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Desmoyokin, a 680 kDa keratinocyte plasma membrane-associated protein, is homologous to the protein encoded by human gene AHNAK

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SUMMARY

We have obtained a monoclonal antibody (33A-3D) that specifically recognize desmoyokin, a 680 kDa desmosomal plaque protein that is well characterized in bovine muzzle epidermis. A cDNA clone (DY6, 3693 bp) was isolated by immunoscreening a mouse keratinocyte expression library with 33A-3D, and it was confirmed that DY6 has a partial coding sequence for desmoyokin. DY6 consists of highly homologous repeats about 128 residues long. Furthermore, the 128-residue repeats exhibit a quasi seven-residue substructure, which we believe will adopt an antiparallel β-sheet structure. Surprisingly, the amino acid sequence showed a significant homology with AHNAK, a newly identified human gene encoding a 700 kDa protein, which was suggested to be down-regulated in neuroblastoma. From its extensive homology, the similarity in both size and structure, and the identical patterns on Southern blot analysis of genomic DNAs, desmoyokin and AHNAK protein are thought to be identical. Although the desmoyokin/AHNAK protein is detected in a variety of cell types at both protein and mRNA levels, its distribution in keratinocytes (associated closely with cell membrane) is quite different from that in cells other than keratinocytes (distributed diffusely in the cytoplasm). These findings suggest that the desmoyokin/AHNAK protein is a ubiquitous molecule with a unique structure and appears to have different distributions (and probably different functions) among different cells.

Key words: AHNAK, desmosomes, desmoyokin, keratinocytes

INTRODUCTION

The desmosome is one of the intercellular adhering junctions seen mainly in epithelial tissues, to which keratin intermediate filaments attach through cytoplasmic attachment plaques (Schwarz et al., 1990). A number of immunohistochemical and biochemical studies have revealed that desmosomes consist of various glycosylated and nonglycosylated proteins (Skerrow and Matoltsy, 1974; Cohen et al., 1983; Cowin and Garrod, 1983; Watt et al., 1984; Moll et al., 1986). The membrane core domain is composed of membrane glycoproteins, i.e. 150 kDa desmoglein (Schmelz et al., 1986), 120 kDa/110 kDa desmocollins I/II (Parrish et al., 1990), and 22 kDa glycoprotein. The attachment plaque is composed of nonglycosylated proteins, i.e. 310 kDa/238 kDa desmoplakins I/II (Mueller and Franke, 1983; O'Keefe et al., 1989; Virata et al., 1992), 240 kDa desmocalmin (Tsukita and Tsukita, 1985), 83 kDa plakoglobin (Cowin et al., 1986; Franke et al., 1983), 78 kDa band 6 protein (Franke et al., 1983) and 466 kDa plectin (Wiche et al., 1983, 1991). cDNAs for most of these desmosomal components have recently been isolated, and both desmoglein and desmocollins I/II are now known to be cell adhesion molecules of the cadherin family (Koch et al., 1990; Wheeler et al., 1990; Nilles et al., 1991; Collins et al., 1991; Mechanic et al., 1991; Parker et al., 1991). The structures and functions in various plaque proteins, namely, desmoplakins I/II (Green et al., 1990), plakoglobin (Franke et al., 1989) and plectin (Wiche et al., 1991), have also been extensively studied by using their isolated cDNA clones.

In addition to these well-characterized constituents, desmoyokin, a high molecular mass protein of 680 kDa, has been identified as a new desmosomal plaque protein that is found only in the stratified epithelium (Hieda et al., 1989). Using low-angle rotary shadowing isolated desmoyokin molecules have been visualized by electron microscopy and shown to have a characteristic dumbbell shape, and to be approx. 170 nm in length with the central rod domain being approx. 100 nm long. An immunogold electron microscope study showed that the monoclonal anti-

body (mAb) specific to desmoyokin heavily labeled the periphery of the attachment plaques, suggesting that the molecule may work as a stabilizer for desmosomes.

In the present study, we have obtained a mAb during the screening of a number of mAbs produced from mouse immunized with a bovine muzzle epidermis desmosome preparation. On the basis of several criteria this mAb was considered to recognize desmoyokin (Hieda et al., 1989). Consequently, we have isolated cDNA clones by immunoscreening a mouse expression cDNA library with the mAb and confirmed that the cDNA encodes desmoyokin. Analysis of the amino acid sequence revealed extensive repeats of unique segments of 128 residues, which suggests a distinct structure and function for desmoyokin in the assembly of desmosomes. Surprisingly, a homology search demonstrated that the deduced amino acid sequence showed significant homology with a 700 kDa protein encoded by a human gene AHNAK, which has been suggested to be down-regulated in neuroblastoma (Shtivelman et al., 1992). Further studies revealed that the desmoyokin/AHNAK protein is expressed in a variety of cell types, although this protein is distributed diffusely in the cytoplasm of all the cells examined, other than keratinocytes.

These results indicate that the desmoyokin/AHNAK protein is a ubiquitous protein, but that its distribution and function in keratinocytes may be different from those in other types of cells.

MATERIALS AND METHODS

mAbs

mAb 33A-3D (mouse IgM) was isolated by the standard hybridoma technique using spleen cells from mice immunized with bovine desmosome preparation (Gorbsky and Steinberg, 1981; Konohana et al., 1987), and by ELISA screening using desmosome preparation-coated plates (Cohen et al., 1983). Production and characterization of anti-desmoyokin mAb (Yo-12086; mouse IgM) have been reported (Hieda et al., 1989). mAbs were obtained from ascites fluid, after the hybridomas were injected peritoneally into pristane-primed Balb/c mice. Anti-desmoplakins I/II mAb (11-5F; mouse IgG) (Parrish et al., 1987) used as a control was a generous gift from Dr D. R. Garrod, Manchester University, UK.

Cell cultures

In the present study, the following cell lines were used: Pam, a mouse keratinocyte cell line (Yuspa et al., 1980); KU8, a human squamous cell carcinoma cell line (Tsukamoto, 1989); DJM1, a human squamous cell carcinoma cell line (Kitajima et al., 1989); HCC-T, a human hepatoma cell line (Saito et al., 1989), Balb-3T3, a mouse fibroblast cell line; B16, a mouse melanoma cell line; Nagai, a human neuroblastoma cell line (a generous gift from T. Kato, Nagoya City University, Japan).

Immunofluorescence test

Indirect immunofluorescence with monoclonal or polyclonal antibodies was performed on bovine snout or normal human skin sections, or on cultured cells of a variety of cell types as previously described (Hieda et al., 1989), using fluorescein-conjugated anti-mouse immunoglobulins or rabbit immunoglobulins antisera (DAKO, Copenhagen, Denmark) as secondary antibodies.

Immunoblot analysis

Extraction of normal human epidermis and cultured cells, and immunoblot analysis, were performed as described previously (Hashimoto et al., 1990).

Construction and screening of cDNA library

cDNA was synthesized using mRNA extracted from Pam cells, as described previously (Amagai et al., 1990). The cDNA was ligated with *Eco*RI linkers and inserted into the *Eco*RI site of λZAPII (Stratagene, La Jolla, CA), then packaged using Gigapack II Gold packaging extract (Stratagene). Approximately 5×10^5 independent recombinants were screened by immunostaining with the avidin-biotin system using mAb 33A-3D. Positive clones were plaque-purified through at least three rounds of re-screening. The cDNA inserts from the positive clones were subcloned into the plasmid vector pBluescript II SK− (Stratagene) for further characterization.

Northern blot analysis

Total RNA was isolated from various cultured cells with LiCl buffer (Amagai et al., 1990). Twenty μg of each RNA was resolved in a 1% agarose gel containing formaldehyde, transferred to a GeneScreenPlus membrane (DuPont/NEN Research Products, Boston, MA), and hybridized at 42°C in 50% formamide with ^{32}P-labeled insert cDNA (Amagai et al., 1990). The same membrane was re-hybridized with human EGF receptor cDNA probe pE7, *Ban*III 2.4 kbp fragment (Merlino et al., 1985). Membranes were exposed to the imaging plate and analysed using the BAS 2000 image analyser system (Fuji Photo Film Co. Ltd., Japan).

Southern blot analysis

Genomic DNAs (4 μg each) from human placenta and mouse cell line A9 were digested with either *Eco*RI or *Hin*dIII, fractionated on a 0.7% agarose gel, transferred to a GeneScreenPlus membrane, and hybridized at 65°C with ^{32}P-labeled insert cDNA.

