Protein Kinase Cδ Increases Kruppel-like Factor 4 Protein, which Drives Involucrin Gene Transcription in Differentiating Keratinocytes*

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Background: KLF4 is an important regulator of terminal differentiation and barrier formation in epidermis, but its mechanism of action is not well understood.

Results: PKCδ increases KLF4, which increases hINV expression by interaction at specific elements in the hINV promoter.

Conclusion: KLF4 drives expression of involucrin in response to differentiation stimuli.

Significance: This study provides detailed information regarding the mechanism of KLF4 regulation of keratinocyte differentiation.

KLF4 is a member of the Kruppel-like factor family of transcriptional regulators. KLF4 has been shown to be required for normal terminal differentiation of keratinocytes, but the molecular mechanism whereby KLF4 regulates genes associated with the differentiation process has not been studied. In the present study, we explore the impact of KLF4 on expression of involucrin, a gene that is specifically expressed in differentiated keratinocytes. KLF4 overexpression and knockdown studies show that involucrin mRNA and protein level correlates directly with KLF4 level. Moreover, studies of mutant KLF4 proteins indicate that transcriptionally inactive forms do not increase involucrin expression. PKCδ is a regulator of keratinocyte differentiation that increases expression of differentiation-associated target genes, including involucrin. Overexpression of KLF4 augments the PKCδ-dependent increase in involucrin expression, whereas KLF4 knockdown attenuates this response. The KLF4 induction of human involucrin (hINV) promoter activity is mediated via KLF4 binding to a GC-rich element located in the hINV promoter distal regulatory region, a region of the promoter required for in vivo involucrin expression. Mutation of the GC-rich element, an adjacent AP1 factor binding site, or both sites severely attenuates the response. Moreover, loss of KLF4 in an epidermal equivalent model of differentiation results in loss of hINV expression. These studies suggest that KLF4 is part of a multiprotein complex that interacts that the hINV promoter distal regulatory region to drive differentiation-dependent hINV gene expression in epidermis.

KLF4 is a member of the Kruppel-like factor transcription factor family that recognizes GC-rich DNA enhancer elements

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‡ The abbreviations used are: KLF4, Kruppel-like factor 4; hKLF4, human KLF4; hINV, human involucrin; DRR, distal regulatory region; PRR, proximal regulatory region; KSFM, keratinocyte serum-free medium; TPA, 12-O-tetradecanoylphorbol-13-acetate; MOI, multiplicity of infection.
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entiation. The epidermis is a stratifying epithelium in which the basal layers contain stem cells that undergo intermittent proliferation. These cells then give rise to cells that differentiate to form the suprabasal differentiated layers and that express genes associated with terminal differentiation (18–20). Information available in the literature suggests that genes associated with differentiation should be increased by KLF4 (3, 12, 21); however, this has not been studied at the molecular level. Involutin is a model for the study of gene expression during keratinocyte differentiation (22–26). It is expressed in the suprabasal epidermal layers, but is absent in basal cells (22, 27, 28). Activation of hINV transcription relies on a variety of mechanisms, but a prominent mechanism is PKCδ activation (13, 29–32). We have demonstrated that PKCδ activity drives hINV expression via activation of a MEKK1, MEK3/MEK6, and p38/ERK signaling cascade that elevates Sp1 and AP1 transcription factor level and binding to the hINV promoter to activate transcription (22, 24–26, 29, 33–38). 12-O-Tetradecanoylphorbol-13-acetate (TPA), a diacylglycerol analog that activates PKC isoforms, is a pharmacologic agent that activates keratinocyte differentiation via this pathway (23, 24, 39), as do some other naturally occurring agents (40).

Our previous study showed that PKCδ activation increases KLF4 mRNA and protein level in normal human keratinocytes (13). In the present study, we extend these studies and examine the effect of the elevation of KLF4 on expression of hINV, an important marker of keratinocyte differentiation. Our results show that KLF4 interacts via a GC-rich element in the hINV promoter distal regulatory region (DRR) to drive transcription. Thus, these findings present the first detailed evidence that KLF4 interacts with response elements on a keratinocyte differentiation-expressed gene to drive transcription.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Keratinocyte serum-free medium (KSFM) and trypsin were purchased from Invitrogen. Sodium butyrate was from Calbiochem, and actinomycin D was purchased from EMD Chemicals (Gibbstown, NJ). Dimethyl sulfoxide and phorbol ester (TPA), a diacylglycerol analog that activates PKC isoforms, is a pharmacologic agent that activates keratinocyte differentiation via this pathway (23, 24, 39), as do some other naturally occurring agents (40).

