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Molecular Cloning and Expression of Human Keratinocyte Proline-Rich Protein (hKPRP), an Epidermal Marker Isolated from Calcium-Induced Differentiating Keratinocytes

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We isolated a human gene encoding keratinocyte proline-rich protein (hKPRP). hKPRP gene is located in the region of epidermal differentiation complex on chromosome 1q21, and its ~2.5 kb mRNA encodes 579 amino acid protein with high proline content (18%). The mRNA level of hKPRP was markedly increased at both 7 and 14 d after treatment with 1.2 mM calcium in cultured normal human epidermal keratinocytes. *In situ* hybridization demonstrated that hKPRP was expressed in upper granular layer of normal epidermis with characteristic intermittent pattern. In psoriatic lesion, hKPRP expression was increased as compared with normal skin and showed continuous pattern. Immunohistochemical analysis also confirmed the expression of hKPRP at the protein level. Western blot analysis showed that hKPRP protein of ~70 kDa size was significantly increased by calcium in a time-dependent manner. In mouse tissue blot assays, the expression of KPRP was detected in stomach and skin tissues, and began at 17.5 embryonic days. Additionally, hKPRP expression was detected in the periderm of human fetal skin from 16 wk estimated gestational age. Together, these results suggest that hKPRP is an epidermal marker expressed in stratified squamous epithelia and has a potential role in keratinocytes differentiation.

Key words: calcium/epidermal differentiation/human keratinocyte proline-rich protein
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The skin is an organ that lies at the interface between the organism and the environment (Eckert, 1989). It provides the physical barrier against environmental insults, such as chemicals, pathogens, and UV light (Chuong *et al*, 2002). This protective function is largely dependent on the cornified cell envelope (CE), a specialized insoluble structure formed beneath the plasma membrane in terminally differentiated stratified squamous epithelium (Kalinin *et al*, 2001; Kalinin *et al*, 2002). Most of CE-built proteins are provided by skin keratinocytes through a sophisticated differentiation process that involves the sequential expression of lots of genes. These include transglutaminase 1 and 3 (TGase 1 and 3), involucrin, cornifin, loricrin, filaggrin, and small proline-rich proteins (SPR) (Rice and Green, 1977; Fuchs, 1993; Steinert and Marekov, 1995; Steinert and Marekov, 1997; Nemes and Steinert, 1999). Interestingly, many of these genes have evolutionarily conserved structure, and are clustered on chromosome 1q21 in humans (Volz *et al*, 1993), chromosome 3 in mice (Cabral *et al*, 2001), and chromosome 2 in rats (Kong *et al*, 2003). Despite intensive investigation during the last decades, however, many genes related to keratinocytes differentiation remain to be discovered.

In an effort to identify the genes related to keratinocytes differentiation, we previously performed suppression subtractive hybridization (SSH) using a calcium-induced keratinocytes differentiation model. One gene, previously named as 7-4-14, showed remarkable induction by calcium treatment in normal human epidermal keratinocytes (NHEK) (Seo *et al*, 2004). In this study, we isolated the full-length cDNA of that gene and named as human keratinocytes proline-rich protein (hKPRP), based on the sequence analysis. We demonstrated that hKPRP is expressed in stratified squamous epithelial layer in a differentiation-specific manner, and believed that hKPRP is a novel marker for epidermal differentiation.

Abbreviations: CE, cornified cell envelope; EGA, estimated gestational age; hKPRP, human keratinocyte proline-rich protein; NHEK, normal human epidermal keratinocytes; SSH, suppression subtractive hybridization

Results

Isolation of hKPRP, a gene associated with the keratinocyte differentiation We previously obtained a partial cDNA fragment that showed remarkable induction by cal-

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cium treatment in NHEK (Seo *et al*, 2004). Partial sequence obtained from SSH was analyzed against NCBI Map viewer, and result showed that this gene was located in the region of epidermal differentiation complex (EDC) on chromosome 1q21 (Fig 1). In order to isolate the full length cDNA, we performed 5′- and 3′-RACE and obtained 2.5 Kb cDNA. Sequence analysis against BLAST search revealed that this gene encoded a hypothetical protein (accession number XM_060108). Its open reading frame predicted a protein of 579 amino acids with 64 kDa molecular mass (Fig S1). One characteristic feature of this protein was that its proline content was relatively high (18%), within the carboxyl-terminal half proline content reached 26%. This putative protein has high homology (64%) to a rat keratinocyte proline-rich protein (accession number AF548002) (Fig 2), thus we named it as human keratinocyte proline-rich protein (hKPRP, GenBank accession number AY960854). Amino acid sequence analysis using Prosite (http://au.expasy.org/prosite) revealed that hKPRP has eight putative *N*-myristoylation sites, gluatime-rich region (aa 4–209), and proline-rich region (aa 257–510).

