Characterization of the antigens recognized by two monoclonal antibodies reactive with basal-layer keratinocytes of human epidermis

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Two monoclonal antibodies, GR3 and GR4, reactive with the basal-layer keratinocytes of human epidermis, were derived by immunization of Balb/c mice with glycoproteins isolated from cultured keratinocytes by lectin-affinity chromatography. Immunoprecipitation of Triton X-100 extracts from human keratinocytes metabolically labelled with D-[1-¹⁴C]glucosamine revealed that GR3 recognized a major glycoprotein with migration properties identical with those of a glycoprotein (reduced form M_r 126 000) which was previously shown to be implicated in intercellular adhesion [Roberts and Brunt (1985) *Biochem. J.* **232**, 67–70]. In their unreduced forms the antigens recognized by GR3 and GR4 both migrated as two bands with M_r values of 118 000 and 147 000. Comparison of ¹²⁵I-labelled glycoproteins

immunoprecipitated by GR3, GR4 and integrin antibodies revealed that, under reducing conditions, the major band immunoprecipitated by both GR3 and GR4 co-migrated with the α_3 and β_1 integrin chains. In addition the immunoprecipitate obtained with GR4 contained an additional band co-migrating with the α_2 integrin chain. Sequential immunoprecipitation studies with GR3, GR4 and integrin antibodies confirmed that GR3 is directed against the α_3 integrin chain, whereas GR4 is directed against the β_1 chain. These studies also indicate that some of the α_3 integrin chains on keratinocytes may be associated with a β -chain not recognized by the antisera against the β_1 integrin chain used in this study.

INTRODUCTION

The protective function of the epidermis is dependent on an ordered sequence of events involving mitosis of cells in the basal layer and terminal differentiation of the resulting daughter cells as they leave the basal layer to form in turn the spinous, granular and cornified layers. The production of new keratinocytes in the basal layer is balanced by shedding of terminally differentiated cells from the cornified layer. Changes in the expression of keratins and other proteins such as involucrin have been widely used as markers of differentiation. Positional changes in the differentiating keratinocytes are also accompanied by changes in membrane glycoproteins which have been detected using fluorescein-conjugated lectins [1]. These changes in membrane glycoproteins may have a role in modulating the adhesive interactions of keratinocytes within the different layers and thus impart positional control.

Several families of adhesion receptors have now been identified in various tissues. These include integrins, adhesion molecules of the immunoglobulin superfamily, cadherins, and selectins [2]. A glycoprotein of M_r 126 000 was previously shown to have a function in keratinocyte intercellular adhesion [3], but it was uncertain to which class of adhesion receptor this molecule belonged. Monoclonal-antibody technology has featured prominently in the identification, characterization and classification of adhesion molecules. An understanding of the role of membrane glycoproteins in modulating epidermal architecture would be aided by the availability of monoclonal antibodies which interact specifically with different regions of the epidermis. In the present study the production and characterization of two monoclonal antibodies which react selectively with the basal region of the epidermis are described. It is shown that these two antibodies, which immunoprecipitate a major glycoprotein with properties similar to the previously described intercellular adhesion glycoprotein of M_r 126 000, are directed against the α_3 and β_1

integrin chains. There are now many monoclonal antibodies to the integrins available commercially which can be used to probe their function, but the approach adopted in the present study differed in that the antibodies were raised with the aim of identifying intercellular adhesion glycoproteins active in specific regions of the human epidermis.

MATERIALS AND METHODS

Antibodies

The murine monoclonal antibodies J143 and AJ2 directed against the α_3 and β_1 integrin chains respectively were generously provided by Dr. L. Old (Sloan-Kettering Institute, New York, NY, U.S.A.) [4]. A murine monoclonal antibody 12F1 directed against the α_2 integrin chain was kindly donated by Dr. V. Woods (University of California, San Diego, CA, U.S.A.) [5]. Rabbit anti-mouse IgG, fluorescein-conjugated rabbit anti-mouse IgG and peroxidase-conjugated rabbit anti-mouse IgG were purchased from Sigma Chemical Co Ltd., Poole, Dorset, U.K. Biotin-conjugated sheep anti-mouse IgG and biotinylated peroxidase–streptavidin complex were obtained from Amersham International, Amersham, Bucks., U.K.

