Biochimica et Biophysica Acta, 689 (1982) 351–362
Elsevier Biomedical Press

BBA 71231

ANALYSIS OF THE PROTEINS, GLYCOPROTEINS AND GLYCOSAMINOGLYCANS OF FIBROBLAST ADHESIONS TO SUBSTRATUM

SUSAN A. BAYLEY and DAVID A. REES

Unilever Research, Colworth Laboratory, Sharnbrook, Bedford MK44 1LQ (U.K.)

(Received January 28th, 1982)

Key words: Cell adhesion; Glycoprotein; Glycosaminoglycan; Focal adhesion; Fibroblast

The focal adhesion preparations which remain attached to a glass substratum when fibroblast bodies are removed by a gentle stream of buffer have been analysed by gel electrophoresis coupled with other selective methods of analysis. The results are consistent with the presence of three classes of macromolecular components. (i) Muscle and associated proteins amongst which actin was abundant with significant amounts of tropomyosin, some myosin and traces of α-actinin. Some vimentin was present but no vinculin. We detected a major new protein component, as yet unidentified, with a molecular weight in the region of 50000–55000 which is not desmin or tubulin and could have an important function at the focal adhesion. (ii) Glycoproteins which are a specialised subset of those in the whole plasma membrane and included a family which bind ricin and therefore contain β-galactose end groups, together with a series having carbohydrate chains which bound neither ricin nor concanavalin A. The relative proportion of ricin-binding glycoproteins compared to concanavalin A-binding glycoproteins was higher than in whole plasma membranes. (iii) Glycosaminoglycans, with hyaluronate identified as the major component by column chromatography and its susceptibility to *Streptomyces* hyaluronidase.

Introduction

Many fibroblast types develop specialised areas on the cell underside to mediate their attachment when spread on serum coated substrata. By comparison of interference reflection and high voltage electron microscope images of the same cell, Heath and Dunn [1] showed that such focal adhesions occur at the ends of the actin bundles known as stress fibres. In our own previous work we showed that cell bodies may be removed with a stream of buffer, to leave the adhesions attached to the substratum [2] and so provide very convenient material for immunochemical and ultrastructural study [2,3]. Immunofluorescence microscopy has revealed components of our isolated focal adhesions which react with antibodies to proteins of the actomyosin system including actin, myosin, α-actinin and tropomyosin and to the 10 nm filament proteins, and that they contain certain classes of glycoproteins [2]. However, this approach only allows the detection of previously identified macromolecules against which antibodies have already been prepared. Also it is possible that some integral components of adhesion sites may be inaccessible to antibody protein reagents. Therefore, we have now begun a complementary analysis of these preparations by biochemical methods. This task is technically difficult in view of the small amounts of material, the complexity of the mixture, and the need to distinguish products of cellular origin from those from the medium, and we are

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

therefore approaching it in a number of stages. In this first report, we describe results from analysis by one-dimensional gel electrophoresis with selective labelling and staining methods.

In a study which is to some extent analogous, Culp et al. [4–8] have analysed residual material ('substrate-attached material: SAM') on the substratum after fibroblast detachment. Their studies and ours are complementary; we have made a preliminary classification of glycoproteins of membrane origin and have made detailed correlations with intereference reflection and immunofluorescence images, whereas they have made a more extensive analysis of glycosaminoglycans, proteoglycans and other matrix components [8]. Before attempting to correlate our results in detail with theirs, it is important to note a number of rational differences in materials and procedures. Their method for cell detachment involves agitation with ethyleneglycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid (EGTA), but we chose to avoid this reagent in case it removes any internal or external molecules associated with the adhesions. Scanning electron microscopy shows that their preparations contain sealed vesicles [8] whereas focal adhesions from our method are shown by transmission electron microscopy to consist of open structures composed of plasma membrane, electron dense material on the inner surface and strands of material bridging the outer surface to the substratum [3]. They used 3T3 cells and lines derived from them, grown on plastic in medium containing 10% serum when matrix components are evidently secreted in substantial quantities; we have used 16C cells allowed to respread on glass with serum levels (0.1%) at which growth is arrested and the cells are presumable in G_0 phase and synthesis of matrix material is diminished so that it does not swamp analysis of membrane components. For all these reasons, the biochemical compositions of the two preparations may show significant differences which could be significant for cellular function [36].

The cell surface and transmembrane molecules involved in these cell-substratum interactions have yet to be identified but there is mounting evidence that glycoproteins could be involved. Pouyssegur et al. [9] have shown that an adhesion defective mutant of 3T3 Balb/c cells has impaired glycoprotein synthesis. On adhesion the cells remain rounded in shape and microfilament bundles are not formed [10] until the biochemical lesion (in the ability to acetylate glucosamine 6-phosphate) is by-passed by addition of *N*-acetylglucosamine to the culture medium, when normal spreading and formation of bundles are restored. Studies with lectins that bind to the carbohydrate portion of glycoproteins also suggest that glycoproteins are important in attachment and, in particular, receptors for the *Ricinus communis* lectins have been implicated by studies on ricin-resistant mutants of BHK cells [11–13]. In agreement with this, we have found that fluorescent-labelled ricin stains isolated focal adhesions strongly against a faint background staining of the serum layer [2]. These sites are also stained on intact cells confirming that the receptors are not intracellular but situated between the cell and the substrate. In contrast, fluorescent-labelled concanavalin A stains focal adhesions weakly and the immediate surrounding areas more strongly. This suggests that ricin receptors may be concentrated in the focal adhesion and that con A receptors may be relatively excluded. Further observations by fluorescence microscopy have shown that the ricin receptors can be distinguished from fibronectin in its distribution on the undersides of cells [3].