Our previous study showed that PKCδ activation increases KLF4 mRNA and protein level in normal human keratinocytes (13). In the present study, we extend these studies and examine the effect of the elevation of KLF4 on expression of hINV, an important marker of keratinocyte differentiation. Our results show that KLF4 interacts via a GC-rich element in the hINV promoter distal regulatory region (DRR) to drive transcription. Thus, these findings present the first detailed evidence that KLF4 interacts with response elements on a keratinocyte differentiation-expressed gene to drive transcription.

Promoter Activity—For hINV promoter activity analysis, 2 µg of hINV promoter reporter plasmid was mixed with 4 µl of FuGENE 6 reagent diluted with 96 µl of KSFM. The mixture was incubated at 25 °C for 15 min and then added to a 50% confluent culture of primary human epidermal keratinocyte maintained in 2 ml of KSFM in a 9.6-cm² dish. For co-transfection experiments, 2 µg of p21Cre1 promoter reporter plasmid and 1 µg of KLF4 expression plasmid were mixed, treated with FuGENE 6, and added to cells as indicated above. After 24 h, the cells were harvested, and extracts were prepared for assay of luciferase activity.

Electroporation and siRNA-mediated Knockdown—Keratinocytes were electroporated with siRNA or plasmids using the Amaxa electroporator and the VPD-1002 nucleofection kit. For electroporation, keratinocytes were harvested with trypsin and replated 1 day prior to the day of electroporation. On the day of electroporation, 1 × 10⁶ of the replated cells were harvested with trypsin and resuspended in KSFM. The cells are collected at 2000 rpm, washed with 1 ml of sterile phosphate-buffered saline (pH 7.5), and suspended in 100 µl of keratinocyte nucleofection solution. The cell suspension, which included 3 µg of gene-specific siRNA, was mixed by gentle pipetting and electroporated using the T-018 settings. Warm KSFM (500 µl) was added, and the suspension was transferred to a 21.3-cm² culture dish containing 3.5 ml of KSFM. When required, cells were electroporated a second time with luciferase reporter or expression plasmid. This was accomplished by harvesting the cells with trypsin and resuspending in KSFM. The cells were collected, washed with PBS, and resuspended in nucleofection solution as above. The nucleofection suspension, which included 2 µg of plasmid, was electroporated using the T-018 settings. The cells were plated and maintained for various times before extracts were prepared for assay. Our electroporation method delivers nucleic acid reagents with greater than 90% efficiency.

Immunological Analysis—Equivalent amounts of protein were electrophoresed on a 4–15% denaturing polyacrylamide gradient gel and transferred to nitrocellulose. The membranes were blocked, incubated with a specific primary antibody, washed, and exposed to an appropriate horseradish peroxidase-conjugated secondary antibody. Chemiluminescent detection was used to visualize secondary antibody binding.
Real-time PCR—Total RNA was isolated (illustra RNAspin mini kit, GE Healthcare) and reverse-transcribed. Quantification was performed by using the LightCycler 480 system (Roche Applied Science). PCR primers were designed to quantify PKCδ, KLF4, and hINV transcripts using LightCycler 480 SYBR Green I, and signals were normalized using cyclophilin A as control primers. Primers for detection of mRNA levels are cyclophilin A (forward, 5'-CATCTGACGCTGCCAGAC-TGA-3', reverse, 5'-TTATGCTTTCTTCTGTTG-3'), hINV (forward, 5'-CCTCGCTTACTGAG-3', reverse, 5'-GGGAGGAGCTTGAGTGG-3'), PKCδ (forward, 5'-GGCCACATCAGATTGCCGACTTT-3', reverse, 5'-ACTGGCCAATGAGCACATCTGACA-3'), and KLF4 (forward, 5'-GAAATTCGCCGCTCAGATGAACT-3', reverse, 5'-TTCTCTTCTGGCAGTGTGGGTCAT-3').