Cell culture and metabolic labelling

Human keratinocytes were cultured from newborn-child foreskins as previously described [6]. Cells at the first or second passage were cultured in 50 mm plastic dishes (Flow Laboratories, Irvine, Ayrshire, Scotland) and labelled with D-[1-¹⁴C]glucosamine (4 μ Ci/ml) at 37 °C for 24 h.

Extraction of keratinocytes

Keratinocytes were extracted twice with 0.5 ml of 0.5% Triton X-100 in 50 mM Tris/HCl (pH 7.5)/0.14 M NaCl/1.5 mM

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MgCl₂/2 mM phenylmethanesulphonyl fluoride at 23 °C for 15 min.

Lectin-affinity chromatography

Keratinocyte extracts were adjusted to 5 mM CaCl₂/5 mM MnCl₂ and applied to the first of three columns of 1 ml of concanavalin A-agarose (Sigma Chemical Co.) and one column of 1 ml of wheat-germ-agglutinin-agarose (Sigma Chemical Co. Ltd) arranged in tandem. Unbound material was removed by washing with 0.5 % Triton X-100 in 50 mM Tris/HCl (pH 7.5)/0.14 M NaCl/1.5 mM MgCl₂/5 mM CaCl₂/5 mM MnCl₂/2 mM-phenylmethanesulphonyl fluoride. The columns were then separated and the glycoproteins bound by the first concanavalin A-agarose column and the wheat-germ-agglutinin-agarose column were eluted with 2 % α -methyl-D-mannoside and 2 % *N*-acetyl-D-glucosamine respectively in the above buffer. The two additional concanavalin A-agarose columns were included to ensure that no concanavalin A-reactive glycoproteins passed through to the wheat-germ agglutinin-agarose. The column eluates were dialysed against water then freeze-dried and extracted twice with acetone (2 ml) to remove the Triton X-100.

Immunization of mice and hybridoma production

Balb/c mice (3 months old) were immunized subcutaneously with glycoproteins emulsified in Freund's complete adjuvant. Glycoproteins isolated from approx. 2×10^6 keratinocytes were used for each immunization. The primary immunization was followed by three further subcutaneous immunizations with glycoproteins emulsified in Freund's incomplete adjuvant at intervals of 1, 3 and 2 months. A test bleed was carried out 10 days after the third immunization. The mice were boosted 3 days before they were killed by intraperitoneal injection with glycoproteins in the absence of adjuvant.

Spleen cells were fused with non-secreting mouse myeloma SP2/0-Ag14 cells using poly(ethylene glycol) 1500 in physiological saline as described by Liddell and Cryer [7]. The reaction was stopped by slow step-wise addition of RPMI medium. Cells were spun down at 1000 *g* for 10 min, resuspended in hypoxanthine/aminopterin/thymidine (HAT) medium (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.) containing 20 % foetal-calf serum and distributed into three 96-well microtitre plates (NUNC, Gibco BRL). After incubation at 37 °C in 5 % CO₂ for 12 days the HAT medium was replaced by RPMI containing 20 % foetal-calf serum. Supernatants from growing hybridomas were screened for antibodies binding to epidermal glycoproteins by an e.l.i.s.a. and by indirect immunofluorescence on frozen sections of normal skin. Hybridomas selected for further study were expanded to 1 ml cultures and cloned twice by limiting dilution using mouse thymocytes as feeder cells [7]. Colonies producing antibodies of the desired specificities were expanded in RPMI containing 10 % foetal-calf serum and ascitic fluid was produced by intraperitoneal injection of $(2-4) \times 10^6$ hybridoma cells into Balb/c mice preconditioned 7 days earlier with an intraperitoneal injection of 0.5 ml of 2,6,10,14-tetramethylpentadecane (Pristane; Sigma).

The immunoglobulin subclass of the monoclonal antibodies was determined by Ouchterlony diffusion of culture supernatants using a commercial kit (The Binding Site, Birmingham, U.K.).

E.l.i.s.a.

