Journal of Cellular Biochemistry 18:157-167 (1982)
Cellular Recognition 563-573

Expression of Adhesion-Related Membrane Components in Adherent Versus Nonadherent Hamster Melanoma Cells

Karen A. Knudsen, Caroline H. Damsky, and Clayton A. Buck

The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

The existence of integral membrane components that are involved in cell-substratum adhesion has been postulated. Using an immunochemical approach developed in this laboratory, we provide further evidence for the role in cell-substratum adhesion of integral membrane glycoproteins within a molecular weight region of 120,000–140,000. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of material enriched approximately 100-fold in adhesion-related components revealed the 120,000–140,000 M_r glycoproteins in an adherent hamster melanoma cell line. These glycoproteins are greatly reduced in a nonadherent variant. Induction of adhesion in these cells by exposure to BudR is accompanied by re-expression of the surface adhesion antigens.

Key words: cell adhesion, surface membrane antigens, nonadherent melanoma cells

Adhesion of cells to a substratum is a fundamental characteristic important to many cells. However, adhesion variants that display altered cell-substratum interactions and altered cell morphology are commonly observed, especially among transformed cells. Recent studies have shown that normal morphology and adhesion can be restored to these cells in many cases by the addition of fibronectins [1, 2, reviews]. Following the identification of domains of fibronectin responsible for binding such things as collagen, glycosaminoglycans, and cells, it was shown that both the collagen and cell binding sites must coexist on the same molecule for adequate cell-substrate adhesion to occur [3]. A corollary of this observation is that the integral membrane cell surface binding site for fibronectin, or for any adhesion molecule associated with the extracellular matrix, must be functionally intact for cellular adhesion to occur. Adhesion variants with implied defects in integral surface membrane components have been described by other laboratories [4,5]. In this paper, we analyze a nonadherent variant of a hamster melanoma cell line and show that, when compared with adherent cells, it has a greatly reduced expression

Received April 16, 1981; accepted September 15, 1981.

0730-2312/82/1802-0157$03.50

of integral membrane glycoproteins suggested by previous work from this laboratory [6–8] to be involved in cell–substratum adhesion. When induced to adhere to the substratum, these nonadherent cells are found to express surface membrane adhesion antigens. The data demonstrate that cellular adhesion is correlated with the presence of a highly restricted population of glycoproteins in the molecular weight region of 120,000–140,000.

METHODS

Cells and Cell Culture

Two variants of the hamster melanoma cell line RPMI 3460 were used routinely: an adherent line, CM1 (cell monolayer) (Fig. 1A), and nonadherent line, CS1 (cell suspension) (Fig. 2A).* These cell lines were derived (The Wistar Institute) by Dr. Richard Farishian and are described elsewhere [9]. Cells were grown under conditions previously described [6] in either 490 cm^2 tissue culture roller bottles (Corning, Corning, New York) or 150 cm^2 tissue culture flasks. Cells were labeled metabolically by the addition of 20 μCi [D-^{14}C]glucosamine hydrochloride (New England Nuclear, Boston, Mass) for 48 h. All cells, adherent and nonadherent, were treated with 1 mM EDTA during harvest.

The observation that the nonadherent cells, which grow as a suspension of aggregated and single cells, can be induced by BudR (2×10^{-6} M) to adhere to the substratum was described by Dr. Nebojsa Avdalovic at The Wistar Institute [10]. Nonadherent cells induced to adhere to the substratum by BudR are referred to as adherent, BudR-treated cells. In addition to treating the nonadherent cells with BudR, various culture conditions were tested for their effect on altering cell morphology. In one set of experiments, the wells of microtiter plates were coated with the following: 1) human CIG (Collaborative Research, Waltham, Massachusetts) prepared and used according to the manufacturer's instructions, 2) heat inactivated fetal calf serum (0.1 ml), 3) concanavalin A (1–10 μg), and 4) rat-tail collagen and 0.1% gelatin (gifts of Dr. Alan F. Horwitz, University of Pennsylvania). Reagents (1) and (2) were exposed to the culture plate for 2 h at room temperature and then removed before the cells were added. Reagents (3) and (4) were dried onto the surface of the culture plates. In other experiments, nonadherent cells were grown in the presence of cell-free medium conditioned by adherent cells and on cell-free extracellular matrix remaining after removal of adherent cells by 1 mM EDTA.