KLF4 mRNA Half-life—To analyze KLF4 mRNA decay kinetics, keratinocytes were infected with 15 MOI of Ad5-EV or Ad5-PKCδ for 24 h prior to the addition of 5 μg/ml actinomycin D. At 0, 0.5, 1, 2, 3, and 4 h after actinomycin D addition, RNA was isolated (illustra RNAspin mini kit, GE Healthcare) and analyzed for KLF4 and cyclophilin A mRNA content by quantitative real-time PCR using primers described in the previous section. Values for each mRNA level at each time point are presented as the mean ± S.D. derived from triplicate quantitative RT-PCR reactions of independent samples. First-order decay constants (k) were determined by nonlinear regression analysis (Prism version 3.03, GraphPad) of plots measuring the percentage of KLF4 mRNA remaining versus time of actinomycin D treatment. KLF4 mRNA decay constants are presented as the mean ± S.D. of three independent time-course experiments, permitting pairwise statistical assessment using the Student’s t test. Differences were considered significant if p < 0.005.

Chromatin immunoprecipitation assay (ChIP)—ChIP assays were conducted as described (13). Enrichment of KLF4-associated DNA sequences in immunoprecipitated samples and input samples was detected by quantitative RT-PCR using sequence-specific primers and LightCycler 480 SYBR Green I master mix. ChIP primers were as follows: hINV AP1-5/Sp1 binding site located at nucleotides −2218/−2055 (forward, 5'-TCAGCTGT-TATCCACTGCGCTCTTTT-3', reverse, 5'-TCACACCGGT-CTAAGGGGTAGCA-3'), and hINV promoter control located at nucleotides −1040/−919 (forward, 5'-CCTCTCA-GGGAGAGATTGACATGA-3', reverse, 5'-CAACAGT-GCACCACGACTGTGGA-3').

Epidermal Equivalents—Normal human keratinocytes (1.5 × 10⁶), growing in KSFM (25), were harvested with trypsin and electroporated with 3 μg of siRNA. The cells were re-plated and expanded in KSFM for 72 h, and 2 × 10⁶ cells were re-electroporated with 3 μg of siRNA and then allowed to settle overnight onto Millicell-PCF chambers (diameter = 12 mm, 0.4-μm pore size) in KSFM (Millipore, Billerica, MA). The cells were then shifted to EpiLife medium containing 1.4 mM calcium chloride and 5 μg/ml of vitamin C and cultured at the air–liquid interface. Fresh medium was added every 2 days, and after 4 days, the epidermal skin equivalents were harvested for preparation of histological sections and analysis of RNA for quantitative RT-PCR analysis.
KLF4 Regulates Keratinocyte Differentiation

Expression of differentiation-responsive epidermal genes has not been examined. Involucrin is an extensively studied model of differentiation-associated gene expression in epidermis and in epidermal keratinocytes (22, 49–53). Fig. 2 (A and B) shows that KLF4 expression increases hINV protein and mRNA level, suggesting that KLF4 may regulate hINV gene transcription. To explore this further, we monitored the impact of KLF4 expression and knockdown on hINV promoter activity. Cells were transfected with the hINV full-length promoter construct, pINV-2473 and hKLF4(1-470) (wild type); an hKLF4 mutant lacking the zinc finger domain, hKLF4(1-388); and a mutant encoding only the zinc finger domain, hKLF4(335–470) (13). Treatment with wild-type KLF4 increases transcription, but treatment with the inactive mutants, hKLF4(1-388) and hKLF4(335–470), does not (Fig. 2C). hKLF4(1-470) and hKLF4(1-388) were confirmed to be expressed at similar levels by immunoblot, thereby confirming that the difference in hINV promoter activity is not due to a difference in expression of these proteins (Fig. 2C). Because anti-KLF4 binds to KLF4 within amino acids 1–180, expression of hKLF4(335–470) could not be monitored. We also assessed the impact of knockdown of endogenous KLF4 on promoter activity. As shown in Fig. 2D, reduction in KLF4 level is associated with reduced hINV promoter activity.