Expression of fusion protein

Escherichia coli strain XL1-Blue was transformed with pBluescript II SK− inserted with insert cDNA either in-frame or in the opposite direction, and then the fusion protein was induced by addition of isopropyl-β-thiogalactopyranoside (Maniatis et al., 1989). Whole bacterial pellet was lysed with Laemmli's sample buffer (Laemmli, 1970) under reducing conditions, and subjected to immunoblot analysis.

Preparation of anti-fusion protein polyclonal antibodies

In order to obtain highly purified recombinant protein for preparing polyclonal antibodies, the cDNA was subcloned in-frame into expression vector pGEX-3X (Pharmacia, Uppsala, Sweden) (Smith and Johnson, 1988), which was subsequently transfected to XL1-Blue. After fusion protein was induced by an addition of isopropyl-β-thiogalactopyranoside, it was purified with a glutathione S-transferase/Sepharose 4B column (Pharmacia), according to the manufacturer's instructions, and used for immunizing rabbits.

Sequence analysis

After an insert of a cDNA clone was subcloned into phage M13mp18 or mp19 (Yanisch-Perron et al., 1985), a series of deletion mutants were produced with the exonuclease III/mung bean nuclease deletion system (Stratagene) and single-stranded DNAs were sequenced in both directions by the dideoxy chain-termination method (Sanger et al., 1977). A search for homologous sequences was carried out with the databases EMBL/Gen-

Bank/DDBJ (Release 11) using Microgenie software (Beckman Instruments, Inc., Palo Alto, CA). Dot matrix comparisons were performed with the programs COMPARE and DOTPLOT using a software package from the University of Wisconsin Genetics Computer Group (UWGCG, Madison, WI). Alignments of homologous regions and derived computations (percentage of identical and similar amino acids; Dayhoff, 1979) were done both manually and using the programs GAP (UWGCG) and ALIGN (DNA Star Inc.; Lipman and Pearson, 1985). Homologous residues were considered as follows: D/E; K/R; S/T; L/V/I/M/Y/F/A. Secondary structure predictions were performed using the AASAP package, which incorporates the methods of Chou and Fasman (1978) and Garnier et al. (1978). Fourier transform analyses to examine the possibility of a periodic distribution of residues (e.g. charged and apolar) were carried out as described by Parry (1975) and McLachlan and Stewart (1976).

RESULTS

Production and characterization of mAbs

We isolated a hybridoma cell line by screening with ELISA. By using immunofluorescence of frozen sections of bovine snout, the mAb (33A-3D) produced by the cell line showed clear and slightly patchy cell surface staining in the entire epidermis (Fig. 1A), which was indistinguishable from the staining pattern produced by the anti-desmoplakins I/II mAb (Fig. 1B). When frozen sections of normal human skin were used, the 33A-3D mAb showed linear cell surface staining in only the upper epidermis (Fig. 1D). This staining pattern was considerably different from the granular cell surface staining in the entire epidermis produced by the anti-desmoplakins I/II mAbs (Fig. 1E). The 33A-3D mAb also showed linear cell boundary staining with considerable cytoplasmic staining in both Pam cells (data not shown) and KU8 cells (Fig. 1G). This staining pattern was also quite different from the dispersed dot-like cell surface staining, without cytoplasmic staining by the anti-desmoplakins I/II mAb (Fig. 1H). All of these staining patterns shown by the 33A-3D mAb were indistinguishable from those shown by the previously characterized anti-desmoyokin mAb Yo-12086 (data not shown).

In immunoblot analysis of an extract from normal human epidermis, from Pam cells and from KU8 cells, the 33A-3D mAb specifically reacted with a protein band, the molecular mass of which was estimated to be approx. 680 kDa using crosslinked phosphorylase *b* as a molecular mass marker (Fig. 2A). The strongest reactivity was constantly seen in the Pam cell extract. Furthermore, the protein band that reacted with the 33A-3D mAb in the Pam cell extract showed exactly the same mobility as the protein band that reacted with the Yo-12086 mAb (Fig. 2B).

Isolation, identification and characterization of cDNA clones

Because the 33A-3D mAb always showed the strongest reactivity in the Pam cell extract, by immunoblotting, we decided to use a λZAPII expression cDNA library constructed from poly(A)$^+$ RNA extracted from Pam cells for immunoscreening with the 33A-3D mAb. Of the 5×10^5 recombinant clones, nine positive clones were isolated. One of these (3.7 kbp cDNA insert designated DY6) was characterized further.

Northern blot analysis with DY6 showed similar messages extremely large in size in mRNAs from both cultured Pam cells and KU8 cells (Fig. 3A). The intensities of the signals were quite different between the two cell lines, i.e. although the signal for the Pam cells was seen after a very short exposure, prolonged exposure was required to detect the signal for the KU8 cells. pE7 probe for human EGF receptor exhibited its 10 kb message only in the KU8 cell mRNA. Although precise size determination for such a large mRNA was difficult, the mRNAs hybridized with DY6 were considered to be around 20 kb in size. The size of the mRNA thus seems to be large enough to encode the entire 680 kDa protein.

With immunoblotting using lysate of bacteria transformed by pBluescript inserted in-frame with DY6, the 33A-3D mAb showed clear reactivity with several protein bands (Fig. 2C). The upper band with the strongest reactivity and with a molecular mass of approx. 150 kDa seemed to be the intact fusion protein. The lower bands were considered to be its degradation products. The Yo-12086 mAb also clearly labeled these protein bands in almost the same pattern. None of the negative controls, including the culture supernatant of parental cells for hybridoma and mouse IgM mAb against unrelated antigen, showed any positive reactivity. Furthermore, neither the 33A-3D nor Yo-12086 mAb showed positive reactivity in the lysate of bacteria transformed by the plasmid in which DY6 was inserted in the opposite direction.

The fusion protein that was produced by pGEX-3X inserted in-frame with DY6 and purified with a glutathione S-transferase column was also specifically recognized by both the 33A-3D and Yo-12086 mAbs (data not shown). The rabbit antiserum raised against this fusion protein showed exactly the same immunofluorescence staining patterns as those shown by the 33A-3D and Yo-12086 mAbs in all bovine muzzle sections (Fig. 1C), normal human skin sections (Fig. 1F) and KU8 cells (Fig. 1I). The anti-fusion protein polyclonal antibodies also showed clear reactivity with the 680 kDa protein bands, the same protein bands recognized by the mAbs, with immunoblotting of extracts of all normal human epidermis, Pam cells and KU8 cells (data not shown).

Taking all these results together, DY6 was considered to encode part of the desmoyokin molecule.

DNA sequencing and homology search

The nucleotide and deduced amino acid sequences of DY6 are shown in Fig. 4. DY6 is 3693 nucleotides long and has an open reading frame over its entire length. The other two possible frames, as well as the three frames in the opposite direction, all showed multiple stop codons. The nucleotide sequence encodes a polypeptide of 1231 amino acids with a molecular mass of approx. 132 kDa, which is approximately one-fifth of the intact desmoyokin molecule.

However, comparison with previously reported sequences showed that the amino acid sequence of DY6 has extensive homology (80.8%) with that of a 700 kDa protein encoded by the human gene AHNAK (Shtivelman et al., 1992) (Fig. 5). The molecular structure predicted by

the analysis of the AHNAK gene is compatible with the dumbbell-like molecule of desmoyokin suggested by the electron microscope study (Hieda et al., 1989). Northern blot analysis of AHNAK RNA showed a single transcript of about 18 kb in various human and rodent cell lines (Shtivelman et al., 1992) that is similar to the messages shown by DY6 in the present study. Furthermore, DY6 detected a single fragment of approx. 24 kbp on Southern blot analysis of *Eco*RI-digested genomic DNA from both mouse cells and human tissue (Fig. 6A). DY6 hybridized with two *Hin*dIII fragments of mouse genomic DNA and three *Hin*dIII fragments of human genomic DNA (Fig. 6B). These patterns on Southern blots of DY6 are identical to those of AHNAK (Shtivelman et al., 1992). (The pattern of