Preparation of Antisera

The antisera used to perturb cell–substratum adhesion were raised in goats and prepared as described previously [6]. Briefly, anti-M serum was raised against the surface membranes of hamster fibroblasts and was found to round and detach hamster fibroblasts at a dilution of 1:50. Anti-GP serum was raised against lectin-purified glycoproteins extracted from hamster fibroblasts; it rounds and detaches

*These cell lines were incorrectly designated in preliminary results reported by Knudsen, Damsky, and Buck in the J Supramol Struct Cell Biochem Suppl 5:308, 1981.

hamster fibroblasts at a dilution of 1:3,000. The sera were heated at 56°C for 30 min to inactivate complement.

Absorption of Anti-GP Serum With Cells

Adherent or BudR-treated cells were harvested with 1 mM EDTA in PBS, washed with PBS, and dispensed in different numbers into 1.5-ml, conical-shaped, plastic vials. Nonadherent cells were collected and washed first with 1 mM EDTA in PBS and then with PBS before dispensing into vials. The cells were pelleted by gentle centrifugation and resuspended in 0.15 ml of PBS. An equal volume (0.15 ml) of anti-GP serum diluted 1:500 in MEM containing 2 × each of amino acids, vitamins, glucose, fetal calf serum, penicillin, and streptomycin was added to each vial. The cells were incubated in this mixture for 1 h at 4°C and then removed by centrifugation. The supernate was centrifuged again at high speed and assayed for remaining activity by adding it to adherent hamster fibroblasts (BHK_{21}/C_{13}) in the wells of microtiter culture plates overnight. The percentage of rounded cells was then determined.

Antiserum Neutralization Assay/Blocking Assay

The blocking assay used has been previously described [7,8]. Briefly, the assay detects solubilized adhesion-related antigens in various cell fractions by testing their ability to compete with sites on the surface of adherent cells for antibodies in anti-M serum, thereby inhibiting the anti-M-induced rounding and detachment of cells. A final antiserum dilution of 1:50 was found to round 100% of either adherent hamster fibroblasts [7] or adherent melanoma cells (Fig. 1B) overnight. The effect of anti-M serum was considered to be completely blocked when the cells appeared as they do in Figure 1C.

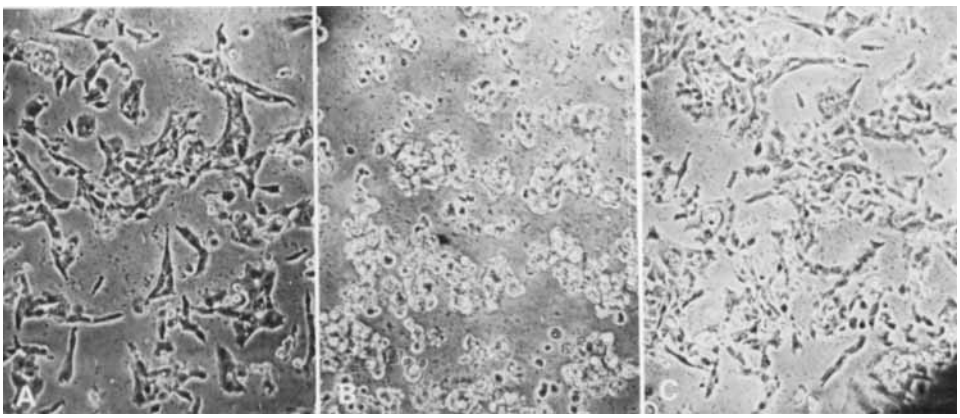

Fig. 1. Blocking assay. Adherent hamster melanoma cells (A) were induced to round and detach cells from the substratum (B) by a 1:50 dilution of anti-M serum (raised in goats against surface membranes isolated from hamster fibroblasts). The effect of the anti-M serum can be blocked by 14 μg of protein from a detergent (NP40) extract of adherent cells (C).

