Identification of an epidermal cell-adhesion glycoprotein

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Glycoproteins which mediate intercellular adhesion were studied by comparing the effects of trypsin and the neutral proteinase, Dispase, on human keratinocytes metabolically labelled with D-[1-¹⁴C]glucosamine or L-[1-³H]fucose. Whereas digestion of keratinocytes with trypsin/EDTA resulted in loss of both cell–substratum and intercellular adhesion, only cell–substratum adhesion was disrupted by incubation with Dispase. Analysis of the radiolabelled glycoproteins by polyacrylamide-gel electrophoresis revealed that a glycoprotein of M_r 126000 was cleaved by trypsin/EDTA, but not by Dispase. Surface labelling of keratinocytes with galactose oxidase/ NaB^3H_4 confirmed that this glycoprotein was exposed on the cell surface. Addition of 1 mM-Ca^{2+} prevented dispersion of keratinocytes by trypsin and concomitantly protected the glycoprotein of M_r 126000 from digestion. These results indicate that this glycoprotein has an important role in mediating intercellular adhesion of keratinocytes.

INTRODUCTION

An elucidation of the molecular basis of cellular adhesion would aid our understanding of a number of important biological processes, including morphogenesis, wound healing and the invasive and metastatic behaviour of tumour cells. The epidermis is an interesting tissue in which to study adhesion phenomena, because the differentiation of epidermal keratinocytes is accompanied by a regular sequence of changes in the nature of their adhesive interactions which are essential for the normal functioning of the epidermis (Skerrow, 1978). Rheinwald & Green (1975) have shown that human epidermal keratinocytes cultured in the presence of irradiated 3T3 feeder cells grow as stratified colonies that retain many of the properties of the intact epidermis. Mitosis is restricted to the basal layer, and cells that leave it undergo terminal differentiation. Keratinocytes are normally subcultured by incubation with trypsin and EDTA, which results in loss of both cell–substratum and cell–cell adhesion to yield a single-cell suspension (Rheinwald & Green, 1975). In contrast, keratinocytes incubated with Dispase, a neutral proteinase isolated from *Bacillus polymyxa* (Irie, 1976), are released as a sheet of cells by cleavage of cell–substratum adhesive molecules but not the intercellular adhesive molecules (Kitano & Okada, 1983). This property of Dispase has been utilized to produce sheets of epidermal cells for skin grafting (Green *et al.*, 1979). In the present investigation we have compared the effects of trypsin/EDTA and Dispase on the glycoproteins of human epidermal keratinocytes in order to identify the glycoproteins mediating intercellular adhesion. We show that a cell-surface glycoprotein with M_r 126000 appears to play a prominent role in cell–cell adhesion of epidermal keratinocytes.

EXPERIMENTAL

Materials

D-[1-¹⁴C]Glucosamine hydrochloride (61 Ci/mol), L-[1-³H]fucose (6.23 Ci/mmol), [¹⁴C]methylated protein M_r

markers and NaB^3H_4 (10 Ci/mmol) were supplied by Amersham International (Amersham, Bucks., U.K.). Dispase I was purchased from Boehringer Corp. (London) Ltd. (Lewes, East Sussex, U.K.). Trypsin (1:250) was obtained from Difco (West Molesey, Surrey, U.K.). Dulbecco's modified Eagle's medium, Eagle's minimum essential medium and foetal-calf serum were obtained from Flow Laboratories (Irvine, Scotland, U.K.). Epidermal growth factor, galactose oxidase, cortisol, insulin, *N*-ethylmaleimide, mitomycin C, phenylmethanesulphonyl fluoride, transferrin and tri-iodothyronine were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Cholera toxin was obtained from Becton Dickinson (Wembley, Middx., U.K.).