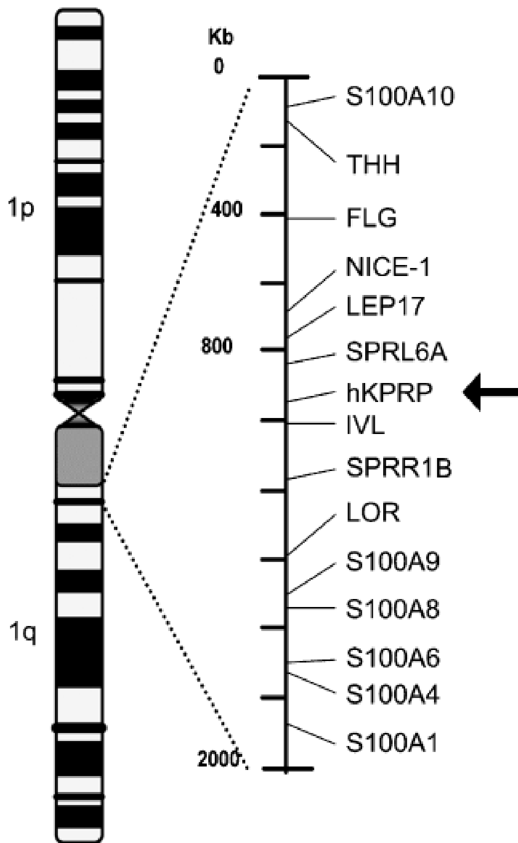

Figure 1
Map of the 2 Mb epidermal differentiation complex (EDC). The q21 region of chromosome 1 is expanded to illustrate the EDC. The gene for hKPRP is located between SPRL6A and IVL genes, indicated by arrow. S100A10, S100 calcium binding protein A10; THH, trichohyalin; FLG, filaggrin; NICE-1, NICE-1 protein; LEP17, late envelope protein 17; SPRL6A, small proline rich-like 6A; hKPRP, human keratinocyte proline-rich protein; IVL, involucrin; SPRR1B, small proline-rich protein 1B; LOR, loricrin; S100A9, S100 calcium binding protein A9; S100A8, S100 calcium binding protein A8; S100A6, S100 calcium binding protein A6; S100A4, S100 calcium binding protein A4; S100A1, S100 calcium binding protein A1.

Detection of hKPRP mRNA In our previous report (Seo *et al*, 2004), the mRNA expression was detected using a partial cDNA fragment obtained from SSH that covered 3′-untranslated region of hKPRP. To further verify whether the expression of hKPRP was related to the calcium-induced keratinocytes differentiation, we performed Northern blot analysis using a partial cDNA fragment that included 5′-end of hKPRP (nt 19–499). As shown in Fig 3*A*, hKPRP mRNA was detected at 7 and 14 d after treatment with calcium of NHEK cultured *in vitro*. Real-time PCR also confirmed that hKPRP mRNA is highly induced by calcium (Fig 3*A*, *lower panel*). Interestingly, this expression pattern was very similar to that of loricrin (Seo *et al*, 2004), suggesting its possible involvement during the late events of keratinocytes differentiation, such as CE formation.

To localize the hKPRP expression, we performed *in situ* hybridization using both sense and anti-sense riboprobes (Fig 3*B*). Consistent with northern blot data, the expression of hKPRP was detected at upper granular layer of normal