Keratinocyte glycoproteins ($\sim 10 \mu\text{g/ml}$) in PBS were adsorbed to 96-well microtitre plates (Greiner Labortechnik Ltd., Dursley,

Glos., U.K.) overnight at 4 °C. Sites that had not reacted were blocked with 150 μl of 1 % BSA in PBS for 2 h at 23 °C. Hybridoma supernatants (100 μl) or dilutions of ascitic fluid were added to the wells and incubated for 2 h at 23 °C. The wells were then washed three times with 200 μl of 0.05 % Tween 20 in PBS and incubated for 2 h at 23 °C with 100 μl of peroxidase-conjugated rabbit anti-mouse IgG at a dilution of 1 in 625 in 0.05 % Tween 20/0.04 % BSA in PBS. After washing the wells four times with 200 μl of 0.05 % Tween 20 in PBS the bound peroxidase was detected by addition of 100 μl of *o*-phenylenediamine (1 mg/ml) in 0.1 M citrate/phosphate buffer, pH 5.0, containing 0.03 % H₂O₂. The reaction was stopped after 5 min by the addition of 50 μl of 2.5 M H₂SO₄, and the absorption was read at 492 nm on a microtitre plate reader.

Monoclonal antibodies were examined for direct competition in e.l.i.s.a.'s using the procedure described by Friguet et al. [8].

Immunostaining

Biopsies of normal human skin were embedded in OCT (R. A. Lamb, Acton, London, U.K.), snap-frozen in *n*-hexane cooled in solid CO₂ and stored in liquid N₂. Cryostat sections (7 μm thick) were cut, mounted on slides and air-dried. For immunofluorescence the sections were washed in PBS for 10 min, then incubated with hybridoma supernatants (150 μl) or ascitic fluid diluted in RPMI containing 10 % foetal-calf serum for 30 min at 37 °C in a humidified chamber. The slides were then washed for 30 min in PBS and incubated for 30 min at 37 °C with 150 μl of fluorescein-conjugated rabbit anti-mouse IgG (1-in-50 dilution in PBS). The slides were again washed for 30 min in PBS, air-dried, mounted in Hydromount (National Diagnostics, Aylesbury, Bucks., U.K.) and examined with a Nikon fluorescence microscope. For immunoperoxidase staining a modification of the avidin-biotin-peroxidase method of Hsu et al. [9], with cobalt and nickel enhancement [10], was used. Cryostat sections (7 μm) on poly-L-lysine (Sigma)-coated slides were fixed in 4 % (v/v) formaldehyde/5 % (w/v) sucrose in PBS containing 1 mM Mg²⁺ and 1 mM Ca²⁺ for 30 min at 23 °C, permeabilized in 0.5 % Triton X-100/0.1 M glycine in PBS containing 1 mM Mg²⁺ and 1 mM Ca²⁺ for 5 min and non-specific binding blocked by incubation with 10 % sheep serum in PBS at 37 °C for 30 min. Incubation with monoclonal antibodies appropriately diluted in 10 % sheep serum in PBS was carried out at 37 °C for 1 h, and the slides were washed in PBS for 15 min. The slides were then incubated with biotinylated sheep anti-mouse Ig (diluted 1 in 100 in PBS) for 1 h at 23 °C, washed in PBS for 15 min and incubated with biotinylated-peroxidase-streptavidin complex (diluted 1 in 125 in PBS) 30 min at 23 °C. After washing the slides in PBS for 20 min, immobilized peroxidase was revealed by incubation with 0.5 mg/ml 3,3'-diaminobenzidine hydrochloride (Sigma) in PBS/0.01 % H₂O₂/0.003 % cobalt chloride/0.003 % nickel chloride for 5 min. The sections were washed in water, dehydrated and mounted in Styrolite mounting medium (BDH Laboratory Supplies, Poole, Dorset, U.K.).

Radioiodination

Polypropylene 1.5 ml Eppendorf tubes were coated with 25 μg of 1,3,4,6-Tetrachloro-3 α ,6 α -diphenylglycouril (Sigma) [11] by evaporation of 50 μl of a 0.5 mg/ml solution in methylene chloride and then rinsed with 1 ml 0.25 M phosphate buffer, pH 7.4. Radioiodination was carried out by addition of 5 μl of Na¹²⁵I (500 μCi) (Amersham International) to keratinocyte glycoproteins in 120 μl of 0.1 M phosphate buffer, pH 7.5, in the coated Eppendorf tubes and mixing at 23 °C for 10 min. The

mixture was desalted on a Sephadex G-25 column (10 ml) equilibrated with 0.5% Triton X-100/50 mM Tris/HCl (pH 7.5)/0.14 M NaCl/1.5 mM $MgCl_2$ /5 mM $CaCl_2$ /5 mM $MnCl_2$ /2 mM phenylmethanesulphonyl fluoride/0.2% BSA (Buffer A) and then fractionated on a concanavalin A-agarose column (2 cm $\times$ 0.6 cm) (Sigma). The radioiodinated glycoproteins retained by this column were eluted with 2% α -methyl D-mannoside in elution buffer A and used for immunoprecipitation.

