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CD24 (heat stable antigen, nectadrin), a novel keratinocyte differentiation marker, is preferentially expressed in areas of the hair follicle containing the colony-forming cells

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SUMMARY

We have isolated, by subtractive and differential hybridization from a library constructed from keratinocyte colony-forming cells (K-CFCs), a cDNA coding for the rat CD24 (nectadrin, heat stable antigen). CD24, a glycoprotein thought to be involved in cell-cell adhesion and signalling, is highly expressed in keratinocytes located in the bulge area of the rat vibrissa which contains the most K-CFCs. CD24 is also expressed in the outer epithelial sheath of human hair follicles and in glabrous epidermis. However, its expression is not restricted to K-CFCs as demonstrated by cell sorting experiments, and it is thus not a specific marker of clonogenic keratinocytes. Rather, its preferential distribution in keratinocytes located in the most innervated area of the rat vibrissal follicle, i.e. the bulge, suggests that its function could be related to the tactile role of the hair follicle.

Key words: CD24, Keratinocyte, Hair follicle

INTRODUCTION

In the epidermis, proliferative keratinocytes are located in the basal layer and, to some extent, in the suprabasal layer. Two different populations can be distinguished by cell kinetic experiments. Cells belonging to the first population are slow-cycling when labeled with ^{3}H-TdR and are likely to be stem cells. Cells belonging to the second population are rapidly cycling and are thought to be transient amplifying cells with restricted growth potential (Potten, 1983; Lavker and Sun, 1982, 1983; Weinstein et al., 1984; Régnier et al., 1986; Potten and Morris, 1988; Fuchs, 1990; Cotsarelis et al., 1990). Slow cycling ^{3}H-TdR-retaining keratinocytes have also been identified in the bulge area of mouse pelage hair follicles, i.e. in the upper part of the follicle. On the contrary, rapidly cycling keratinocytes have been identified in the hair matrix, i.e. in the epithelial part of the hair follicle bulb (Cotsarelis et al., 1990). Multiplying keratinocytes which can initiate colonies in vitro are defined as clonogenic or keratinocyte-colony forming cells (K-CFCs) (Rheinwald and Green, 1975) and some of them have the growth potential expected from stem cells (Barrandon and Green, 1985; Rochat et al., 1994; Jones and Watt, 1993, 1995). Interestingly, most follicular clonogenic keratinocytes are also located in defined areas, i.e. in the bulge of the rat vibrissal follicle (Kobayashi et al., 1993) and in the lower third of the outer epithelial sheath of human scalp follicles (Limat and Noser, 1986; Lenoir et al., 1988; Rochat et al., 1994) while very few K-CFCs are found in the hair bulb (Kobayashi et al., 1993). In an attempt to identify genes expressed specifically in clonogenic keratinocytes but not in transient amplifying cells (Barrandon, 1993), we therefore took advantage of this segregation and constructed a cDNA library from a clone of K-CFCs, derived from the bulge area of a rat vibrissal follicle. This library was then subtracted with cDNAs prepared from freshly microdissected hair matrices which contain many proliferative keratinocytes but very few K-CFCs (Kobayashi et al., 1993). Interestingly, a cDNA which hybridized preferentially to transcripts expressed in the bulge area encodes the rat CD24 (mouse heat stable antigen, nectadrin), a small and highly glycosylated protein (35-45 kDa) which had been originally described as a pre-B lymphocyte marker (Dupperay et al., 1990; Kay et al., 1990, 1991; Wenger et al., 1991).

CD24 is, however, expressed in other cell types like T-cells (Crispe and Bevan, 1987), neurons (Rougon et al., 1991; Kadmon et al., 1992), muscle cells (Figarella-Branger et al., 1993), and carcinoma cells (Jackson et al., 1992). It is synthesized as a pro-peptide of 76 and 80 amino acids in mouse and human cells, respectively (Kay et al., 1991; Wenger et al., 1991), which is then processed. Removal of a signal peptide and of a carboxy terminal tail leads to the formation of a smaller peptide of about 30 amino acids (3 kDa) which is then N- and O- glycosylated and anchored to the cell membrane by a glycosylphosphatidylinositol (Kay et al., 1990; Wenger et al., 1991; Jackson et al., 1992). Variations in the carbohydrate

contents of CD24 account for the different molecular masses (35 to 65-70 kDa) that have been reported. Moreover, differences in its glycosylation may reflect cell or tissue functional specificities (Kay et al., 1990, 1991; Wenger et al., 1991; Kadmon et al., 1992; Jackson et al., 1992).

The function of CD24 has not yet been clearly demonstrated, but it has been suggested that it is involved in cell adhesion and signalling. For instance, the CD24 sugar backbone may act as a ligand of lectin-like molecules (Kay et al., 1991; Shirasawa et al., 1993) such as P-selectin (Sammar et al., 1994). Furthermore, CD24 participates in cell-cell and cell-matrix interactions of neural cells either via homotypic interactions or via heterotypic interactions with the L1 neural cell adhesion molecule (Kadmon et al., 1992, 1995; Hubbe and Altvogt, 1994). Several observations indicate that CD24 may also be involved in signal transduction. In pre-B-lymphocytes, membrane-bound CD24 complexes are specifically associated with a tyrosine-kinase activity (Stefanova et al., 1991). Also, intracellular calcium concentrations are significantly increased when lymphocytes are treated with an anti-CD24 antibody (Fischer et al., 1990). This is also observed in cerebellar neurons and neuroblastoma N_2a cells (Kadmon et al., 1995).

The expression of CD24 in the areas of the hair follicle containing the clonogenic keratinocytes is then of special interest. Therefore we selected human keratinocytes expressing CD24 and cultivated them under clonal conditions. We found that membrane-bound CD24 was not restricted to K-CFCs and that it was not a specific marker of clonogenic keratinocytes. The function of CD24 in keratinocytes remains thus to be clarified. We suggest that its function may be related to the tactile role of the hair follicle, possibly through neuron-keratinocyte signalling, as it is an adhesion and signalling molecule preferentially expressed in the most innervated area of the rat vibrissal follicle (i.e. the bulge).

MATERIALS AND METHODS

Materials

Vibrissal follicles were obtained from mystacial pads of Fisher 344 inbred rats and microdissected as described (Kobayashi et al., 1993). Rat epidermal keratinocytes and dermal fibroblasts were obtained from rat foot pads. KR4cl2 keratinocytes were obtained from a KCF-C cloned from the bulge area of a rat vibrissal follicle (A. Rochat et al., unpublished results). Human hair follicle keratinocytes (YH16P1 and YH16P3) were obtained as described (Rochat et al., 1994). Human epidermal keratinocytes (YF29) and dermal fibroblasts (AFF11) were obtained from the foreskin of newborns.

Cell culture

Swiss 3T3 cells were cultivated in DMEM supplemented with 10% calf serum. Rat and human dermal fibroblasts were cultivated in DMEM supplemented with 10% fetal calf serum (FCS), Pre-B lymphomblastoid cells (NAML6), were cultivated in RPMI supplemented with 10% FCS. Media were from Gibco BRL and sera were from HyClone Laboratories Inc.

Rat and human keratinocytes were cultivated on a feeder layer of lethally irradiated mouse 3T3 fibroblasts (Rheinwald and Green, 1975) as described previously (Kobayashi et al., 1993; Rochat et al., 1994).

RNA purification

RNAs were extracted according to the method of Chomczynski and Sacchi (1987) from bulge- and matrix-containing fragments (from which the follicular papillae were removed mechanically) obtained from one hundred microdissected vibrissal follicles. RNAs were isolated from cultured cells following the same procedure.