Purification Scheme

The purification scheme outlined in Figure 3 was developed in this laboratory and is described in detail elsewhere [6–8,11]. Note that both the adherent and nonadherent cells were exposed to 1 mM EDTA in PBS prior to washing them with PBS and extracting them with NP40 detergent.

Protein Determination

Protein concentrations were measured by the method of Lowry et al [12], using bovine serum albumin as a standard. Sodium dodecyl sulfate (SDS) was added at a final concentration of 0.5% to the NP40-containing blanks, standards, and samples to prevent the formation of a white precipitate.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were analyzed by slab SDS-PAGE according to the method of Laemmli [13]. Separation was accomplished using 6.5% acrylamide gels. Samples were warmed at 37°C for 3 min in buffer containing 2% SDS and 5% 2-mercaptoethanol. Gels were subjected to 30 mamp constant current, until pyronin-Y tracking dye reached the bottom of the gel, stained with Coomassie blue, destained, and photographed. The gels were then soaked in En^{3}Hance (New England Nuclear, Boston, Massachusetts), dried, and exposed to XR-1 Kodak X-ray film at −60°C for 10–14 days.

RESULTS

Manipulation of Cell-Substratum Adhesion of Adherent Hamster Melanoma Cells

Adherent hamster melanoma cells could be reversibly rounded and detached from the substratum with either of two antisera raised against material from hamster fibroblasts. In fact, the dilution of antiserum necessary to cause 100% of the cells to round overnight in the blocking assay was the same for both fibroblasts

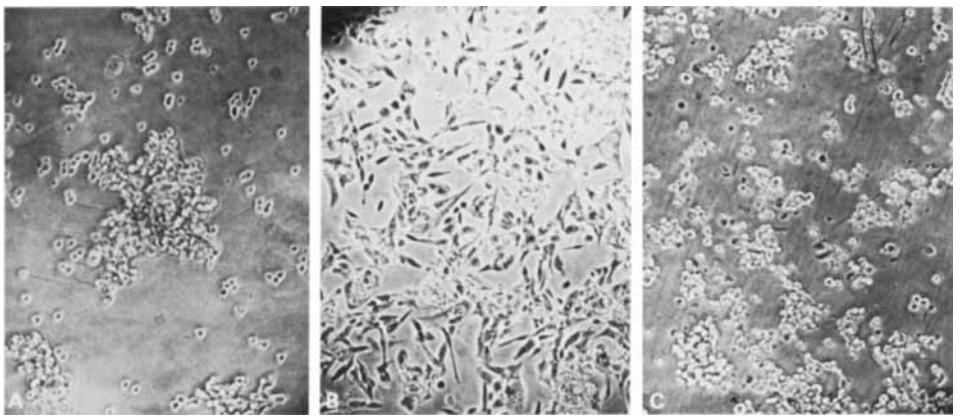

Fig. 2. Manipulation of cell-substratum adhesion in a nonadherent hamster melanoma cell line. Nonadherent cells (A) could be induced to adhere to the substratum by the addition of BudR at a concentration of 2×10^{-6} M (B). The adherent, BudR-treated cells were rounded and detached by a 1:50 dilution of anti-M serum (C).

