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Cross-Linked Polypentapeptide of Tropoelastin: an Insoluble, Serum Calcifiable Matrix†

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ABSTRACT: The synthetic, cross-linked polypentapeptide of tropoelastin has been shown to calcify from serum alone even when separated from the serum medium by a dialysis membrane with a low-molecular-weight cut off. By microprobe analysis, it appeared that the only serum elements required for the calcification were calcium and phosphorus. Furthermore, thin sections of the calcified matrix showed the calcification to occur throughout the matrix, and thereby verified that it is a bulk property of the matrix and not an interfacial property. To our knowledge this is the first demonstration of an insoluble, synthetic polypeptide to function as a serum calcifiable matrix and by doing so it opens the door to potential medical applications.

The vascular elastic fiber is a primary site of pathological calcification of the arterial wall (Martin et al., 1963; Urry, 1974b). Tropoelastin, the precursor protein of the fibrous core of the elastic fiber (Smith et al., 1975, 1968; Sandberg et al., 1969), and α-elastin, a chemical fragmentation product of fibrous elastin (Partridge et al., 1955; Partridge and Davis, 1955), exhibit an interesting property that has been utilized in studies on calcification. Both molecular systems are soluble in aqueous solutions at lower temperature. However, the solutions become turbid when the temperature is raised to that of the body and settling occurs to form two phases. The more dense, protein-rich phase is called the coacervate. Because the coacervates are the stable state at body temperature, because they contain a similar volume percent of water as fibrous elastin (Partridge, 1967), and because they exhibit filamentous structures in negatively stained electron micrographs (Cox et al., 1973, 1974; Volpin et al., 1976) with periodicities similar to those of native fibrous elastin (Gotte et al., 1974, 1976), coacervation of tropoelastin is viewed as a key step in elastogenesis and the coacervates are considered to be models for relaxed fibrous elastin (Urry, 1976). Coacervates of α-elastin, in which all carboxylate groups have been methylated and amino groups formylated, have been shown to calcify from a barbital buffer calcifying medium (Starcher and Urry, 1973) and from a serum medium (Starcher et al., 1974; Cox et al., 1975). Coacervates of tropoelastin, free or chemically blocked by O-methylation and N-formylation, calcify from a serum

† From the Laboratory of Molecular Biophysics and the Cardiovascular Research and Training Center, University of Alabama Medical Center, Birmingham, Alabama 35294. *Received March 10, 1976.* This work was supported in part by National Institutes of Health Grant HL-11310.