Construction and subtraction of a cDNA library from KR4cl2 keratinocytes

mRNAs were purified from cultured KR4cl2 cells and from freshly isolated matrices according to standard procedures (Maniatis et al., 1989). Double strand cDNAs were synthesized according to the manufacturer's guidelines (λZAP-cDNA Synthesis Kit, Stratagene). KR4cl2 cDNAs were then subtracted with a 10 molar excess of matrix cDNAs devoid of cloning ends and previously digested by *Rsa*I, *Alu*I and *Xho*I restriction enzymes. KR4cl2 and digested matrix cDNAs were then hybridized for 24 hours in 50% formamide, 5× SSC, 10 mM $NaPO_4$, pH 7, 1 mM EDTA, 0.1% SDS, 1 μg yeast tRNA and ligated into λZap vector. A total of 495 independent clones were isolated and hybridized differentially using single strand [^{32}P]cDNA probes synthesized randomly either from KR4cl2 or from matrix mRNAs. Clones which hybridized preferentially with either one of the probes were selected and further purified by sequential differential hybridizations. pBluescript plasmid pSKII$^+$ DNA was obtained according to the manufacturer's instructions (λZAP-cDNA Synthesis Kit, Stratagene).

DNA sequencing

Both cDNA strands were sequenced according to the standard dideoxynucleotide chain termination procedure (Sanger et al., 1977) using Sequenase version 2.0 (USB). Analyses of DNA sequences were performed in the GenBank EMBL data bases using GCG programes.

Northern blotting

RNA samples (10 μg) were processed as described (Magnaldo et al., 1995).

In situ hybridization

Freshly isolated vibrissal follicles were snap-frozen in liquid nitrogen and kept at −80°C until further processing. Frozen sections (6 μm) were obtained and processed for in situ hybridization as described (Magnaldo et al., 1995). A subfragment of the 3G12/CD24 cDNA was amplified by PCR using the following primers: forward, 5′ TTTGGATCCTGCAACCCAAACATCGGTTGCA 3′; reverse, 5′ TTTAAGCTTCCTTTAAGCCAGTCGAATTTC 3′ and cloned into *Bam*HI and *Hin*dIII restriction sites of pSKII$^+$. The amplified fragment encompassed nucleotides 90 to 390 of the 3G12 cDNA. A subfragment of the human CD24 cDNA was amplified by RT-PCR using mRNAs obtained from YH16P3 human follicular keratinocytes and the following primers: forward, 5′ ATGGCCAGAGCAATGGTGGCCAGG 3′; reverse, 5′ TCTTAAGAGTAGAGATGCAGAAGA 3′, and encompassed nucleotides 57 to 301 of the published sequence (Kay et al., 1991). Riboprobes were generated from the 3G12 subfragment, 2SB1 human CD24 cDNA, and a human K5 cDNA as described (Magnaldo et al., 1995).

Immunodetections

Polyclonal antibody against rat CD24 core peptide, a synthetic peptide corresponding to amino acids 38 to 51 (Q-S-I-S-A-A-P-N-P-T-N-A-T-T) (Fig. 1) of the core peptide, was synthesized by Neosystem laboratories (France). Two female New Zealand rabbits were immunized with a total of 1.6 mg of the ovalbumin-coupled peptide. Sera were then tested by indirect immunofluorescence on frozen sections of rat foot pads and by western blotting using protein extracts prepared from rat epidermal keratinocytes.

Western blotting

Total proteins were extracted from cultured cells in 8 M urea, 50 mM

Tris-HCl pH 7.6, 0.1 M β-mercaptoethanol, 1 mM DTT, 100 μg/ml paramethyl sulfonide fluoride (Sybert et al., 1985), separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PDVF; Immobilon-P, Millipore). Membranes were washed in phosphate buffered saline (PBS), 3% non fat dried milk (blotto, Régilait), 0.5% Tween-20, then in PBS, 1.5% blotto, 0.1% Tween-20 (solution I) and saturated overnight in PBS, 1% bovine serum albumin (BSA), 0.1% Tween-20 (solution II). Membranes were then incubated for one hour with an anti-human CD24 monoclonal antibody (ALB9, Immunotech) diluted to 1/200 in PBS 0.1% BSA. They were then washed three times in solution I, once in solution II, incubated for 30 minutes with a peroxidase-conjugated secondary anti-mouse IgG antibody diluted 1/5,000 in PBS 0.1% BSA (Amersham), and then washed again as in the previous step and developed using ECL reagents (Amersham).

Immunostaining

Cytocentrifuged cells and frozen sections of mouse vibrissal follicles were immunostained as described (Bernerd et al., 1992). Antibodies were diluted in PBS as follows: anti-human CD24 antibody, 1/25; 193-4-3 anti-mouse CD24, 1/500; FB1 anti-K14 keratin monoclonal antibody (Parent et al., 1994), undiluted; anti-K10 RKSE60 monoclonal antibody (Sanbyo), 1/10. Cells and sections were observed and photographed with an epifluorescence microscope (Zeiss Axioskop).

Frozen sections (8 μm) obtained from microdissected human hair follicles were fixed in 3% paraformaldehyde, 0.3% glutaraldehyde in PBS as advised for GPI anchored proteins (Mayor et al., 1994) and immunostained using the ABC Vectastain kit (Vector, USA) according to the manufacturer's recommendations. Sections were counter-stained with Mayer's haematoxylin.

Cell sorting

Confluent cultures of human keratinocytes (YF29, passage V) were trypsinized. Cells (1.8×10^6) were incubated in CFAD containing 10% complement-inactivated bovine serum (CFAD-DBS) and 200 μl of undiluted sterile ALB9 monoclonal antibody (Immunotech) for 1.5 hours at 4°C with gentle stirring. Sheep anti-mouse IgG coated magnetic beads (8×10^7, M-450, Dynal) were washed twice in sterile PBS/0.1% BSA. Cells were then washed twice with CFAD-DBS and incubated with a 44-fold excess of magnetic beads in CFAD-DBS for 30 minutes at 4°C. Cells coated with beads were then separated with a magnet and the supernatant containing the uncoated cells was harvested and kept at 4°C. The latter procedure was repeated twice to harvest all uncoated cells which were then pooled and referred to as the CD24-negative cell population (YF29/CD24$^-$). Cells coated with beads were resuspended in CFAD-DBS and incubated at room temperature for 45 minutes in the presence of 2 units of Detachbead CD19 (Dynabeads M-450 Pan-B DYNAL). Detached cells were separated and harvested as described above. They were referred to as the CD24-positive cell population (YF29/CD24$^+$). The colony-forming ability of the different cell populations (unfractionated cells, CD24$^+$ and CD24$^-$ cells) was assayed by clonal analysis. Cells (10^2 and 10^3) from each population were cultured on a feeder layer of irradiated 3T3 cells for 12 days before cultures were fixed in formalin and stained with 1% Rhodamine B.

Unfractionated cells, CD24$^+$ and CD24$^-$ cells were cytocentrifuged as described (Rochat et al., 1994) and immunostained using anti-CD24 (ALB9), anti-K14 (FB1) and anti-K10 (RKSE60) monoclonal antibodies.

