Journal of Cellular Biochemistry 67:216–222 (1997)

Detection and Cloning of Epidermal Zinc-α_2-Glycoprotein cDNA and Expression in Normal Human Skin and in Tumors

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Abstract Zinc-α_2-glycoprotein (Zn$\alpha_2$gp) is almost ubiquitous in body fluids, and its antibody labels the corresponding secretory epithelia. We have found that Zn$\alpha_2$gp is also expressed in human epidermis. We cloned the Zn$\alpha_2$gp cDNA by screening our cDNA library, derived from epidermal keratinocytes, with a probe for prostate Zn$\alpha_2$gp. It had complete nucleic acid sequence homology with that from prostate, including the signal peptide. Just as Zn$\alpha_2$gp expression is higher in more differentiated breast tumors, so in skin tumors the highest mRNA levels occurred in the normal controls, the lowest in basal cell carcinomas (the least differentiated epidermal tumor type), and intermediate levels in squamous cell carcinomas and Merkel cell carcinomas. A similar increase in Zn$\alpha_2$gp gene expression with differentiation was observed when epidermal keratinocytes were cultured in media that varied in cellular maturation potential. J. Cell. Biochem. 67:216–222, 1997. © 1997 Wiley-Liss, Inc.

Key words: stratified epithelia; carcinomas; cell differentiation; gene expression; keratinocytes

Zinc-α_2-glycoprotein (Zn$\alpha_2$gp) was initially purified from plasma [Bürgi and Schmid, 1961]; it can be precipitated by adding zinc ions and it displays electrophoretic mobility in the α_2-region of the plasma globulins, hence its name. It has been detected in most body fluids, including blood, seminal plasma, breast milk, synovial fluid, saliva, urine, and sweat [Poortmans and Schmid, 1968; Frenette et al., 1987; Ohkubo et al., 1990; Sánchez et al., 1992]. The antibody to this protein labels a wide variety of secretory epithelia in various human glands [Tada et al., 1991]. Northern blot analyses reveal the gene to be expressed in liver, breast, prostate, kidney, pancreas, and several tumors [Schmid and Takahashi, 1964; Freije et al., 1991]. Zn$\alpha_2$gp has been cloned and the complete genomic sequence determined for prostate [Ueyama et al., 1993] and breast [Freije et al., 1993]. The cDNAs differ only in some minor substitutions and are ascribed to a single active gene and one or two pseudogenes. The corresponding amino acid sequences appear to be similar for these two tissues, as also for blood plasma [Araki et al., 1988], consisting of a single polypeptide chain of 278 amino acids. The molecular weight is in the range 38-41 kDa, depending on the tissue. Its fluctuation is associated with variations in glycosylation in different tissues, mostly about 12-18% carbohydrate except that seminal plasma is unglycosylated [Ohkubo et al., 1990]. Direct sequencing of Zn$\alpha_2$gp is blocked at its N-amino acid terminus, pyroglutamic acid [Araki et al., 1988], again except in seminal plasma. Zn$\alpha_2$gp has considerable homology with major histocompatibility complex (MHC) class Iα chains in its amino acid se-

Contract grant sponsor: National Institute for Dental Research; Contract grant number: DE08477.

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Received 14 March 1997; accepted 24 June 1997

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quence and domain structure [Araki et al., 1988]. It lacks the coding information for the transmembrane and cytoplasmic domains present in MHC class I genes; this is consistent with its occurrence as a soluble protein in body fluids [Ohkubo et al., 1990]. The physiological function of Zn$\alpha_2$gp remains uncertain. Zn$\alpha_2$gp has not been associated previously with stratified epithelia. We report here the cloning and cDNA sequencing of Zn$\alpha_2$gp from epidermal keratinocytes and its gene expression in vivo and in vitro.

MATERIALS AND METHODS

Cell Culture

Confluent keratinocyte cultures from foreskin epidermis were cultured in KGM until they reached confluence, then cultured for three additional days in three different media: Keratinocyte Growth Medium (KGM) from Clonetics (San Diego, CA), KGM + 2 mM Ca^{2+}, and KGM + 2 mM Ca^{2+} + 10% fetal calf serum [Brysk et al., 1995a]. Supplementation of the serum containing medium with 1000 U/ml interferon-γ (Genentech, South San Francisco, CA) was also used [Brysk et al., 1995b].