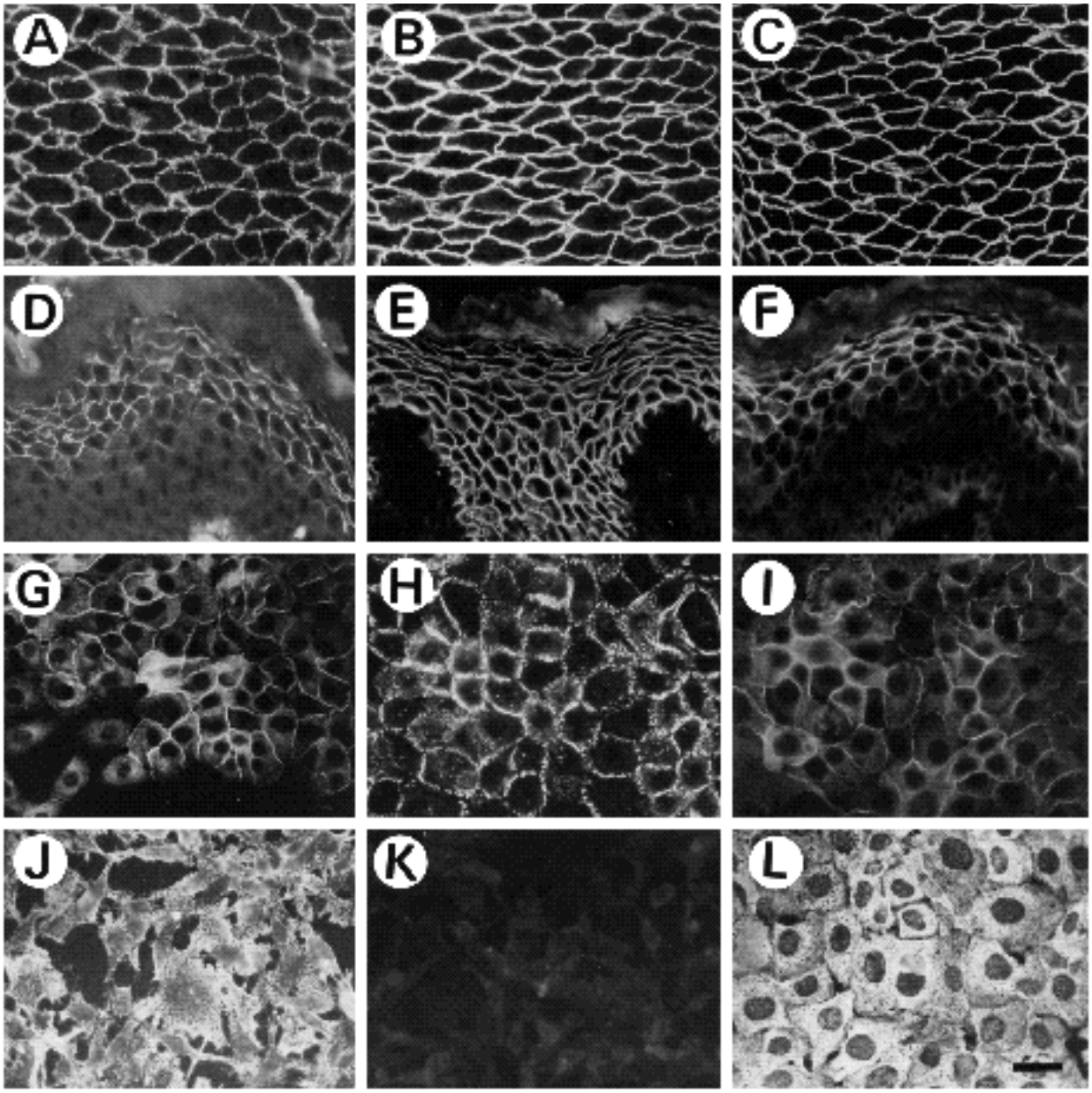

Fig. 1. Results of immunofluorescence. All the 33A-3D mAb (A), the anti-desmoplakins I/II mAb (B) and the polyclonal antibodies against fusion protein produced by DY6 (C) showed clear and slightly patchy cell surface staining of the entire epidermis in frozen sections of bovine muzzle epidermis. In frozen sections of normal human skin, both the 33A-3D mAb (D) and the anti-fusion protein polyclonal antibodies (F) showed linear cell surface staining in the upper epidermis only. This staining pattern was quite different from the granular cell surface staining of the entire epidermis produced by the anti-desmoplakins I/II mAb (E). In cultured KU8 cells, both the 33A-3D mAb (G) and the anti-fusion protein polyclonal antibodies (I) showed linear cell boundary staining with considerable cytoplasmic staining, which was also very different from the dispersed dot-like staining without cytoplasmic staining produced by the anti-desmoplakins I/II mAb (H). The 33A-3D mAb showed a very strong granular cytoplasmic staining without any cell boundary staining in B16 cells (J), whereas anti-desmoplakins I/II mAb showed no reactivity in these cells (K). The anti-fusion protein polyclonal antibodies also showed strong cytoplasmic staining in 3T3 cells. Bar, 10 μm.

*Hin*dIII fragments of mouse genomic DNA detected by DY6 is identical to that of bovine DNA detected by AHNAK, but not to that of mouse DNA, shown in the Fig. 3 of the paper by Shtivelman et al. (1992). Dr Shtivelman kindly informed us that the lines containing mouse and bovine DNAs were mislabeled.) These results strongly suggest that the protein encoded by DY6 is a mouse homologue of the AHNAK protein.

Evidence was presented that the AHNAK protein was expressed in a variety of cell types, and in each case was suspected to reside within the nucleus (Shtivelman et al., 1992; and a personal communication from Dr E. Shtivelman and Dr J. M. Bishop). Therefore, we further examined the expression and distribution of desmoyokin/AHNAK protein in other cell lines of various origins.

Both the 33A-3D and Yo-12086 mAbs showed clear cell boundary staining with considerable cytoplasmic staining in another keratinocyte cell line, DJM1 (data not shown), which was very similar to the staining patterns in Pam cells and KU8 cells. In contrast, these mAbs showed very strong granular cytoplasmic staining without any cell surface staining in all HCC-T cells, Balb-3T3 cells, B16 cells (Fig. 1J), and Nagai cells. Anti-fusion protein polyclonal antibodies showed exactly the same staining patterns as those shown by the mAbs in all of these cell lines (Fig. 1L). However, anti-desmoplakins I/II mAb showed no positive staining in any of these non-epithelial cells (Fig. 1K).

Immunoblot analysis also showed that the 33A-3D mAb reacted with approx. 680 kDa proteins in all DJM1, HCC-T, 3T3, B16 and Nagai cells (Fig. 2D). Both the Yo-12086