RESULTS

Cloning of a rat CD24 cDNA

To isolate genes that were expressed specifically in keratinocyte colony-forming cells, a cDNA library was constructed using polyadenylated transcripts prepared from a K-CFC clone, KR4cl2, isolated from the bulge of a rat vibrissal follicles. mRNAs were also prepared from freshly microdissected matrices as they contain multiplying cells but very few

A

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   1 GGCACGAGTCTCCACGCACCGGAGCATCCAGAGAGTCGCCGCGCGCCGACGGAGCGGAC
  61 ATGGGCAGAGCGATGGTGGTCAGGCTAGGGCTGGGGTTGCTGCTTCTGGCACTGCTCCTA
   1 M  G  R  A  M  V  V  R  L  G  L  G  L  L  L  L  A  L  L  L
 121 CCCACGCAGATTTATTGCAACCAAACATCGGTTGCACCATTTTCCGGGAACCAGAGTATC
  21 P  T  Q  I  Y  C  N  Q  T  S  V  A  P  F  S  G  N  Q  S  I
 181 TCTGCTGCCCCAAATCCAACTAATGCTACCACCAGATCTGGGTGTAGCTCCCTGCAGTCC
  41 S  A  A  P  N  P  T  N  A  T  T  R  S  G  C  S  S  L  Q  S
 241 ACAGCCGGTCTCCTGGCCCTCTCTCTCTCTCCTACATCTCTACTGTTAGAGACTCAGG
  61 T  A  G  L  L  A  L  S  L  S  L  L  H  L  Y  C  76
 301 CCAAGAAACGTCTCTACTTCCCCATCTAGACCCACCCCGAATGGCAACCACAAGTCCAAT
 361 GTGAGTGACCAGGAAGAGACAGGTCCACATCAAATCGGCTGTTACCATTACCTCAACAGA
 421 AAACACTGAGAATCCGAAATTCGACTGGCTTAAAGGACGCGTGAAGGGTTTGAGAGCTGA
 481 GAGATGCCAATATGGGATCTGCTGGAGTTTTATGTCCGAGATGAAGACAAGCATCCAAAA
 541 TTGACGTGATTTGCTCGAAATGTGGCTTAAGAAATGTGGACACTTAAAACTCTCACTCGA
 601 AATCGGGCGTAGGTTTGATGTAGAGGGAGAAGGATGGGGTGTGGAATGGAGGTCCATTAT
 661 TTTCATTGGTTCCTTCGACCTATAAGGCCATAATGCAGTTAGGTGGTATTCAAAAGCTTC
 721 TCTGATGCTATTTCTGTGTATGTTGGAAGGAACTACCAGGTGTTATTTTAAATCCCCGAT
 781 GTGTTGTTTTGTTACTACTAATTTAATAGGCTAAACTCTAGGTGAAGTCCCACGTCGTTG
 841 ACCTCTAGTTCTTCTCTCCGGAACAGAACGTTTTACACCCTCCTTCTGTGGCTTTAGGTC
 901 TGACATATTGTATTTGACCTTTGTTAGTAATTGACACGGGCCAGGCAACGATGGATTGGA
 961 ACCCACCCCCAAATCCAGCCACCCCTGAGTAAATCTGATTTCAAAAGTCACAACGGCAAA
1021 GCATTTCCCAGTGTCTTTTCTCACTCACCACGGGTACTAAGTTCACTTAGGTGAGCTGGT
1081 TCTCCAGAGAGCAGAATTCTGTTGATCTCGGCTAATCTCCAAACGCTGCGTTTTATTTTG
1141 GCATATGTCGAGGGCTTTTCATGATTTAAAGCGTGGTCTGTTAGCAAGGCCAAAATTATG
1201 AGTCTTGAAATTCTACAGGCAAATATGCAACCCAGCATTTGGGATGTGAGGACTGGAAGC
1261 TCTCACAGAATTACACAAAGTCTTTTATACAATTTCTGTAAATACTTTTTTTTTTTTAAA
1321 GAGAAAAACAAAACCATGGATCGGAATAACCACGTTTGGAATACTTTGGTTATCAGTTCA
1381 TATTTTTAGATAGTTTGACATTGGTCCTGTGCCTGAGAGGGGGTTTGGTTCTACAGTAAG
1441 TTTTTTTTTCCCCCAATTTCCTTGATACACACATACCTTCTAAAACACAGACATTTCCTC
1501 AAAAAAAAAAAAAAATTCCTTTTGTTCGCATGGTCACACACTGATGCTTGCCATTCTGGG
1561 ATCTAATACGGTCACAGTAGTCTTGATAACCAGAATCATTTTTTTCTCCGTCTTTAGAAA
1601 CCTACCCGGGAACAAGGAGACCTCACAGACCCGAAGCTACTGTGTGTGTGAATGAACACT
1661 CCCCTTGCCTCATACCCGAATGCTGTACATCTATTTGATTGTATATTGTGTTTGTGTATT
1721 TATGCTTTGATTCATAGTAACTTCTCATGTTATGGAATTGATTTGCATTGAACACAAACT
1781 GTAAATAAAAGAAAGAAATGGCTG(Ax40)
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B

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          ****** ******************
Human     MGRAMVA RLGLGLLLLALLLPTQIYS
Mouse     MGRAMVA RLGLGLLLLALLLPTQIYC
Rat       MGRAMVV RLGLGLLLLALLLPTQIYC

          ..*. .  .  .   ..  .***.***
Human     SET TTGTSSNSSQSTSNSGLAPNPT NAT
Mouse     NQT SVAPFPGNQNISA----SPNPS NAT
Rat       NQT SVAPFSGNQSISA----APNPT NAT

          *. .. ..*****.*. .*******.
Human     T KAAGGALQSTASL F VVSLSLLHLYS80
Mouse     T RGGGSSLQSTAGL L ALSLSLLHLYC76
Rat       T RSGCSSLQSTAGL L ALSLSLLHLYC76
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Fig. 1. Nucleotide and amino acid sequences of the rat CD24. (A) Nucleotides are numbered on the left of each line. The 3G12 cDNA is 1,863 nucleotides in length. The putative translational initiator ATG codon (position 61), the putative polyadenylation AATAAA signal (position 1,783) and the putative signals for rapid RNA degradation ATTTA (Brewer, 1991) are underlined. The deduced amino acid sequence is composed of 76 residues (bold characters). (B) Alignment of the putative CD24 rat peptide along with the mouse and human CD24 peptide sequences. Stars indicate identical residues; points indicate similarities. Note the high ratio of similar residues in the amino and carboxy-terminal ends of the three peptides; the core region is less conserved between the rat and human sequences. Amino acids 1 to 26 of the different CD24 peptides are likely to correspond to the signal peptide. Amino acids 54 to 58 of the rat CD24 predict a GPI-displaced tail, as suggested by the human and mouse CD24 peptide sequences (Wenger et al., 1991; Kay et al., 1991). The rat CD24 core peptide is thus highly homologous to mouse CD24 (90.8%) but less homologous to human CD24 (68.4%).