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hKPRP: 1   MCDQQQIQCRLPLQQCCVKGPSFCSSQSPFAQSQVVVQAPCEMQIVDCPASCPVQVCQ-- 58
           MCDQQ+IQC  P+ QCCVKG SF  SQ P+A +QV+V+APCEMQ ++C A CP+QV Q
rKPRP: 1   MCDQQEIQCCGPIPQCCVKGSSFGPSQFPYANNQVLVEAPCEMQFLECAAPCPIQVSQTP 60

hKPRP: 59  -------VSDQAPCQSQTTQVKCQSKTKQVKGQAQCQSKTTQVKGQAASQSQTSSVQSQA 111
                  V  QAPC+  TT VKCQ+KT QVK    CQ KTT++K QA  Q+Q S VQ QA
rKPRP: 61  CQSSTTEVKGQAPCK--TTNVKCQTKTTQVK----CQPKTTEIKCQAPCQAQVSCVQCQA 114

hKPRP: 112 PCQSEVSYVQCEASQPVQTCFVECAPVCYTETCYVECPVQNYVPCPAPQPVQMYRGRPAV 171
           PCQS+VSYVQ    QP QT +VECAPV YTET +VE PV NYVP PAPQP   Y   P++
rKPRP: 115 PCQSQVSYVQVP--QPPQTYYVECAPVYYTETRFVEYPVSNYVPVPAPQPGYTYVECPSL 172

hKPRP: 172 CQPQGR--FSTQCQYQGSY-------------SSCGPQFQSRATCNNYTPQFQLRPSY-- 214
            Q QG+  FST+ QYQGSY             SSCGPQ QS+A+ +   PQFQ RPSY
rKPRP: 173 GQGQGQGSFSTRYQYQGSYGSCTSQSQRGSYSSCGPQHQSQASYSYCEPQFQSRPSYTN 232

hKPRP: 215 ------------------------SSCFPQYRSRTSFSPCVPQCQTQGSYGSFTEQHRSR 250
                                   S+C  Q RS TSFS C PQCQ QG+YGSFT Q +S+
rKPRP: 233 CGTQRQSQASFGSCTSQLQSRASYSNCSSQRRSGTSFSTCAPQCQGQGTYGSFTAQRKSQ 292

hKPRP: 251 STSRCLPPPRRLQLFPRSCSPPRRFEPCSSSYLPLRPSEGFPNYCTPPRRSEPIYNSRC- 309
           S SRCLP  RRLQ   RSCSPPR  EPC SS LP R S G  NYCTPPRRSEPIY S C
rKPRP: 293 SASRCLPS-RRLQPSYRSCSPPRHSEPCYSSCLPSRCSSGSYNYCTPPRRSEPIYGSHCS 351

hKPRP: 310 PRRPISSCSQRRGPKCRIEISSPCCPRQVPPQRCPVEIPPIRRRSQSCGPQPSWGASCPE 369
           PR   S CSQR GPKCRIEISSPCCPRQVPPQRCPV+IPPIR RS+SC  QPSWG SCP+
rKPRP: 352 PRGRPSGCSQRCGPKCRIEISSPCCPRQVPPQRCPVQIPPIRGRSRSCPRQPSWGVSCPD 411

hKPRP: 370 LRPHVEPRPLPSFCPPRRLDQCPESPLQRCPPPAPRPRLRPEPCISLEPRPRPLPRQLSE 429
           LRP  EP   P  C P+RLD+ PES  +RCP PAPRP  RPEPC S EPRP P PR   E
rKPRP: 412 LRPCAEPHAFPRPCRPQRLDRSPESSWRRCPVPAPRPYPRPEPCPSPEPRPCPRPRPRPE 471

hKPRP: 430 PCLYPEPLPALR------------------PTPRPVPLPRPGQCEIPE--PRPCLQPCEH 469
           PC  PEP P  R                  P+P P P PRP  C  PE  PRPC +PC
rKPRP: 472 PCPSPEPRPRPRPDPCPSPELRPRPRPEPCPSPEPRPRPRPDPCPSPEPRPRPCPEPCPS 531

hKPRP: 470 PE--------------------------------------------------------PC 473
           PE                                                        PC
rKPRP: 532 PEPRPCPPLRRFSEPCLYPEPCSVSKPVPCPVPCPAPHPRPVHCETPGRRPQPSPRSQPC 591

hKPRP: 474 PRPEPIPLPAPCPSPEPCRETWRSPSPCWGPNPVPYPGDLGCHESSPHRLDTEAPYCGPS 533
           P PEP+P P PC SP PC +    PSPC G NPVPY  +LGCHES+P RLDTE P
rKPRP: 592 PHPEPMPRPVPCSSPVPCGDPIHCPSPCSGHNPVPYSQELGCHESNPCRLDTEGP--SSY 649