KLF4 Is Required for Activation of hINV Gene Expression—To examine the interplay between PKCδ and KLF4, we examined the impact of KLF4 knockdown on PKCδ- and TPA-dependent activation of hINV gene expression. TPA is a strong inducer of keratinocyte differentiation and hINV expression (39). TPA is a diacylglycerol analog that is known to activate calcium- and phospholipid-dependent PKC isoforms (54). Fig. 3 (A and B), shows that KLF4 siRNA reduces the ability of PKCδ and TPA to increase hINV mRNA level, and Fig. 3C shows that KLF4 siRNA reduces TPA-stimulated hINV promoter activity. Consistent with these findings, KLF4 overexpression enhances the PKCδ- (Fig. 3D) and TPA- (Fig. 3E) dependent increase in hINV promoter activity.

KLF4 Activation of hINV Expression Requires the DRR—Our next goal was to identify hINV promoter regulatory sites responsible for the KLF4-dependent increase in transcription. Previously identified functional domains, including the DRR and the proximal regulatory region (PRR), are indicated in Fig. 4A. We tested the response of a series of truncated hINV promoter-luciferase reporter constructs to challenge with KLF4 expression plasmid (37). Activity of the full-length promoter construct, pINV-2473, was increased by KLF4, but the truncated constructs did not respond (Fig. 4B). These studies suggest that a KLF4-responsive element is present in the segment spanning nucleotides −2473 to −2136. This region encodes the hINV promoter DRR (39). pINV-41 encodes the hINV minimal promoter (39) and, as expected, has low activity and does not respond to KLF4 stimulation.

The DRR encodes previously characterized AP1 and GC-rich response elements located at nucleotides −2122/−2107 (38, 39). Because the KLF4-associated increase in hINV promoter activity is abolished in the absence of this segment (Fig. 4B), we determined whether the AP1-5 or the GC-rich response elements are required for the KLF4-dependent increase. Fig. 5A shows a schematic of the hINV luciferase promoter reporter plasmids used in this experiment. Fig. 5B shows that mutation of either the AP1-5 or the GC-rich response elements, or both elements, reduces KLF4-stimulated activity of the full-length promoter. This reduction is also observed (Fig. 5C) using a con-
struct, pINV(−2473/−2088), that encodes a smaller segment of the promoter containing only the DRR region (nucleotides 2473/−2088) linked to the minimal promoter (Fig. 5A). This finding indicates that the DRR is necessary and sufficient for the response and rules out a requirement for the PRR AP1-1 site (39). This eliminates the possibility that the PRR has a role in this regulation. pINV-41 encodes the hINV basal promoter (39) and, as expected, does not respond.

We next examined KLF4 interaction at the DRR using chromatin immunoprecipitation. Cells were infected with empty or KLF4-encoding virus, and after 48 h, extracts were prepared for chromatin immunoprecipitation. Fig. 6A shows increased KLF4 interaction at the DRR (nucleotides 2218/−2055) in KLF4-expressing cells. In contrast, as a control, we examined KLF4 interaction at a DNA region (nucleotides −1040/−919) that does not encode AP1 or GC-rich elements. As shown in Fig. 6B, KLF4 does not bind to this segment.

KLF4 Knockdown Suppresses Keratinocyte Differentiation and Reduces hINV Expression—To assess the impact of KLF4 knockdown on hINV expression during differentiation, we used an epidermal equivalent model that mimics in vivo epidermal differentiation (55). Keratinocytes were electroporated with control or KLF4 siRNA and then plated into Millicell chambers to test for the ability to form a stratified epidermal equivalent. Fig. 7A confirms that KLF4 siRNA reduces KLF4 mRNA and protein level. Fig. 7B shows that cells treated with control siRNA produce a well differentiated epidermal equivalent that includes a multiple stratified layer and a cornified layer. In contrast, KLF4 siRNA-treated cells form a disordered stratified structure in which suprabasal cells retain their...
nuclei (arrows), showing that KLF4 knockdown impedes the differentiation process. Counting of nucleated suprabasal cells reveals 8/110062 suprabasal nucleated cells in control siRNA-treated cultures, versus 41/11003 in KLF4 siRNA-treated cells (n = 5, p < 0.05). This is evidence of reduced differentiation. Fig. 7C shows that hINV mRNA level is reduced in these cultures, thus confirming that KLF4 is required for hINV expression under in vivo-like differentiation conditions.