In this paper we investigate the protein, glycoprotein and glycosaminoglycan compositions of adhesion preparations and compare them with whole plasma membranes. We identify a number of macromolecules that may be characteristic of adhesion sites, and which include a number of glycoproteins that bind ricin strongly and concanavalin A weakly and may therefore be important in the attachment of cells to substrate.

Materials and Methods

Cell culture

16C cells (Colworth strain of an established spontaneously transformed line of rat dermal fibroblasts) were cultured as previously described [14]. For preparation of isolated focal adhesions, cells were seeded onto glass coverslips (64 × 76 mm) precoated with a glycoprotein of low molecular weight (140000) which promotes cell spreading from chick serum ('SF 140') [15]. The precoating

was by overnight incubation with 420 μg per coverslip of SF 140 in Hanks' balanced salts solution, followed by rinsing three times with 10 ml Dulbecco's minimum essential medium (MEM; Gibco-Biocults, special formulation). For seeding, cells were detached from confluent cultures by treatment with 0.05% EGTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (Dulbecco) for 10 min, then shaking into Dulbecco's minimum essential medium containing 0.1% serum; they were plated onto the precoated coverslips at a cell density of about $5 \cdot 10^6$ cells per coverslip. These cultures were incubated for 24 h at 37°C in an atmosphere of 10% v/v CO_2 in air.

Metabolic labelling

Cells seeded at a density of $0.75 \cdot 10^6$ cells per 8 oz medicine bottle in 10 ml Dulbecco's minimum essential medium plus 10% serum, with 10 μCi (U-^{14}C)-labelled protein hydrolysate (spec. act. 50 mCi/matom carbon), 10 μCi D-[1-^{14}C]glucosamine hydrolysate (spec. act. 50–60 mCi/mmol) were incubated at 37°C for 48 h. Metabolically labelled membranes were prepared (as below) from such cells. For metabolically labelled focal adhesion preparations, cells were plated and allowed to spread as above on coated coverslips, with addition of a further 10 μCi of the appropriate precursor to each coverslip.

Adhesion preparations

As previously [2], a stream of phosphate-buffered saline was forced over the coverslips from a Pasteur pipette until all cells detached. The back of the coverslip was then scraped with a rubber policeman and the whole coverslip was rinsed with phosphate-buffered saline to remove cell bodies. For electrophoresis, the material from six coverslips for each gel track was scraped into 10 ml 0.2% sodium dodecyl sulphate (SDS). In most experiments the sample was freeze-dried and the SDS concentration reduced by extraction with acidified acetone [16]. The residue was then dissolved in 100 μl Laemmli sample buffer [17] containing dithiothreitol and boiled for 5 min. Alternatively, the solution in SDS was concentrated by vacuum dialysis and then boiled with sample buffer containing dithiothreitol.

In some experiments a mixture of 2 mM benzamidine hydrochloride, 40 μg/ml leupeptin, 20 μg/ml pepstatin, 10 μg/ml chymostatin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EGTA and 5 mM *N*-ethylmaleimide, was used to inhibit possible proteolysis during isolation. Since some of these inhibitors alter the appearance of focal adhesions and the response of the cells to the detachment conditions, inhibitory-free buffer was first used to detach the cells (this takes a maximum of 5 min), before the immediate immersion of coverslips in buffer which contained the inhibitors. Inhibitors were present in all subsequent buffers.

Preparation of plasma membranes

Plasma membranes were isolated by the method of Thom et al. [18]. In some experiments the protease inhibitors detailed above were added to all solutions. The samples were prepared for electrophoresis by boiling for 5 min in Laemmli sample buffer containing dithiothreitol.

Electrophoresis and fluorography

SDS-polyacrylamide gel electrophoresis was in 3–15% polyacrylamide slab gels using the procedure described by Laemmli [17]. The proteins in the gels were fixed with 50% ethanol, 10% acetic acid then stained overnight with a 0.1% Coomassie brilliant blue solution containing 40% ethanol and 20% acetic acid. Gels were destained in 5% ethanol, 7.5% acetic acid. For fluorography, gels were impregnated with Enhance (New England Nuclear) then dried and placed in contact with Kodak X-Omat H film which had been prefogged to reduce exposure times [19]. For molecular weight determinations Biorad high and low molecular weight markers and muscle proteins in chicken gizzard extracts were used. For comparison between gels run on different occasions, the position of each band was related to these markers and to the staining pattern with Coomassie blue on the same track. Wherever possible (including the lectin staining of gels of adhesion preparation versus whole membranes), comparisons were confirmed by re-running samples on adjacent gel tracks.

Preparation of iodinated lectins

The solutions of iodinated lectins were diluted to 250 ml with buffer A, and carrier protein (bovine

serum albumin) was added to a final concentration of 5 mg/ml; gels which had been stained with Coomassie blue were equilibrated with buffer A then immersed in these solutions for 24 h [21]. They were then washed with buffer A for several days with many changes of buffer. Gels were dried and autoradiographed. In control experiments, gel slices incubated with the appropriate inhibitory sugars always confirmed the specificity of labelling. The sensitivity was enhanced by use of Ilford fast tungstate intensifying screens and exposure at −80°C [22].

Preparation of glycosaminoglycans for ion exchange chromatography

Adhesion preparations from six coverslips of cells metabolically labelled with [^{3}H]glucosamine were scraped into 15 ml 0.1 M Tris-HCl, 2 mM $CaCl_2$, pH 7.9, containing 300 μg/ml pronase. 2 mg Hyaluronic acid (Miles) and 2 mg chondroitin sulphate (Miles) were added as carrier. After incubation at 80°C for 15 min then cooling and maintaining at 37°C, a further 3 mg pronase was added twice a day for 7 days. The sample was cooled in ice and trichloroacetic acid was added to a final concentration of 10% w/v. After 1 h in ice the precipitate was removed by centrifugation at 100000 × *g* for 1 h and the supernatant solution was neutralised with sodium hydroxide then freeze-dried. The product was dissolved in 1 ml 10 mM Tris-HCl, pH 8.4, and dialysed to equilibrium against this buffer.

