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INITIAL STUDIES OF THE MOLECULAR ORGANIZATION OF THE CELL-SUBSTRATE ADHESION SITE

MARTHA K. CATHCART * and LLOYD A. CULP **

Department of Microbiology, Case Western Reserve University, Cleveland, OH 44106 (U.S.A.)

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Summary

Using selective extraction reagents and non-penetrating probes, studies have been initiated on the molecular organization of substrate-attached material, adhesion sites which pinch off from the cell surface of normal Balb/c 3T3 or SV40-transformed Balb/c 3T3 (SVT2) cells and which remain bound to the serum-coated substrate during EGTA-mediated detachment of cells. Extraction of SVT2 adhesion sites with non-ionic detergents resulted in (a) only small amounts of leucine-radiolabeled protein and glucosamine-radiolabeled polysaccharide being solubilized; (b) selective solubilization of 80% of the adhesion site actin, and (c) solubilization of 95% of the phospholipid from these membranous pools. ATP in combination with potassium chloride extracted 60% of the actin. The 3T3 and SVT2 adhesion site proteins which are accessible to lactoperoxidase-catalyzed iodination were also determined. Many of the serum-derived proteins, bound to the substrate, were iodinated during iodination treatment of serum-coated or substrate-attached material-coated substrates, whereas the cellular proteins in the adhesion sites were not iodinated even though they were present in larger quantity as revealed by Coomassie blue staining. Iodination of cells, followed by their EGTA-mediated detachment and reattachment to fresh serum-coated substrates, indicated that the principal iodinated cell surface component deposited in new adhesion sites is the large

* Present address: Immunology Research, Cleveland Clinic Foundation, Cleveland, OH 44106, U.S.A.
** To whom correspondence should be directed.
Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; LETS, large external transformation-sensitive glycoprotein [33], also commonly referred to as fibronectin [34]; SDS, sodium dodecyl sulfate; SV40, Simian virus 40; TEMED, *N,N,N',N'*-tetramethylenediamine.

external transformation-sensitive glycoprotein (even though large external transformation-sensitive glycoprotein is not the only principal iodinated cell surface component of these cells). These studies further establish the selective enrichment in this adhesive material of specific cell surface components and indicate that they are tenaciously bound at the interface between the serum coating and the undersurface of the adhesion site membranous pools.

Introduction

Detachment of cells from their tissue culture substrate with the Ca^{2+}-specific chelating agent EGTA leaves the cell-substrate adhesion sites bound to the substrate [1,2]. These sites are pinched off from the cell as it rounds up and detaches. Oncogenic virus-transformed cells have been reported to exhibit altered adhesion properties in vitro [3—7], and the study of cell-substrate adhesion sites from normal and transformed cells may help to determine the molecular basis for this altered adhesion. First, however, it is necessary to investigate the general molecular organization of the adhesion site.

The adhesion site material has been biochemically characterized and several of its components have been identified including the LETS glycoprotein, microfilament- and filament-associated proteins, several distinct glycosaminoglycans and many phospholipid moieties [8—11]. To approach the problem of determining the general organization of the adhesion site components on the substrate, we studied the relative extractability of the protein and polysaccharide components from SVT2 adhesion sites through the use of many selectively acting reagents. These reagents have been shown to selectively dissociate red cell membrane components [12,13]. In addition, a cell surface-specific protein radiolabeling technique was used to determine whether any of these protein components are exposed in this adhesive material.

Materials and Methods

Cell growth

Balb/c 3T3 (clone A31) and SV40-transformed Balb/c 3T3 (clone SVT2) murine cell lines have been described previously [14]. The cells were maintained in tissue culture between their tenth and twentieth passages. Cells were routinely cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2 in minimal essential medium containing a four-fold concentration of vitamins and amino acids supplemented with streptomycin (0.25 mg/ml), penicillin (25 units/ml), and 10% donor calf serum. For experimental purposes cells were grown in plastic tissue culture dishes. The cells were determined to be free of Mycoplasma according to a radiolabeling assay [14].

Radiolabeling procedures

Protein radiolabeling. Cells were inoculated into tissue culture dishes for 6 h and then the medium was changed to medium containing a decreased concentration of leucine and either 5 μCi/ml L-[4,5-^{3}H]leucine (60 Ci/mmol) or 0.5 μCi/ml L-[U-^{14}C]leucine (348 Ci/mol) for 72 h as described previously [9].