**DISCUSSION**

Keratinocyte differentiation is a complex process that requires the coordinated activation of a variety of genes (22). Previous studies show that PKCδ is an important driver of cell differentiation (13) and that PKCδ stimulates a MEKK1, MEK3/MEK6, p38 cascade that triggers differentiation by increasing Sp1 and AP1 transcription factor level (22). In particular, the expression of involucrin, a marker of differentiation, is increased via AP1 and Sp1 factor interaction with DNA elements in the hINV promoter DRR (23–26). However, it is unlikely that these transcription factors are the only one involved in regulating involucrin gene expression.

KLF4 is an important candidate regulator that has not been extensively studied at the mechanistic level in epidermis. Previous studies in mouse transgenic mouse models indicate that KLF4 regulates keratinocyte differentiation (3, 10, 12, 21). Specifically, KLF4 impacts the terminal stages in differentiation. KLF4 knock-out mice die shortly after birth due to loss of skin barrier function, which is accompanied by loss of integrity of late stage differentiation structures such as the cornified envelope (12). These defects are retained when KLF4 knock-out mouse skin is grafted onto nude mice, suggesting that the absence of KLF4 creates an intrinsic defect in the keratinocytes (12). Consistent with these findings, overexpression of KLF4 in mouse epidermis, using the keratin 5 promoter, accelerates barrier formation, which is associated with increased epidermal stratification and increased expression of cornified envelope markers (10). Although global profiling has identified potential KLF4 tar-

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**FIGURE 4.** KLF4 activation of hINV promoter requires the DRR. A, the hINV promoter upstream regulatory region showing functionally important (37–39, 50, 52, 53) AP1 (AP1-1 and AP1-5) and GC-rich (Sp1 binding) (22, 37, 38) response elements. The two biologically important AP1 sites present within the upstream regulatory region are indicated (AP1-5 and AP1-1), as is the Sp1 site. The distances are in nucleotides relative to the transcription start site. B, KLF4 regulation of hINV promoter activity requires the DRR. Keratinocytes were transfected with 2 μg of the indicated hINV reporter plasmid and 1 μg of empty expression vector or KLF4 expression vector for 24 h prior to harvest and assay for luciferase activity. In all cases, the values are the mean ± S.D. (n = 3), and the asterisk indicates a significant increase or decrease, p < 0.005. Similar results were observed in three independent experiments.

**FIGURE 5.** KLF4 activation of hINV promoter activity requires the DRR AP1 and GC-rich response elements. A, schematic showing key regulatory elements in the hINV promoter. pINV-2473 is the full-length promoter. pINV(-2473/-2088) is a construct in which the DRR region (nucleotides -2473/-2088) is linked to the hINV minimal promoter (-41/-1). The dashed line indicates the fusion. The functionally important AP1 (AP1-1 and AP1-5) and GC-rich element are indicated. The distances are in nucleotides relative to the transcription start site. B and C, keratinocytes (KERn) were transfected with 2 μg of the indicated reporter plasmid and 1 μg of empty vector or hKLF4-expression vector, and after 24 h, the cells were harvested, and extracts were prepared for luciferase activity assay. The values are mean ± S.D., n = 3. In all cases, the asterisk indicates a significant reduction in luciferase activity as determined using the Student’s t test, p < 0.005.
Mechanism of KLF4 Regulation of Involucrin Expression—We previously showed that hINV expression occurs specifically in differentiated cells and involves interaction of AP1 and Sp1 transcription factors at specific sites in the hINV promoter distal regulatory region (22). This is observed in cultured cells (36, 40, 59), and the DRR is required to drive suprabasal expression in vivo when the human gene is placed in transgenic mice (49, 50, 52, 53, 58). Expression is activated by agents and kinases that stimulate keratinocyte differentiation including calcium, phorbol ester, and protein kinase C activity (22). The role of KLF4 in driving expression of differentiation-associated gene in epidermis has not been examined at the molecular level. In the present study, we explore the idea that KLF4 may drive expression of involucrin. We first show that KLF4 expression increases hINV mRNA and protein level, but that this increase is not observed with a transcriptionally inactive KLF4 mutant. The fact that vector-mediated KLF4 increases expression of involucrin, a marker of differentiation, is consistent with the proposed role of KLF4 in driving keratinocyte terminal differentiation (12). Moreover, knockdown of endogenous KLF4 reduces basal hINV mRNA level.