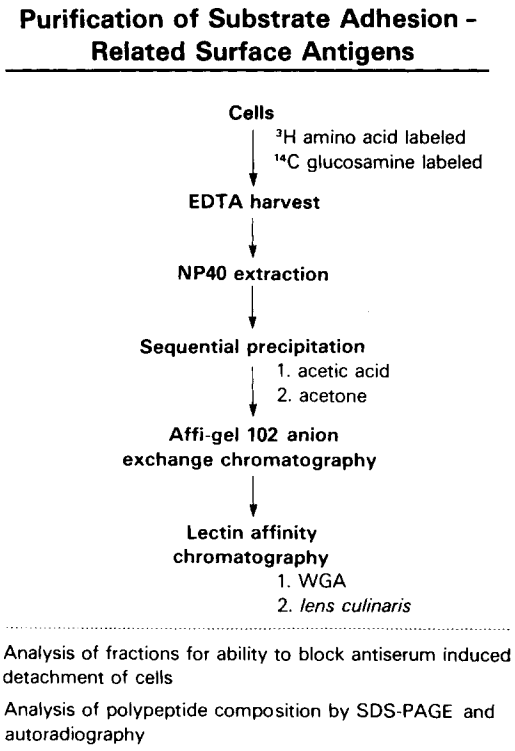

Fig. 3. Purification scheme. The protocol for isolating integral membrane glycoproteins involved in cellular adhesion is outlined above. Details concerning this scheme are described elsewhere [7,8,11].

and melanoma cells. A 1:50 dilution of anti-M serum completely rounded both fibroblast [6,7] and melanoma cells (Fig. 1B). Similarly, a 1:3,000 dilution of anti-GP produced the same effect. Furthermore, the antiserum-induced rounding and detachment of either cell type could be blocked by a similar amount of protein in the detergent extract of the same (Fig. 1C) or heterologous cell type [11]. Therefore, the two antisera, originally raised against material from hamster fibroblasts and used to perturb cell-substratum adhesion of the fibroblasts, also proved to be useful as probes for surface components involved in the adhesion of hamster melanoma cells.

Manipulation of Cell-Substratum Adhesion in Nonadherent Hamster Melanoma Cells

A variety of extracellular matrix-associated components were added to the nonadherent melanoma cells and tested for their ability to induce adhesion and spreading of the cells. They included gelatin, rat-tail collagen, fetal calf serum, concanavalin A, medium conditioned by adherent melanoma cells, the matrix left after adherent cells were removed with EDTA, and human fibronectin. No component tested would induce the nonadherent cells to adhere to, or to spread on, the substratum. Under all of the above conditions, the cultures appeared exactly as in Figure 2A. However, continuous exposure to BudR (2×10^{-6} M) in the dark induced a proportion of the cells to adhere to, and to spread on, the substratum

(Fig. 2B). As with normal adherent cells, adhesion of these BudR-treated cells could be perturbed by anti-M serum at a dilution of 1:50 (Fig. 2C).

Absorption of Anti-GP Serum With Adherent and Nonadherent Hamster Cells

Because nonadherent cells were not induced to adhere by adding a variety of substratum-associated components, we investigated the possibility that the cells were not expressing the integral surface membrane constituents necessary for establishing adhesion and cell spreading. Therefore, we compared the ability of the nonadherent and adherent cells to bind adhesion-related antibodies from an antiserum (anti-GP) that induces rounding and detachment of cells. The results show that greater than 10-fold more nonadherent cells than adherent cells were required to remove 50% of the rounding activity from anti-GP serum (Fig. 4). In a similar experiment, the ability of adherent, BudR-treated cells to remove activity from anti-GP serum was compared with that of untreated nonadherent cells (data not shown). In both experiments, the expression of cell surface adhesion antigens was greatly reduced in the nonadherent cells as compared with either the adherent parent cells or the nonadherent cells induced to adhere by the presence of BudR.