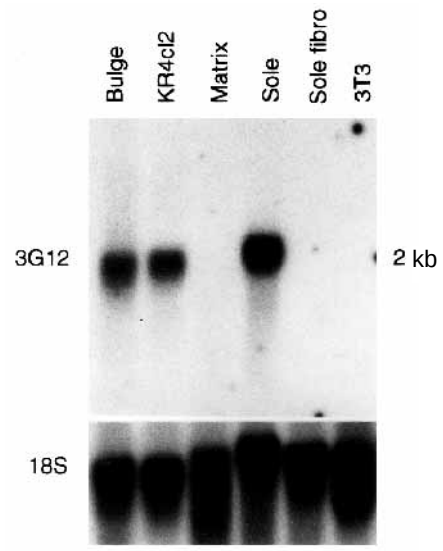

Fig. 2. CD24 is expressed in the bulge of the rat vibrissal follicle. Total RNAs were extracted from freshly microdissected rat vibrissal follicles (bulge and matrix-containing fragments), rat sole epidermis, and rat clonogenic KR4cl2 keratinocytes (KR4cl2). They were then analysed by northern blot using a rat CD24 cDNA probe. CD24 mRNAs are preferentially expressed in epithelial parts containing most of the K-CFC i.e. the bulge and the sole. Note that CD24 mRNAs were not detected in microdissected matrices. Also CD24 mRNAs were not detected in cultured fibroblasts i.e. rat sole fibroblasts and mouse 3T3 cells, when compared to cultured KR4cl2 clonogenic keratinocytes.

clonogenic cells (Kobayashi et al., 1993). These mRNAs were reverse transcribed, and the cDNAs were then used to subtract the KR4cl2 cDNA library. A total of 495 independent clones were obtained and then hybridized differentially using cDNA probes prepared from either KR4cl2 cells, or from freshly microdissected matrices. Twelve KR4cl2-positive/matrix-negative cDNA clones were selected. Clone 5B4 corresponded to the K5 keratin which is expressed in the upper part of the hair follicle including the bulge, but not in the matrix area (Kopan and Fuchs, 1989). Clone 4G1 corresponded to K19 keratin which is expressed in the outer epithelial sheath of human hair follicles (Lane et al., 1991; Narisawa et al., 1994). These cDNAs were used as internal controls of the cloning procedure. Clone 3G12 was 1,863 base pairs in length (Fig. 1A) and had high sequence identities with the mouse and human CD24 cDNAs (89.6 and 76.5% identity, respectively; Kay et al., 1991; Wenger et al., 1991). The 5′ part of 3G12 cDNA was also almost identical to a shorter sequence (length: 1,445 bp) recently identified as the rat CD24 cDNA sequence (Shirasawa et al., 1993). The 3G12 cDNA was therefore considered as encoding the rat CD24 core peptide.

A putative ATG codon for translational initiation was located at nucleotide position 60 and a putative TAG stop codon was located at nucleotide 292. The deduced protein sequence was 76 amino acids in length. The primary peptide sequence of the rat CD24 was highly similar to its murine counterpart and, to a lesser extent, to human CD24. The amino-terminal portion corresponding to the putative signal peptide and the carboxy terminal portion were the most conserved sequences (Fig. 1B). Removal of these portions would give rise

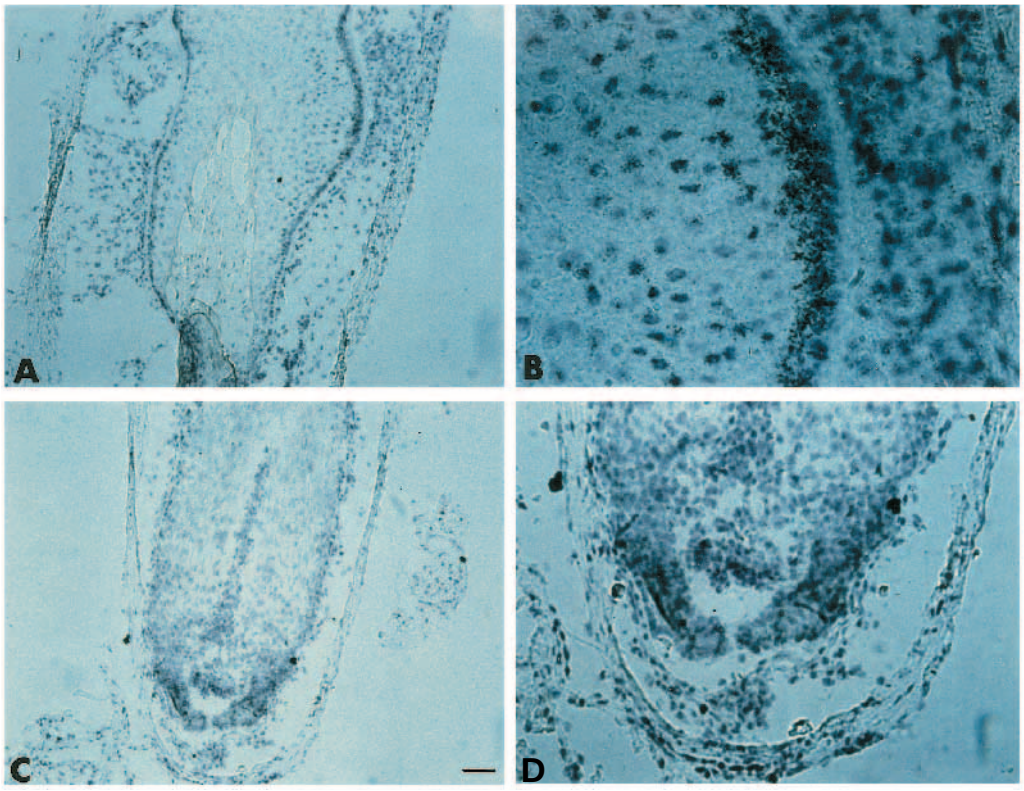

Fig. 3. In situ localization of CD24 mRNAs in the rat vibrissal follicle. Longitudinal frozen sections of rat vibrissal follicle were hybridized using sense and anti-sense riboprobes generated from a subfragment of a rat CD24 cDNA. (A,B) The bulge-containing area; note that the basal cells and some suprabasal cells are strongly labelled with the anti-sense probe. (C,D) The matrix-containing area; note that only a few cells located at the tip of the bulb are labelled. No significant signal was obtained with the sense probe. Bar, 50 μm.

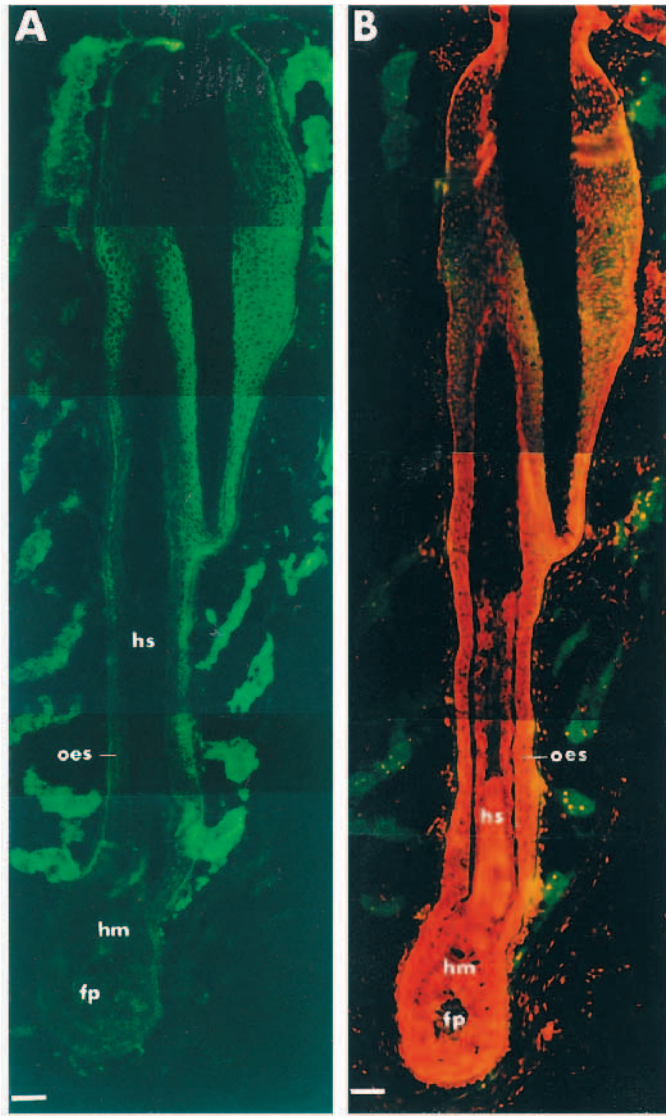

Fig. 4. Location of CD24-expressing keratinocytes in mouse vibrissal follicle. Longitudinal serial frozen sections of a mouse vibrissal follicle were labelled with the 193-4-3 rat monoclonal antibody (A) and counterstained with propidium iodide (B). oes, outer epithelial sheath; fp, follicular papilla; hm, hair matrix; hs, hair shaft. Note that labelled keratinocytes (A, green; B, yellow-orange) are located in the upperpart of the follicle, i.e. the bulge area, and not in the bulb area. Bars, 100 μm.

to a shorter peptide of about 30 amino acids as described for the mouse and the human CD24.