Cell, substrate-attached cellular, and substrate-attached serum proteins were radiolabeled by lactoperoxidase- catalyzed ^{131}I iodination using glucose oxidase as the hydrogen peroxide generator. The technique was modified from Schenkein et al. [15]. Radiolabeling prior to cell removal was performed on cells which covered approximately 80% of the tissue culture substrate. Subsequent to three rinses in phosphate-buffered saline containing 100 mg/l of $MgCl_2$ and 100 mg/l $CaCl_2$, the cells were exposed to radiolabeling medium for 10 min. The radiolabeling medium consisted of lactoperoxidase (0.2 mg/ml), KI (10^{-6} M), $Na^{131}I$ (100 μCi/ml, carrier free), glucose oxidase (0.1 unit/ml) and glucose (5 mg/ml) in saline containing the divalent cations. After the 10 min incubation period, the medium was removed and the cells were rinsed with saline containing 10^{-3} M KI. Cell and substrate-attached fractions were isolated for further analysis. To radiolabel substrate-attached material subsequent to cell removal, cells were detached with EGTA, the substrate was rinsed once with saline and twice with distilled water, and exposed to radiolabeling medium as for cells above. The substrate-attached material was then solubilized with SDS for further study. Adsorbed serum components were prepared by incubating tissue culture dishes with medium containing serum (without cells), for the same period of time as those with cells. They were treated with EGTA and rinsed in the same manner as for substrate-attached material preparations. The adsorbed serum proteins were then radiolabeled by lactoperoxidase-catalyzed ^{131}I iodination as indicated previously.

Polysaccharide radiolabeling. Cells were inoculated into fresh dishes and incubated for 6 h in non-radioactive medium. After this period the medium was changed to medium containing 1 μCi/ml D-[1-^{3}H]glucosamine (specific activity: 7.3 Ci/mmol).

Preparation of cellular and substrate-attached fractions

Cells. Cells, still attached to the tissue culture substrate, were gently rinsed three times with saline and then released by incubation in EGTA (0.5 mM in saline) for 30 min on a gyratory shaker (37°C). The cell suspension was carefully pipetted over the tissue culture substrate surface to ensure detachment of all cells. The cells were then pelleted out of the EGTA solution by centrifugation and were then washed with saline for subsequent analyses.

Substrate-attached material. Following cell release with EGTA, the tissue culture substrates were rinsed once with phosphate-buffered saline and then twice with distilled water. The substrate-attached material was then quantitatively extracted with 0.2% SDS (w/v, in distilled water) by incubating at 37°C for 30 min on a gyratory shaker.

Extraction of substrate-attached material

To extract adhesion site components using selectively acting reagents, the substrate-attached material layer, subsequent to saline and water washes, was incubated for 30 min at 37°C on a gyratory shaker with the extracting reagent. After this incubation the extracting solution was withdrawn, the tissue culture plate was rinsed several times with saline and distilled water, and the remaining material was solubilized with 0.2% SDS. Amounts of material solubilized or resistant to the reagent were determined as described in Results.Phenylmethyl-

sulfonyl fluoride (PMSF, 2 mM), a serine protease inhibitor, was added to the extracting solutions.

Polyacrylamide gel electrophoresis

Electrophoresis of samples on SDS-polyacrylamide slab gels was performed according to the ORTEC method as described previously [9]. Substrate-attached material extracts were concentrated approximately 1000-fold by vacuum dialysis, dialyzed overnight against sample buffer, and electrophoresed on 8, 12 or 20% polyacrylamide slab gels. Following electrophoresis, the gels were impregnated with 4,5-diphenyloxazole and fluorographed according to the quantitative method of Laskey and Mills [17]. The radioactivity present in gel bands was quantitated by scanning the fluorogram with a Joyce-Loebl microdensitometer; peaks were then cut out and weighed.

In the samples containing NP40 or Triton, most of the detergent was removed before electrophoresis by extracting the sample twice with ether. This was necessary because these detergents caused distortion of the protein banding patterns. Ether extraction was shown to remove 95% of the detergent leaving 99—100% of the protein in the water phase. In ,control experiments, ether extraction of substrate-attached material solubilized in SDS resulted in no change in the electrophoretic banding pattern of adhesion site proteins.