Purification of Adhesion-Related Antigens From Adherent and Nonadherent Hamster Melanoma Cells

To try to correlate adherence with particular integral membrane components, adhesion-related antigens were isolated from [^{14}C]glucosamine-labeled adherent, nonadherent, and BudR-treated cells using the purification scheme developed in this laboratory (Fig. 3) to isolate surface membrane glycoproteins involved in cellular adhesion of both hamster fibroblasts [7] and mouse epithelial cells [8]. The blocking assay (Fig. 1) was used to detect antigens of interest. In

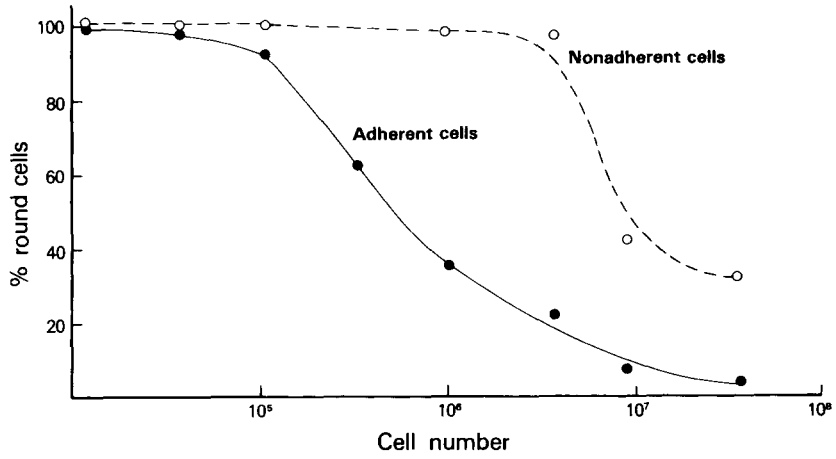

Fig. 4. Comparison of the ability of adherent cells and nonadherent cells to remove cell rounding activity from anti-GP serum. Different numbers of adherent cells (• — •) and nonadherent cells (o---o) were incubated at 4°C with anti-GP serum (1:1,000) for 1 h. The absorbed antiserum was then tested for its ability to round adherent cells.

TABLE I. Blocking Activity From Adherent and Nonadherent Hamster Melanoma Cells

	NP40 extract			Lens eluate			
Cells	Total μg protein (10^{-3})	μg protein to block	Total blocking units[a]	Total μg protein	μg protein to block	Total blocking units	(%) recovery of activity
Adherent	136	14	9,714	50	0.15	333	3.4
Non-adherent	136	200	680	40	2.00	20	2.9
BudR-treated	136	11	12,363	56	0.12	466	3.7

[a]One unit of blocking activity is defined as the minimum amount of protein required to inhibit the effect of anti-M in the blocking assay. The total blocking units are calculated by dividing the total amount of protein in a cell fraction by the minimum amount of protein from that fraction required to inhibit the effect of anti-M in the blocking assay.

each case, approximately 2×10^9 cells were extracted; this yielded a similar amount of protein. A total of 136 mg of protein from the extract of each cell type was fractionated according to Figure 3. At the end of the purification protocol, the various fractions generated were tested for activity in the anti-M blocking assay and were assayed for protein content. The results (Table I) indicate that the NP40 extract of the adherent cells contained about 14 times more total blocking activity than the extract from the nonadherent cells. On the other hand, the NP40 extract of cells induced to adhere with BudR expressed similar blocking activity to that extracted from the normally adherent cells. The small amount of blocking activity that could be detected in the NP40 extract of nonadherent cells was recovered in the same lectin-purified fraction as the blocking activity from adherent cells. Blocking activity was not detected in any other fraction in the three cell types suggesting that the biochemical characteristics of molecules responsible for the blocking activity do not differ greatly between adherent and nonadherent cells. Whereas the percentage of recovery of blocking activity after the purification protocol is similar for adherent, nonadherent, and BudR-treated cells, the absolute amount of blocking activity in the lectin-purified fraction was approximately 20-fold reduced in the nonadherent cells when compared with either of the adherent cell types. Between 0.12–0.15 μg of protein of lectin-purified material from either type of adherent cell was required to block the effects of anti-M serum in the blocking assay, whereas 2 μg of protein of lectin-purified material from the nonadherent cells was required to produce the same blocking effect.