CD24 is preferentially expressed in the keratinocytes of the bulge area of the rat and mouse vibrissal follicles

CD24 transcripts were detected in freshly microdissected rat vibrissal bulges and KR4cl2 keratinocytes, but not in freshly microdissected rat vibrissal matrices, using northern blotting with short exposures (Fig. 2). However, CD24 transcripts could be detected at a low level in matrices after long exposures. Furthermore, CD24 mRNAs were also detected in sole skin but not in sole fibroblasts and 3T3 cells (Fig. 2). These results demonstrated first that the CD24 gene was expressed in keratinocytes but not in fibroblasts; second, that it was preferentially expressed in the keratinocytes located in the area of the vibrissal follicles containing most of the colony-forming cells i.e. the bulge; and third, that CD24 is not expressed in differentiating hair cells (trichocytes).

The distribution of CD24 transcripts was then analysed by in situ hybridization. Longitudinal sections of rat vibrissal follicles were hybridized with sense and antisense riboprobes generated from a 300 nucleotide subfragment (nucleotides 90 to 390) of the 3G12 cDNA. Keratinocytes located in the outer epithelial sheath of the bulge area were strongly labelled with the antisense probe (Fig. 3B). Most basal cells and those of the first suprabasal layer were labelled, whereas only a few cells in the suprabasal layers were labelled. In the bulb, a few cells located at the tips of the hair bulb were labelled (Fig. 3C,D). No significant hybridization signals were detected in the keratinocytes located elsewhere in the follicular matrix. These results demonstrate that the keratinocytes expressing CD24 are located in the bulge and the tip of the bulb, the areas of the vibrissal follicle thought to contain stem cells (Cotsarelis et al., 1990; Reynolds and Jahoda, 1991; Kobayashi et al., 1993).

Antibodies recognizing either the leucine-alanine-proline sequence of the core peptide (ALB9, Immunotech) or the glycosylated motifs (Weber et al., 1993; BA-1, Boehringer) of human CD24 did not label rat keratinocytes. Therefore, we used a rat monoclonal antibody directed against mouse CD24 (kindly provided by G. Rougon) to immunostain cryosections of mouse vibrissal follicle. CD24-labelled cells were located mostly in the bulge area and not in the matrix (Fig. 4).

CD24 mRNAs are expressed in the developing hair follicle

CD24 transcripts were also detected by in situ hybridization in the developing pelage hair follicles of the back of E18 and E20 rat embryos (Fig. 5). At E18, CD24 was highly expressed in the whole epithelial part of the round-shaped hair germs. In contrast, CD24 expression was much weaker in the interfollicular epidermis (Fig. 5B,C). Interestingly, at E20, CD24 expression became more restricted to the upper part of the elongated follicles (Fig. 5D,E), reminiscent of the pattern observed in adult follicles. These results indicate that the CD24 gene is expressed at an early stage of hair follicle development (Shirasawa et al., 1993). Thus, expression of the CD24 gene may be linked to the segregation of stem cells in the upper part of the follicle.

An isoform of CD24 mRNA is preferentially expressed in clonogenic keratinocytes cultured from the intermediate part of human hair follicles

To study the expression of CD24 mRNA in human hair follicles, a 244 bp cDNA segment was amplified by RT-PCR using mRNAs isolated from cultured human follicular keratinocytes. Primers (see Materials and Methods) were designed to hybridize to sequences located at the borders of the coding region of human CD24 mRNA (Kay et al., 1991). The amplified DNA fragment was sequenced and identical to that of the published human cDNA (Kay et al., 1991). This partial human cDNA was then used as a probe in northern blot analysis and in library screenings to isolate a full length human cDNA clone (see below).

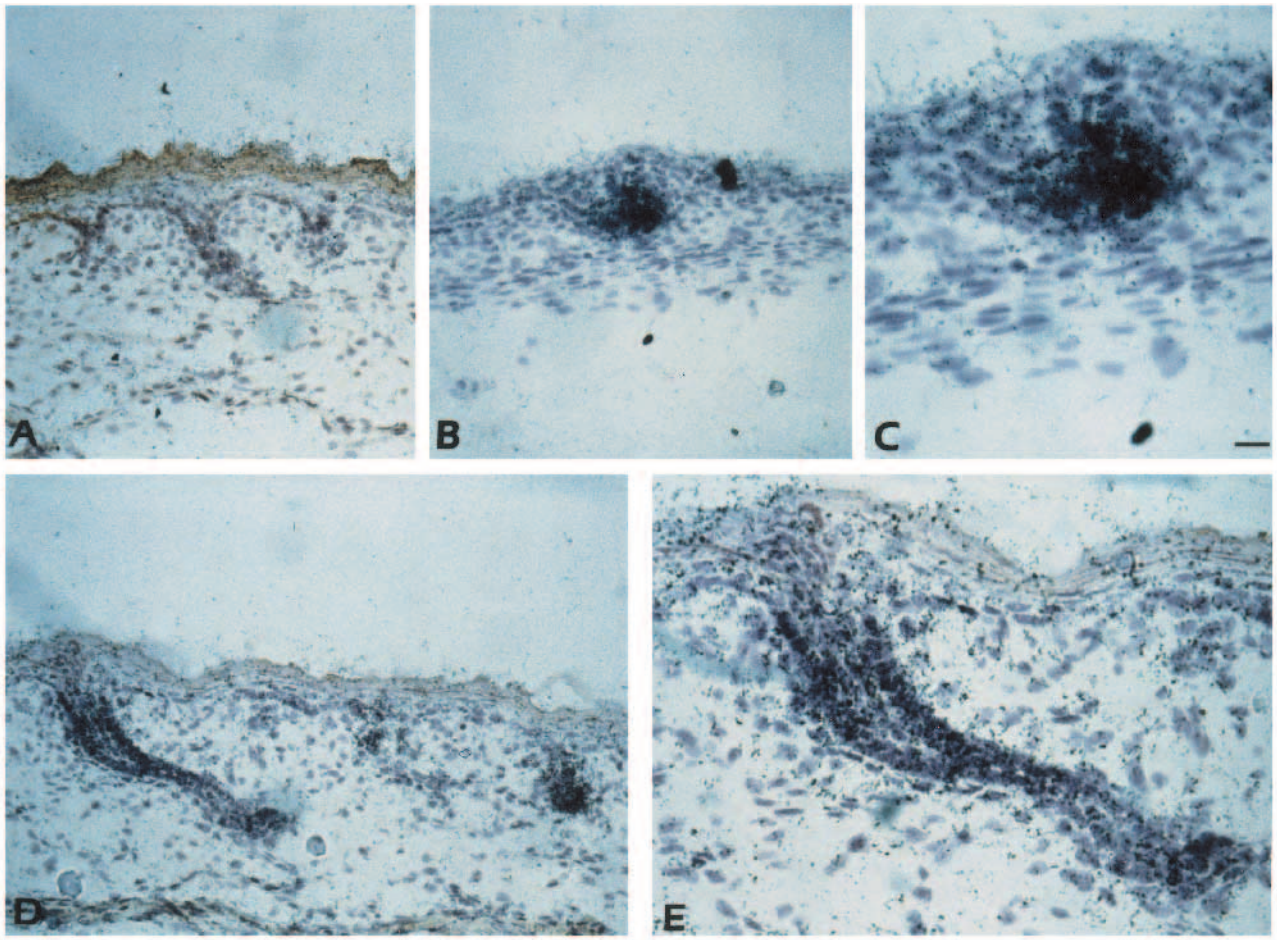

Fig. 5. CD24 expression in the developing hair follicle of the rat. In situ hybridizations were performed using the 3G12 cRNA probes on frozen sections of embryonic back skin. (A) Negative control using the sense RNA probe; (B,C) E18 embryo; (C,D) E20 embryo. Note the strong concentration of CD24 mRNAs in hair buds. In the developed follicle, the CD24 mRNAs are redistributed in the upper part of the outer epithelial sheath and are thus reminiscent of the hybridization pattern observed in the adult vibrissal follicles (Fig. 2B). Bar: (A,B,D), 28 μm; (C,E), 14 μm.