Cell cultures

Human keratinocytes isolated from newborn foreskin were grown in the presence of 3T3 cells pretreated with mitomycin C (4 $\mu\text{g/ml}$) for 2 h (Rheinwald, 1980). The culture medium used in most of this study was Dulbecco's modified Eagle's medium supplemented with 5% (v/v) foetal-calf serum, cortisol (0.4 $\mu\text{g/ml}$), 0.1 nm-cholera toxin, transferrin (5 $\mu\text{g/ml}$), insulin (5 $\mu\text{g/ml}$), epidermal growth factor (10 ng/ml) and 20 pM-tri-iodothyronine (Watt & Green, 1982). In some experiments, which are indicated in the text, keratinocytes were cultured in Eagle's minimum essential medium under the conditions described by Dykes *et al.* (1982). Cultures were grown in 35 mm-diameter culture dishes at 37 °C in CO_2 /air (1:19). First- or second-passage cultures were grown until they were 80–100% confluent, and then the 3T3 cells were removed with EDTA (Rheinwald, 1980) before radiolabelling of the keratinocytes.

Radiolabelling of keratinocytes

Metabolic labelling with D-[1-¹⁴C]glucosamine (4 $\mu\text{Ci/ml}$) or L-[1-³H]fucose (25 $\mu\text{Ci/ml}$) was carried out by addition of the radioisotope to fresh medium (0.75 ml) and incubation at 37 °C for 24 h. Externally exposed glycoproteins on keratinocytes were labelled by digestion

Abbreviation used: SDS, sodium dodecyl sulphate.

with galactose oxidase, followed by reduction with NaBH_4 as described previously (Roberts & Jenner, 1983).

Enzyme digestions

Keratinocytes were digested with 0.04% Dispace in phosphate-buffered saline (15 mM-sodium phosphate/140 mM-NaCl), pH 7.4, containing 0.1% glucose at 37 °C for 40 min. Cell layers were digested with 0.125% trypsin/0.01% EDTA in phosphate-buffered saline, pH 7.4, at 37 °C for 20 min. Trypsin was inactivated by addition of foetal-calf serum to a final concentration of 20%, and the cells were washed three times with 0.14 M-NaCl. In some experiments keratinocytes were treated with 0.125% trypsin/1 mM- Ca^{2+} or 0.01% EDTA alone at 37 °C for 20 min.

Extraction of keratinocytes

Two different methods of extraction were used. (a) The labelled keratinocytes were solubilized in 0.2 ml of electrophoresis sample buffer [2% (w/v) SDS in 0.05 M-Tris/HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 0.001% Bromophenol Blue, 0.14 M-2-mercaptoethanol and 2 mM-phenylmethanesulphonyl fluoride] at 100 °C for 5 min. (b) The cells were extracted three times with 0.5 ml of 0.5% Triton X-100 in 50 mM-Tris/HCl (pH 7.5)/0.14 M-NaCl/10 mM-EDTA/10 mM-N-ethylmaleimide/1.5 mM- MgCl_2 /2 mM-phenylmethanesulphonyl fluoride at 20 °C for 15 min. The residue was solubilized in electrophoresis sample buffer (0.2 ml) and heated at 100 °C for 5 min. The first Triton X-100 extract was freeze-dried, extracted with acetone (2 ml) and then solubilized in electrophoresis sample buffer at 100 °C for 5 min.

Polyacrylamide-gel electrophoresis

Samples were analysed by SDS/polyacrylamide-gel electrophoresis in a discontinuous buffer system (Laemmli, 1970) with gel slabs containing a linear gradient of 5–15% w/v polyacrylamide in the separating gel and a 5% (w/v) stacking gel. Proteins were detected with Coomassie Blue R 250, and radiolabelled glycoproteins were detected by autoradiography or fluorography as described previously (Roberts & Jenner, 1983). The gels were calibrated with [^{14}C]methylated protein M_r markers, namely myosin (200000), phosphorylase b (92500), bovine serum albumin (69000), ovalbumin (46000), carbonic anhydrase (30000) and lysozyme (14300).

RESULTS

Cultures of human epidermal keratinocytes were radiolabelled with D-[1- ^{14}C]glucosamine and then digested with Dispace or trypsin/EDTA as described in the Experimental section. As shown in other studies, digestion with trypsin/EDTA resulted in rapid dispersal of the cells to yield a single-cell suspension (Rheinwald & Green, 1975), whereas digestion with Dispace caused detachment of the cells from the substratum in the form of a sheet of cells (Green *et al.*, 1979). Electrophoretic analysis of the proteins extracted from keratinocytes digested with trypsin/EDTA or Dispace revealed no qualitative differences (Fig. 1a). However, detection of the radiolabelled glycoproteins by autoradiography revealed that a glycoprotein with M_r 126000 was removed by digestion with trypsin/EDTA, but not by