Comparison of SDS-PAGE Analysis of Lectin-Purified Material From Adherent and Nonadherent Hamster Melanoma Cells

Material from each of the three cell types eluted from the immobilized Lens culinaris lectin (last step in the purification scheme) was analyzed by SDS-PAGE. Because of the limited amount of ^{14}C-labeled material that was recovered after fractionating the extract of nonadherent cells, only ~3,000 cpm were available for SDS-PAGE analysis. A similar number of ^{14}C counts from the lectin-purified fraction from adherent and BudR-treated cells was applied for the purpose of comparison (Fig. 5). The total amount of blocking activity, however, differed

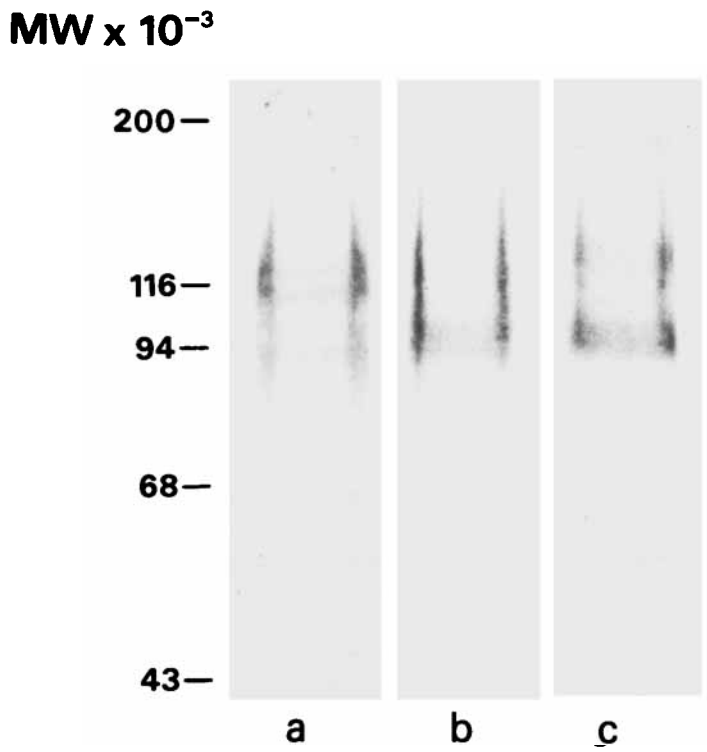

Fig. 5. SDS-PAGE analysis of lectin-purified glycoproteins isolated from adherent and nonadherent cells. The autoradiogram depicts the [^{14}C]glucosamine-labeled components in the fractions eluted from immobilized Lens culinaris lectin from (a) adherent cells, (b) nonadherent cells, and (c) adherent BudR-treated cells. The number of cpm in each lane was approximately the same: a) 3,200, b) 3,200, c) 3,900. The amount of protein also did not vary greatly: a) 10μg, b) 24 μg, c) 24 μg. The number of blocking units varied greatly, however: a) 66, b) 12, c) 200. One unit of blocking activity is defined as the minimum amount of protein required to block the effect of anti-M serum in the blocking assay.

greatly. The samples from adherent and BudR-treated cells contained, respectively, 5.5 and 16 times more blocking activity than the sample from the nonadherent cells. Figure 5 shows that the major ^{14}C-labeled components in the lectin-purified fraction from the adherent cell line are in the molecular weight region of 120,000–140,000, with a minor band appearing in the region of 94,000 M_r. In contrast, the major ^{14}C-labeled components in lectin-purified material from the nonadherent cells are found at approximately 90,000 M_r. ^{14}C-Labeled material from the higher molecular weight region is greatly reduced in nonadherent cells. On the other hand, when material purified from nonadherent cells induced to adhere to the substratum by exposure to BudR was analyzed, ^{14}C-labeled components in the higher molecular weight region were again detected. The lower molecular weight band was also detected in this fraction. This might result from the fact that re-ex-