Northern blot experiments were performed using RNA prepared from cultured follicular keratinocytes isolated from the intermediate part (P3) of a hair follicle which contains most of the clonogenic keratinocytes (YH16P3), and from the lowest part (P1) of the same follicle (YH16P1) i.e. the bulb (Rochat et al., 1994). A 2.4 kb message was detected in RNAs isolated from both YH16P1 and YH16P3 keratinocytes but in much greater amounts in the latter (Fig. 6). Similarly, a 2.4 kb message was detected in normal human foreskin keratinocytes (YF29) and in a human pre-B-lymphoblastoid cell line (NAML6). Interestingly, three smaller bands of approximately 2, 0.8 and 0.5 kb were also detected in these cells (Fig. 6). No CD24 transcripts could be detected in human dermal fibroblasts (AFF11).

The 2 kb mRNAs were predominant in human brain, liver and muscle extracts (data not shown) but they were also detected, although at very low levels, in NAML6 cells and in cultured human keratinocytes. The 2.4 kb CD24 transcripts were detected predominantly in human cultured follicular keratinocytes (Fig. 6), spleen, kidney and pancreas (data not shown), demonstrating that the 2.4 kb mRNAs were not specifically expressed by follicular keratinocytes. A cDNA library was constructed from YH16P3 hair follicle keratinocytes and screened using the 244 bp CD24 probe described above. Three different cDNA clones were isolated. 4SB1 cDNA was 2.152 kb in length. 3SB1 and 2SB1 cDNAs were 0.8 and 0.5 kb, respectively. Sequencing of these cDNA clones revealed that each overlapped with the 5′ part of the human CD24 published sequence (Kay et al., 1991; Jackson et al., 1992). However the 0.8 (3SB1) and 0.5 kb (2SB1) CD24 cDNAs were shorter in their 3′ portion whereas 4SB1 was much larger in its 3′ end compared to the human composite CD24 cDNA sequence published previously (Kay et al., 1991). These data demonstrated that all the different CD24 cDNAs had the same coding region, thus indicating that the same primary translation product should be present in all cells expressing the CD24 gene. The three transcripts detected in human cells may result from alternative polyadenylation as described in the mouse (Wenger et al., 1991).

Distribution of CD24 mRNA and protein in human hair follicles

Most clonogenic keratinocytes are located in the intermediate part of the outer epithelial sheath of the human hair follicle

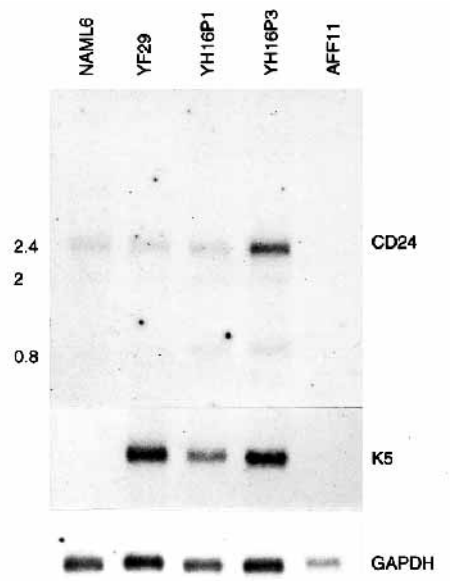

Fig. 6. CD24 gene is expressed in cultured human keratinocytes. Total RNAs were extracted from cultured human keratinocytes isolated from foreskin (YF29), hair follicles (YH16P3 and YH16P1), from human dermal fibroblasts (AFF11) and the pre-B human lymphoblastoid cell line NAML6. They were then analysed by northern blot using a human CD24 cDNA probe generated by RT-PCR. The same blot was then sequentially probed using human K5 keratin and GAPDH cDNAs as loading controls. Three CD24 mRNAs (2.4, 2, and 0.8 kb) were detected. Note that the 2.4 kb is the major form, both in cultured keratinocytes and in the pre-B human lymphoblastoid cell line NAML6. Note also that CD24 is expressed at a higher level in cultured follicular keratinocytes isolated from the intermediate part (YH16P3) of a hair follicle, than in cultured keratinocytes isolated from the lowest part (YH16P1) of the same follicle.

(Rochat et al., 1994). To localize cells expressing CD24 transcripts in human hair follicles, in situ hybridization was performed using riboprobes generated from 2SB1 cDNA. Labeled keratinocytes were located in the lower part of the outer epithelial sheath, principally in the basal and suprabasal layers. No significant signal could be detected in the keratinocytes located in the hair bulb nor in those located in the upper part of the follicle. These results were in concordance with those of the northern blot analysis (Fig. 6).

Proteins extracted from human keratinocytes were analysed by western blotting with an anti-CD24 monoclonal antibody (ALB9) binding to a short leucine-alanine-proline sequence of the human core peptide (Weber et al., 1993). These experiments demonstrate the presence of a CD24 related protein of approximatly 60 kDa in cultured human keratinocytes (Fig. 7).

The keratinocytes expressing CD24 in human hair follicles were then localized by immunocytochemistry using the same monoclonal antibody. $CD24^+$ keratinocytes were confined to the lower part of the outer epithelial sheath, mainly in the basal and suprabasal layers. No significant signal was detected in the keratinocytes located in the hair bulb or the upper part of the follicle (Fig. 8A,B). These results were consistent with those obtained by in situ hybrization. The location of the $CD24^+$ keratinocytes in human hair follicles was thus reminiscent of the location of the clonogenic keratinocytes (Rochat et al., 1994).

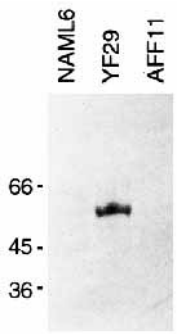

Fig. 7. CD24 core peptide is present in human cultured keratinocytes. Total proteins were extracted from YF29 keratinocytes, NAML6 preB lymphoblastoid cells and AFF11 fibroblasts, and were analysed by western blot using an antibody recognizing a short peptide sequence of the human CD24 core peptide (Weber et al., 1993). A single band of about 60 kDa was detected in keratinocytes. Several very faint bands ranging from about 65 to 50 kDa were detected in NAML6 cells after longer exposure of the blot. Size standards (in kDa) are shown at the left.

Are $CD24^+$ keratinocytes clonogenic?