Materials

Materials were purchased from the following sources: acrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED), and bisacrylamide from Eastman Organic Chemicals; dimethylsulfoxide from Fisher Scientific Co., ; D-[1-^{3}H]-glucosamine, L-[4,5-^{3}H]leucine and L-[U-^{14}C]leucine from Amersham/Searle Corp.; Nonidet P40 from Particle Data Laboratories, Ltd.; rabbit skeletal muscle myosin and actin from Abbot Clark, Dr. P. Vignos or Dr. R. Lasek, 2,5-diphenyloxazole (PPO) from Research Products International; sodium deoxycholate from Fisher Scientific; SDS from BioRad Laboratories; Triton X-100 from Research Products International; Tween from Schwartz Mann; sodium[^{131}I]iodide from E.R. Squibb and Sons, Inc.; lactoperoxidase, B grade lyophilized, from Calbiochem. Corp.; glucose oxidase from Boehringer Mannheim, Inc.; MEM X4 from Grand Island Biologicals Co.; donor calf serum from Kansas City Biologicals.

Results and Discussion

Substrate adhesion sites are tenaciously bound

The results presented in Fig. 1 reveal that the kinetics of release of proteins from the SVT2 adhesion site vary with the extracting reagent. All of the reagents except 5 M urea achieved maximal extraction levels in 5—15 min under these conditions. The bile salt, sodium deoxycholate only extracted approximately 40% of the [^{3}H]leucine-radiolabeled material. On the other hand, the three non-ionic detergents, nonidet P40 (NP40), Triton X-100 (Triton), and Tween 40, did not extract more than 7% of the total radiolabeled protein.

The chemically similar non-ionic detergents NP40 and Triton were capable

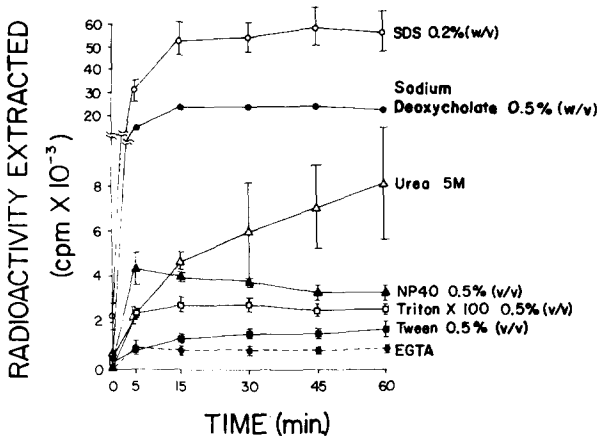

Fig. 1. Kinetics of reagent extraction of adhesion site protein. SVT2 cells were radiolabeled with [^{3}H]-leucine for 72 h and substrate-attached material was prepared as indicated in Materials and Methods. This material was extracted with various reagents at 37°C on a gyratory shaker and small aliquots were withdrawn at several time points to determine amounts of radioactivity solubilized. The radioactivity in each fraction was determined, adjusted for volume and plotted versus time. The data are from a representative experiment with duplicate samples (as shown by error bars). The sodium deoxycholate data are from a representative experiment without duplicate samples.

of extracting approximately 25% of the [^{3}H]glucosamine-radiolabeled material (Fig. 2), most of which is glycosaminoglycan [8,10]. Urea which is noted for its ability to perturb protein conformation was not an efficient extractor of glucosamine-radiolabeled material. SDS was the only reagent tested which effectively removed all of the protein and polysaccharide components from the substrate as determined by complete combustion of the plastic and detection of $^{14}CO_2$ or ^3H_2O from leucine-radiolabeled cultures (Cathcart, M.K. and Culp, L.A., unpublished results).