Analysis of glycosaminoglycans by ion-exchange chromatography

Ion-exchange chromatography was by the method of Winterbourne and Mora [23]. Samples were applied to a DEAE-cellulose column (0.6 × 14 cm), equilibrated with 10 mM Tris-HCl, pH 8.4, then elution was with a 20 ml linear gradient of 0 to 0.6 M sodium chloride in 10 mM Tris-HCl, pH 8.4. Fractions (300 μl) were collected and monitored by scintillation counting. Carrier glycosaminoglycans were located using the phenol sulphuric method [24].

Characterisation of the major glycosaminoglycan

Fractions constituting the major peak of tritiated material were pooled and freeze dried, then dissolved in 1 ml distilled water for dialysis against 0.5 M sodium acetate, 0.1 M sodium chloride (pH 5.0). Half of this material was treated with 80 turbidity reducing units of *Streptomyces* hyaluronidase (Miles) for 18 h at 37°C, then compared with the material before digestion by chromatography on Sephadex G-50 (fine) (0.6 × 14 cm column). For control experiments, commercial hyaluronic acid and chondroitin sulphate were submitted to the same overall procedure.

Results

Major proteins in adhesion and membrane preparations

An established transformed cell line, 16C, was used for adhesion preparation. This line forms well-defined focal adhesion structures [2,3] and was preferable to normal cells or lines since 16C cells secrete relatively little matrix material to complicate the analysis and examination of the adhesion preparations by electron microscopy showed that the plane of shear from the cell bodies was more consistent with little contamination from membrane from other parts of the cell. Initially we grew cells for adhesion preparation in 10% serum but examination by polyacrylamide gel electrophoresis often showed the major proteins staining with Coomassie blue to the serum-derived with bovine serum albumin especially predominant. The much fainter bands of cell-derived proteins could not be visualised easily against this background. However, 16C cells had the further convenient property that they spread to form focal adhesions on coverslips coated with SF-140, at a serum level in the medium of only 0.1% and cell-derived products in the adhesion preparations thus prepared could then be analysed very reproducibly. Normal cells are less amenable to spreading at low serum levels.

16C cells form well-defined focal adhesions in both high and low serum which can be visualised by interference reflection microscopy and shown by immunofluorescence microscopy to be the termini of actin bundles. The focal adhesions prepared on coated coverslips in low serum gave similar immunofluorescence staining with antibodies specific to actin, myosin and α-actinin and also similar staining with fluorescent lectins, to those

reported by Badley et al. [2] for focal adhesions prepared from cells grown in 10% serum on uncoated coverslips. Therefore 16C cells in both high and low serum form focal adhesions that have many similarities to those of normal cells. Since this work was begun, progressive refinement of the experimental methods and increasing experience of them, has now made it possible to prepare and analyse focal adhesion preparations from a wider range of sources; as a result we can confirm that gel electrophoresis of adhesion preparations from 16C cells grown in 10% serum and from BHK cells also grown in 10% serum, show patterns of cell-derived proteins and glycoproteins with no major differences from the results reported in detail below for 16C cells in 0.1% serum. However, for ease of handling and clarity of interpretation, we continue to use 16C cells at the low serum level.

The adhesions appear as dark areas using interference reflection microscopy but after scraping into SDS nothing could be visualised on the coverslip suggesting that solulibization of the adhesive material is complete. Also the release of cytoplasmic components for artifactual binding to substratum is minimal since the detached cell bodies remain viable and do not take up Trypan blue which is readily taken up by damaged cells.

Adhesion preparations were examined by electrophoresis under reducing conditions on 3–15% polyacrylamide gels. Samples that were prepared for electrophoresis by freeze-drying followed by SDS extraction gave indistinguishable lectin staining (discussed below) and protein patterns to samples that were concentrated by vacuum dialysis. The former method was therefore routinely used since it allowed larger loadings to be put onto gels. The product of adhesion preparations from six large coverslips was used for each gel track and the bands observed with Coomassie staining were faint. Each preparation contained only a few micrograms of protein and this was predominantly SF-140. To improve the sensitivity as well as to determine which proteins were of cellular origin, the cells were pre-labelled for three days with ^{14}C-labelled protein hydrolysate before preparation of the adhesions and electrophoretic analysis using autoradiographic detection (Fig. 1). Approx. 10^5 cpm, which represents 1% of the total incorporation into the isolated cells, was loaded onto each gel track. Both staining and autoradiography showed similar protein distributions except that one of the most prominent proteins in the Coomassie pattern (molecular weight 70000) was not labelled. Since, under similar conditions, SF 140 shows two components with molecular weights of 43000 and 70000, we conclude that the 70000 protein is due to the SF 140 used to precoat the coverslips. Components with molecular weights of 43000 were observed by both methods so that, although some of this material must be a fragment of SF 140, there is also a cellular protein in this position, probably actin. Each of the other bands staining with Coomassie blue was radiolabelled, showing that the contamination with serum components is otherwise minimal. The consistency between results from five independently isolated adhesion preparations was good. In each case 20 to 25 bands stained with Coomassie blue and most of these were observed in all of the preparations. The pattern was unaffected by the presence of protease inhibitors during isolation. In agreement with the previous immunochemical analysis, bands were observed which comigrated with fibronectin and vimentin, both of which were minor components, and with muscle proteins, of which actin was most abundant; further bands suggested a high concentration of tropomyosin, some myosin and traces of α-actinin. (Some of the more minor of these components, although clearly visible on the original autoradiograms, fail to reproduce satisfactorily in Fig. 1 for photographic reasons.) No band was observed at the position of vinculin which has been shown by immunofluorescence to be present in the region of the focal adhesion in intact cells [25,26]. A major component with a molecular weight between 50000 and 55000 was observed. This does not comigrate with desmin or vimentin and is unlikely to be tubulin since in immunochemical studies with anti-tubulin no staining of the focal adhesions or surrounding areas was observed [2]. This appears to represent a newly identified protein component of adhesion preparations.