Construction of cDNA Library and Screening

We have prepared a cDNA library from keratinocytes grown for 3 days in KGM medium supplemented with 2 mM Ca^{2+}, 10% fetal calf serum (FCS), and 1,000 U/ml of IFN-γ. The cells were rinsed with Dulbecco's phosphate-buffered saline (DPBS), then lysed in situ with 5 M guanidinium isothiocyanate containing 10 mM EDTA and 0.2 M β-mercaptoethanol. The lysate was layered over a cushion of 2 M CsCl and centrifuged at 31,000 rpm for 20 h. The sedimented RNA was washed with 0.1% sodium dodecyl sulfate (SDS), precipitated, and subjected to affinity chromatography on poly A^+ by the method of Aviv and Leder [1972]. Five µg of poly A^+ was reverse transcribed using AMV reverse transcriptase at 42°C for 1 h. The second-strand cDNA was generated by incubating this mixture with RNase H and DNA polymerase. The ends were then blunted with T4 DNAse polymerase and linked with *Eco*RI and *Xho*I sequences. These were ligated into the phage vector Unizap II (Stratagene, La Jolla, CA), packaged, and grown on a lawn of the host bacteria PLK MRF (Stratagene). The recombinant plaques were transferred onto 150 mM nitrocellulose filters and hybridized with a Zn$\alpha_2$gp cDNA fragment labeled with gamma ^{32}P; the fragment came from polymerase chain reactiom (PCR) amplification using prostate Zn$\alpha_2$gp primers. The clones showing positivity were subjected to secondary screening and amplified.

Gene Sequencing

Sequencing was carried out by the method of Sanger et al. [1977]. The plasmid Bluescript (Stratagene) containing a Zn$\alpha_2$gp cDNA insert was denatured with 0.2 N NaOH at 37°C for 30 min, neutralized with 0.2 M NaAc, and purified by precipitation with ethanol. Two µg of the denatured plasmid was mixed with 0.2 µg of T3 or T7 primers, and heat-denatured for 5 min at 65°C. The resulting mixture was subjected to dideoxy sequencing using ddNTPs and Sequenase enzyme by methods prescribed by the supplier (USB Specialty Biochemicals, Cleveland, OH). The reaction mixture was resolved in a polyacrylamide-urea gel and autoradiographed. The collected sequence information underwent homology analysis, using the software program PC GENE (IntelliGenetics, Campbell, CA).

Northern Blot Analysis

Twenty µg of the total RNA was electrophoresed through a 1.2% formaldehyde-agarose gel, transferred to a nylon filter, and cross-linked by ultraviolet (UV) radiation for 1 min. The filter was then pre-hybridized in a solution of 6 $\times$ SCC (1 $\times$ SCC = 150 mM NaCl and 15 mM sodium citrate, pH 7), 5$\times$ Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% BSA), 0.1% SDS, and 100 ng/ml denatured salmon sperm DNA at 42°C for 4 h. Hybridization to the Zn$\alpha_2$gp cDNA probe, labeled with ^{32}P-deoxy-CTP (Amersham, Arlington Heights, IL), was performed for 16-18 h at 42°C.

The filter was washed in 2 $\times$ SCC and 0.1% SDS at room temperature and at 68°C for 15 min each, then twice in 0.2% $\times$ SCC and 0.1% SDS at 68°C for 15 min, and autoradiographed at −70°C for 3 days using Kodak X-Omat X-ray film (Eastman Kodak, Rochester, NY).