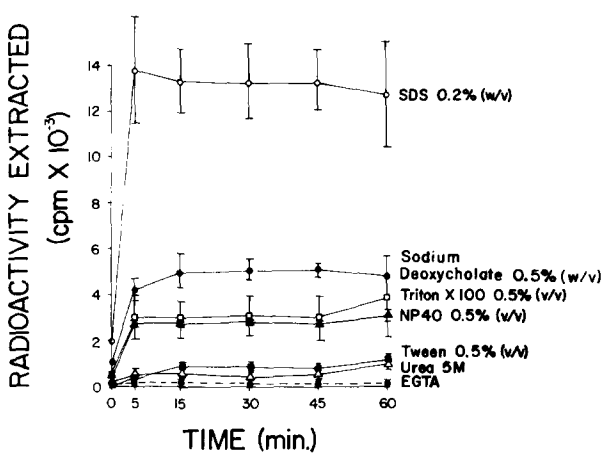

Fig. 2. Kinetics of reagent extraction of adhesion site polysaccharide. SVT2 cells were radiolabeled with [^{3}H]glucosamine for 72 h and substrate-attached material was prepared according to Materials and Methods. The kinetics of extraction of several reagents was determined as in Fig. 1. The data are from a representative experiment with error bars depicting duplicate samples.

The results of the extraction studies presented in Figs. 1 and 2 indicate that most of the protein and polysaccharide in the adhesion sites are tenaciously bound to the tissue culture substrate and require treatment with a strong anionic detergent for their removal. Their binding is not strictly dependent on the integrity of the phospholipid matrix, since non-ionic detergents were minimally effective in solibilizing protein and polysaccharide. The more easily solubilized polysaccharide material may be hyaluronate and chondroitin proteoglycan material which can be enzymatically removed from substrate-attached material with minimal solubilization of heparan sulfate, the LETS glycoprotein and the cytoskeletal proteins [31].

Triton selectively solubilizes a portion of the actin pool

Polyacrylamide slab gel electrophoresis of the adhesion site proteins extracted by Triton and those left adherent subsequent to Triton treatment

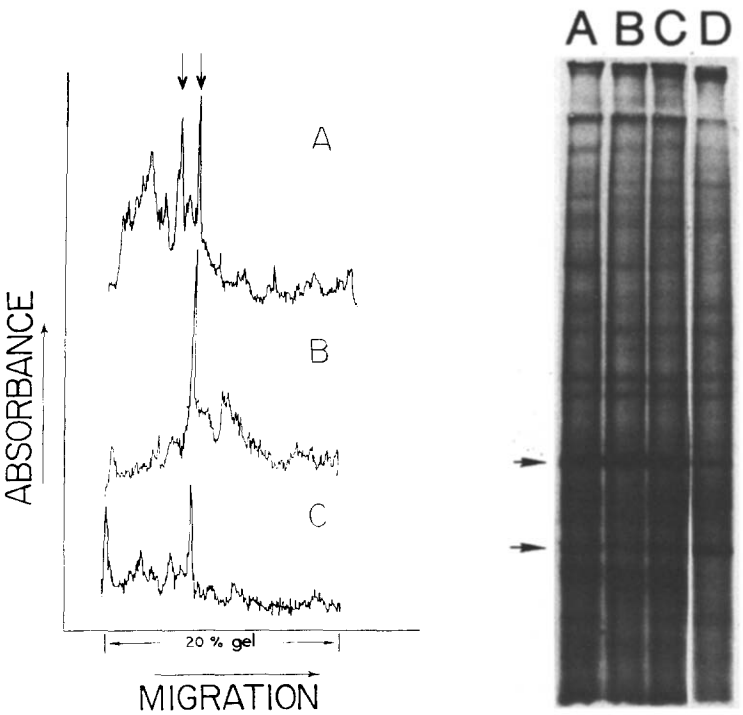

Fig. 3. Selective extraction of actin from adhesion sites. SVT2 substrate-attached material, radiolabeled with [^{14}C]leucine, was extracted with (A) SDS; (B) ATP + KCl, or (C) Triton. The extracts were concentrated, electrophoresed on 20% polyacrylamide slab gels and fluorographed according to Materials and Methods. The fluorographs were scanned on a Joyce-Loebl microdensitometer. The left-hand arrow marks the position of the 56 000 protein and the right-hand arrow marks the actin electrophoretic position as found in substrate adhesion sites.

Fig. 4. Triton extraction of adhesion site protein. SVT2 cells were radiolabeled with [^{14}C]leucine and substrate-attached material was prepared, extracted with the appropriate reagents, and electrophoresed on 12% polyacrylamide slab gels according to Materials and Methods. Well A represents the material resistant to buffer pH 4.0; well B, material resistant to Triton in distilled H_2O, pH 4.0; cell C, material resistant to Triton in saline (pH 7.4), and well D, non-extracted, SDS-solubilized substrate-attached material. The upper arrow marks the position of the 56 000 protein and the lower arrow marks the electrophoretic position of actin in the adhesion sites.

