Efimova *et al*, 2003). The only other examples of a MAPK complex of this type is in HeLa cells or 293T cells (Zhang *et al*, 2001; Sanz-Moreno *et al*, 2003). These authors describe an ERK1/2–p38α complex that assembles in a stimulus-dependent manner in HeLa cells (Zhang *et al*, 2001) and in a stimulus-independent manner in 293T cells (Sanz-Moreno *et al*, 2003).

Other Agents also Alter Activity of the ERK–p38δ Complex

The above studies show that phorbol ester, okadaic acid, and calcium regulate hINV gene expression by interfacing with the MAPK cascade. Other agents also operate through this mechanism, including green tea polyphenols. Green tea polyphenols are antioxidants that have important cancer chemopreventive properties (Ahmad and Mukhtar, 1999). Balasubramanian *et al* (2002) showed that treatment with a biologically active green tea polyphenol, (−)-epigallocatechin-3-gallate (EGCG), results in an increase in hINV promoter activity. This activation requires the hINV promoter AP1-1 site within the PRR. This response to EGCG is associated with an increase in AP1 factor level and increased binding of Fra-1 and JunD to the AP1-1 site, and is inhibited by the dominant-negative forms of Ras, MEKK1, MEK3, and p38. Moreover, EGCG treatment shifts the p38δ/ERK activity ratio in favor of p38δ, a response that is characteristic of the MAPK pathway that regulates hINV expression (Balasubramanian *et al*, 2002). Thus, it appears that a wide variety of structurally diverse agents activate involucrin gene expression via regulation of the ERK1/2–p38δ complex.

POU Domain Proteins Suppress hINV Promoter Activity

The POU domain transcription factors are a superfamily of homeodomain proteins that regulate cell differentiation and proliferation (Scholer, 1991; Wegner *et al*, 1993). POU domain protein act by binding to specific DNA sequence to regulate gene expression; however, POU domain factors can also regulate gene expression in a binding site-independent manner (Yang *et al*, 1994). Involucrin promoter activity is suppressed by a host of POU homeodomain proteins, including Oct1, Oct2, Brn4, SCIP, Skn1a, and Skn1i (Welter *et al*, 1996; Chapman and Latchman, 1998). This suppression is POU protein DNA binding site independent. Moreover, activity of the hINV minimal promoter is suppressed by POU protein expression, suggesting that POU proteins may be interact with the basal transcription machinery. An interaction of POU domain proteins with the basal transcriptional apparatus has been observed in several systems (Arnosti *et al*, 1993; Zwilling *et al*, 1994). Studies by Welter *et al* (1996) suggest that POU domain proteins may suppress hINV promoter activity by suppressing the activity of an unknown factor involved in basal transcription. The physiological importance of this regulation is not known, although POU domain proteins are expressed in epidermis (Andersen *et al*, 1993; Yukawa *et al*, 1993, 1996; Faus *et al*, 1994).

At the beginning of the 1990s, it was appreciated that the gene expression of several gene products, including involucrin, was increased during epidermal differentiation. The extent of this knowledge was defined by studies assessing mRNA level and by runoff studies assessing transcriptional activity. Moreover, knowledge of cell signaling mechanisms was largely defined using pharmacologic inhibitors. A brief review of our present understanding of the mechanisms that regulate involucrin gene expression provides one example of how rapidly our understanding has improved. It can only be anticipated that this rapid rate of progress will continue during the next decade.

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