Plasma membranes prepared by the method of Thom et al. [18] have been shown by electron microscopy to consist of large membrane vesicles essentially free of contaminating orgenelles. There is no detectable activity of $NADH_2$-lipoamide

oxidoreductase, an endoplasmic-reticulum marker, and the activity of the mitochondria marker succinate dehydrogenase is 99% reduced [18]. Comparison of membranes isolated from cells grown in 10% serum with adhesions prepared from cells seeded in 0.1% serum onto SF-140 coated coverslips is justified since, as discussed earlier, adhesions prepared by either regime appear very similar. Plasma membrane loadings of approx. 20 μg protein gave complex patterns showing more than 40 bands on electrophoresis (Fig. 1), and metabolic-labelling using approx. 10^5 cpm per gel track showed again that each of the bands which stained by Coomassie blue contained cell-derived material. These patterns were unaffected when protease inhibitors were added at the lysis stage and subsequent steps during isolation. Coomassie blue staining showed twelve major components which were present in similar amounts. Of these, components comigrating with fibronectin, myosin light and heavy chains, α-actinin, vimentin, actin, tropomyosin and the 50000–55000 protein discussed above could be identified. Components were apparent which comigrated with each of the proteins from the focal adhesion preparations, but many additional bands were present, especially with molecular weights greater than 70000. The relative proportions of common components varied between the two preparations. For instance actin and the 50000–55000 protein which were most

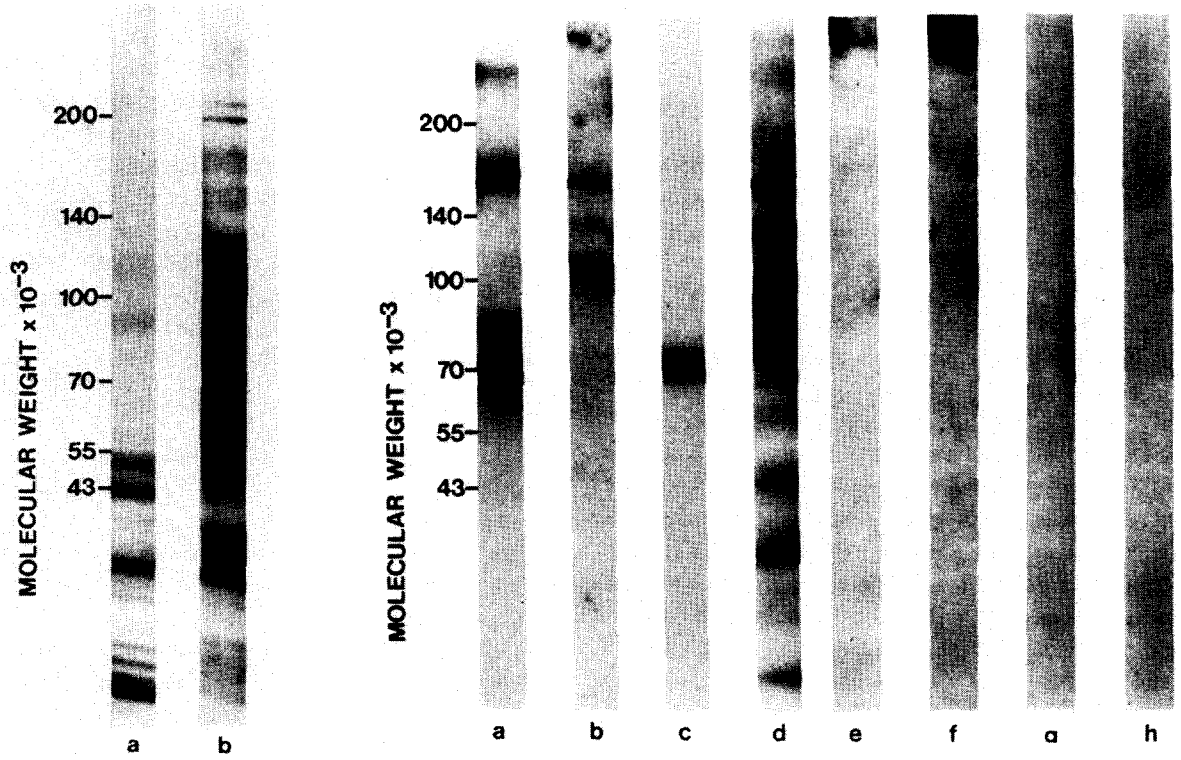

Fig. 1. (Left). Comparison of the total cellular protein composition of (a) an adhesion preparation and (b) a membrane preparation. Proteins were metabolically labelled with ^{14}C-labelled protein hydrolysate and detected by fluorography of 3–15% gradient polyacrylamide gels. The fluorographs shown are of sections of different gels, and therefore the molecular weight scale shown is very approximate.

Fig. 2. (Right). Detection of lectin receptors in 3–15% gradient polyacrylamide gels of adhesion and membrane preparations by direct binding of ^{125}I-labelled lectins followed by autoradiography. The autoradiographs, which are of sections of different gels, show (a) an adhesion preparation reacted with ^{125}I-labelled ricin, (b) a membrane preparation reacted with ^{125}I-labelled ricin, (c) an adhesion preparation reacted with ^{125}I-labelled concanavalin A and (d) a membrane preparation reacted with ^{125}I-labelled concanavalin A, (e–h) duplicate samples of (a–d) incubated with lectins in the presence of inhibitory sugar.

abundant in the adhesion preparations were present only in amounts similar to other muscle proteins in the membrane preparation. Thus the focal adhesion is a specialised area of membrane at which certain proteins are concentrated at the expense of others.