hKPRP: 534 SYNQGQESGAGCGPGDVFPERRGQDGHGDQGNAFAGVK----GEAKSAYF 579
           S++QGQES   C  G VF   RG  G GDQGN + G+     G  + AYF
rKPRP: 650 SFSQGQESNGCCVSGGVFSGSRGLSGCGDQGNTYRGMNCGACGGTQGAYF 699
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Figure 2
Sequence alignment between human keratinocytes proline-rich protein (hKPRP) and rat keratinocytes proline-rich protein (rKPRP). Similarity of amino acid sequence between hKPRP and rKPRP is evaluated. The alignment shows 64% identity between these two proteins.

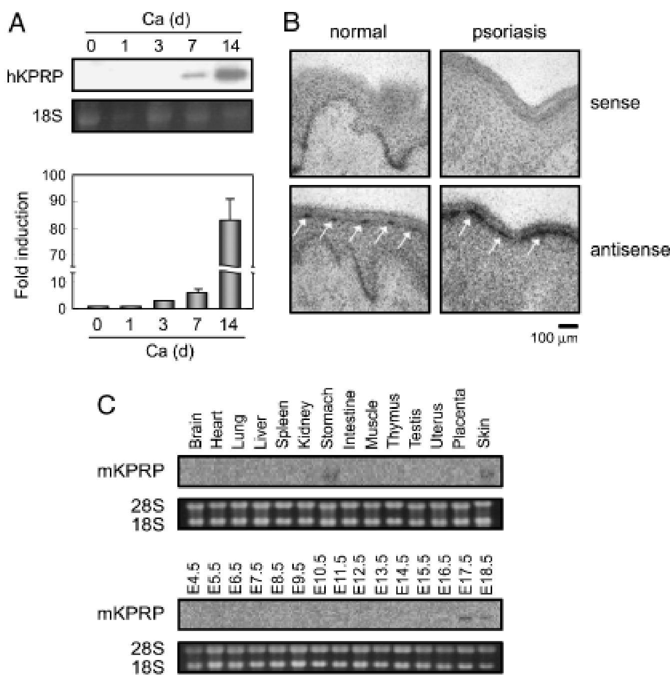

Figure 3
Detection of human keratinocytes proline-rich protein (hKPRP) mRNA. (*A*) Lower panel showed the result of quantitative real-time PCR analysis. Two micrograms of total RNA were reverse transcribed with M-MLV reverse transcriptase and used for PCR amplification. The data of real-time PCR was corrected according to the quantity of β-actin and expressed as a fold induction. Data are represented as mean ± SE of triplicated experiments ($p < 0.01$). (*B*) *In situ* hybridization. Fourteen micrometer sections of human normal skin and psoriatic lesion were hybridized with sense and antisense hKPRP riboprobes. The hKPRP-specific hybridization signals are strongly detected in the granular layer of the epidermis of both the normal and psoriatic skin. Arrows indicate the cells that express hKPRP. Pictures were taken under the magnification of × 40 (*scale bar*: 100 μm). (*C*) Tissue distribution and developmental expression of mouse KPRP. Using mouse KPRP probe that was made by RT-PCR, northern hybridization was performed. About 3.0 kb band was seen in stomach and skin tissues (*upper panel*), and the expression of mouse KPRP begins at E 17.5 d (*lower panel*). Ethidium bromide-stained gel was photographed as a loading control.

skin, with a characteristic intermittent pattern. As psoriasis is a well-known disease related to keratinocytes differentiation, we also investigated the localization of hKPRP in psoriatic lesion. Interestingly, the expression of hKPRP was highly increased as compared with normal skin and showed continuous pattern.

Next, we examined at which tissues KPRP was expressed. To this end, we used mouse tissue blot obtained commercially and mouse KPRP probe that was made by RT-PCR based on the predicted sequence (accession number XM_485267). As shown in Fig 3*C*, mouse KPRP mRNA of ~ 3.0 kb was detected in stomach and skin. We further determined the KPRP expression in developmental stage using mouse embryo blot. KPRP began to express from E17.5 d (Fig 3*C*, *lower panel*).