RT-PCR Analysis of mRNA Levels

Total RNAs were isolated from fresh epidermis, tumor biopsies, and cultured cells. The

reverse transcriptase polymerase (RT-PCR) technique was performed, as described previously [Arany and Tyring, 1996]. Briefly, a reverse transcription reaction was run, the resultant cDNA mixture was aliquoted, and PCR reactions were employed with specific primer pairs, including glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as a constitutively expressed internal control. The specificity of PCR was confirmed by gene-specific oligonucleotide hybridization after agarose gel electrophoresis and Southern transfer. The results are presented as densitometer scans of the autoradiograms, measuring mRNA levels relative to G3PDH expression (separately for each). Each bar represents the mean of five runs; the standard deviation is indicated atop the bar. Student's t-test was run; appropriate confidence limits are quoted in the figure legends.

Extraction of Epidermal Zn$\alpha_2$gp

Split thickness fresh human cadaveric skin was obtained from the Skin Bank at this institution. Epidermis was separated from dermis by overnight incubation at 4°C in a buffer of 25 mM Tris–HCl, pH 7.5, containing 1 M NaCl. The epidermis was separated from the dermis with tweezers, then dialyzed against several changes of 25 mM Tris–HCl, pH 7.5, to elute out excess NaCl. It was then homogenized in 25 mM Tris–HCl, pH 7.5, containing 2 mM phenylmethyl sulfonyl fluoride. The homogenate was centrifuged for 30 min at 9,000 rpm, and the soluble extract was concentrated, then further purified by lectin-affinity chromatography on Con A–Sepharose (Vector, Burlingame, CA). The bound proteins were eluted with 1 M methyl-α-D-mannopyranoside in a buffer of 25 mM Tris–HCl containing 0.5 mM $MnCl_2$, 0.5 mM $CaCl_2$, 0.05 M NaCl, and 0.1% NaN_3, dialyzed, and concentrated. The proteins were further fractionated by gel filtration on a G-3000 SW preparative high-performance liquid chromatography (HPLC) column. The protein peak at 30-50 kDa was analyzed for Zn$\alpha_2$gp by Western blotting.

Western Blotting

The Zn$\alpha_2$gp fraction was analyzed by electrophoresis on 10% SDS-polyacrylamide gels (Protean Minigel system, BioRad) and stained with Coomassie blue. A duplicate gel was electrophoretically transferred to nitrocellulose membranes in a buffer of 20% methanol, 25 mM Tris–HCl, and 192 mM glycine. The blots were quenched in TBST (10 mM Tris–HCl, pH 8.8, 150 mM NaCl, 0.05 % Tween 20), containing 3% nonfat dried milk, for 30 min, then washed twice for 10 min in the buffer alone. The blot was then incubated for 1 h with a 1 : 500 dilution of rabbit anti-Zn$\alpha_2$gp IgG (kindly provided by Dr. Iwao Ohkubo) in 0.5% dried milk in TBST, then washed twice for 10 min each with unsupplemented TBST. It was then incubated for 1 h in a 1 : 500 dilution of peroxidase-conjugated sheep antirabbit IgG (Cappel, ICN Biomedicals, Costa Mesa, CA), and the color developed by incubation with 4′-chloro-1-naphthol.

RESULTS AND DISCUSSION

We cloned the Zn$\alpha_2$gp cDNA from a library we had generated from cultured epidermal keratinocytes. The nucleotide sequence of epidermal Zn$\alpha_2$gp (Fig. 1) shows nearly complete homology to the sequence published for Zn$\alpha_2$gp cloned from a prostatic cDNA library [Ueyama et al., 1991]. Minor variations in individual bases (doubly underlined in Fig. 1) occur at positions 310, 1045, and 1124. The cDNA of Zn$\alpha_2$gp from liver is reported to match that from prostate [Ueyama et al., 1991]. The epidermal nucleotide sequence differs appreciably more from the sequence published for Zn_2gp from breast [Freije et al., 1993], though the discrepancy is still minor. The published complete gene sequences have been interpreted as showing that there is a single active gene for Zn$\alpha_2$gp, with indications of a pseudogene in breast [Freije et al., 1993] and two pseudogenes in prostate [Ueyama et al., 1993]; these two independent studies were concurrent and do not cross-reference each other. The N-terminal amino acid encoded by the epidermal nucleotide sequence is glutamine. We measured gene expression by Northern blot analysis of mRNA, isolated from fresh epidermis, to assess the size of the epidermal Zn$\alpha_2$gp cDNA (Fig. 2). At 1.2 kb, it is in agreement with published reports for Zn$\alpha_2$gp from other organs.