Comparison of lectin receptors in adhesion and membrane preparations

The components of membrane and adhesion preparations were separated by electrophoresis then lectin receptors were identified by autoradiography of gels that had been exposed to either ^{125}I-labelled concanavalin A or ^{125}I-labelled ricin. Three independently isolated adhesion preparations were stained with different ricin baths, and in each case ricin binding components with molecular weights of 70000, 80000 and 160000 were observed. In two of the experiments there was an additional 43000 component and in the third another 300000 component (Fig. 2). Some non-cell derived contaminants must be suspected since under reducing conditions, SF-140 is broken into 43000 and 70000 fragments, both of which are ricin and concanavalin A receptors and, as discussed earlier, the 70000 component of adhesion preparations does not become metabolically labelled with ^{14}C-labelled protein hydrolysate; the 43000 ricin binding component must include the SF-140 fragment, but the results from metabolic labelling cannot exclude the possibility that it is cell-derived in part. The 160000 ricin receptor does not bind concanavalin A (see below) so is probably not unreduced SF-140.

When whole plasma membrane preparations were compared with the focal adhesion preparations by this method the labelling pattern was quite different. Short autoradiographic exposure revealed three bands of apparent molecular weight 120000, 140000 and 160000 (Fig. 2). Longer periods of exposure revealed another six bands of much lower intensity, clustered in the molecular weight range 50000 to 100000. The 160000 species therefore appears to be common and the 80000 ricin receptor of focal adhesions may be a minor membrane component.

Similar analysis of adhesion preparations with ^{125}I-labelled concanavalin A showed only one receptor with a molecular weight of 70000 (Fig. 2), and since SF-140 binds concanavalin A as well as ricin, this is identified as the contaminating fragment of SF-140. Closer examination of longer exposures showed additional bands at 150000, 120000, 80000 and 43000 but these were very faint and also inconsistent because in one experiment the 80000 component, and in another the 43000 component, were absent. We conclude that concanavalin A receptors are present in adhesion preparations in much lower concentrations relative to ricin receptors. This result had already been suggested by fluorescence microscopy [2] which also suggested that ricin receptors were located more specifically at the adhesion plaque itself, with the concanavalin A receptors being distributed more diffusely.

Concanavalin A, like ricin, stained many more bands on gel slices from the electrophoretic analysis of membrane preparations (Fig. 2). On average eleven components were stained with roughly equal intensity, these had molecular weights between 150000 and 10000, and only 150000 and 120000 components were common with the adhesion preparations.

It is interesting that no staining was observed with either lectin to correspond with fibronectin (220000) which would be expected to bind both concanavalin A and ricin, and has been implicated in cell surface/substratum interactions. However, the result does correlate with the observation of Burridge [27] that for some other transformed cell lines the staining of the fibronectin band by lectins is reduced in comparison to normal cells.

Control experiments showed that the addition to the lectin baths of 0.15 M hapten (lactose for ricin and methyl α-mannoside for concanavalin A), completely inhibited staining of gels from both adhesion and membrane preparations (Fig. 2).

As a control experiment, coverslips precoated with SF-140 were incubated with medium containing serum, in the absence of cells. They were then treated and analysed as for focal adhesion preparations; staining with either ^{125}I-labelled concanavalin A or ^{125}I-labelled ricin showed only one faint band corresponding to the 70000 fragment of SF-140, with no staining to correspond to the 43000 fragment (which is always less intense than the 70000 band). These low recoveries relative to the levels of the same components in focal adhe-

sion preparations would suggest that the amount of SF-140 which is retained is increased in the presence of cellular material. Without the independent evidence from metabolic labelling, control experiments could not therefore distinguish between cell-derived and non-cell-derived glycoproteins.

The autoradiographs after lectin staining of glycoproteins both from whole membrane preparations and focal adhesion preparations, were compared with the gels stained beforehand with Coomassie blue. Protein bands were detected corresponding to almost all the radioactive bands but most of these were minor protein components and therefore, as discussed earlier, cannot be seen in Fig. 1; exceptions were the 300000 ricin receptor and the minor 120000 and 150000 concanavalin A receptors, all from focal adhesions, which showed no corresponding staining with Coomassie blue. That the proteins detected could be of cellular origin was confirmed by comparison with the gel autoradiographs from preparations which had been metabolically labelled with ^{14}C-labelled protein hydrolysate as discussed earlier.

Both concanavalin A and ricin label an 80000 component, which resolves to a doublet with Coomassie blue. The definition of the autoradiographs is not precise enough to determine whether both lectins bind to the same band. However, as discussed earlier, much more ricin binding than concanavalin A binding is found in this region.

Carbohydrate components of adhesion and membrane preparations

To obtain further information on the glycoproteins present in the adhesion preparations as compared with whole plasma membranes, the metabolic labelling studies were repeated with [^{14}C]glucosamine. Gel electrophoresis of adhesion preparations followed by autoradiography showed much simpler patterns than from the labelling with protein hydrolysate. The most striking feature is the large amount of material of very high molecular weight which is retained in the 3% stacking gel, most of which was removed before taking the photograph (Fig. 3). Of the total amount of glucosamine label incorporated into the cell layer ($6 \cdot 10^5$ cpm), 10% was retained in the adhesion preparations ($6 \cdot 10^4$ cpm). (The corresponding figure from