(Triton resistant) revealed that this detergent is capable of extracting relatively few protein components from the adhesion site material. Fig. 3 demonstrates that the major component which Triton extracted was the 42 000 dalton actin component (indicated by the right-hand arrow). This component has been conclusively shown to be actin by several criteria [9]. Comparative analysis of the Triton-resistant material and untreated SDS-solubilized material revealed that Triton extracted approximately 80% of the total adhesion site actin (Table I, Fig. 4). This was observed when Triton was made up in distilled water (final approx. pH 4) or in phosphate-buffered saline (pH 7.4); however, acidic conditions alone were found to extract 60% of the actin component (Table I, Fig. 4). The calculations in Table I were made by monitoring the change in the actin/56 000 protein ratio after extraction. These two proteins were selected because they are two very prominent and well-isolated components on the gel scans, thus minimizing error. Fig. 3C reveals that a very small portion of the 56 000 protein can be solubilized by Triton X-100 (depicted by the left-hand arrow in Fig. 3C); therefore, the values of extracted actin obtained for this reagent from the actin/56 000 protein ratio are slightly lower than the actual values. Triton extraction seems to affect actin uniquely, since the other proteins in substrate-attached material including myosin were not appreciably solubilized.

Although Triton removed only a small portion of the adhesion site protein and polysaccharide components, additional studies revealed that Triton is capable of extracting 95% of the adhesion site [^{32}P]phospholipids [11]. The small portion of the phospholipid which was resistant to extraction was not composed of only one or a few phospholipid classes but contained each of the adhesion site phospholipid species. (This may represent nonspecifically trapped material.) Therefore, binding of protein and polysaccharide in this adhesive material is not dependent on the majority of the phospholipid.

TABLE I

RATIOS OF EXTRACTION-RESISTANT PROTEIN COMPONENTS

SVT2 cells were radiolabeled with [^{14}C]leucine for 72 h and substrate-attached material fractions were prepared as indicated in Materials and Methods. The [^{14}C]leucine-radiolabelled substrate adhesion sites were extracted with 0.5% Triton, ATP + KCl (3 mM, 0.5 M), KCl alone (0.5 M) or ATP alone (3 mM). The resistant material was solubilized with SDS, electrophoresed on 12 or 20% polyacrylamide slab gels and fluorographed. The fluorograph was scanned on a microdensitometer and the appropriate peaks were cut out and weighed for quantitation. The ratios represent the weight of the peak corresponding to actin divided by the weight of the peak corresponding to the 56 000 mol.wt. protein (presumably the 10 nm filament protein). The percentage of actin extracted was obtained by:

$$\left(1 - \frac{\text{reagent-resistant ratio}}{\text{total substrate-attached material ratio}}\right) \times 100$$

Sample	Ratios actin to the 56 000 mol.wt. protein	% actin extracted
Substrate-attached material	1.216	— *
Triton-resistant substrate-attached material	0.259	79
ATP + KCl-resistant substrate-attached material	0.436	64
KCl-resistant substrate-attached material	0.712	41
ATP-resistant substrate-attached material	0.957	21

* When substrate-attached material is extracted with 0.2% SDS, 100% of the proteins are solubilized (see text.

The selective extraction of actin with Triton is interesting in light of the findings of Osborn and Weber [18] and Brown et al. [19], who reported that treatment of murine 3T3 or chick embryo fibroblasts with 0.5% Triton results in the extraction of most of the cell protein (approximately 80%), leaving a cytoskeleton containing actin attached to the tissue culture dish. It was determined that all of the non-extracted actin was present in bundles of filaments; no separate 7-nm actin filaments were detected in this material [19]. As a result of their experimental observations, Brown et al. [19] proposed that 50% of the cellular actin may be organized in bundles. Furthermore, Hynes and Destree [20] have reported that 50% of the actin remains bound subsequent to extraction of substrate-bound NIL cells with the chemically similar non-ionic detergent NP40. All of these investigators have found that a 55—58 000 protein (most likely the 10 nm filament protein [19]) was resistant to these extraction conditions. Our results agree with these studies in finding two classes of actin in substrate-attached material; one soluble in Triton and another resistant to Triton extraction. However, the quantities of actin in each of these classes is very different from whole cells studies. Unlike actin the 56 000 protein in this material, which appears to be the subunit protein of 10-nm filaments [1], resists extraction by Triton as well as extraction by ATP + KCl (see below). It has been suggested that the small amount of actin, resistant to extraction procedures, is linked to the non-extractable 10-nm filaments [21,22].