Fig. 1. Effect of digestion with Dispace and trypsin/EDTA on (a) proteins and (b) D-[1- ^{14}C]glucosamine-labelled glycoproteins of human keratinocytes

Keratinocytes were incubated in medium containing D-[1- ^{14}C]glucosamine for 24 h, and extracts were analysed by SDS/polyacrylamide-gel electrophoresis as described in the text. (a) Proteins were stained with Coomassie Blue R 250 and (b) D-[1- ^{14}C]glucosamine-labelled glycoproteins were detected by autoradiography. Lane 1, keratinocytes without proteinase digestion; lane 2, keratinocytes digested with 0.04% Dispace for 40 min at 37 °C; lane 3, keratinocytes digested with 0.125% trypsin/0.01% EDTA for 20 min at 37 °C. The M_r values of protein standards run in parallel are given on the right of the gel. The arrowhead shows the position of the glycoprotein with M_r 126000.

digestion with Dispace (Fig. 1b). In replicate experiments this was the only reproducible difference detected in the glycoprotein profiles after digestion of keratinocytes with the two proteinases. Similar experiments in which keratinocytes were metabolically labelled with L-[1- ^3H]fucose showed that fucose was incorporated into the glycoprotein of M_r 126000 and that again this was the only detectable difference between the glycoproteins from keratinocytes digested with Dispace and trypsin/EDTA (Fig. 2). Comparison of the electrophoretic patterns of L-[1- ^3H]fucose-labelled glycoproteins solubilized in electrophoresis buffer with and without 2-mercaptoethanol (Fig. 2, lanes 2 and 1 respectively) revealed that the glycoprotein of M_r 126000 migrated with an apparent M_r of 141000 before reduction. It is noteworthy that this was the most prominent difference in the glycoprotein pattern of the two samples and that the migration of most of the glycoproteins was not altered by reduction with 2-mercaptoethanol.

Extraction of cultured cells with non-ionic detergents such as Triton X-100 removes most of the proteins and

Fig. 2. Fluorograph of L-[1-³H]fucose-labelled glycoproteins from human keratinocytes separated by SDS/polyacrylamide-gel electrophoresis

Keratinocytes were incubated in medium containing L-[1-³H]fucose for 24 h and then extracted directly with electrophoresis sample buffer or digested with proteinases before solubilization. Lane 1, keratinocytes extracted with electrophoresis sample buffer without 2-mercaptoethanol; lane 2, keratinocytes extracted with electrophoresis sample buffer containing 2-mercaptoethanol; lane 3, keratinocytes digested with 0.125% trypsin/0.01% EDTA for 20 min at 37 °C; lane 4, keratinocytes digested with 0.04% Dispase for 40 min at 37 °C. In lane 2 the arrowhead shows the position of the glycoprotein with M_r 126 000, and in lane 1 the arrowhead shows the position of the unreduced form of this glycoprotein.

glycoproteins from the cytoplasm and plasma membranes, leaving the cytoskeleton and nuclear residue adherent to the substratum (Osborn & Weber, 1977). In a previous study (Roberts & Jenner, 1983) we showed that extraction of D-[1-¹⁴C]glucosamine-labelled keratinocytes with 0.5% Triton X-100 left an 'adherent cytoskeleton' containing eight glycoproteins, with M_r 99 000–232 000. In the present study we found that these glycoproteins were removed from the cytoskeleton by both Dispase and trypsin/EDTA (Fig. 3b). This indicates that any adhesive function these glycoproteins have is probably restricted to that of cell–substratum adhesion.

The glycoprotein of M_r 126 000 was soluble in 0.5% Triton X-100 (Fig. 3a). Confirmation that this glyco-

Fig. 3. Effects of proteinases on (a) 0.5%-Triton-X-100-soluble and (b) 0.5% Triton-X-100-insoluble glycoproteins of human keratinocytes

Human keratinocytes were cultured in Eagle's minimum essential medium containing D-[1-¹⁴C]glucosamine for 24 h. The keratinocytes were extracted with 0.5% Triton X-100 and the soluble extract and insoluble residue were analysed separately by SDS/polyacrylamide-gel electrophoresis followed by autoradiography. Lane 1, control keratinocytes not digested with proteinases; lane 2, keratinocytes digested with 0.04% Dispase for 40 min at 37 °C; lane 3, keratinocytes digested with 0.125% trypsin/0.01% EDTA for 20 min at 37 °C. The arrowhead shows the position of the glycoprotein with M_r 126 000.

protein was exposed on the external cell surface was obtained by radiolabelling keratinocytes with galactose oxidase/ NaB^3H_4 . Extraction of the keratinocytes with 0.5% Triton X-100, followed by electrophoresis and fluorography, revealed that the glycoprotein of M_r 126 000 was a prominent surface component (Fig. 4).