Rabbit anti-mouse IgG was iodinated in a similar manner to the keratinocyte glycoproteins, but with the omission of the final concanavalin A-agarose fractionation step.

Immunoprecipitation

Immunoabsorbents were prepared by mixing 60 mg of protein A-Sepharose CL-4B (Sigma) with 200 μ l of rabbit anti-mouse IgG in 1 ml of buffer A at 4°C for 6 h. The mixture was centrifuged, the supernatant discarded, and the pellet washed twice with 1 ml of buffer A. The final pellet was resuspended in 1 ml of buffer A and mixed with ascitic fluid containing mouse monoclonal antibodies at a ratio of 100 μ l of protein A-Sepharose CL-4B suspension to 20 μ l of ascitic fluid for 16 h at 4°C. The immunoabsorbents were isolated by centrifugation and washed once with 1 ml of buffer A. Radiolabelled keratinocyte glycoproteins in buffer A were preabsorbed for 16 h at 4°C with protein A-Sepharose CL-4B which had been primed with rabbit anti-mouse IgG. The suspension was centrifuged (16000 g, 5 min), and aliquots of the supernatant were mixed with the immunoabsorbents for 4 h at 4°C. After centrifugation the supernatants were treated with more immunoabsorbents in sequential immunoprecipitation studies. The immunoabsorbent pellets were washed three times with 1 ml of buffer A and once with 1 ml of 50 mM Tris/HCl, pH 7.5, containing 2 mM phenylmethanesulphonyl fluoride. The bound antigens were released from the immunoabsorbents by heating in electrophoresis sample buffer (2% SDS in 0.05 M Tris/HCl, pH 6.8, containing 10% glycerol, 0.001% Bromophenol Blue and 2 mM phenylmethanesulphonyl fluoride) at 100°C for 5 min. Samples examined under reducing conditions contained 4% 2-mercaptoethanol in the electrophoresis sample buffer.

PAGE

Samples were analysed by SDS/PAGE in a discontinuous buffer system [12] with gel slabs containing a linear gradient of 5–15% polyacrylamide in the separating gel and a 5% stacking gel. Proteins were detected with Coomassie Blue R-250, and radio-labelled glycoproteins were detected by autoradiography or fluorography as described previously [13]. Osray M3 X-ray film (Agfa-Gevaert, Dunstable, Beds., U.K.) was used for autoradiography of D-[1- ^{14}C]glucosamine-labelled glycoproteins and pre-flashed Fuji RX X-ray film with a Hi-Speed-X intensifying screen [Fuji Photo Film (U.K.) Ltd., London, U.K.] at -70°C was used to detect ^{125}I -labelled glycoproteins.

Immunoblotting

Keratinocyte extracts were subjected to SDS/PAGE and transferred to nitrocellulose (Hybond-C, Amersham International) as described by Towbin et al. [14]. The nitrocellulose membrane was washed with 0.05% Tween 20 in 10 mM Tris/HCl (pH 7.5)/0.14 M NaCl and blocked with 1% casein in 10 mM Tris/HCl (pH 7.5)/0.14 M NaCl for 5 h at 20°C. Incubation of the nitrocellulose blot with monoclonal antibodies was carried out with 1-in-50 dilutions of ascitic fluid in 1% casein/10 mM Tris/HCl (pH 7.5)/0.14 M NaCl for 16 h at 20°C. After ex-

tensive washing with 10 mM Tris/HCl (pH 7.5)/0.14 M NaCl, bound antibodies were detected by incubation with radioiodinated rabbit anti-mouse Ig in 1% casein/10 mM Tris/HCl (pH 7.5)/0.14 M NaCl for 4 h at 23°C. Finally the nitrocellulose blot was washed extensively, dried, and subjected to autoradiography.

RESULTS

Isolation of keratinocyte glycoproteins and immunization of mice

Human keratinocytes were extracted with 0.5% Triton X-100 and fractionated on coupled columns of concanavalin A-agarose and wheat-germ-agglutinin-agarose. The glycoproteins retained by these two columns were released with their specific sugars and each used to immunize five Balb/c mice, and the resulting immune sera were tested for their ability to immunoprecipitate glycoproteins extracted from D-[1- ^{14}C]glucosamine-labelled keratinocytes. Autoradiographs of these immunoprecipitates after separation by SDS/PAGE are shown in Figure 1.