ATP + KCl extracts adhesion site actin

Non-muscle cell actin is generally obtained by extraction with concentrated KCl or by first acetone drying to denature myosin and then treating with ATP and a reducing agent [23]. ATP dissociates actin and myosin as well as serving as the energy source which drives their reaction. Substrate-attached adhesion sites were extracted with various concentrations of KCl, ATP, ATP + KCl and ATP + mercaptoethanol. The most effective of these extraction procedures for maximally solubilizing actin proved to be ATP (3 mM) + KCl (0.5 M) at pH 8.0 (Fig. 3B). Experiments using KCl alone or ATP + KCl buffers indicated that ATP and KCl have an additive effect on the extraction of actin from the adhesion site (Table I, Fig. 5). KCl extracted approximately 40% of the total actin, ATP alone extracted about 20% and ATP + KCl extracted approximately 60%. Neither AMP + KCl nor GTP + KCl (Fig. 5) extracted more than KCl alone. AMP alone, GTP alone and the buffer (pH 8.0) did not extract detectable levels of actin.

Results of sequential treatments of substrate-attached adhesion sites with Triton followed by ATP + KCl (and vice versa) revealed that the actin extracted by ATP + KCl is included as part of the actin which is solubilized by Triton. ATP + KCl extraction, like Triton, uniquely affects the actin component. The 56 000 molecular weight protein (indicated by the left arrow in Fig. 3) is relatively resistant to Triton or ATP + KCl treatment.

ATP + KCl most likely acts on F -ctin-myosin and -tropomyosin interactions since solutions of KCl have been reported to dissociate tropomyosin from actin filaments [24] and ATP is an inhibitor of the F actin-myosin interaction [25]. ATP + KCl may be unable to extract the actin which is protected by or in association with the lipid matrix and therefore Triton, which solubilized the lipids,

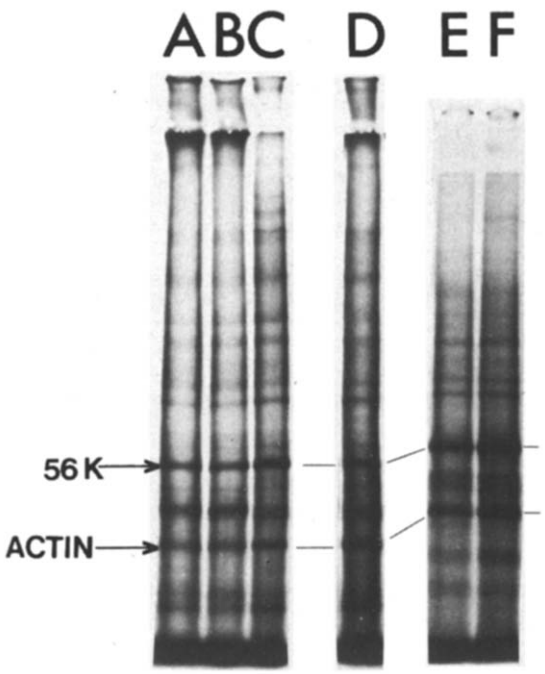

Fig. 5. ATP + KCl extraction of adhesion site protein. SVT2 substrate-attached material, radiolabeled with [^{14}C]leucine, was extracted with reagents and the reagent-resistant material was solubilized with SDS, electrophoresed on 12% polyacrylamide gels and fluorographed according to Materials and Methods. The samples consist of the resistant material following treatments with: (A) GTP + KCl; (B) AMP + KCl; (C) KCl; (E) ATP, and (F) ATP + KCl. The sample in well D is unextracted and SDS-solubilized adhesion site material. The upper arrow marks the 56 000 component and the lower arrow marks the electrophoretic position of rabbit skeletal muscle actin marker and actin in substrate-attached material.