The protective effect of Ca^{2+} on the glycoprotein of M_r 126 000 was examined by digesting surface-labelled keratinocytes with trypsin in the presence of 1 mM- Ca^{2+} . Under these conditions keratinocytes were not dispersed after 20 min at 37 °C, and the glycoprotein of M_r 126 000 remained intact. Incubation of keratinocytes with 0.01% EDTA alone at 37 °C for 20 min did not cause detachment of the cells, nor did it remove the glycoprotein of M_r 126 000. These results indicate that the glycoprotein of M_r 126 000 is an intercellular adhesive molecule which, under the conditions of digestion used in this study, is protected from trypsin digestion by Ca^{2+} .

DISCUSSION

There are now several lines of evidence implicating cell-surface glycoproteins in the processes of cell–cell and cell–substratum adhesion (Hughes & Pena, 1981; Roos, 1984). In the present study we have shown that destruction of a cell-surface glycoprotein of M_r 126 000

Fig. 4. Surface labelling of keratinocytes with galactose oxidase/ NaB^3H_4

Keratinocytes were surface labelled with galactose oxidase/ NaB^3H_4 as described in the text. The labelled keratinocytes were extracted with 0.5% Triton X-100 and the soluble glycoproteins were analysed by SDS/polyacrylamide-gel electrophoresis and detected by fluorography. The arrowhead shows the position of the glycoprotein with M_r 126000.

is correlated with loss of intercellular adhesion in epidermal keratinocytes. Differential identification of the glycoproteins involved in cell-substratum and cell-cell adhesion was achieved by comparison of the glycoproteins cleaved by the neutral proteinase Dispase with those cleaved by trypsin/EDTA. Loss of cell-substratum adhesion occurs with both proteinases, but only trypsin/EDTA disrupts intercellular adhesion. Addition of Ca^{2+} protected the glycoprotein of M_r 126000 from digestion by trypsin and prevented dispersion of the keratinocytes.

Desmosomes are ultrastructurally recognizable regions of cell-cell contact, which are believed to have a major role in the adhesion of epithelial cells. The relationship of the intercellular adhesive glycoprotein of M_r 126000 to desmosomal components is unclear at present. It does not correspond in size to either of the two major cell-surface desmosomal glycoproteins (desmocollins) isolated from bovine nasal epithelium (Cowin *et al.*, 1984), but this may be due to species differences.

However, it is probable that desmosomal adhesion is not the only form of intercellular adhesion between keratinocytes (Skerrow, 1978). Antibodies in the sera of patients with the autoimmune disorder pemphigus vulgaris react with an intercellular component of the living cell layers of stratified squamous epithelia to induce cell separation. The pemphigus antigen has been characterized as a glycoprotein of M_r 130000 (Stanley *et al.*, 1982), which is not a component of desmosomes (Gorbsky *et al.*, 1983).

The intercellular adhesive glycoprotein of M_r 126000 does have some similarity to cell-adhesion molecules characterized in other cell types. Glycoproteins with a similar size and Ca^{2+} -dependent properties have been reported to mediate cell-cell adhesion of teratocarcinoma cells (Ogou *et al.*, 1983), early embryonic cells (Peyrieras *et al.*, 1983), hepatocytes (Gallin *et al.*, 1983) and mammary carcinoma cells (Damsky *et al.*, 1983). Edelman (1983) has proposed that cell-cell recognition occurs by means of local surface modulation of a small number of cell-adhesion molecules rather than by expression of large numbers of different cell-surface markers. Further studies are required to establish whether the intercellular adhesive glycoprotein of M_r 126000 has any relationship to the cell-adhesion molecules, the desmocollins or the pemphigus antigen.

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