Detection of hKPRP protein To investigate the hKPRP expression at the protein level, we raised polyclonal antibody against synthetic peptide covering the middle portion of hKPRP (aa 415–429). Western blot analysis showed that hKPRP protein of ~ 70 kDa was highly increased by calcium treatment of NHEK in a time-dependent manner (Fig 4*A*). This data was well consistent with Northern blot result. To further verify that hKPRP was related to the keratinocytes differentiation, we used another model system in which immortalized keratinocytes HaCaT was induced to differentiate by calcium and ionophore A23187 (Fuchs, 1990; Zhao *et al*, 1992). As expected, hKPRP protein level was also increased by treatment of calcium and ionophore A23187 (Fig 4*B*).

To localize the hKPRP expression, we performed immunohistochemical staining (Fig 4*C*). The results showed that the expression of hKPRP was highly differentiation-specific in normal skin. Particularly, very strong signal was seen in the uppermost part of granular layer. In psoriatic lesion, the expression of hKPRP was significantly increased as compared with normal skin, showing consistent result with *in situ* hybridization data. In addition, we investigated hKPRP expression in several skin diseases including epidermal cyst and lichen planus. The expression of hKPRP was found in upper granular layer of epidermal cyst skin. In contrast, hKPRP expression was not detected in the lesion of lichen planus. We also examined the hKPRP expression in atopic dermatitis and it was rarely detected (data not shown).

To investigate the spatiotemporal expression of hKPRR in developmental stage, immunohistochemical staining was performed with human fetal skin. It has been previously documented that the embryonic epidermis consists of the periderm, intermediate, and basal layers until 22 wk estimated gestational age (EGA), but they were replaced with the granular, spinous, and basal layers from 24 wk EGA (Holbrook and Odland, 1980; Lee *et al*, 1999). As shown in Fig 4*D*, the expression of hKPRP began at 16 wk EGA in the periderm and not in intermediate layer. Further, hKPRP was continuously expressed in the granular and horny layers of the epidermis at 24 wk EGA and thereafter.

Discussion

Although a number of genes required for keratinocyte differentiation have been already discovered, it is likely that many of important molecules remain undisclosed. In this study, we have cloned a novel keratinocytes differentiation related gene hKPRP by a PCR-based cDNA subtraction method. Based on the sequence analysis, hKPRP is though to be a homolog of rat KPRP isolated by Kong *et al* (2003). The full-length cDNA of hKPRP is about 2.5 kb and predicted to encode 579 amino acid protein with a calculated molecular mass of 64 kDa and an isoelectric point (IP) of 8.72, which is little different from rat KPRP with 699 amino acids, 76.4 kDa molecular mass and 8.4 IP. One remarkable feature of hKPRP is a high content of proline, similar to the SPR. Despite its high proline content, however, hKPRP is unlikely to be related with SPR. The SPR have a common structure of head, tail, and internal repeat domains, and the amino acid sequences of each domain are highly conserved in SPR family (Cabral *et al*, 2001). The amino acid sequences of head and tail of hKPRP do not correspond to those of SPR, however, and there are no internal repeats of consensus sequence. In addition, locus for hKPRP is out of SPR