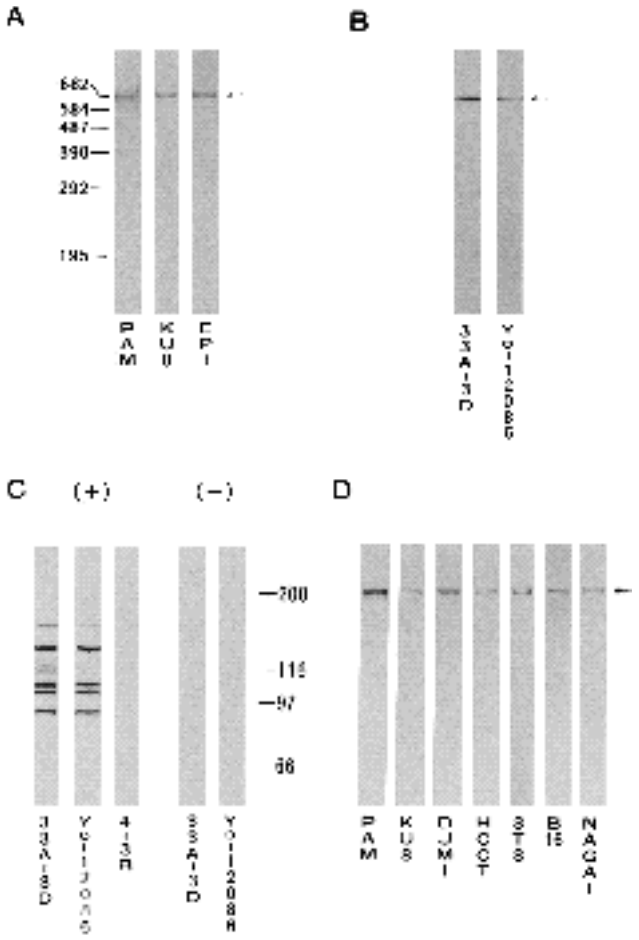

Fig. 2. Results of immunoblot analyses. (A) Extracts of Pam cells (lane PAM), KU8 cells (lane KU8) and dispase-separated normal human epidermis (lane EPI) were fractionated by SDS-PAGE, and blotted sheets were allowed to react with the 33A-3D mAb. At all antigen sources the mAb reacted with a single protein band with a molecular mass of approx. 680 kDa (arrow). The migration of cross-linked phosphorylase *b* as the molecular mass marker (kDa) is shown on the left. (B) The blots of fractionated Pam cell extract are shown to react with both the 33A-3D and Yo-12086 mAbs. Both mAbs reacted with the 680 kDa protein band (arrow). (C) Results of immunoblotting of the fusion protein produced by DY6 with mAbs. In the lysate of bacteria with pBluescript II inserted in-frame with DY6 (lanes (+)), both the 33A-3D and Yo-12086 mAbs showed strong reactivity with several protein bands with the same pattern, indicating that both mAbs may react with the same or a close epitope that is repeated in this highly repetitive domain of this protein. Although two of the protein bands are hardly seen in the Yo-12086 mAb lane, these bands could be seen weakly but clearly in the original blot. Mouse IgM mAb 4-3B against unrelated antigen, however, showed no reactivity. In the lysate of bacteria with the vector inserted in the opposite direction to DY6 (lanes (−)) no reactivity was shown by either the 33A-3D or Yo-12086 mAb. (D) The 33A-3D mAb reacted with similar protein bands with molecular mass of approx. 680 kDa (arrow) in all extracts of Pam cells (lane PAM), KU8 cells (lane KU8), DJM1 cells (lane DJM1), HCC-T cells (lane HCCT), 3T3 cells (lane 3T3), B16 cells (lane B16), and Nagai cells (lane NAGAI).

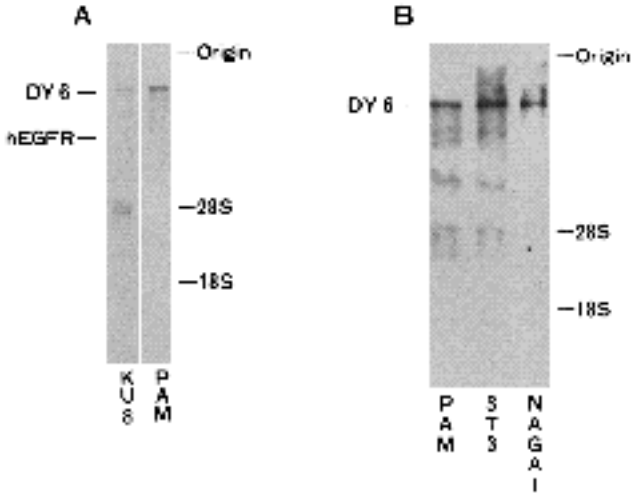

Fig. 3. Northern blot analysis for DY6. (A) A 20 μg sample of each total RNA extracted from KU8 cells (lane KU8) and Pam cells (lane PAM) was electrophoresed, transferred to a nylon membrane, and hybridized with DY6. A strong single message of approx. 20 kb was identified by DY6 in RNA from Pam cells. After prolonged exposure a similar message was identified by DY6 in RNA from KU8 cells. The positions of messages identified by DY6 (DY6) and a message identified by re-hybridization with human EGF receptor probe in RNA only from KU8 cells (hEGFR, the signal is not seen in this figure) are indicated on the left. The positions of the origin of the gel and of the 28 S and 18 S rRNAs are indicated on the right. (B) The approx. 20 kb mRNAs (DY6) were also hybridized with DY6 in the lanes loaded with 20 μg of total RNAs from Pam cells (lane PAM), 3T3 cells (lane 3T3) and Nagai cells (lane NAGAI).

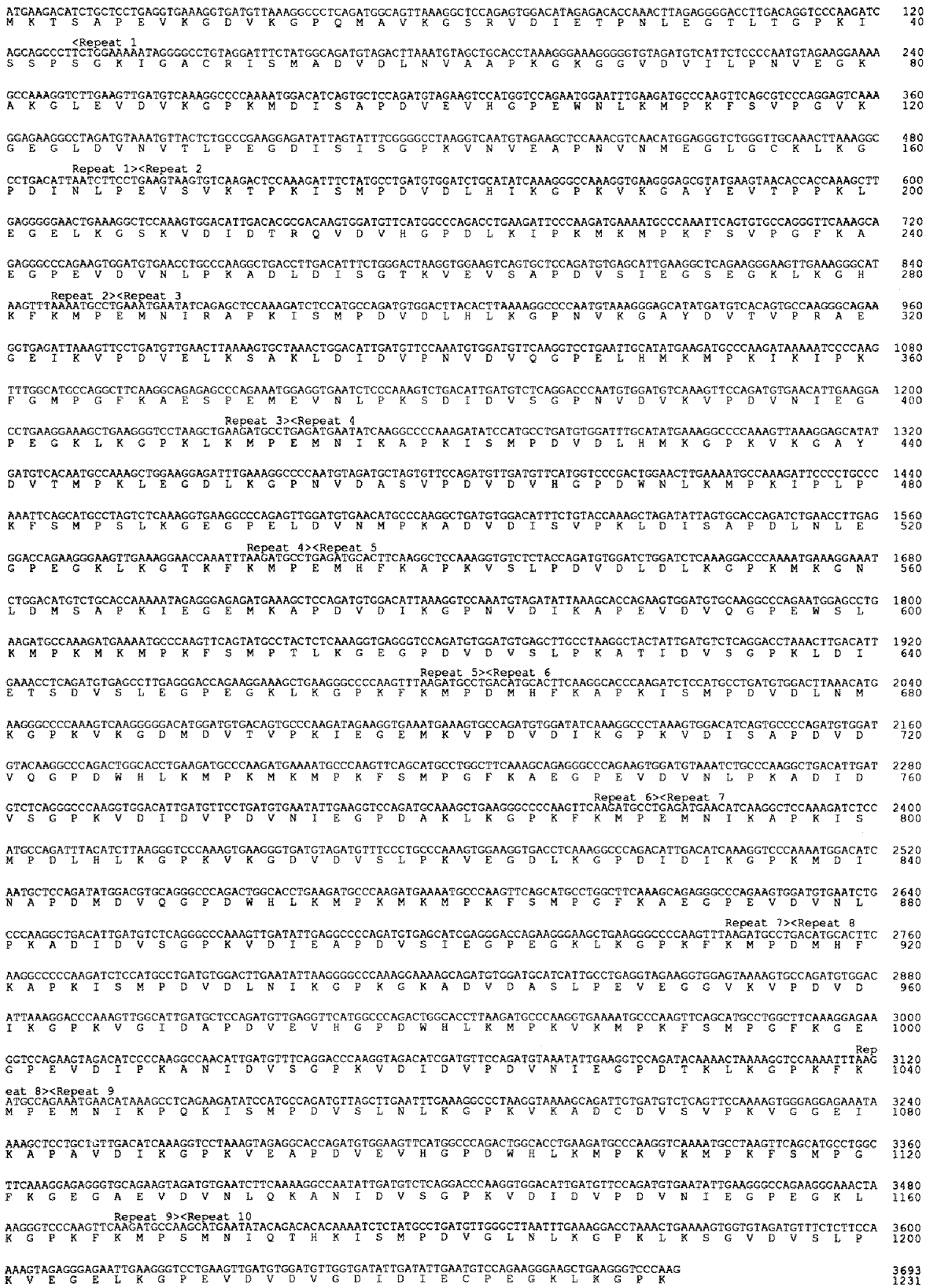

Fig. 4. Nucleotide and deduced amino acid sequences of DY6. Nucleotide and amino acid residues are numbered from the 5′ end of this cDNA. The boundaries between each of the ten 128-residue repeats are indicated above the sequences. These sequence data are available from EMBL/GenBank/DDBJ under accession number X65157.

mAb and anti-fusion protein polyclonal antibodies also showed the same staining pattern in all of these cell lines (data not shown).

Furthermore, by northern blot analysis of the total RNAs extracted from either 3T3 cells or Nagai cells, DY6 also hybridized with mRNAs of similar size to those seen in Pam cells (Fig. 3B), which further confirmed the expression of desmoyokin/AHNAK protein in these nonepithelial cells.

Analysis of the deduced amino acid sequences of desmoyokin

Almost all of the amino acid sequence can be accounted for by about 10 consecutive repeats, each about 128 residues in length. Within each repeat there are four segments, which we have designated C, A, B and A′ (Fig. 7).