can solubilize the ATP + KCl extractable actin and an additional 20% of the total actin. Or, it is possible that the additional Triton-extractable actin is due to the solubilization of surface-associated, non-polymerized G actin. Some actin associated with the cell surface may be non-filamentous [25]. Our findings are similar to those of Gruenstein et al. [32] who found that extraction of 3T3 and HeLa cell membranes, under conditions favoring the depolymerization of actin, resulted in the extraction of 80% and 60% respectively, of the membrane-associated actin. In these respects the actin in adhesion sites behaves like membrane-associated actin. No myosin was solubilized with the ATP + KCl or Triton. This may indicate that myosin is not bound strictly to structures containing actin.

Cells impair iodination of substrate-attached proteins

Cells do not adhere directly to their in vitro tissue culture substrates. Instead, they adhere to a layer of serum components adsorbed to the substrate from the medium [27—30]. Lactoperoxidase catalyzed ^{131}I incorporation into substrate-attached material (cellular adhesion sites plus adsorbed serum components) prior to removal of cells was found to be only approximately 10% of the levels found when the iodination was performed on serum-adsorbed substrates

TABLE II

IODINATION OF SUBSTRATE-ATTACHED CELLULAR AND SERUM PROTEINS

SVT2 cells were grown until approximately 80% of the tissue culture substrate was covered with cells. I. The cells were radiolabeled with ^{131}I as indicated in Materials and Methods, than detached with EGTA and the adherent material was solubilized with SDS. II. Substrate-attached material was iodinated following EGTA-mediated cell detachment and then solubilized with SDS. III. Adsorbed serum components were radiolabeled with ^{131}I as indicated in Materials and Methods with complete or incomplete radiolabeling medium as indicated. The radioactivity incorporated into each fraction was determined. The data are from a representative experiment with triplicate samples. The error is approximately ±10%. —, experiments were not done.

Radiolabeling conditions	Complete labeling medium (cpm/35 mm dish)	Incomplete labeling medium	
		Without glucose oxidase	Without lactoperoxidase
I. Before cell detachment	88 266	—	—
II. After cell detachment	751 924	—	—
III. Adsorbed serum layer only	693 091	34 092	35 337

alone or on substrate-attached material after EGTA-mediated detachment of cells (Table II). This quantitative difference seems to indicate that the cells block the iodination of protein components in the substrate-attached cellular or serum-adsorbed material, probably by making the enzymes inaccessible to these potential substrates.

The control experiments (Table II), consisting of iodination in the absence of lactoperoxidase or glucose oxidase, indicate that the iodination is a specific enzyme-mediated radiolabeling. Only 5% or less of the total incorporation of ^{131}I took place in the absence of the enzymes. The iodination studies were performed on both the Balb/c 3T3 and SVT2 cells; however, no differences were discovered between these cell lines. The data presented here are from work with the SVT2 cell line.

Adhesion site surface proteins differ from those of whole cells

In addition to quantitative differences in incorporated ^{131}I into substrate-attached components in the two radiolabeling conditions, qualitative differences in the protein components are major. Those proteins iodinated in adhesion sites subsequent to cell removal cannot be distinguished from the iodinated adsorbed serum proteins (Fig. 6B and C). On the other hand, adhesion sites iodinated prior to cell detachment (Fig. 6D) have several iodinated components which differ from the radiolabeled serum components, and these proteins are present in different proportions from total cell surface proteins (Fig. 6A). This implies that the surface of the adhesion site has a specialized topographical distribution of surface components.

The 220 000 glycoprotein (LETS or fibronectin, indicated by the top arrow in Fig. 6) is a major iodinated species in cells but a minor one in substrate-attached material (Fig. 6A and D). It is interesting that this component which is radiolabeled in cells can subsequently be 'chased' into cell-substrate adhesion sites on reattachment of EGTA-suspended cells to a fresh tissue culture substrate (Fig. 6E), and that it is the major iodinated component in these newly