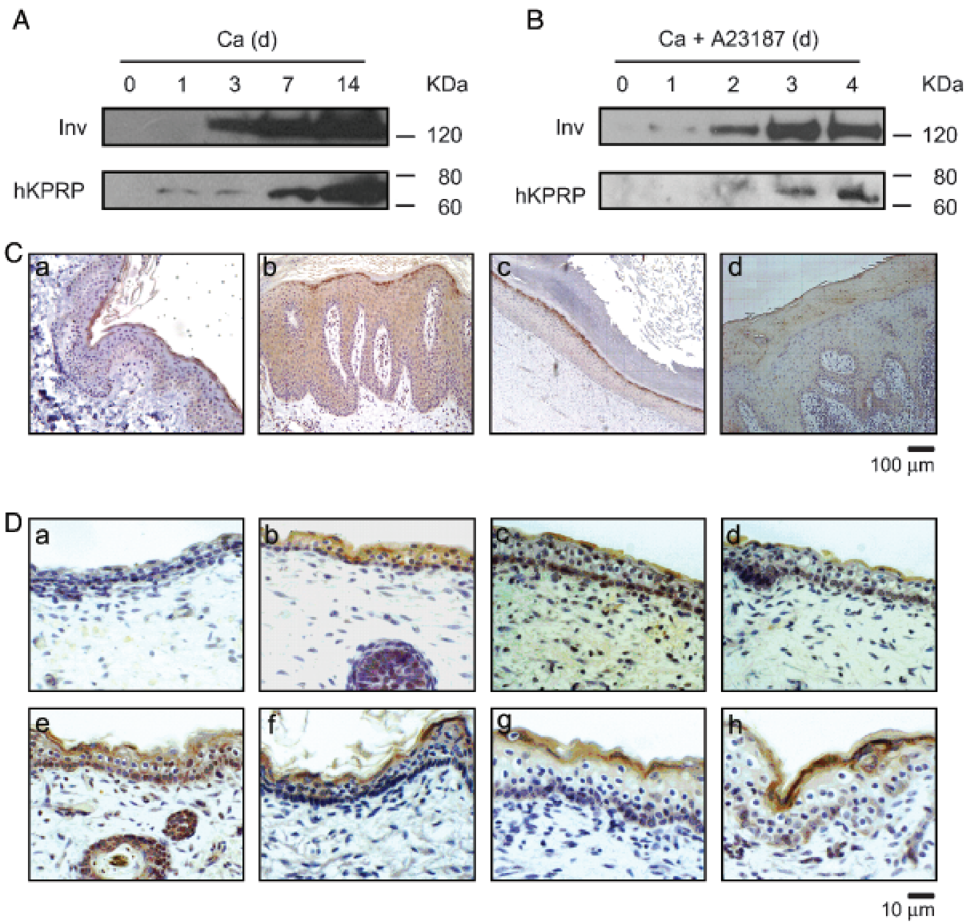

Figure 4
Detection of human keratinocytes proline-rich protein (hKPRP) protein. (*A*) Western blot analysis. Normal human epidermal keratinocytes were cultured and differentiated by addition of 1.2 mM calcium (Ca) for the indicated time points. Proteins (20 μg per lane) were separated on 10% polyacrylamide gels. Duplicate blots were probed with anti-involucrin (Inv) antibody as a positive control, and anti-hKPRP antibody. (*B*) HaCaT keratinocytes were induced to differentiate by treatment with 0.3 mM calcium and ionophore A23187 (1 μM) for the indicated time points. Western blot was performed as in (*A*). (*C*) Immunohistochemical staining. Paraffin-embedded tissue sections of skin specimens were stained with anti-hKPRP antibody. a, normal skin; b, psoriatic lesion; c, epidermal cyst lesion; d, lichen planus lesion. Pictures were taken under the magnification of $\times 40$ (*scale bar*: 100 μm). (*D*) The expression of hKPRP during human fetal development. Paraffin-embedded tissue sections of human fetal skin were stained with anti-hKPRP antibody. (*a*) 14 wk estimated gestational age (EGA), (*b*) 16 wk, (*c*) 17 wk, (*d*) 18 wk, (*e*) 20 wk, (*f*) 24 wk, (*g*) 27 wk, and (*h*) 32 wk. Pictures were taken under the magnification of $\times 200$ (*scale bar*: 10 μm).

multigene cluster within the EDC on chromosome 1q21, suggesting that there is no relationship between hKPRP and SPR in terms of evolution via gene amplification.

Amino acid sequence analysis revealed that there are eight putative *N*-myristoylation sites in hKPRP protein. Protein myristoylation is critical in many biological pathways such as signal transduction, apoptosis, and alternative extracellular protein export (Maurer-Stroh *et al*, 2002). It has been suggested that the myristoyl group acts as not only a simple hydrophobic anchor, but also a modulator of calcium-sensitive process. For example, recoverin, a known myristoylated protein that controls the lifetime of the photoexcited rhodopsin (Senin *et al*, 1995), undergoes conformational change and becomes an active form upon binding of calcium (Ames *et al*, 1997). Therefore, it could be speculated that the myristoyl-binding sites of hKPRP protein might play some specific roles in the epidermal differentiation by cooperating with calcium. Domain analysis revealed that hKPRP protein contains also the glutamine-rich region and the proline-rich region at N-terminal and C-terminal respectively, suggesting that it could be linked by transglutaminases and become a component of the CE.