Several of the glycoproteins retained by concanavalin A-agarose elicited a strong antibody response in all five mice immunized. One of these corresponded with a glycoprotein of M_r 126000 previously shown to be involved in intercellular adhesion [3]. The immune response of mice immunized with glycoproteins retained by wheat-germ-agglutinin-agarose was more variable, but two of the mice did mount a strong antibody response to a diffuse glycoprotein band of M_r > 200000. A previous study [6] has identified differentiation-related sialoglycoproteins which migrate in this region.

Hybridoma production

Spleen cells from two mice (immunoprecipitates of sera shown in lanes 2 and 6 of Figure 1) were fused with the non-immunoglobulin-secreting cell line SP2-0Ag14 in two separate experiments. The hybridoma supernatants were screened initially by an e.l.i.s.a. using microtitre plates coated with keratinocyte glycoproteins retained by concanavalin A-agarose. Some of the

Figure 1 Characterization of antisera against keratinocyte glycoproteins isolated by lectin-affinity chromatography

Glycoproteins from detergent extracts of D-[1- ^{14}C]glucosamine-labelled keratinocytes were immunoprecipitated by sera from mice immunized with concanavalin A-binding glycoproteins (lanes 2–6) or wheat-germ-agglutinin-binding glycoproteins (lanes 7–11). Lane 1 contains an aliquot of the original detergent extract. The immunoprecipitates were analysed by SDS/PAGE on a 5–15% polyacrylamide-gradient gel under reducing conditions followed by autoradiography. The positions and M_r values ($\times 10^{-3}$) of protein standards run in parallel are shown on the right.

Figure 2 Immunostaining of human skin with monoclonal antibodies GR3 and GR4

Frozen sections of adult human skin were stained with monoclonal antibodies (a) GR3 and (b) GR4 using the immunoperoxidase procedure as described in the Materials and methods section. The scale bars represent 20 μm .

monoclonal antibodies produced were directed against concanavalin A leached off the affinity columns during preparation of the antigen used for immunization. Consequently the hybridoma supernatants which were positive by e.l.i.s.a. were also screened by indirect immunofluorescence on frozen sections of normal skin. Selected hybridomas were cloned twice by limiting dilution, expanded and ascites produced in pristane-primed mice. Strong pericellular immunostaining of the basal cells of the epidermis was observed with monoclonal antibodies GR3 (Figure 2) and GR4, and these were chosen for further study. Ouchterlony subtyping revealed that GR3 is of the IgG2a subtype and GR4 is of the IgG1 subtype.

Characterization of the antigens recognized by GR3 and GR4

Immunoprecipitation of D-[1- ^{14}C]glucosamine-labelled keratinocyte extracts followed by SDS/PAGE revealed that GR3 recognizes a glycoprotein which, in its reduced form, migrates as a major band of M_r 126000 and a minor band of M_r 35000 (Figure 3). SDS/PAGE of immunoprecipitates under non-reducing conditions revealed that the antigens recognized by GR3 and GR4 both migrate as two bands with M_r values of 118000 and 147000 (see Figures 5 and 6 below).

Studies of direct competition between the two antibodies GR3 and GR4 using an e.l.i.s.a. showed that the signal produced was

Figure 3 D-[1- ^{14}C]Glucosamine-labelled glycoproteins immunoprecipitated by monoclonal antibody GR3

Glycoproteins from detergent extracts of D-[1- ^{14}C]glucosamine-labelled keratinocytes were immunoprecipitated by serum from a mouse immunized with concanavalin A-binding glycoproteins (lane 1) an unrelated monoclonal antibody (lane 2) and monoclonal antibody GR3 (lane 3). The immunoprecipitates were analysed by SDS/PAGE on 5–15% gradient gels under reducing conditions, followed by autoradiography. The position and M_r values ($\times 10^{-3}$) of protein standards run in parallel are shown on the right. The arrowhead marks the position of the origin, and the arrows show the positions of the glycoprotein bands of M_r 126000 and 35000.