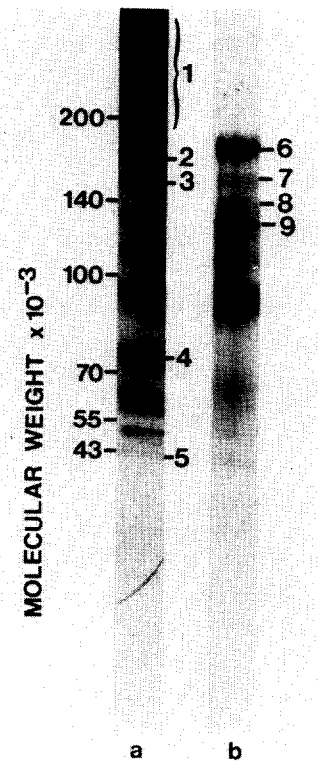

Fig. 3. Fluorographs showing the localisation of metabolically incorporated [^{14}C]glucosamine in 3–15% polyacrylamide gels of (a) an adhesion preparation and (b) a membrane preparation. The fluorographs are of sections of different gels. Numbers indicate (1) glycosaminoglycan; adhesion preparation glycoproteins comigrating with: (2) 160000 ricin receptor, (3) 150000 concanavalin A receptor, (4) 80000 concanavalin A and ricin receptors, (5) 43000 concanavalin A and ricin receptors; membrane preparation glycoproteins comigrating with (6) 160000 ricin receptor, (7) 150000 concanavalin A receptor, (8) 140000 ricin receptor, (9) 120000 concanavalin A and ricin receptors.

protein labelling was much lower, around 1%.) Most of the glucosamine label is retained in the stacking gel; the identity of this material as a glycosaminoglycan will be discussed later.

In addition to the components in the stacking gel, eleven further labelled bands were observed, all of relatively higher molecular weight relative to the distribution of components detected after protein labelling; several of the bands were not detected after labelling with ^{14}C-labelled protein hydrolysate. The largest of the distinct bands comigrated with fibronectin (obscured by the heavy glycosaminoglycan labelling under the conditions

of Fig. 3, but clearly visible under other conditions (not shown)), and other bands were in positions corresponding to the 160000 ricin receptor, 150000 concanavalin A receptor, 80000 and 43000 concanavalin A and ricin receptors. Again, the 70000 concanavalin A/ricin receptor was not labelled, consistent with its identification as a fragment of SF-140. Since the 300000 ricin receptor coincides in position with the edge of the glycosaminoglycan, it is not possible to determine whether there is a band to correspond to it. In agreement with results from metabolic labelling of protein, the 120000 concanavalin A receptor was not detected showing that, if indeed it is a cell-derived protein, it can only be present in adhesion preparations at very low levels. Glucosamine label was, however, incorporated into the band which comigrated with the 43000 ricin/concanavalin A receptor, showing that, in addition to actin and the SF-140 fragment, a cellular glycoprotein is also present at this position. It is not clear whether this cellular glycoprotein binds either lectin because the presence of the SF-140 fragment is sufficient to account for the lectin staining behaviour of this band. Seven other glycoproteins were detected which do not bind either ricin or concanavalin A, one of which comigrated with fibronectin but the identities of the others are unknown.

Analysis of whole plasma membranes after metabolic labelling with [^{14}C]glucosamine, and loading approx. 10^5 cpm/track gave much simpler patterns than those after labelling with ^{14}C-labelled protein hydrolysate (Fig. 3). The most prominent band comigrated with the 160000 ricin receptor. Also clear were bands comigrating with the other major ricin receptors (140000 and 120000) and two concanavalin A receptors (150000 and 120000). More diffuse staining occurred in the molecular weight ranges 90000–100000 and 50000–60000 which could therefore include some of the other ricin and concanavalin A receptors identified above. No radioactivity was detected in the lower molecular weight regions where some concanavalin A receptors had been detected (see above) so these receptors must be minor constituents only of the membrane.

This pattern of carbohydrate components in whole membranes is therefore different from that in adhesion preparations. Although there are some common bands (160000, 150000, 120000 and 90000) each pattern shows several additional bands not present in the other. The glycoprotein that comigrates with the 80000 ricin receptor and is prominent in the adhesion preparations, is absent from the membrane preparations – providing confirmation by autoradiography of the results given by lectin staining.

Identification of glycosaminoglycans present in adhesion preparations

To investigate the nature of the high molecular weight material which dominated the gel electrophoresis pattern after glucosamine labelling, adhesion preparations were treated with pronase to release any glycosaminoglycan chains which were bound in the form of proteoglycans. The samples were then prepared and subjected to ion exchange chromatography as described in the Methods Section. Most of the cell-derived (i.e. metabolically labelled) material coeluted with carrier hyaluronic acid (Fig. 4). In addition there was a smaller peak which eluted in a position corresponding to heparan sulphate [23] and an even smaller but

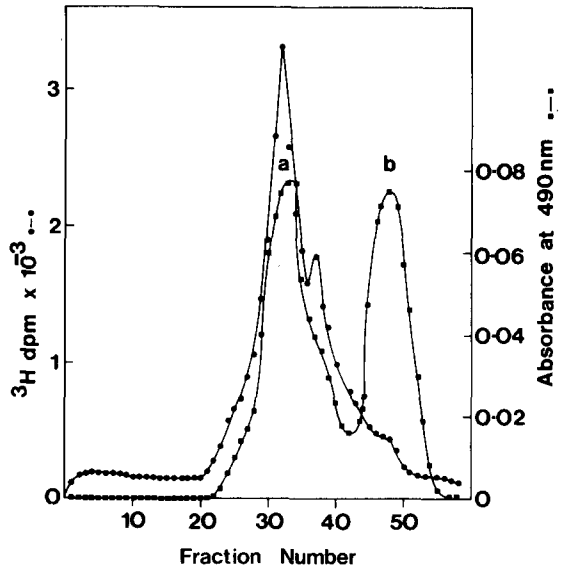

Fig. 4. Ion-exchange chromatography of glycosaminoglycans. Glycosaminoglycans from adhesion preparations (●———●) and standards (■———■) were applied to a DEAE-cellulose column and eluted as described in the text. Peaks a and b correspond to hyaluronic acid and chondroitin sulphate, respectively.