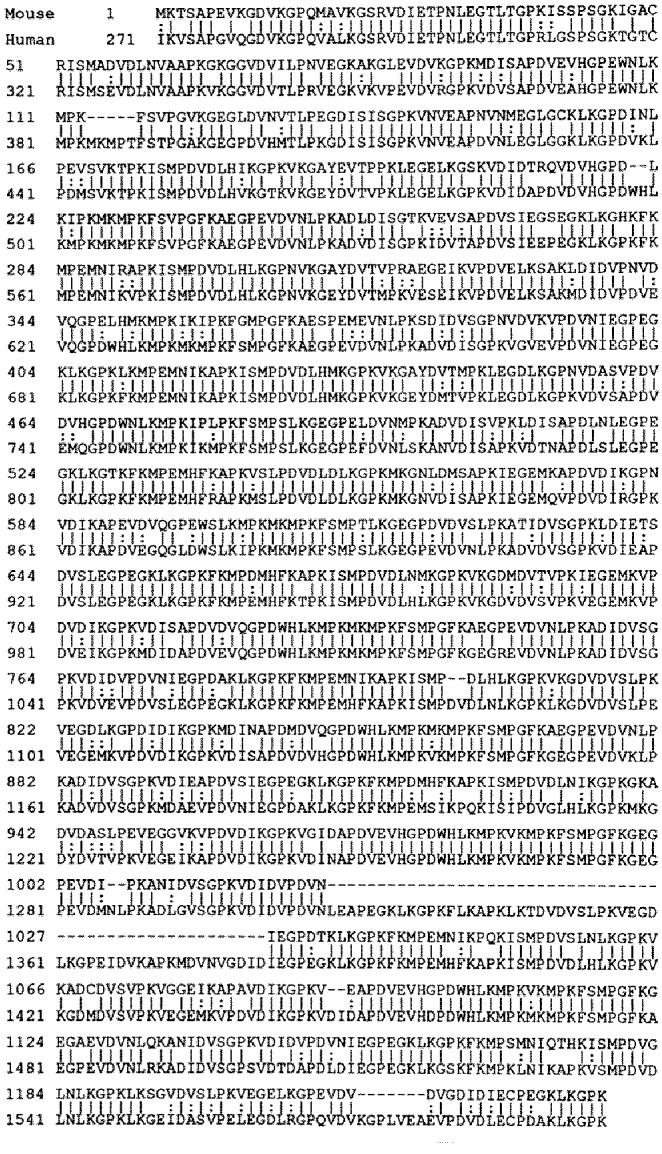

Fig. 5. Comparison of amino acid sequences between DY6 (mouse) and AHNAK (human). Identity is indicated by vertical bars, homologous residues are indicated by colons, and gaps introduced in order to maximize alignment are indicated by dashes.

Segments A and A′ are both 28 residues in length, segment C is 46 residues long and segment B is 26 residues long. Dot matrix comparison also shows the very high degree of internal homology in DY6 (Fig. 8A). Although there is very great similarity between A and A′, there are key conserved differences in several positions. For example, a D (residue 12 in the consensus sequence of segment A), V (residue 13), W (residue 18), H (residue 19) and M (residue 22) in segment A are replaced by an N, I, G, K and G, respectively, in segment A′. When all of the C, A, B and A′ segments are compared, the average degree of internal identity (or similarity when conservative substitutions are allowed) is as follows: C, 60%(78%); A, 62%(80%); B, 68%(85%) and A′, 65%(77%).

Within the 128-residue repeats there is a quasi seven-residue substructure of the form (G P K/D V D ap K) (Fig. 8B and Fig. 9). The secondary structure corresponding to these repeats is most unlikely to be α-helical, since proline is a major helix-disrupting amino acid. Also, glycine does not favour an α-helical structure, and where it is present it commonly terminates an α-helix. The most probable structure, therefore, is a series of β-turns (as many as 18) separated by β- or β-like extended chain. The β-turn, which is a structure in which the protein chain folds back on itself in a tight loop, occurs commonly on the surface of a protein, and the likelihood of its occurrence can be calculated on the basis of the analysis of such structures by Chou and Fasman (1978) from crystalline globular protein data. We find that the first four residues of the quasi seven-residue repeats (G P K/D V in the consensus sequence shown in Fig. 9) have a very great probability of adopting a β-turn. These four residues are underlined in Fig. 7.

The short β-like strands all have an odd number of residues (1, 3, 5 or 7). Interestingly, also, the conserved

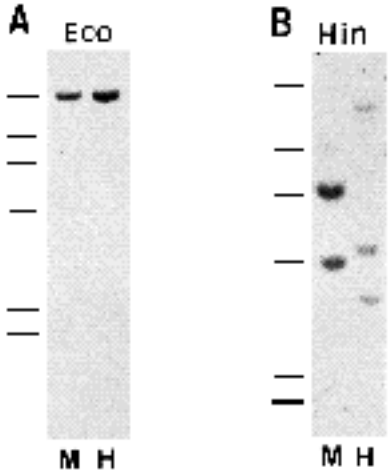

Fig. 6. Southern blot analysis. (A) Genomic DNAs from mouse cell line A9 (lane M) and human placenta (lane H) were digested with *Eco*RI and subjected to Southern blot analysis. The DY6 detected a single fragment of about 24 kbp in each lane. (B) When genomic DNAs were digested with *Hin*dIII, DY6 detected two bands in the mouse DNA (lane M) and three bands in the human DNA (lane H). Bars at the left of each panel indicate molecular sizes, from top to bottom, 23 kbp, 9.4 kbp, 6.6 kbp, 4.4 kbp, 2.3 kbp and 2.0 kbp.

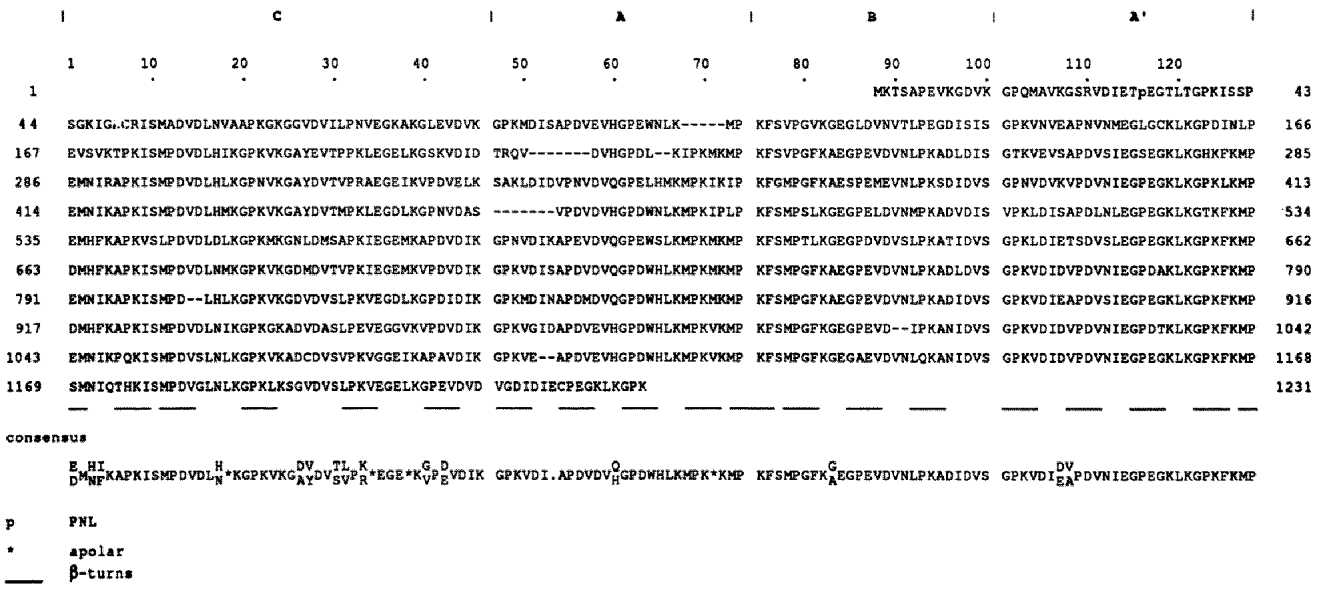

Fig. 7. Alignments of the 128-residue repeats with subdomains of C, A, B and A′. The consensus sequence is shown at the bottom of the panel. The symbols * and • used in the consensus sequence denote an apolar residue and a non-conserved residue, respectively. The first four residues of the quasi seven-residue repeats, which are postulated to form a β-turn (consensus sequence:G P K/D V), are shown by lines below the last repeat.

apolar residues are always separated by two residues (or a multiple) and the conserved charged residues are never immediately adjacent. If the β-like structure is correct (and such conformations have 2 residues per turn) then one face of the strand will be totally apolar and the other will be very highly charged (Richardson, 1981) (Fig. 10). The 18