Figure 4 Comparison of glycoproteins immunoprecipitated by GR3, GR4 and integrin antibodies

Immunoprecipitation of iodinated glycoproteins from keratinocytes with monoclonal antibodies GR3, GR4, AJ2 [Anti- β_1 integrin chain], 12F1 [Anti- α_2 integrin chain] and J143 [Anti- α_3 integrin chain]. The immunoprecipitates were analysed by SDS/PAGE on 5–15% gradient gels under reducing conditions, followed by fluorography. The positions and M_r values ($\times 10^{-3}$) of protein standards run in parallel are shown on the right, and the positions of the integrin chains are shown by the arrows.

additive, thus demonstrating that the two antibodies recognized different epitopes. Immunoblotting studies were carried out to determine which of the chains represented by the two bands of M_r 118000 and 147000 carried the antigenic determinants. Monoclonal antibody GR4 reacted with the chain of M_r 118000, but monoclonal antibody GR3 failed to react with either chain on immunoblots.

The characteristics of the antigens recognized by GR3 and GR4 suggested that they were integrins. In order to examine this possibility, keratinocyte glycoproteins isolated by concanavalin A-agarose affinity chromatography were iodinated with ^{125}I and immunoprecipitated with GR3, GR4 and monoclonal antibodies directed against integrin chains. Comparison of the immuno-

Figure 5 Integrins immunoprecipitated after preclearing with GR3 or anti- α_3 integrin

Iodinated glycoproteins from keratinocytes were sequentially immunoprecipitated by (a) two treatments with monoclonal antibody GR3, followed by GR4, J143 [anti- α_3 integrin chain] and AJ2 [anti- β_1 integrin chain]; (b) two treatments with monoclonal antibody J143, followed by GR4 and then GR3. The immunoprecipitates were analysed by SDS/PAGE on 5–15% gradient gels under non-reducing conditions, followed by fluorography.

Figure 6 Integrins immunoprecipitated after preclearing with GR4 or anti- β_1 integrin

Iodinated glycoproteins from keratinocytes were sequentially immunoprecipitated by (a) two treatments with monoclonal antibody GR4, followed by GR3; (b) two treatments with monoclonal antibody AJ2 [anti- β_1 integrin chain], followed by GR4 and then GR3. The immunoprecipitates were analysed by SDS/PAGE on 5–15% gradient gels under non-reducing conditions, followed by fluorography.

precipitates by electrophoresis under reducing conditions revealed that the antigen recognized by GR3 migrated as a band with the same mobility as the α_3 and β_1 integrin chains (Figure 4). Immunoprecipitates obtained with GR4 contained an additional band migrating with the same mobility as the α_2 integrin chain (Figure 4), and in this respect it was comparable with the immunoprecipitate obtained with a monoclonal antibody directed against the integrin β_1 chain.

Sequential immunoprecipitation studies on ^{125}I -labelled keratinocyte glycoproteins followed by SDS/PAGE under non-reducing conditions showed that anti-(integrin chain α_3) (monoclonal antibody J143) and GR3 removed most of the β_1 integrin chain, with the exception of a small amount of antigen (Figure 5) which, when reduced and re-examined by electrophoresis, was found to have the characteristics of $\alpha_3\beta_1$ integrin.

Anti-(integrin chain β_1) (monoclonal antibody AJ2) removed all the GR4-reactive antigen, but not all the GR3-reactive antigen (Figure 6b). Similarly, GR4 did not remove all the GR3-reactive antigen (Figure 6a). Reduction of the material remaining after sequential immunoprecipitation with anti-(integrin chains β_1 and

GR4) followed by electrophoresis revealed a single band corresponding to the α_3 and β_1 chains, but no band corresponding to the α_2 chain. These results demonstrate that monoclonal antibodies GR3 and GR4 are directed against the α_3 and β_1 integrin chains respectively. In addition, some of the α_3 integrin chains isolated from keratinocytes appear to be associated with a β -chain variant not recognized by antisera directed against the β_1 integrin chain.

Immunoprecipitates obtained with antibodies directed against the α_3 - and β_1 -chains contain an additional faint band of $M_r > 200\,000$ (Figures 5 and 6). This may be a disulphide-linked form of the $\alpha_3\beta_1$ heterodimer, as it disappears on reduction of the immunoprecipitates without the appearance of bands other than the α_3 - and β_1 -chains (Figure 4).