The occurrence of Zn$\alpha_2$gp in a stratified epithelium is a new finding. Previous reports have associated Zn$\alpha_2$gp with various glandular epithelia. In particular, Zn$\alpha_2$gp has been detected by means of immunohistochemical staining in the secretory portion of both the eccrine and

Human Skin Zn-alpha2-glycoprotein sequence

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1     GCAAGA  ATG GTG CCT GTC CTG CTG TCT CTG CTG CTG CTT CTG GGT CCT
              Met Val Pro Val Leu Leu Ser Leu Leu Leu Leu Leu Gly Pro

49    GCT GTC CCC CAG GAG AAC CAA GAT GGT CGT TAC TCT CTG ACC TAT ATC
      Ala Val Pro Gln Glu Asn Gln Asp Gly Arg Tyr Ser Leu Thr Tyr Ile     13
                  ↑
97    TAC ACT GGG CTG TCC AAG CAT GTT GAA GAC GTC CCC GCG TTT CAG GCC
      Tyr Thr Gly Leu Ser Lys His Val Glu Asp Val Pro Ala Phe Gln Ala     29

145   CTT GGC TCA CTC AAT GAC CTC CAG TTC TTT AGA TAC AAC AGT AAA GAC
      Leu Gly Ser Leu Asn Asp Leu Gln Phe Phe Arg Tyr Asn Ser Lys Asp     45

193   AGG AAG TCT CAG CCC ATG GGA CTC TGG AGA CAG GTG GAA GGA ATG GAG
      Arg Lys Ser Gln Pro Met Gly Leu Trp Arg Gln Val Glu Gly Met Glu     61

241   GAT TGG AAG CAG GAC AGC CAA CTT CAG AAG GCC AGG GAG GAC ATC TTT
      Asp Trp Lys Gln Asp Ser Gln Leu Gln Lys Ala Arg Glu Asp Ilu Phe     77

289   ATG GAG ACC CTG AAA GAC ATC GTG GAG TAT TAC AAC GAC AGT AAC GGG
      Met Glu Thr Leu Lys Asp Ile Val Glu Tyr Tyr Asn Asp Ser Asn Gly     93

337   TCT CAC GTA TTG CAG GGA AGG TTT GGT TGT GAG ATC GAG AAT AAC AGA
      Ser His Val Leu Gln Gly Arg Phe Gly Cys Glu Ile Glu Asn Asn Arg    109

385   AGC AGC GGA GCA TTC TGG AAA TAT TAC TAT GAT GGA AAG GAC TAC ATT
      Ser Ser Gly Ala Phe Trp Lys Tyr Tyr Tyr Asp Gly Lys Asp Tyr Ile    125

433   GAA TTC AAC AAA GAA ATC CCA GCC TGG GTC CCC TTC GAC CCA GCA GCC
      Glu Phe Asn Lys Glu Ile Pro Ala Trp Val Pro Phe Asp Pro Ala Ala    141

481   CAG ATA ACC AAG CAG AAG TGG GAG GCA GAA CCA GTC TAC GTG CAG CGG
      Gln Ile Thr Lys Gln Lys Trp Glu Ala Glu Pro Val Tyr Val Gln Arg    157

529   GCC AAG GCT TAC CTG GAG GAG GAG TGC CCT GCG ACT CTG CGG AAA TAC
      Ala Lys Ala Tyr Leu Glu Glu Glu Cys Pro Ala Thr Leu Arg Lys Tyr    173

577   CTG AAA TAC AGC AAA AAT ATC CTG GAC CGG CAA GAT CCT CCC TCT GTG
      Leu Lys Tyr Ser Lys Asn Ile Leu Asp Arg Gln Asp Pro Pro Ser Val    189

625   GTG GTC ACC AGC CAC CAG GCC CCA GGA GAA AAG AAG AAA CTG AAG TGC
      Val Val Thr Ser His Gln Ala Pro Gly Glu Lys Lys Lys Leu Lys Cys    205