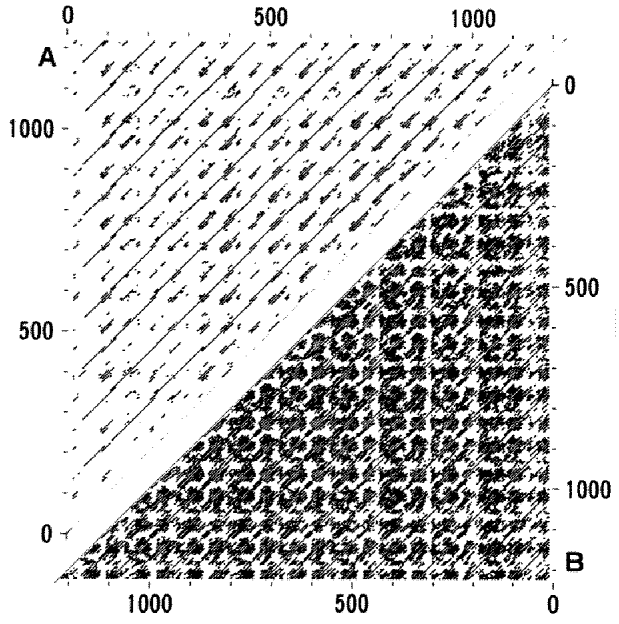

Fig. 8. Searches for internal homology within DY6 using COMPARE and DOTPLOT. (A) Comparison with a window of 30 amino acids and a stringency of 21.0 shows the 128-residue repeats. (B) Comparison with a window of 20 amino acids and a stringency of 11.0 clearly shows the quasi seven-residue repeats.

β-turns and β-strands that could form an antiparallel β-sheet structure, and which correspond to a single 128-residue repeat, are unlikely to be planar as drawn, would almost certainly have a right-handed twist, and could fold up into a quite compact structure with the apolar residues forming the core and the charged residues the outer surface. Conversely, two such sheets might aggregate via their apolar faces, thus leaving the charged aspects of the structure on the surface and in a position where they could be hydrated or interact with other structures.

The ratio of charged (D E K R) to apolar residues (L I V M F Y A) for a DY6 repeat is about 0.92, a value consistent with a compact rather than an elongated structure. It seems likely therefore that each repeat forms an approximately spherical structure (diameter is 3.2 nm on the basis of the calculated volume) and that these repeats form an extended rod domain. The AHNAK data suggest that there are about 33 repeats in the rod domain, thus enabling an estimate to be made of the length of this domain (i.e. 33×3.2=106 nm).

The charged residues are not randomly disposed on the charged side of the β-sheet and there is evidence that the acidic and the basic residues are clumped into alternating zones of positive and negative charge (two of each per 128-residue repeat) (Fig. 10).

DISCUSSION

In a previous report (Hieda et al., 1989), the characteristics of desmoyokin in bovine muzzle and tongue tissues were well established by electron microscope studies as a dumbbell-shaped molecule localized at the periphery of desmosomal attachment plaques. This localization was also con-

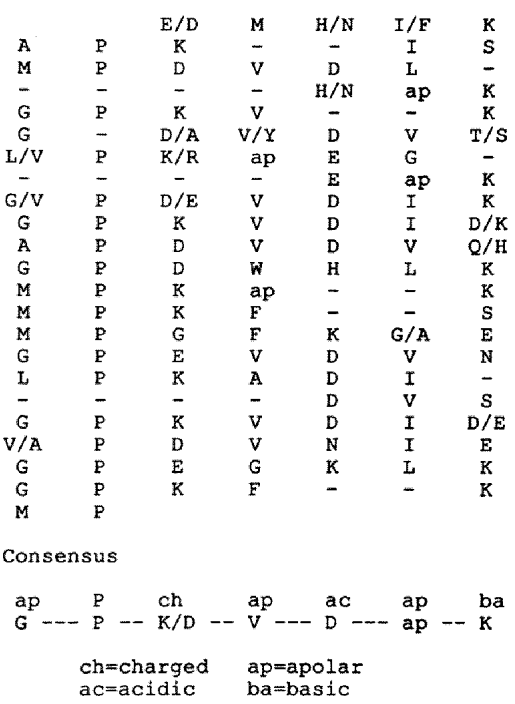

Fig. 9. A quasi seven-residue substructure seen within the 128-residue repeats. The consensus sequence is shown below the panel. ap, apolar residues.

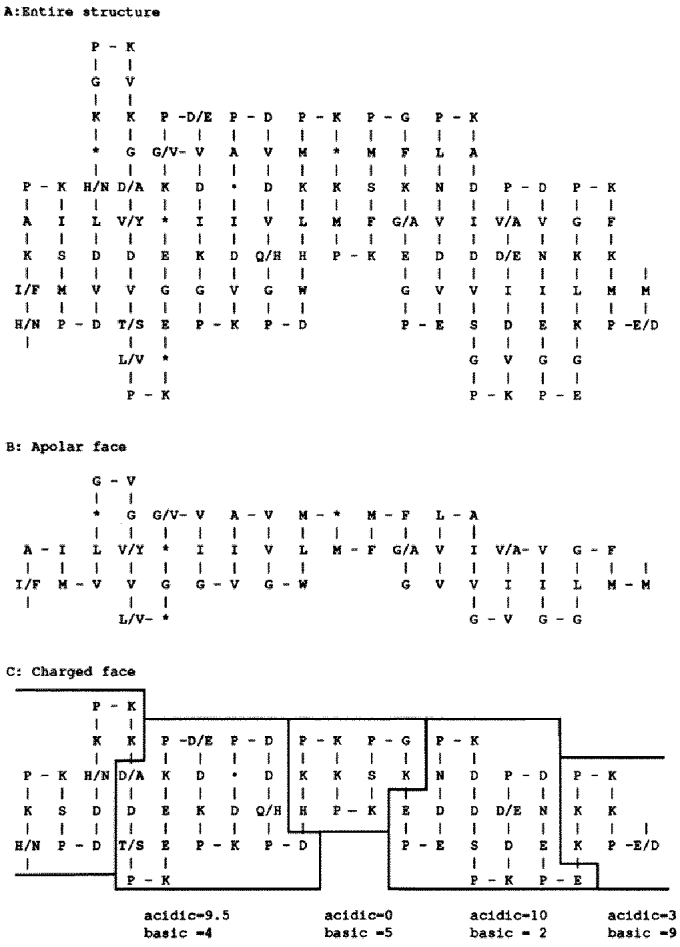

Fig. 10. Secondary structure with 18 β-turns and β-like strands suggested by the quasi seven-residue substructure within the 128-residue repeats. (A) An entire sheet structure with the apolar residues forming one face and the charged residues the other face. (B) Apolar face. (C) Charged face. The acidic and the basic residues are clumped into two alternating zones of positive and negative charge. The symbols * and • denote an apolar residue and a non-conserved residue, respectively.

firmed in the present study by demonstrating indistinguishable immunofluorescence staining patterns in bovine muzzle epidermis between the anti-desmoplakins I/II mAb and the anti-desmoyokin monoclonal and polyclonal antibodies. However, considerable differences in staining pattern or distribution between desmoplakins I/II and desmoyokin seen in other epithelial tissues, such as human epidermis or cultured keratinocytes, made us suspect that the desmoyokin is restricted to the desmosome in keratinocytes other than bovine muzzle epidermis, although definite association of desmoyokin with the plasma membrane was confirmed in some types of keratinocyte examined in this study.

We have cloned and characterized DY6, a mouse cDNA clone that encodes part of desmoyokin. Several lines of evidence have confirmed this conclusion. The 33A-3D mAb used for immunoscreening the cDNA library specifically recognized the 680 kDa protein, shown by immunoblotting studies of several different tissues, the reactivity of which was exactly the same as that of the Yo-12086 mAb, a well-characterized mAb to desmoyokin (Hieda et al., 1989). DY6 hybridized with a mRNA (approx. 20 kb) that is large enough to encode the entire 680 kDa desmoyokin molecule. Immunoblotting methods revealed that both the 33A-3D and Yo-12086 mAbs specifically recognize the fusion proteins produced by DY6. Finally, the rabbit antiserum raised against a fusion protein produced by DY6 showed exactly the same patterns of reactivity as those shown by anti-desmoyokin mAbs, by both immunofluorescence and immunoblotting studies of various keratinocytes.