DISCUSSION

Culture of human keratinocytes under conditions which promote differentiation results in the synthesis of a complex array of cell-surface glycoproteins [6]. In order to facilitate an immunological approach to the analysis of these glycoproteins and to elucidate their role in controlling epidermal structure, the glycoproteins have been fractionated on coupled columns of concanavalin A and wheat-germ agglutinin linked to agarose. The glycoproteins retained by these columns were then used to immunize mice, and the resulting immune sera examined for their ability to immunoprecipitate radiolabelled keratinocyte extracts. The majority of keratinocyte glycoproteins were retained by concanavalin A-agarose and elicited a strong immune response in all mice immunized (Figure 1). Included in these was a cell-surface glycoprotein of $M_r\ 126\,000$ in its reduced form which has previously been shown to play a role in intercellular adhesion [3]. Glycoproteins retained by wheat-germ agglutinin-agarose, but not by concanavalin A-agarose, included a prominent glycoprotein which migrated as a broad band of $M_r > 200\,000$. This glycoprotein, against which only two of five mice mounted a strong immune response, may be related to the differentiation-induced sialoglycoproteins reported previously [6].

One of the main objectives of this investigation was to produce monoclonal antibodies which would aid in the characterization of glycoproteins involved in intercellular adhesion in different regions of the epidermis. On the basis of the retention of the cell-surface glycoprotein of $M_r\ 126\,000$ by concanavalin A-agarose, mice immunized with this fraction were chosen for hybridoma production. Two monoclonal antibodies, GR3 and GR4, were isolated, which reacted specifically with the basal layer of the epidermis. GR3 immunoprecipitated a glycoprotein which, in its reduced form, migrated as a major band of $M_r\ 126\,000$ and a minor band of $M_r\ 35\,000$. Sequential immunoprecipitation analysis carried out to establish the identity of the antigens recognized by GR3 and GR4 showed that these monoclonal antibodies reacted with the α_3 and β_1 integrin chains respectively.

These studies also revealed that some of the α_3 integrin chains on keratinocytes were associated with a β -chain not recognized by antisera directed against the β_1 integrin chain. Further work will be required to establish whether this is a new β -chain or a conformational variant of the β_1 -chain.

Whilst this study was in progress, several reports appeared describing the expression of integrin chains on human keratinocytes [15–21] and their aberrant expression in hyperproliferative disorders [22,23]. The three most prominent integrin heterodimers expressed are $\alpha_6\beta_4$, which is localized to the basal aspect of basal keratinocytes, and $\alpha_2\beta_1$ and $\alpha_3\beta_1$, which have been variously reported to be exposed on the entire periphery of basal cells or preferentially concentrated on the lateral and apical regions of

these cells. Some investigations have also reported the expression of small amounts of $\alpha_1\beta_1$ [20,25] $\alpha_v\beta_5$ [20,25,26] and the main fibronectin receptor, $\alpha_5\beta_1$ [18,20,27], which is up-regulated during keratinocyte activation and migration [28].

The integrin $\alpha_3\beta_1$ has been reported to function as a low-affinity receptor for collagen, fibronectin and laminin in other cell types [29] and it has been shown that monoclonal antibodies to this integrin inhibit the binding of keratinocytes to laminin [20,30] and to fibronectin [18,30]. Recently an epithelial basement-membrane glycoprotein named epiligrin [31] or kalinin [32] has been reported to be a ligand for $\alpha_3\beta_1$ integrin.

Carter et al. [33] have shown that epiligrin is the ligand of an adhesion supercomplex resembling hemidesmosomes and composed of $\alpha_3\beta_1$ focal adhesions and $\alpha_6\beta_4$ stable anchoring contacts. Blocking the $\alpha_3\beta_1$ receptor on keratinocytes was shown to enhance their migration on fibronectin and collagen matrices [34].

The concentration of $\alpha_3\beta_1$ and $\alpha_2\beta_1$ integrins on the lateral and apical surfaces of basal keratinocytes suggested that these integrins may have a role in cell-cell adhesion as well as cell-substrate adhesion [16]. In functional assays, cell-cell adhesion of keratinocytes was shown to be inhibited by monoclonal antibodies to the β_1 integrin chain [27,30] and α_3 integrin chain [30]. Recent studies by Symington et al. [35] suggest that the $\alpha_3\beta_1$ and $\alpha_2\beta_1$ integrins may interact to mediate intercellular adhesion. The present study, in conjunction with a previous study on the identification of an intercellular adhesion glycoprotein of M_r 126000 [3], provides additional evidence for the importance of the $\alpha_3\beta_1$ integrin in intercellular adhesion of keratinocytes.

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