This question could not be reliably answered using freshly microdissected human hair follicles as a large number of them is needed to isolate enough keratinocytes to perform the experiment (Rochat et al., 1994). Therefore, cultured human foreskin keratinocytes were labelled with an anti-human CD24 monoclonal antibody (ALB9) and $CD24^+$ cells were then analysed by clonal assay. $CD24^+$ cells were sorted using magnetic beads. The efficiency of the sorting procedures was monitored by immunofluorescence labelling of the different purified cell compartments with the anti-human CD24 monoclonal antibody (total cell population, $CD24^+$ and $CD24^-$ sorted populations). Ninety-nine per cent of the CD24-sorted cells were also labeled by immunofluorescence demonstrating that the sorting procedure was highly efficient (Fig. 9A). In three different experiments $CD24^+$ cells were not clonogenic whereas $CD24^-$ cells were (Fig. 9B). This was also consistent with the fact that $CD24^+$ keratinocytes were large cells, and $CD24^-$ cells were small cells. Thus $CD24^+$ keratinocytes could not be expected to be clonogenic (Barrandon and Green, 1985). This was also in concordance with the fact that most $CD24^+$ cells were K14-negative (80-85%) whereas most $CD24^-$ keratinocytes were K14-positive (95%) (data not shown). These data thus demonstrate that CD24 glycoprotein is not specifically expressed on the cell surface of human clonogenic keratinocytes.

DISCUSSION

We have isolated a cDNA coding for the rat CD24 (HSA/nectadrin) by subtractive and differential hybridization of a cDNA library prepared from a clone of follicular keratinocytes. The CD24 molecule is a small, extensively glycosylated peptide, with a glycosylphosphatidylinositol membrane anchor in mouse and human B-lymphocytes (Pirrucello and LeBien, 1986; Kay et al., 1991; Wenger et al., 1991; Jackson et al., 1992; Kadmon et al., 1992). The rat CD24 cDNA that we have isolated encodes a 76 amino acid peptide with high

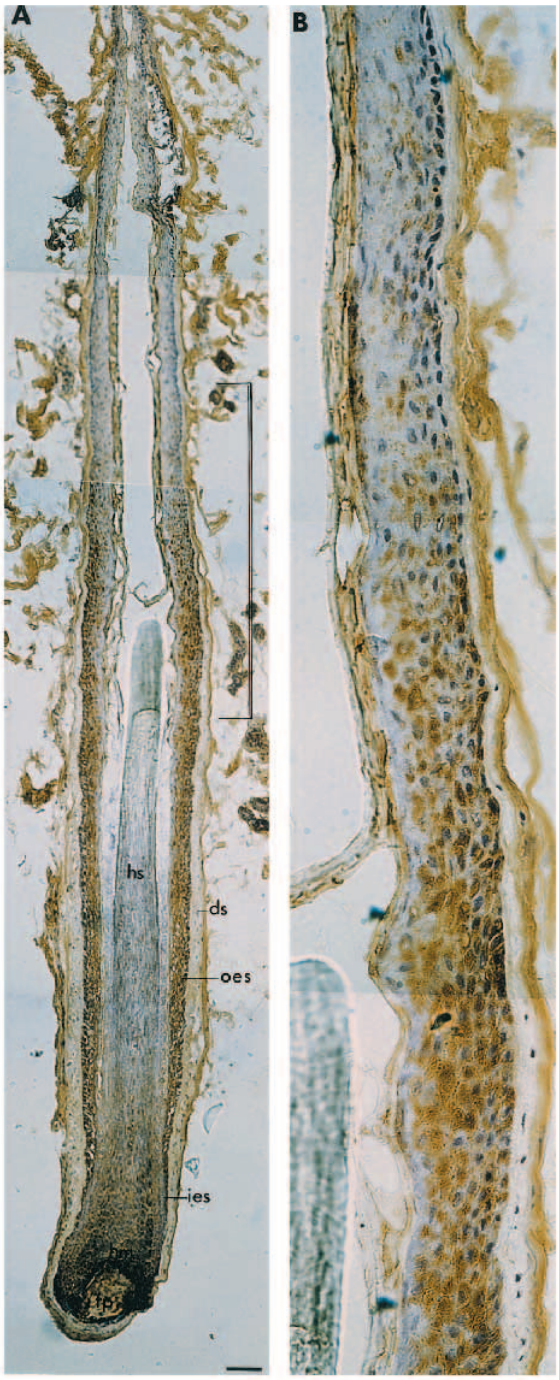

Fig. 8. Location of CD24-expressing keratinocytes in human hair follicle. Longitudinal frozen sections of a human hair follicle were labelled with the ALB9 monoclonal antibody described in Fig. 6. (A) ds, dermal sheath; oes, outer epithelial sheath; ies, inner epithelial sheath; hs, hair shaft. Brackets indicate the portion of the hair follicle magnified in B. Note that the labelling is much more intense in keratinocytes of the intermediate part of the outer epithelial sheath where basal and suprabasal cells are labelled. No labelling was observed in the inner epithelial sheath. A very weak labelling is seen in keratinocytes of the hair matrix. Magnification in B is shown to illustrate that CD24 expression very rapidly fades and becomes undetectable in the upper part of the outer epithelial sheath. The heavy brown coloration in the center of the bulb region was also seen in unstained sections, and is likely due to hair pigment (melanins). Bar: (A), 50 μm; (B), 12.5 μm.

homology to mouse and human CD24 cDNA (Fig. 1). This peptide is likely to undergo post-translational modifications in keratinocytes similar to that in mouse and human lymphocytes. These modifications will then lead to the formation of a small core peptide (3 kDa), which will then be glycosylated and anchored to the cell membrane by a glycosylphosphatidylinositol (Kay et al., 1991; Wenger et al., 1991; Jackson et al., 1992). The molecular mass of the protein isolated from human keratinocytes and detected with an anti-human CD24 monoclonal antibody (ALB9) by western blot analysis strongly supports this hypothesis (Fig. 7).

Several pieces of evidence have been put forward indicating that CD24 is involved in cell adhesion and signalling. For instance, a tyrosine-kinase activity has been reported in membrane-bound CD24 complexes isolated from pre-B lymphocytes (Stefanova et al., 1991). CD24 has also been demonstrated as a costimulator of T-cell clonal expansion (Liu et al., 1992). Interestingly, the intracellular calcium concentration is significantly increased when lymphocytes, cerebellar neurons, and neuroblastoma N_2a cells are treated with an anti-CD24 antibody (Fischer et al., 1990; Kadmon et al., 1995). This is worth noting because calcium is thought to modulate significantly the proliferation and differentiation of epidermal keratinocytes (Hennings et al., 1980; Hennings and Holbrook, 1983; Watt et al., 1987; Yuspa et al., 1989). Furthermore, there is strong evidence that CD24 is involved in cell adhesion, either via homotypic or heterotypic interactions (Kadmon et al., 1992, 1995; Hubbe and Altevogt, 1994; Sammar et al., 1994). It has also been suggested that the glycosylated backbone of CD24 may act as a ligand of lectin-like molecules (Kay et al., 1991; Shirasawa et al., 1993) such as P-selectin (Sammar et al., 1994). For instance, the adhesion of mouse leukocytes to endothelial cells may involve CD24/P-selectin interactions (Sammar et al., 1994). Also, CD24 (nectadrin) forms a functional complex with the cell adhesion molecule L1 in neurons (Kadmon et al., 1992, 1995). Nevertheless, the exact function of CD24 remains to be further clarified. Mice generated by targeted disruption of CD24 alleles may help to clarify the role of CD24 both in the B-cell lineage and in other cell types (Wenger et al., 1995).