pression of the adhesion phenotype appears to be incomplete (Fig. 2B). Nevertheless, it is clear that the presence of both blocking activity and the adherent phenotype best correlates with the presence of glycoproteins in the region of 120,000–140,000 M_r. Further labeling with ^{125}I (chloramine T method) of the lectin-purified material from the three cell types confirmed that the ^{14}C-labeled components were the only major constituents present (data not shown).

DISCUSSION

The maintenance of cell–substratum adhesion and cellular morphology is a complex process involving the coordinated behavior of molecular components from three compartments: intracellular, membraneous, and extracellular. A perturbation of some critical component, or a deficiency or defect in a component, in any of the three compartments, results in alterations in cell–substratum adhesion or cell morphology. Intracellular and extracellular molecules implicated by both perturbation and localization studies to be involved in cellular adhesive processes include cytoskeletal elements [14–17] and fibronectin [2,review; 18,19], respectively. However, while integral membrane constituents critical to the coordination of the extracellular and intracellular adhesion molecules have been postulated [15, 19], their identity has remained unresolved. By an approach that combines the use 1) of a heterologous anti-membrane antiserum that specifically perturbs adhesion and 2) an antibody neutralization (blocking) assay to detect relevant antigens, we previously identified a highly restricted population of glycoproteins that appears to be important in the maintenance of cell-substratum adhesion of both hamster fibroblasts [7,11] and mouse epithelial cells [8,11]. SDS-PAGE analysis of the purified glycoproteins showed a single, diffuse [^{14}C]glucosamine-labeled band migrating with apparent subunit molecular weight of about 140,000. Further labeling of this fraction with ^{125}I revealed two to three separate bands in the 120,000–160,000 M_r region but no additional bands outside of this region. To date, attempts to fractionate these glycoproteins further have been unsuccessful. In addition, because the blocking activity is irreversibly destroyed by the conditions used for SDS-PAGE, blocking activity cannot be recovered from any region of the gel or by combining material from any regions of the gel. Since we do not as yet have a monospecific antibody that will perturb cell–substratum adhesion, we cannot identify the adhesion glycoprotein(s) by antibody staining of the gel. Therefore, the relevance of the components in the 120,000–140,000 M_r region have to be confirmed by indirect means at this time.

To gain further insight into the identity of the surface membrane molecules critical to cell–substrate adhesion and to provide more evidence for a role in adhesion for the 12,000–140,000 M_r glycoproteins, we extended our studies to include a nonadherent variant of a hamster melanoma cell line. Cell–substratum adhesion of these cells could not be induced by a variety of extracellular adhesion-related components, which suggested to us that the failure of the cells to adhere might result from a missing or altered component in the membrane and not simply from a lack of some extracellular matrix component. In fact, we show in this paper that the nonadherent cells display a greatly reduced expression of surface membrane adhesion antigens. This is demonstrated in two ways. It is shown that the nonadherent cells have a greatly reduced ability to absorb rounding and detaching activ-

ity from anti-GP serum when compared with either the adherent cells (Fig. 4) or to the nonadherent cells induced to adhere to the substratum by BudR (data not shown). It is also demonstrated that greatly reduced anti-M blocking activity is extracted from the nonadherent cells as compared with either type of adherent cell (Table I). It is not possible by our assays to determine whether the reduction in antigenic activity and the loss in functional activity is due to a reduced amount of adhesion antigens on the surface of the nonadherent cells or to either a greatly altered antigen or an antigen that is not organized in a functional form. It is known that the reduction in antigenic activity and the loss of cellular adhesion in the nonadherent cells is not due to an increase in proteolytic activity since co-culturing adherent and nonadherent cells has no effect on the morphology of either cell types (data not shown). In addition, mixing NP40 extracts of the nonadherent and adherent cells in the absence of proteolytic inhibitors does not affect the blocking activity present in the extract of the adherent cells. It remains possible, however, that some cell-associated protease destroys the functional activity of the adhesion antigen without totally destroying its ability to block the effects of anti-M serum in the blocking assay.