So far, it is not known whether hKPRP is a structural protein in the CE or another type of functional protein. We speculate, however, that hKPRP is one of CE components for several reasons below. First, temporal expression pattern of hKPRP was almost same to loricrin expression after calcium treatment of cultured NHEK at both transcriptional and translational levels. Loricrin is a well characterized CE protein that shows late expression in calcium-induced keratinocytes differentiation (Seo *et al*, 2004). Second, *in situ* hybridization and immunohistochemical staining clearly localized the hKPRP expression at the uppermost part of granular layer, implicating that this protein may exert its role during the formation of corny layer. This notion is partly supported by the fact that hKPRP expression looks like slightly increased in the region of thick corny layer of epidermal cyst skin as compared with that of thin corny layer (Fig 4*Cc*). It is not clear, however, why there is no hKPRP expression in lichen planus, which is one of representative skin disease showing thick corny layer with thick granular layer. Third, tissue distribution of hKPRP expression was restricted to the orthokeratinizing epithelia such as stomach and skin, similar to other CE proteins. For example, human loricrin is expressed only in the stratified squamous epithelia of epidermis, palate, stomach, tongue and esophagus (Hohl *et al*, 1993). Another example includes suprabasin expressed in skin, tongue, palate and stomach (Park *et al*, 2002). Fourth, the KPRP expression was first observed at E17.5 in mice, coinciding with beginning of stratification of the epithelia in developmental stage (Koster and Roop, 2004). In addition, hKPRP expression started from 16 wk EGA in human fetal development and persisted postnatally through the epidermal layers. Since it has been suggested that the sequential order of CE proteins expression in human fetal skin was similar to that of their incorporation into the CE in postnatal skin (Lee *et al*, 1999), temporal expression pattern of hKPRP reflects somehow the possibility of

becoming a CE component. Evidence suggests that the immature cornified CE is formed in the early second trimester (~ 16 wk EGA), whereas the mature CE is formed in the late second or early third trimester (Lee *et al*, 1999). Although it has been suggested that the periderm uses different sets of precursor proteins in forming a barrier structure, several well-known mature CE precursors, such as loricrin and small proline rich-like protein 1 (SPRR1), are also included in immature CE formation. Therefore, the expression of hKPRP at 16 wk EGA suggests that this protein may be also included in immature CE formation, together with mature CE formation. Nonetheless, the precise biochemical and functional characteristics of hKPRP remain to be elucidated.

In summary, we isolated a novel gene hKPRP and demonstrated that its expression is closely related to the keratinocytes differentiation. This newly identified gene may be a novel epidermal differentiation complex member and thus a potential candidate for skin diseases. Although it remains unknown whether hKPRP is a structural protein in the CE or another type of functional protein, further analysis of hKPRP in comparison with CE proteins will certainly reveal the crucial molecular mechanisms underlying the regulation of skin homeostasis.

Materials and Methods

Skin samples All skin samples were obtained under the written informed consent of donors, in accordance with the ethical committee approval process of Chungnam National University Hospital. The study was conducted according to Declaration of Helsinki Principles.

Cell culture Skin specimens were briefly sterilized in 70% ethanol, minced, and then treated with dispase for overnight at 4°C. The epidermis was separated and placed in a solution containing 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) (Gibco BRL, Rockville, Maryland) for 15 min at 37°C. After vigorous pipetting, cells were pelleted and resuspended in keratinocyte-serum free medium (K-SFM) supplemented with bovine pituitary extract and recombinant human epidermal growth factor (Gibco BRL). Cells were grown in collagen-coated dishes at 37°C, 5% CO_2 atmosphere. At third passage, cells were switched to the same medium containing 1.2 mM calcium and cultured for 1, 3, 7, and 14 d. Immortalized human keratinocytes HaCaT were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL). At 70% confluency, cells were washed twice with PBS and cultured in K-SFM for 24 h. To induce differentiation, 1 μM A23187 and 0.3 mM calcium were added and further incubated for 1, 2, 3, and 4 d.

SSH Messenger RNA were isolated using an Oligotex mRNA kit (Qiagen, Hilden, Germany). The suppression subtractive hybridization was carried out using a PCR-Select cDNA subtraction kit (Clontech, Palo Alto, California), according to the method described previously (Seo *et al*, 2004). One cDNA clone that showed marked induction by calcium in NHEK was selected for a further study.