CD24 expression in keratinocytes

CD24 transcripts are expressed preferentially in the areas of the rat vibrissal follicle and of the human hair follicle containing clonogenic keratinocytes i.e. the bulge area and the lower third of the outer epithelial sheath, respectively (Kobayashi et al., 1993; Rochat et al., 1994). Furthermore, CD24 is also expressed slightly in the cells located at the tip of the follicular bulb, which contains a few keratinocyte colony-forming cells. These results suggest that CD24 expression may be linked to the ability of a keratinocyte to initiate a colony. However, when cultured human keratinocytes isolated from glabrous epidermis are labelled with an anti-human CD24 antibody (ALB9), $CD24^+$ cells are mostly large, differentiated, and unable to initiate colonies. This demonstrates that the clonogenicity of human epidermal keratinocytes is not linked to the expression of CD24. Furthermore, CD24 is also detected in the upper differentiated layers of human foreskin epidermis (data not shown). However, this is in sharp contrast to the high level of CD24 mRNA expression in rat clonogenic keratinocytes (Fig. 2). This suggests possible func-

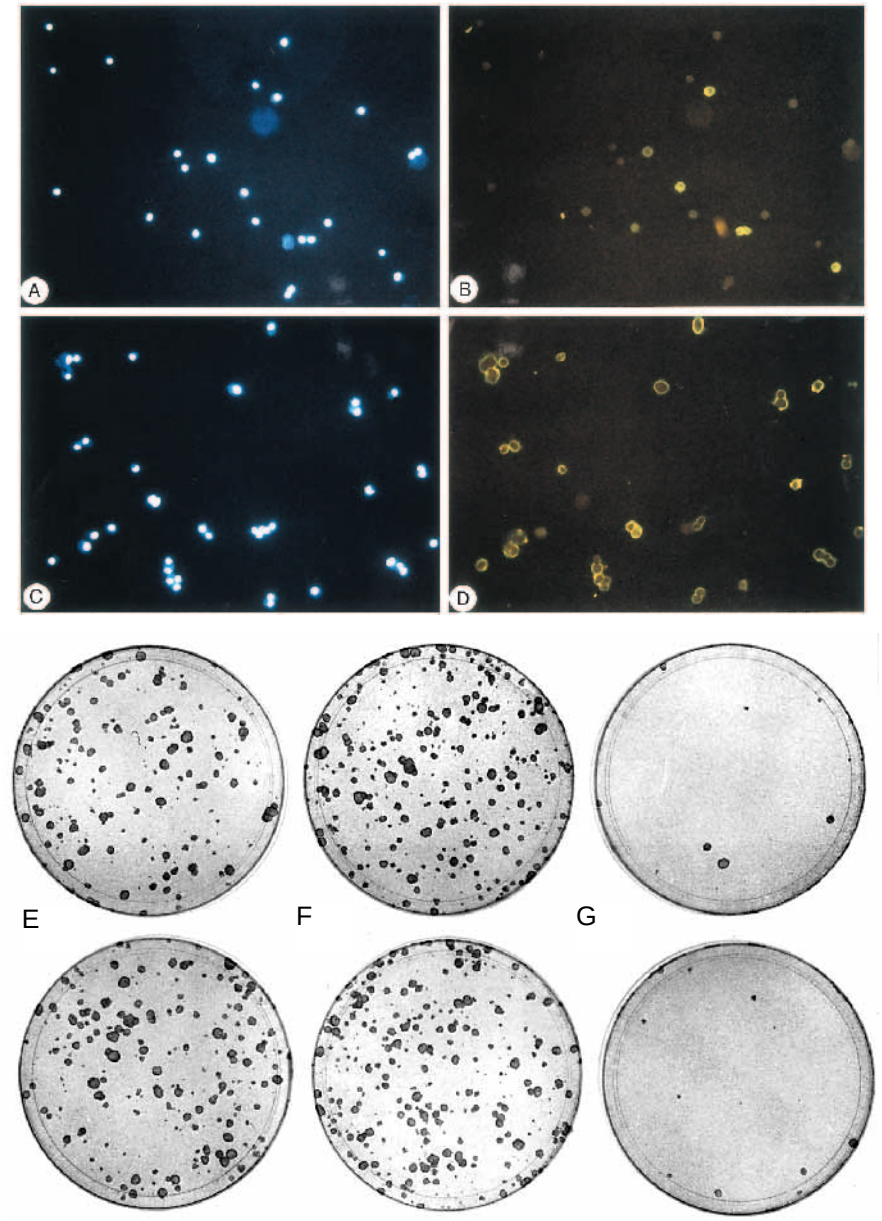

Fig. 9. CD24 positive keratinocytes are not clonogenic. Sorting of the CD24 positive/CD24 negative population was carried out as described in Materials and Methods. (A-D) Unfractionated YF29 cells (A,B) and sorted $CD24^+$ keratinocytes (C,D); populations have been stained using ALB9 mAb against CD24. (A,C) Nuclear staining using Hoechst. (E,F,G) Clonal analysis of original, $CD24^+$ and $CD24^-$ keratinocytes. Note that, for the most part, $CD24^+$ keratinocytes did not form colonies in culture. Conversely, the CD24 negative population of YF29 keratinocytes did form colonies in vitro. Colony-forming efficiencies were: E, unfractionated, 25%; F, $CD24^-$, 44.5%; G, $CD24^+$, 1.2%.

tional differences between species (Kay et al., 1990, 1991). Because antibodies that recognize human CD24 do not label rat cultured keratinocytes, we raised a polyclonal antiserum against the carboxy-terminal end of the rat CD24 peptide, a region highly conserved in mouse, rat and human. All epidermal layers of the sole of the rat were labelled by indirect immunofluorescence using this anti-serum. Thus it is clear that the expression of CD24 is not restricted to the clonogenic keratinocytes in either human or rat epidermis. Interestingly, the labelling was mostly cytoplasmic in the basal and suprabasal layers whereas it was mostly membrane-bound in the upper layers (data not shown). This suggests that CD24 mRNAs are translated in the inner layers of the rat epidermis and that the peptide is processed and bound to the membrane in the more differentiated cells.

Our results demonstrate unambiguously that K-CFCs cannot be distinguished on the basis of the expression of CD24, and thus that CD24 is unlikely to play a direct role in keratinocyte renewal. However, CD24 expression distinguishes further the two different compartments of the hair follicle with distinct proliferative and functional properties. Furthermore, CD24 mRNA expression allows us to discriminate at the molecular level the basal layer from the suprabasal layers of the bulge area. This is particularly interesting since all outer epithelial sheath keratinocytes express K5 and K14 keratins whose expression is usually restricted to proliferative keratinocytes (Kopan and Fuchs, 1989; Coulombe et al., 1989). Interestingly, CD24 is expressed in the developing hair follicle (Shirasawa et al. 1993; our results) suggesting that the pattern of expression of CD24 in the bulge area may be programmed during embryogenesis. Indeed, CD24 transcripts are detected in the epithelial cells of the developing follicles (Fig. 5C). Later, the pattern of expression of CD24 becomes restricted mostly to the keratinocytes located in the upper part of the

elongated follicles (Fig. 5E). This suggests that the expression of CD24 may be somehow linked to the function of the bulge. It is also worth noting that the bulge area is the most innervated region of the vibrissal follicle (Vincent, 1913; Halata, 1993). Therefore, we postulate that CD24 is involved in the sensory function of the follicle, possibly through neuron-keratinocyte signalling. Indeed, the glycosylated backbone of CD24 may confer to the keratinocytes located in the upper part of the vibrissal follicle the rigidity required to transmit the mechanical signal from the whisker to the neuron endings. CD24 null mice may be useful to study the function of CD24 in keratinocytes although no obvious abnormalities of the vibrissa or the pelage have been observed in CD24-deficient mice (P. Nielsen, personal communication; our unpublished results).

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