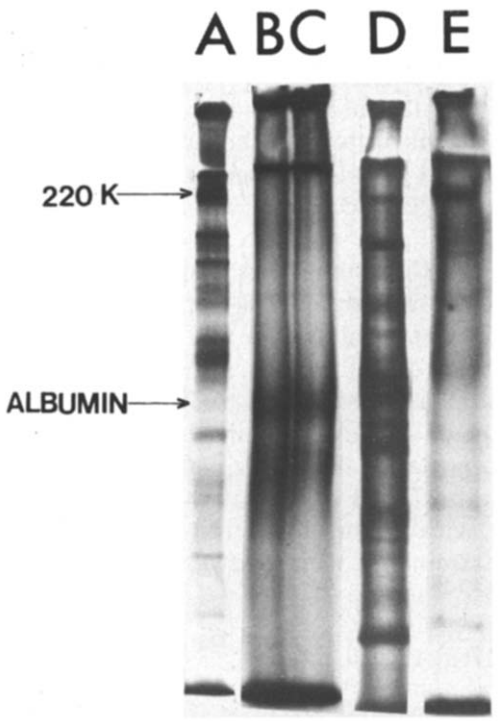

Fig. 6. Iodinated proteins of cell, substrate-attached material and adsorbed serum components. Cell, substrate-attached material and adsorbed serum components (adherent to tissue culture dishes) were radiolabeled by the lactoperoxidase technique as described in Materials and Methods. Samples were solubilized in SDS, electrophoresed on 12% polyacrylamide slab gels and autoradiographed according to Materials and Methods. Wells A and D, cell and substrate-attached material fractions, respectively, from cultures iodinated prior to cell detachment. Well B, adsorbed serum proteins, iodinated on the substrate. Well C, adhesion sites iodinated subsequent to EGTA-mediated cell detachment. Well E, the substrate-attached material fraction obtained after iodinated cells were detached with EGTA and reattached to fresh tissue culture dishes for 2 h. The upper arrow marks the position of the 220 000 LETS glycoprotein and the lower arrow marks the electrophoretic position of purified albumin.

formed adhesion sites even though it is not the only major iodinated component in cells [16]. This glycoprotein is apparently shielded from lactoperoxidase-catalyzed iodination once it is incorporated into the adhesion site. The LETS glycoprotein has been strongly implicated as playing an important role in cell-substrate adhesion [1] and our results provide further support for this hypothesis.

The iodination studies reveal that the exposed substrate-attached proteins radiolabeled subsequent to cell detachment cannot be differentiated from adsorbed serum proteins which have been iodinated, whereas radiolabeling prior to cell release results in the iodination of a different spectrum of proteins, several of which coelectrophorese with cell surface proteins. These results indicate that: the quantity of iodinateable serum protein exposed as a result of cell detachment is far greater than the newly exposed adhesion site surface proteins resulting in a masking effect and a predominance of iodinated serum proteins, or upon cell detachment there is an organizational change in the exposed

surface proteins such as to make them unavailable for surface iodination (lipids or dense carbohydrates could protect the protein species). The present study does not establish the validity of either of these two hypotheses. The present study does indicate, since the amount of substrate-attached protein is equivalent to or greater than the amount of serum protein on the substrate [9], that few if any cellular proteins are intercalated into the upper surface of the isolated adhesion sites where they would be accessible to lactoperoxidase-catalyzed iodination.

The results of these studies emphasize the strength of the adhesive interaction between a cell and its tissue culture substrate. It is apparent that actin organization in the adhesion site is different from that in the whole cell. This may merely be due to EGTA disruption upon cell release; however, our results with adhesion site actin indicate a similar organization to 3T3 membrane-associated actin [32]. The distribution of surface proteins in adhesion sites appears to be considerably different from that of the whole cell surface and the iodination studies implicate a role for the LETS glycoprotein in the adhesive interaction. These observations along with others [1,8—11] provide further evidence that the adhesion site is an extremely specialized portion of the cell surface.

Note added in proof: (Received June 27th, 1979)

Recent studies on the cytoskeletal proteins of cell surface envelopes from Sarcoma 180 ascites tumor cells indicate that extraction of cytoskeletal proteins is independent of the presence of a reducing agent. In addition these investigators found that extraction of the membrane envelopes at low ionic strength and alkaline pH resulted in incomplete extraction of actin and no extraction of myosin. (Moore, P.B. et al. (1978) Exp. Cell Res. 115, 331—342) These results correlate well without our observations on actin organization in substrate adhesion sites.

Acknowledgments

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