673   CTG GCC TAC CAC TTC TAC CCA GGG AAA ATT GAT GTG CAC TGG ACT CGG
      Leu Ala Tyr Asp Phe Tyr Pro Gly Lys Ile Asp Val His Trp Thr Arg    221

721   GCC GGC GAG GTG CAG GAG CCT GAG TTA CGG GGA GAT GTT CTT CAC AAT
      Ala Gly Glu Val Gln Glu Pro Glu Leu Arg Gly Asp Val Leu His Asn    237

769   GGA AAT GGC ACT TAC CAG TCC TGG GTG GTG GTG GCA GTG CCC CCG CAG
      Gly Asn Gly Thr Tyr Gln Ser Trp Val Val Val Ala Val Pro Pro Gln    253

817   GAC ACA GCC CCC TAC TCC TGC CAC GTG CAG CAC AGC AGC CTG GCC CAG
      Asp Thr Ala Pro Tyr Ser Cys His Val Gln His Ser Ser Leu Ala Gln    269

865   CCC CTC GTG GTG CCC TGG GAG GCC AGC TAG GAAGCAAGGGTTGGAGGCAATGT
      Pro Leu Val Val Pro Trp Glu Ala Ser end                            278
918   GGGATCTCAGACCCAGTAGCTGCCCTTCCTGCCTGATGTGGGAGCTGAACCACAGAAATCACA
981   GTCAATGGATCCACAAGGCCTGAGGAGCAGTGTGGGGGGACAGACAGGAGGTGGATTTGGAGA
1044  CTGAAGACTGGGATGCCTGTCTTGAGTAGACTTGGACCCAAAAAATCATCTCACCTTGAGCCC
1107  ACCCCCACCCCATTGTTTAATCTGTAGAAGCTAATAAATAATCATCCCTCCTTGCCTAGCAAA
      AAAAAAAAAAA
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Fig. 1. Nucleotide sequence of human epidermal Zn$\alpha_2$gp cDNA. Nucleotides are numbered in the 5′ to 3′ direction. The deduced amino acid sequence is shown below the nucleotide sequence. An arrow indicates the site of cleavage by signal peptidase. Epidermal Zn$\alpha_2$gp differs from the prostate molecule [Ueyama et al., 1991] by the nucleotide substitution, which is doubly underlined.

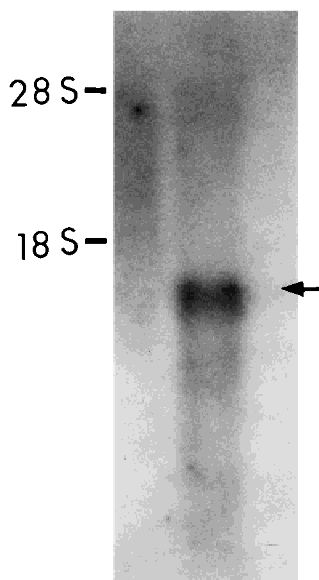

Fig. 2. Expression of Zn$\alpha_2$gp mRNA in human epidermis as analyzed by a Northern blot using a Zn$\alpha_2$gp cDNA probe. The positions of the 28 S and 18 S ribosomal RNA bands are indicated.

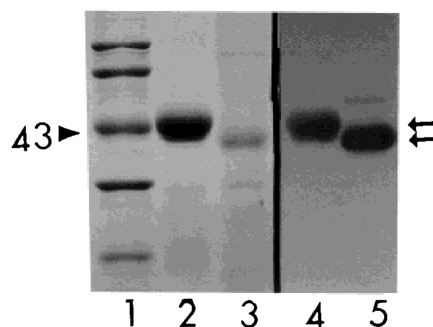

Fig. 3. SDS-PAGE gel **(lanes 1–3)** and a duplicate Western blot **(lanes 4–5)**. **Lane 1**, protein standards (arrow, 43-kDa marker); **lanes 2,4**, Zn$\alpha_2$gp extracted from prostate; **lanes 3,5**, Zn$\alpha_2$gp extracted from epidermis.

apocrine sweat glands and in the sebaceous glands [Mazoujian, 1990; Tada et al., 1991]. As the secretions from these glandular tissues can transiently enter the epidermis, it is important to establish that the epidermis proper is expressing Zn$\alpha_2$gp. Convincing evidence that Zn$\alpha_2$gp is native to the epidermis (as against a contamination by skin gland secretions) is our success in cloning its cDNA from a library derived from cultured epidermal keratinocytes.