reproducible shoulder coeluting with chondroitin sulphate. The amounts of the latter two components were too small for their identities to be further examined at this stage by specific enzymes. However, the pooled material from the major peak was shown to be degraded by *Streptomyces* hyaluronidase since, after digestion, 83% had become included in a G-50 (fine) column whereas it had been totally excluded before this treatment. In control experiments, authentic hyaluronic acid was depolymerised to the same extent but chondroitin sulphate was unaffected. We conclude that the majority of the material that became metabolically labelled in the adhesion preparations with [^{3}H]glucosamine is hyaluronic acid. This result also explains the observation that no high molecular weight protein was observed on gels since hyaluronic acid, unlike the other glycosaminoglycans, is not usually attached to a protein core.

Discussion

By electrophoretic analysis coupled with selective detection methods involving separate metabolic labelling of carbohydrate and protein, staining with Coomassie blue, staining with ^{125}I-labelled lectins, use of a specific enzyme hydrolase, and column chromatography, we have analysed three distinct classes of macromolecular components in our preparations of focal adhesions from 16C cells – namely the proteins, glycoproteins and glycosaminoglycans. The results identify a number of components of adhesion preparations which may have important roles in the attachment of cells to substrates. These include muscle proteins, a 50000–55000 protein of unknown identity and a number of glycoproteins, and in particular 80000 and 160000 ricin receptors, which are a specialised subset of those present in whole plasma membranes. The newly identified molecules, which are discussed in more detail below, will be the subject of further study.

Muscle and associated proteins on the inner face of the adhesion plaque

Predominant amongst the components of focal adhesions that were stained by Coomassie blue, were the muscle proteins previously shown [2,3] to be present at the inner face of the isolated structures. From the electrophoresis analysis, actin appeared to be the most abundant of these, with significant amounts of tropomyosin, some myosin, and traces only of α-actinin. Some vimentin would also seem to be present, again consistent with immunofluorescence. In contrast to Culp and coworkers [8] we identified no desmin as a major component but this is not surprising since the origin of our cells is different and immunofluorescence staining on whole cells shows vimentin as the major and perhaps only intermediate filament protein (Badley, R.A., Bayley, S.A., Woods, A. and Rees, D.A., unpublished data). The 130000 protein, vinculin which seems from many other studies to occur often at sites of actin-membrane association [28] including focal adhesions [25,26], was not detected on our gels. A likely explanation is that, although present near focal adhesions in the whole cell, this protein was washed away with the cell bodies during preparation. Since large amounts of actin are retained in the focal adhesions with no vinculin and little α-actinin, we conclude (in agreement with others [29]) that neither of the latter two proteins is likely to mediate the linkage of actin to the membrane here. We did however detect another protein in significant amounts which is so far unidentified but which has a molecular weight between 50000 and 55000 and does not appear to be desmin, vimentin or tubulin. Metabolic labelling gives no clear evidence that this protein is glycosylated and therefore it is possible that it occurs on the inner rather than the outer face of the membrane. This unidentified protein is equalled only by actin in its relative concentration as estimated from the intensity of Coomassie blue staining.

Since several trace components were present on our gels in the molecular weight range 70000 to 100000 we cannot exclude the possible presence of such known actin-associated proteins as villin [30] and fimbrin [31]. Several proteins of lower molecular weight were present, as observed previously by Culp and co-workers [5] in their substrate-attached material, and attributed by them to histone proteins from a minor proportion of lysed cells. We have no independent evidence on the identity of these components at present.

Our results from the analysis of proteins associated with cellular adhesions, show general similari-

ties to those of Culp and co-workers [5,8] except that their substrate-attached material shows a more complex composition with respect to both cellular and serum proteins and there are some differences in relative proportions e.g. less myosin in our preparations. A major conclusion which is more evident from our system, is that the protein composition of focal adhesions is highly specialized in comparison with the whole plasma membrane – the components are fewer, and, although all present in the plasma membrane, they are in different relative proportions (for fuller details, see Results). This cannot have arisen merely from the adhesion components being in a metabolic pool distinct from the whole membrane, because the labelling experiments were very long term and gave similar protein distributions to Coomassie blue staining.

Cellular glycoproteins of adhesion preparations

Glycoprotein components of adhesion preparations were identified by their metabolic incorporation of labelled glucosamine, and further classified by their ability (or not) to bind ^{125}I-labelled lectins after electrophoretic separation. Only a relatively small proportion of the labelled amino acids incorporated into adhesion preparations were detected in bands corresponding to these glycoproteins. A low relative concentration of external relative to internal protein in macromolecules (i.e. glycoproteins versus muscle-associated proteins) is of course consistent with the image of focal adhesions in the transmission electron microscope, since protein-positive stains show heavy deposits on the inner face and more tenuous structures outside [3].

Receptors for ricin are shown by electrophoresis to be more prominent than receptors for concanavalin A (confirming the fluorescence microscopy), and we identify several ricin-binding components and in particular those with molecular weight of 80000 and 160000 as being characteristic of adhesion preparations. Other ricin receptors were detected in whole plasma membranes but were absent from the adhesion preparations. After metabolic labelling with glucosamine, further glycoprotein bands were seen in the adhesion preparations. These bound neither ricin nor concanavalin A and the nature of their carbohydrate chains has yet to be established.

Of all the cell-derived glycoprotein components, only one can at this stage be provisionally identified, namely fibronectin which appeared as a familar 220000 component and labelled with glucosamine and amino acids but bound neither ricin nor concanavalin A. However, the role of fibronectin at focal adhesions is controversial at present [2,32,35].