Promoter deletion experiments reveal that the KLF4 regulation requires the promoter DRR, which encodes closely juxtaposed AP1 and GC-rich (Sp1) binding sites. These sites have been implicated in regulation of hINV expression in cultured cells (38, 39, 60) and in transgenic mice in vivo (22, 49–53). KLF4 increases activity of the full-length promoter and a promoter segment that encodes only the DRR and flanking sequences. The fact that KLF4 activates the latter construct indicates that the hINV promoter proximal regulatory region is not required for this regulation. This is important because the PRR does have activity in culture models (24, 39). Chromatin immunoprecipitation analysis reveals that KLF4 is enriched at the DRR. It is known that KLF4 interacts at GC-rich elements to drive gene expression and that this interaction requires the KLF4 zinc finger DNA binding domain (1, 61). The Sp1 site of the hINV promoter DRR is a GC-rich sequence (37, 38) that would be predicted to bind KLF4 (1, 61). Indeed mutation of this DNA element results in reduced KLF4-dependent hINV promoter activity. In addition to the GC-rich element, the DRR is required to drive gene expression and that this interaction requires the KLF4 zinc finger DNA binding domain (1, 61). The Sp1 site of the hINV promoter DRR is a GC-rich sequence (37, 38) that would be predicted to bind KLF4 (1, 61). Indeed mutation of this DNA element results in reduced KLF4-dependent hINV promoter activity.
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ester activates this kinase (25, 31, 47, 63, 64). Our present studies show that PKCδ overexpression or treatment with TPA increases KLF4 level in keratinocytes. This is an interesting finding as very little is known about pathways that regulate KLF4 expression. It has been shown that ΔN-p63α suppresses expression in normal epidermal keratinocytes via a mechanism that involves ΔN-p63α direct interaction on the KLF4 promoter (48). ΔN-p63α also regulates KLF4 gene transcription in HaCaT keratinocytes, as determined using a KLF4 promoter-luciferase reporter construct (48). Our actinomycin D inhibitor studies indicate that mRNA stability is not altered in control versus PKCδ-expressing keratinocytes, suggesting that the increase in KLF4 expression is mediated via transcriptional mechanisms. Thus, our results are consistent with previously described transcriptional mechanisms for regulation of KLF4 expression (48).

Moreover, these findings are biologically cohesive as ΔN-p63α expression in basal keratinocytes is designed to maintain cell viability in this layer, and so it is expected that ΔNp63α would suppress expression of the pro-differentiation KLF4 protein (ΔNp63α is absent in the upper epidermis, and this is associated with KLF4 expression) (48). Our results show that PKCδ, a pro-differentiation regulator, increases KLF4 expression in keratinocytes to drive differentiation. Moreover, it is interesting that KLF4 is now implicated in two processes in keratinocytes: suppression of proliferation and enhancement of differentiation. Our recent study shows that PKCδ increases p21Cip1 expression via a mechanism that requires KLF4 (13).

KLF4 Is Required for hINV Expression in Differentiated Keratinocytes—To confirm that KLF4 is required for hINV expression, we used an epidermal equivalent system that mimics in vivo differentiation. In this system, keratinocytes are plated onto a semipermeable membrane and grown at the air-liquid interface. Under these conditions, keratinocytes assemble a highly differentiated multilayered epidermal equivalent that includes a basal proliferative layer, intermediate layers, and a cornified terminal layer (55). Our studies show that knockdown of KLF4 in these cultures severely compromises the differentiation process, resulting in formation of a disordered morphology and the absence of a cornified layer. We further show that hINV expression is markedly reduced. These findings strongly suggest that KLF4 has an in vivo role in controlling hINV gene expression.

Based on these studies, we propose that PKCδ induction of KLF4 expression is a key event in the suprabasal epidermis that drives hINV gene expression and keratinocyte terminal differentiation.

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