For a complementary confirmation that the protein itself is expressed in the epidermis, we isolated a crude Con A-positive fraction in the molecular-weight range of 30–50 kDa and analyzed it both by SDS-PAGE and (after application of a polyclonal antibody to prostatic Zn$\alpha_2$gp) by Western blotting, side by side with Zn$\alpha_2$gp from prostate as control (Fig. 3). The small discrepancy in molecular weights between epidermal Zn$\alpha_2$gp and the prostatic Zn$\alpha_2$gp control can be ascribed to differences in glycosylation. The Zn$\alpha_2$gp proteins from breast fluid, blood plasma, and epidermis are glycosylated, while that from seminal fluid is not. Glycosylation is an established mechanism for protecting proteins from enzymatic degradation. This resistance may be less crucial for the seminal plasma, whose environmental exposure is more restricted and in which Zn$\alpha_2$gp is some six times more abundant than in blood serum [Ohkubo et al., 1990]. Polymorphisms have been observed by starch gel electrophoresis [Schmid and Takahashi, 1964] and isoelectric focusing [Nakayashiki and Katsura, 1989; Nakayashiki et al., 1992]. As the biological function of Zn$\alpha_2$gp is not clear, the implications of the post-translational modifications are the more uncertain.

A significant association has been found between Zn$\alpha_2$gp levels and the histological grade of breast cancer tumors, with higher levels found in well-differentiated tumors than in moderately or poorly differentiated ones, by protein assays [Díez-Itza et al., 1993], as well as at the mRNA level [Freije et al., 1991], and is correlated with clinical prognosis. Analogously, Zn$\alpha_2$gp levels are much higher in benign prostatic hyperplasia than in adenocarcinomas of the prostate, which are characterized by dedifferentiation and a loss of secretory activity [Frenette et al., 1987]. We compared Zn$\alpha_2$gp gene expression in various epidermal tumors, to ascertain whether there was a similar correlation with differentiation; small biopsy samples required use of the more sensitive RT-PCR technique. The expression was indeed highest in the normal nonlesional controls, lowest in basal cell carcinomas (the least differentiated epidermal tumor type, though not the most severe malignancy), and at intermediate levels in squamous cell carcinomas and in Merkel cell carcinomas (Fig. 4). Data sampling and statistical analysis are detailed in the figure legend.

We confirmed directly that Zn$\alpha_2$gp mRNA levels increase with differentiation by comparing them in normal epidermal keratinocytes whose maturation potential was adjusted by varying the composition of the culture media, as we have done previously for other proteins

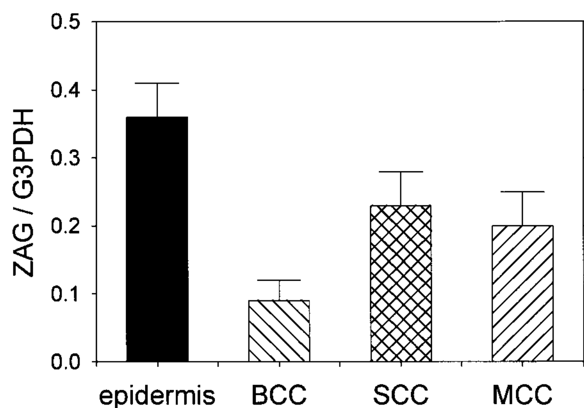

Fig. 4. Gene expression of Zn$\alpha_2$gp in human biopsies of normal epidermis and of epidermal tumors (gene expression indicated as ratio to G3PDH expression). Each histogram bar represents the mean of five runs. The standard deviation is indicated atop each bar. Student's t-test indicates tumors differ from controls with $P = 0.002$ for basal cell carcinoma (BCC), with $P = 0.04$ for squamous cell carcinoma (SCC), and with $P = 0.02$ for Merkel cell carcinoma (MCC); BCC differs from SCC with $P = 0.01$, from MCC with $P = 0.04$; difference between SCC and MCC is not significant ($P = 0.3$).