Glycosaminoglycans

By far the major product metabolically labelled with glucosamine, which dominated the gel pattern as a continuous streak from the origin, was of such high molecular weight that most of it remained in the stacking gel. These properties correspond to glycosaminoglycan or proteoglycan, and in further experiments in which focal adhesions were solubilized with pronase for analysis by ion-exchange chromatography and for testing the sensitivity to hydrolysis by *Streptomyces* hyaluronidase, the main component was shown to be hyaluronate with much smaller amounts of two other components of unproven identity but which had ion-exchange properties consistent with heparan sulphate and chondroitin sulphate, respectively.

Culp and co-workers [8] have made more extensive studies of the glycosaminoglycans, proteoglycans and other matrix components in their system, and our results are qualitatively consistent with theirs. From a current extension of our analysis in which the spatial distributions of hyaluronate and heparan sulphate have been examined in relation to the focal adhesions, we will report (Wilson, G., Woods, A., Rees, D.A., and Höök, M., unpublished data) that a population of heparan sulphate proteoglycan is specifically located at focal adhesions and in transmembrane relationship with their associated actin bundles, whereas hyaluronate is distributed generally over the entire substratum.

Note added in proof: (June 18th, 1982)

In the light of recent developments (Smith, C.G., Woods, A., Rees, D.A. and Wilson, G., unpublished data) we have re-examined our gels for evidence that clathrin might be present at focal adhesions. The results are inconclusive because of the presence of glycoprotein components that run in the same region.

Acknowledgements

We thank Mrs. A. Woods for the preparation of focal adhesions and for helpful discussions, Dr. R.A. Badley for help and advice in exploratory experiments, Miss P. Reed and Dr. R.C. Zobel for the data on adhesion preparations from cells grown in high serum levels, Dr. G. Wilson for assistance and discussions on glycosaminoglycan analysis, and Mr. J.M. Stubbs for help with photography.

References

1 Heath, J.P. and Dunn, G.A. (1978) J. Cell Sci. 29, 197–212
2 Badley, R.A., Lloyd, C.W., Woods, A., Carruthers, L., Allcock, C. and Rees, D.A. (1978) Exp. Cell Res. 117, 231–244
3 Badley, R.A., Woods, A., Smith, C.G. and Rees, D.A. (1980) Exp. Cell Res. 126, 263–272
4 Rosen, J.J. and Culp, L.A. (1977) Exp. Cell Res. 107, 139–149
5 Culp, L.A. (1976) Biochemistry 15, 4094–4104
6 Culp, L.A., Rollins, B.J., Buniel, J. and Hitri, S. (1978) J. Cell Biol. 79, 788–801
7 Rollins, B.J. and Culp, L.A. (1979) Biochemistry 18, 141–148
8 Culp, L.A., Murray, B.A. and Rollins, B.J. (1979) J. Supramol. Struct. 11, 401–427
9 Pouyssegur, J., Willingham, M. and Pastan, I. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 243–247
10 Willingham, M.C., Yamada, K.M., Yamada, S.S., Pouyssegur, J. and Pastan, I. (1977) Cell 10, 375–380
11 Meager, A., Ungkitchanukit, A., Nairn, R. and Hughes, R.C. (1975) Nature 257, 137–139
12 Meager, A., Ungkitchanukit, A. and Hughes, R.C. (1976) Biochem. J. 154, 113–124
13 Edwards, J.G., Dysart, J. McK. and Hughes, R.C. (1976) Nature 264, 66–68
14 Lloyd, C.W., Rees, D.A., Smith, C.G. and Judge, F.J. (1976) J. Cell Sci. 22, 671–684
15 Thom, D., Powell, A.J. and Rees, D.A. (1979) J. Cell Sci. 35, 281–306
16 Lazarides, E. (1976) J. Supramol. Struct. 5, 531–563
17 Laemmli, U.K. (1970) Nature 227, 680–685
18 Thom, D., Powell, A.J., Lloyd, C.W. and Rees, D.A. (1977) Biochem. J. 168, 187–194
19 Laskey, R.A. and Mills, A.D. (1975) Eur. J. Biochem. 56, 335–341
20 Fraker, P.J. and Speck, J.C. (1978) Biochem. Biophys. Res. Commun. 80, 849–857
21 Burridge, K. (1978) Methods Enzymol. 50C, 54–64
22 Laskey, R.A. and Mills, A.D. (1977) FEBS Lett. 82, 314–316
23 Winterbourne, D.J. and Mora, P.T. (1978) J. Biol. Chem. 253, 5109–5120
24 Dubois, M., Gilles, K.A., Hamilton, J.K., Rebens, P.A. and Smith, F. (1956) Anal. Chem. 28, 350–362
25 Geiger, B. (1979) Cell 18, 193–205
26 Burridge, K. and Feramisco, J.R. (1980) Cell 19, 587–595
27 Burridge, K. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4457–4461
28 Geiger, B., Kokuyasu, K.T., Dutton, A.H. and Singer, S.J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4127–4131
29 Burridge, K. and McCullough, L. (1980) J. Supramol. Struct. 13, 53–65
30 Bretscher, A. and Weber, K. (1980) Cell 20, 839–847
31 Bretscher, A. and Weber, K. (1980) J. Cell Biol. 86, 335–340
32 Hynes, R.O. and Destree, A.T. (1978) Cell 15, 875–886
33 Birchmeier, C., Kreis, T.E., Eppenberger, H.M., Winterhalter, K.H. and Birchmeier, W. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4108–4112
34 Fox, C.H., Cottler-Fox, M.H. and Yamada, K.M. (1980) Exp. Cell Res. 130, 477–481
35 Chen, W.-T. and Singer, S.J. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 7318–7322
36 Singer, I.I. and Paradiso, P.R. (1981) Cell 24, 481–492