To help resolve the question as to whether glycoproteins in the region of 120,000–140,000 M_r were involved somehow in the maintenance of cell adhesion and cell morphology, extracts of the adherent cells, the nonadherent cells, and the cells induced to adhere with BudR were subjected to the same purification scheme (Fig. 3). The material eluting from the Lens culinaris column was analyzed by SDS-PAGE to detect any differences between the nonadherent cells and the adherent cells and, if differences did exist, determine whether they were eliminated when adhesion was induced in the nonadherent cells. It was reasoned that such comparisons might suggest which glycoproteins are responsible for anti-M blocking activity and, therefore, are likely to be involved somehow in cellular adhesion. SDS-PAGE analysis of the lectin-purified material extracted from the three cell types and enriched approximately 100-fold in anti-M blocking activity showed that [^{14}C]glucosamine-labeled glycoproteins in the molecular weight region of 120,000–140,000 M_r are the major components in the normally adherent cells, that these components are greatly decreased in the nonadherent variant, and that 120,000–140,000 M_r glycoproteins are again present in the nonadherent cells when they are induced to adhere to the substratum by the presence of BudR. The observation that the presence of glycoproteins of 120,000–140,000 M_r correlates with the adhesive properties of cells adds further support to our contention that this group of glycoproteins plays some as yet unknown but important role in the adhesive process.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Wendy Caudill and Elizabeth Shea and the help of Helen Schorr in preparing the manuscript. The experiment depicted by Figure 4 was done by Karen Butner, a graduate student working briefly in the laboratory. We especially thank Dr. Kathleen Bechtol for her valuable comments on this manuscript. This work was supported by National Cancer Institute grants CA-19144, CA-21124, CA-10815, CA-27909, and CA-16517 and American Cancer Society grant IM-53B.

REFERENCES

1. Yamada KM, Olden K: Nature (London) 275:179, 1978.
2. Grinnell F: Int Rev Cytol 53:65, 1978.
3. Hahn LE, Yamada KM: Cell 18:1043, 1979.
4. Pena SDJ, Hughes RC: Nature (London) 276:80, 1978.
5. Harper PA, Juliano RL: J Cell Biol 87:755, 1980.
6. Wylie DE, Damsky CH, Buck CA: J Cell Biol 80:385, 1979.
7. Knudsen KA, Rao PE, Damsky CH, Buck CA: Proc Natl Acad Sci USA 78:6071, 1981.
8. Damsky CH, Knudsen KA, Dorio RJ, Buck CA: J Cell Biol 89:173, 1981.
9. Farishian RA, Whittaker JR: Arch Biochem Biophys 198:449, 1979.
10. Avdalovic N, Aden D: AACR Proceedings, abstract #780, 1978; pp 195.
11. Damsky CH, Knudsen KA, Buck CA: J Cell Biochem 18:1, 1982.
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
13. Laemmli UK: Nature (London) 227:680, 1970.
14. Wehland J, Osborn M, Weber K: J Cell Sci 37:257, 1979.
15. Geiger B: Cell 18:193, 1979.
16. Geiger B, Tokuyasu KT, Dutton AH, Singer SJ: Proc Natl Acad Sci USA 77:4127, 1980.
17. Badley RA, Woods A, Carruthers L, Rees DA: J Cell Sci 43:379, 1980.
18. Birchmeier C, Kreis TE, Eppenberger HM, Winterhalter KH, Birchmeier W: Proc Natl Acad Sci USA 77:4108, 1980.
19. Wen-Tien C, Singer SJ: Proc Natl Acad Sci USA 77:7318, 1980.