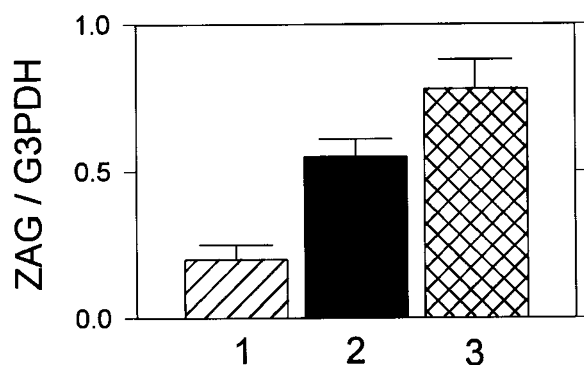

Fig. 5. Upregulation of Zn$\alpha_2$gp gene expression with differentiation in cultured epidermal keratinocytes (display and error bars as in Fig. 3). Student's t-test indicates results with the enriched media differ from pure KGM with $P < 0.001$; they differ from each other with $P = 0.02$.

[Brysk et al., 1995a]. Hennings et al. [1980] showed that keratinocytes cultured in low Ca^{2+} media grow as an undifferentiated monolayer; raising the Ca^{2+} concentration in the same medium triggers differentiation. Supplementation with serum promotes further progress towards terminal differentiation. Consistently, Zn$\alpha_2$gp expression was lowest in cells grown at low Ca^{2+}, intermediate in 2 mM Ca^{2+}, and greatest in the most differentiated cells grown at high Ca^{2+} with serum (Fig. 5).

Clues as to the function of Zn$\alpha_2$gp are fragmentary. Takagaki et al. [1994] specifically associated a Zn$\alpha_2$gp sequence peptide with cell adhesion to extracellular matrices for kidney cell lines but, unaccountably, much less for prostatic and other cells. The amino acid sequence of Zn$\alpha_2$gp has 36–39% homology to MHC class I antigens and somewhat less to class II antigens [Araki et al., 1988]. There is an analogous homology at the nucleotide level (56% identity to HLA-B7) [Freije et al., 1991]. This structural similarity suggests that Zn$\alpha_2$gp may be involved in the immune response, although the expression of its gene is less widespread than that of the MHC class I genes. Zn$\alpha_2$gp lacks the coding information for the transmembrane and cytoplasmic domains present in MHC class I genes; this is consistent with its occurrence as a soluble HLA molecule in body fluids [Poortmans and Schmid, 1968]. In spite of the structural similarity, the expression of Zn$\alpha_2$gp is thought to be under a different control mechanism than that of MHC: the gene for Zn$\alpha_2$gp has been localized to chromosome 7, as against chromosome 6 for MHC [Ueyama et al., 1993; Pendás et al., 1994]. As one function of the skin is as an immune organ, the presence of Zn$\alpha_2$gp in the epidermis may be related to its antigen presentation and immuno-surveillance functions. Indeed, we have shown that Zn$\alpha_2$gp gene expression in epidermal keratinocytes is upregulated by IFN-γ [Brysk et al., 1997], which is not surprising, as IFN-γ induces MHC class I and II expression in epidermal keratinocytes [Basham et al., 1984; Griffiths et. al., 1989]. We have shown that IFN-γ induces terminal differentiation and apoptosis in these cells [Brysk et al., 1995c] and Zn$\alpha_2$gp may play a role in those cellular functions.

ACKNOWLEDGMENTS

We thank Dr. Iwao Ohkubo for kindly providing us his Zn$\alpha_2$gp antibody. This work was supported by grant DE08477 from the National Institute for Dental Research (MMB).

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