The nucleotide sequence of DY6 is 3693 bp long and has an open reading frame that encodes a polypeptide of approx. 132 kDa. However, a homology search revealed that DY6 is homologous to the human gene AHNAK, which encodes a 700 kDa protein (Shtivelman et al., 1992). This protein is considered to be expressed and localized within the nucleus in many types of cells, although it is specifically repressed in neuroblastomas or some particular tumours (Shtivelman et al., 1992). Therefore, we further examined the expression and localization of desmoyokin/AHNAK protein in various cultured cells other than keratinocytes at the protein and mRNA levels using our anti-desmoyokin antibodies and DY6. Both immunoblot and northern blot analyses showed that this protein is expressed in all types of cells examined in the present study. However, immunofluorescence study revealed the considerable difference of distribution of this protein among different cell lines, i.e. a clear association of this protein with cell membrane in all types of keratinocyte examined, and a diffuse cytoplasmic distribution without apparent association with the cell membrane in cells of other origins.

A previous study of immunofluorescence on sections of various tissues indicated that desmoyokin is present only in the stratified squamous epithelia (Hieda et al., 1989). In the present study the anti-desmoyokin antibodies showed no apparent positive reactivity with the cells in the dermis, such as fibroblasts or endothelial cells. However, during the immunofluorescence studies, we noticed that quick freezing of the tissue specimen is critical in order to see the clear cell surface staining with the anti-desmoyokin antibodies in the human epidermis. Prolonged storage of this tissue at −70°C also considerably reduces the reactivity. This reduction of reactivity was not marked in cell surface staining with anti-desmoyokin antibodies in bovine snout epidermis. The mechanisms of these phenomena are not known at present. One explanation may be that desmoyokin is relatively labile when it is diffused throughout the cytoplasm or plasma membrane, whereas it is stable if assembled into the desmosome. This may explain the immunofluorescence results of Hieda et al. (1989), which showed that desmoyokin was observed in the stratified squamous epithelium, but not in any other tissue. Another possible explanation is that, because the diffuse cytoplasmic staining is usually not so marked as the cell membrane staining, the weak cytoplasmic reactivity seen in cells other than keratinocytes may not be easily distinguished from the background staining.

The results from the present study indicate more diverse localization and function of desmoyokin than was expected at the early stages of this study. At present, no definite function for desmoyokin/AHNAK protein is known. In bovine snout epidermis, this protein is restricted to the desmosome and may play some role in plaque assembly (Hieda et al., 1989). On the other hand, it is suggested that down-regulation of this protein in neuroblastoma cells may be relevant to their arrested differentiation (Shtivelman et al., 1992). These authors also showed that the AHNAK protein resides predominantly in the nucleus (Shtivelman et al., 1992; and personal communication from Dr E. Shtivelman and Dr J. M. Bishop).

To investigate the cause of this difference, we compared the reactivity of our anti-desmoyokin antibodies with that of polyclonal antibodies raised against two different synthesized peptides corresponding to the 128-residue repeat of AHNAK (generously provided by Dr E. Shtivelman and Dr J. M. Bishop). These two anti-AHNAK polyclonal antibodies showed clear cell surface staining by immunofluorescence of bovine snout sections, although these antibodies also showed considerable nuclear or cytoplasmic staining. In cultured Pam cells the anti-AHNAK antibodies indeed showed strong staining in the nucleus and the perinuclear area, although the antibodies also showed weak but clear cell boundary staining. In this keratinocyte cell line our anti-desmoyokin antibodies showed much stronger cell boundary staining and some cytoplasmic staining without apparent nuclear staining. In Balb-3T3 cells the anti-AHNAK antibodies also predominantly stained the nuclei, whereas the anti-desmoyokin antibodies showed cytoplasmic staining as well as nuclear staining. No antibodies stained cell membrane of this cell type. These results clearly confirm that the desmoyokin/AHNAK protein does have some role at the cell membrane in keratinocytes, and that this protein has different localizations in different types of cells.

Although there is at present no definite explanation for the different staining patterns between the anti-AHNAK antibodies and our anti-desmoyokin antibodies, the above observations lead us to the hypothesis that different forms of this protein are present in cells. The anti-AHNAK antibodies may have greater affinity for the nuclear form of the molecule, and our antibodies for the cytoplasmic form, although some of both forms is translocated to the cell membrane at least in keratinocytes. The anti-AHNAK antibodies showed clear staining at the nuclei of fibroblasts or endothelial cells in the dermis of bovine snout sections, further indicating the different nature of the different forms. Further studies will be needed to reveal the precise localizations and functions of these different forms.

Analysis of the amino acid sequence revealed a number of unique features. The bulk of the sequence consists of about 10 consecutive repeats of 128 residues. Within the 128-residue repeats there is a quasi seven-residue substructure containing both glycine and proline. This substructure is unusual and clearly indicates that the 128-residue repeat has no significant α-helix content and that the most probable structure is a series of β-turns linking β-like strands. Similar structures were also predicted from the analysis of the AHNAK protein (Shtivelman et al., 1992).

In each 128-residue repeat there could be as many as 18 β-turns and β-like strands, which form a β-sheet conformation. This in turn may fold up into a quite compact structure with its apolar face forming the core and its charged residues the outer surface. Alternatively, two β-sheets might aggregate through their apolar faces. Furthermore, the fact that the acidic and the basic residues are clumped into alternating zones of positive and negative charge strongly suggests that desmoyokin interacts ionically and regularly with other desmoyokin molecules or with other desmosomal constituents. These interactions are likely to be important in the assembly and stabilization of the plaque structure of desmosomes.

The previous electron microscope study showed that desmoyokin has N-terminal, central-rod and C-terminal domains (Hieda et al., 1989). The molecular structure predicted by the analysis of the AHNAK protein, i.e. a large central domain enclosed by the end domains (Shtivelman et al., 1992), is thus compatible with the dumbbell-like molecule of desmoyokin. However, the central rod domain composed of 128-residue repeats is most likely approx. 106 nm long, as suggested by the earlier electron microscope study of desmoyokin (Hieda et al., 1989). We think it unlikely that the central domain is as long as 1.2 μm, as suggested by the analysis of the AHNAK protein (Shtivelman et al., 1992). Crystallization and detailed X-ray analysis of the 128-residue repeats or, alternatively, an NMR structure determination, would be required to confirm the structure proposed for this unique structural motif.

The remarkable structure of desmoyokin/AHNAK protein, as well as its different distribution in different tissues, indicates that further studies will be needed to reveal the range of structural roles and the functions of this protein.

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The sequence data presented are available from EMBL/GenBank/DDBJ under accession number X65157.

REFERENCES

Amagai, M., Hashimoto, T., Tajima, S., Inokuchi, Y., Shimizu, N., Saito, M., Miki, K. and Nishikawa, T. (1990). Partial cDNA cloning of the 230-kDa mouse bullous pemphigoid antigen by use of a human monoclonal anti-basement membrane zone antibody. *J. Invest. Dermatol.* **95**, 252-259.

Cohen, S. M., Gorbsky, G. and Steinberg, M. S. (1983). Immunochemical characterization of related families of glycoproteins in desmosomes. *J. Biol. Chem.* **258**, 2621-2627.

Collins J. E., Legan, P. K., Kenny, T. P., MacGarvie, J., Holton, J. L. and Garrod, D. R. (1991). Cloning and sequence analysis of desmosomal glycoproteins 2 and 3 (Desmocollins): Cadherin-like desmosomal adhesion molecules with heterogeneous cytoplasmic domains. *J. Cell Biol.* **113**, 381-391.

Chou, P. Y. and Fasman, G. D. (1978). Empirical predictions of protein conformation. *Annu. Rev. Biochem.* **47**, 251-276.

Cowin, P. and Garrod, D. R. (1983). Antibodies to epithelial desmosomes show wide tissue and species cross-reactivity. *Nature* **302**, 148-150.

Cowin, P., Kapprell, H.-P., Franke, W. W., Tamkun, J. and Hynes, R. O. (1986). Plakoglobin: a protein common to different kinds of intercellular adhering junction. *Cell* **46**, 1063-1074.

Dayhoff, M. O. (1979). *Atlas of Protein Sequence and Structure*, vol. 5, National Biomedical Research Foundation, Washington, DC.

Franke, W. W., Goldschmidt, M. D., Zimbelmann, R., Mueller, H. M., Schiller, D. L. and Cowin, P. (1989). Molecular cloning and amino acid sequence of human plakoglobin, the common junctional plaque protein. *Proc. Nat. Acad. Sci. USA* **86**, 4027-4031.

Franke, W. W., Mueller, H., Mittnacht, S., Kapprell, H.-P. and Jorcano, J. J. (1983). Significance of two desmosome plaque-associated polypeptides of molecular weights 75000 and 83000. *EMBO J.* **2**, 2211-2215.