Rapid amplification cDNA end (RACE) To isolate the full-length cDNA, we performed 5′- and 3′-RACE with GeneRacer kit with superscript II RT (Invitrogen, Carlsbad, California). As a results, two overlapping clones were obtained that covered amino-terminal and carboxy-terminal regions of hKPRP mRNA. These clones were subcloned into the TA cloning vector, and then sequenced. Two clones were complied to construct a full-length hKPRP cDNA.

Northern blot analysis Total RNA were prepared using an Easy blue RNA isolation kit (Intron, Daejeon, Korea). For northern blot analysis, 10 μg of total RNA were electrophoresed in 1% agarose gels containing formaldehyde and transferred onto Hybond-N^+ membranes (Amersham, Buckinghamshire, UK). Blots were prehybridized for 2 h at 42°C, then hybridized overnight at 42°C in the same solution containing ^{32}P-labeled probe (specific activity is $\sim 1 \times 10^9$ cpm per μg). Following hybridization, blots were washed and analyzed using a Fuji BAS 2500 phosphoimager (Fuji Film, Tokyo, Japan). For tissue distribution and developmental expression of KPRP, mouse RNA blots (Seegene, Seoul, Korea) were hybridized according to manufacturer's recommendation. Probes were prepared by RT-PCR. The following primers were used to amplify each cDNA respectively: hKPRP 5′-GCATCAGGAC-CATGTGTGACC and 5′-GGACAGGGTACATAGTTCTGGA; mouse KPRP 5′-TACCTTACCGCCAAGAACT and 5′-GTATTTCCTTGGTC TCCACA; β-actin 5′-ATCGTGGGCCGCCCTAGGCA and 5′-TGG CCTTACCCTTC AGAGGGG.

Real-time PCR For quantitative measurement, real-time PCR was performed using Rotor-Gene 2000 real-time amplification operator (Corbett Research, Mortlake, Australia). The PCR reactions contained a final concentration of 1 × SYBR Green PCR master mix (Applied Biosystems, Warrington, UK), 10 μM specific primers, and 2.5 ng of cDNA. Relative expression level of hKPRP was computed with respect to the expression level of β-actin according to the method previously reported (Gelmini *et al*, 2001).

***In situ* hybridization** Skin specimens were fixed in 4% paraformaldehyde in PBS for overnight at 4°C, then dehydrated and embedded with OCT compound (Leica Microsystems, Bannockburn, Illinois). Fourteen μm sections were mounted onto gelatin-coated slides, and air-dried. After rehydration, sections were washed with 0.1% diethylpyrocarbonate in PBS for 15 min and incubated with 1 μg per mL of proteinase K for 10 min at 37°C. Riboprobes corresponding to the sense and antisense strands of human hKPRP partial cDNA (about 500 bp) were prepared using T7 and SP6 RNA polymerase (Promega, Madison, Wisconsin) in the presence of [^{35}S]UTP (Amersham). After overnight hybridization at 55°C, sections were rinsed and visualized using Hypercoat nuclear emulsion (Amersham).

Preparation of hKPRP antibody and western blot analysis Polyclonal antibody against hKPRP were raised using the synthetic peptide SLEPRPRPLPRQLSE (aa 415–429 from the deduced protein sequence of hKPRP). For western blot analysis, cell extracts were prepared using Pro-prep protein extraction solution (Intron). Protein samples were run on 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane and incubated with anti-hKPRP antibody. For detection of involucrin, appropriate monoclonal antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, California). Blots were then incubated with peroxidase-conjugated secondary antibody and developed by enhanced chemiluminescence (Amersham).

Immnohistochemistry Paraffin sections of skin specimens were dewaxed, rehydrated, then washed three times with PBS. After treatment with proteinase K for 5 min at 37°C, sections were treated with H_2O_2 for 10 min at room temperature, blocked in 0.1% Tween-20, 1% bovine serum albumin in PBS for 20 min, and followed by reaction with anti-hKPRP antibody (1:5000 dilution) for 30 min. Sections were incubated sequentially with peroxidase-conjugated secondary antibody and visualized with Chemmate envision detection kit (Dako, Carpinteria, California). Sections without primary antibody were used as negative controls.

Statistical analysis Data were evaluated statistically using Student's *t* test or one-way analysis of variance followed by Fisher's least significant difference test for a *post hoc* comparison. Statistical significance was set at $p < 0.01$.

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Supplementary Material

The following material is available online for this article.
Figure S1
Nucleotide sequence and putative amino acid sequence of hKPRP.

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