Garnier, J., Osguthorpe, D. J. and Robson, B. (1978). Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* **120**, 97-120.

Green, K. J., Parry, D. A. D., Steinert, P. M., Virata, M. L. A., Wagner, R. M., Angst, B. D. and Nilles, L. A. (1990). Structure of the human desmoplakins. Implication for function in the desmosomal plaque. *J. Biol. Chem.* **265**, 2603-2612.

Gorbsky, G. and Steinberg, M. S. (1981). Isolation of the intercellular glycoproteins of desmosomes. *J. Cell Biol.* **90**, 243-248.

Hashimoto, T., Ogawa, M. M., Konohana, A. and Nishikawa, T. (1990). Detection of pemphigus vulgaris and pemphigus foliaceus antigens by immunoblot analysis using different antigen sources. *J. Invest. Dermatol.* **94**, 327-331.

Hieda, Y., Tsukita, S. and Tsukita, S. (1989). A new high molecular mass protein showing unique localization in desmosomal plaque. *J. Cell Biol.* **109**, 1511-1518.

Kitajima, Y., Inoue, S. and Yaoita, H. (1989). Effect of pemphigus antibody on the regeneration of cell-cell contact in keratinocyte cultures grown in low to normal Ca^{++} concentration. *J. Invest. Dermatol.* **89**, 167-171

Koch, P. J., Walsh, M. J., Schmelz, M., Goldschmidt, M. D., Zimbelmann, R. and Franke, W. W. (1990). Identification of desmoglein, a constitutive desmosomal glycoprotein, as a member of the cadherin family of cell adhesion molecules. *Eur.J. Cell Biol.* **53**, 1-12.

Konohana, A., Konohana, I., Roberts, G. P. and Marks, R. (1987). Biochemical changes in desmosomes of bovine muzzle epidermis during differentiation. *J. Invest. Dermatol.* **89**, 353-357.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lipman, D. J. and Pearson, W. R. (1985). Rapid and sensitive protein similarity searches. *Science* **227**, 1435-1441.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

McLachlan, A. D. and Stewart, M. (1976). The 14-fold periodicity in α-tropomyosin and interaction with actin. *J. Mol. Biol.* **103**, 271-298.

Mechanic, S., Raynor, K., Hill, J. E. and Cowin, P. (1991). Desmocollins form a distinct subset of the cadherin family of cell adhesion molecule. *Proc. Nat. Acad. Sci. USA* **88**, 4476-4480.

Merlino, G. T., Ishii, S., Whang-Peng, J., Knutsen, T., Xu, Y.-H., Clark, A. J. L., Stratton, R. H., Wilson, R. K., Ma, D. P., Roe, B. A., Hunts, J. H., Shimizu, N. and Pastan, I. (1985). Structure and localization of genes encoding aberrant and normal growth factor receptor RNAs from A431 human carcinoma cells. *Mol. Cell. Biol.* **5**, 1722-1734.

Moll, R., Cowin, P., Kapprell, H.-P. and Franke, W. W. (1986). Biology of disease. Desmosomal proteins: New markers for identification and classification of tumors. *Lab. Invest.* **54**, 4-25.

Mueller, H. and Franke, W. W. (1983). Biochemical and immunological characterization of desmoplakins I and II, the major polypeptides of the desmosomal plaque. *J. Mol. Biol.* **163**, 647-671.

Nilles, L. A., Parry, D. A. D., Powers, E. E., Angst, B. D., Wagner, R. M. and Green, K. J. (1991). Structural analysis and expression of human desmoglein: A cadherin-like component of the desmosome. *J. Cell Sci.* **99**, 809-821.

O'Keefe, E. J., Erickson, H. P. and Bennett, V. (1989). Desmoplakin I and desmoplakin II. Function and characterization. *J. Biol. Chem.* **264**, 8310-8318.

Parker, A. E., Wheeler, G. N., Arnemann, J., Pidsley, S. C., Ataliotis, P., Thomas, C. L., Rees, D. A., Magee, A. I. and Buxton, R. S. (1991). Desmosomal glycoproteins II and III. Cadherin-like junctional molecules generated by alternative splicing. *J. Biol. Chem.* **266**, 10438-10445.

Parrish, E. P., Marston, J. E., Mattey, D. L., Measures, H. R., Venning, R. and Garrod, D. R. (1990). Size heterogeneity, phosphorylation and transmembrane organization of desmosomal glycoproteins 2 and 3 (desmocollins) in MDCK cells. *J. Cell Sci.* **96**, 239-248.

Parrish, E. P., Steart, P. V., Garrod, D. R. and Weller, R. O. (1987). Antidesmsomal mAb in the diagnosis of intracranial tumors. *J. Pathol.* **153**, 265-273.

Parry, D. A. D. (1975). Analysis of the primary structure of α-tropomyosin from rabbit skeletal muscle. *J. Mol. Biol.* **98**, 519-535.

Richardson, J. S. (1981). The anatomy and taxonomy of protein structure. *Advan. Prot. Chem.* **34**, 167-339.

Saito, H., Morizane, T., Watanabe, T., Kagawa, T., Iwabuchi, N., Kumagai, N., Inagaki, Y., Tsuchimoto, K. and Tsuchiya, M. (1989). Establishment of a human cell line (HCC-T) from a patient with hepatoma bearing no evidence of hepatitis B or A virus infection. *Cancer* **64**, 1054-1060.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Nat. Acad. Sci. USA* **74**, 5463-5467.

Schmelz, M., Duden, R., Cowin, P. and Franke, W. W. (1986). A constitutive transmembrane glycoprotein of Mr 165000 (desmoglein) in epidermal and non-epidermal desmosomes. I. Biochemical identification of the polypeptide. *Eur. J. Cell Biol.* **42**, 177-183.

Schwarz, M. A., Owaribe, K., Kartenbeck, J. and Franke, W. W. (1990). Desmosomes and hemidesmosomes: constitutive molecular components. *Annu. Rev. Cell Biol.* **6**, 461-491.

Shtivelman, E., Cohen, F. E. and Bishop, J. M. (1992). A human gene (AHNAK) encoding an unusually large protein with a 1.2-μm polyionic rod structure. *Proc. Nat. Acad. Sci. USA* **89**, 5472-5476.

Skerrow, C. J. and Matoltsy, A. G. (1974). Isolation of epidermal desmosomes. *J. Cell Biol.* **63**, 515-523.

Smith, D. B. and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. *Gene* **67**, 31-40.

Tsukamoto, T. (1989). Establishment and characterization of a cell line (KU-8) from squamous cell carcinoma of the penis. *Keio J. Med.* **38**, 277-293.

Tsukita, S. and Tsukita, S. (1985). Desmocalmin: A calmodulin-binding high molecular weight protein isolated from desmosomes. *J. Cell Biol.* **98**, 1565-1571.

Virata, M. L. A., Wagner, R. M., Parry, D. A. D. and Green, K. J. (1992). Molecular structure of the human desmoplakins I and II amino terminus. *Proc. Nat. Acad. Sci. USA* **89**, 544-548.

Watt, F. M., Mattey, D. L. and Garrod, D. R. (1984). Calcium induced reorganization of desmosomal components in cultured human keratinocytes. *J. Cell Biol.* **99**, 2211-2215.

Wheeler, G. N., Parker, A. E., Thomas, C. L., Ataliotes, P., Poynter, D., Arnemann, J., Rutman, A. J., Pidsley, S. C., Watt, F. M., Rees, D. A., Buxton, R. S. and Magee, A. I. (1990). Desmosomal glycoprotein DGI, a component of intercellular desmosome junctions, is related to the cadherin family of cell adhesion molecules. *Proc. Nat. Acad. Sci. USA* **88**, 4796-4880.

Wiche, G., Becker, B., Luber, K., Weitzer, G., Castanon, M. J., Hauptmann, R., Stratowa, C. and Stewart, M. (1991). Cloning and sequencing of Rat Plectin indicates a 466-kDa polypeptide chain with a three-domain structure based on a central alpha-helical coiled coil. *J. Cell Biol.* **114**, 83-99.

Wiche, G. R., Krepler, R., Artlieb, U., Pytela, R. and Denk, H. (1983). Occurrence and immunolocalization of plectin in tissues. *J. Cell Biol.* **97**, 887-901.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.

Yuspa, S. H., Hawley-Nelson, P., Koehler, B. and Stanley, J. R. (1980). Survey of transformation markers in differentiating epidermal cell lines in culture. *Cancer Res.* **40**, 4694-4703.

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