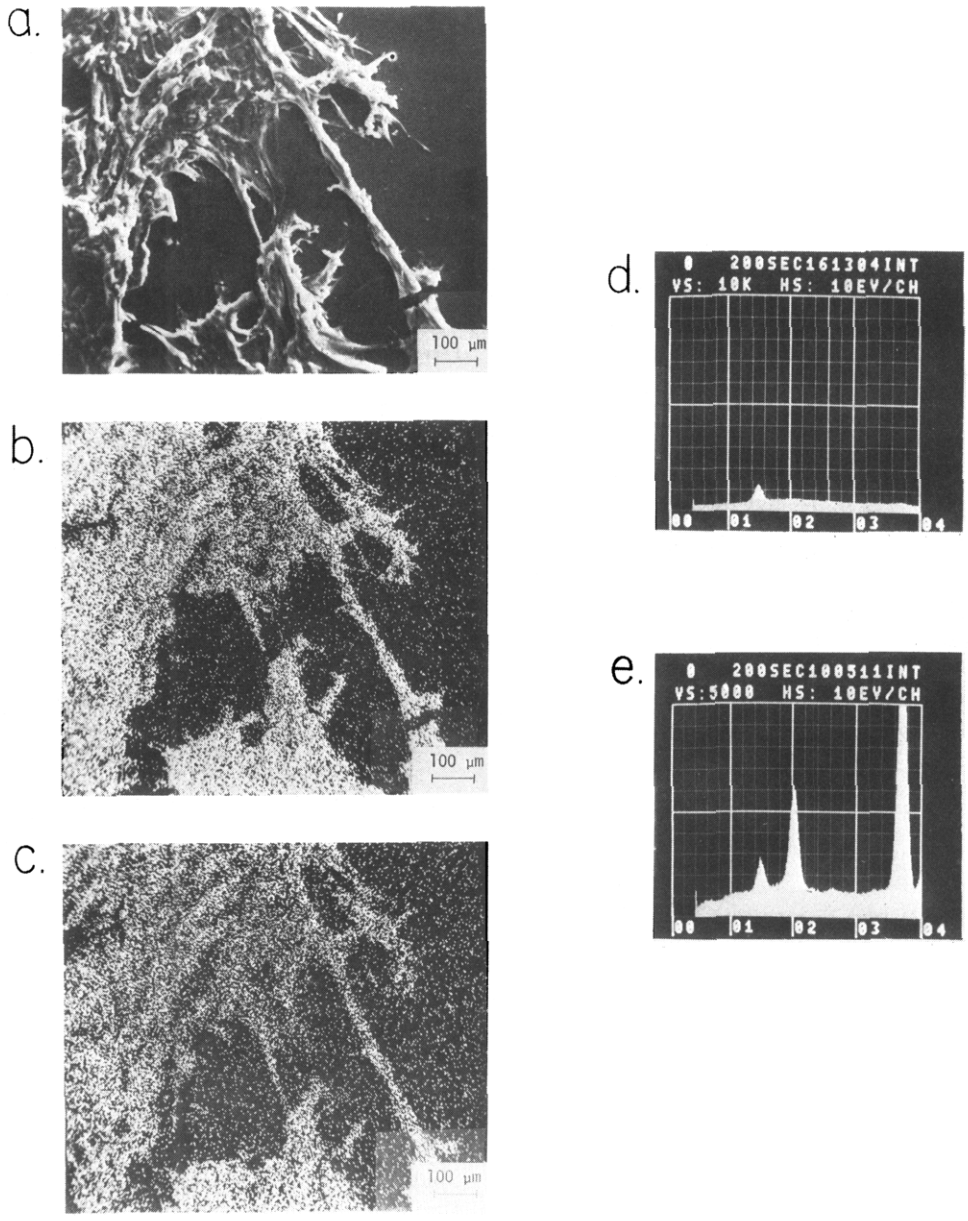

FIGURE 1: SEM, x-ray spectra, and elemental maps of the serum calcified cross-linked polypentapeptide (X-PPP). The sample was calcified from serum containing added 3.0 mM $CaCl_2$ and KH_2PO_4. Figure a is the low magnification (100×) 0° tilt secondary electron image obtained at 25 kV, while b and c are the corresponding calcium and phosphorus elemental maps, respectively. It is evident that the calcium and phosphorus concentrate in the synthetic material. The x-ray spectrum, d, of the background support indicates no elements other than the aluminum coating, while spectrum, e, of the X-PPP has a major calcium peak at 3.69 keV, a major phosphorus peak at 2.01 keV and a peak at 1.4 keV from the aluminum coating.

medium (Starcher et al., 1974), and it has been shown that calcification of blocked α-elastin coacervates in serum occurs throughout the bulk of the coacervate rather than being limited to the aqueous solution–coacervate interface (Urry et al., 1976a).

Recently, tropoelastin has been shown to contain repeating peptide sequences (Foster et al., 1973)—a tetrapeptide (Val_1-Pro_2-Gly_3-Gly_4), a pentapeptide (Val_1-Pro_2-Gly_3-Val_4-Gly_5), and a hexapeptide ((Ala_1-Pro_2-Gly_3-Val_4-Gly_5-Val_6). These peptides, their oligomers, and high polymers have been synthesized in this laboratory and their conformations were extensively studied in solution (Urry and Long, 1976; Urry et al., 1974a, 1975a,b; Urry and Ohnishi, 1974b,c). The polypentapeptide coacervates (Urry et al., 1974b) as does a slight variant of the polyhexapeptide (Urry et al., in preparation). With a degree of polymerization of the order of 20, there remains a very substantial solubility of the polypentapeptide in the equilibrium solution above the coacervate. For this reason it has not proven practicable to attempt calcification studies on the polypentapeptide coacervate. Recently, however, we have synthesized (Okamoto and Urry, 1976) and characterized (Urry et al., 1976b) a cross-linked polypentapeptide coacervate that forms an insoluble matrix suitable for calcification studies using a serum medium.

In the present effort, we report the calcification of the synthetic cross-linked polypentapeptide using a serum medium directly applied to the matrix in one set of experiments and in a second set separated from the matrix by a dialysis membrane.

Methods

In obtaining the cross-linked polypentapeptide, one polypentapeptide was synthesized with one out of about seven Val_4 residues replaced by a Lys residue and a second polypenta-

peptide was synthesized with one out of five Val_4 residues replaced by a Glu residue. The two high polymers were combined and made to flow as a mixed coacervate to which was added 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate to achieve peptide bond formation between the γ-carboxyl of the Glu residue and the ϵ-amino of the Lys residue and the terminal α-amino group. The insoluble product was extensively washed and gave an amino acid analysis of Val, 39.86; Pro, 21.95; Gly, 43.02; Glu, 2.05; Lys, 1.00 (Urry et al., 1976b and Okamoto and Urry, 1976).

Calcification Procedures. The calcification of the cross-linked polypentapeptide was followed in three different systems: (1) in serum with added $CaCl_2$ and KH_2PO_4, (2) in serum alone, and (3) in serum, with dialysis tubing separating the polypentapeptide from the serum. For systems one and two, the synthetic peptide was applied with a spatula to a small Plexiglas block (Rohm and Haas, Philadelphia, Pa.) and allowed to dry with heating at 40 °C. The polypeptide adhered to this support throughout all of the incubation time for each experiment. In the case of system three, the cross-linked material was applied directly to the inside of ¼ in. width dialysis tubing with a 12 000 mol wt cut off (Arthur H. Thomas Co., Philadelphia, Pa.).

In the first set of experiments, 1 ml of sterile bovine serum (Microbiological Associates, Bethesda, Md.), which had previously been incubated at 37 °C for 24 h, was made 1.5 mM in one experiment and 3 mM in another experiment with respect to added $CaCl_2$ and KH_2PO_4 (Starcher et al., 1974). Both salts were added as sterilized aqueous solutions. This serum was added to the polypentapeptide, which had been autoclaved for 20 min at 115 °C for the 1.5 mM ion addition but not for the 3 mM addition. The samples were incubated with shaking at 37 °C for 3.5 days for the 1.5 mM experiment and 2 days for the 3mM experiment. After incubation, the serum was pipetted off and each sample was rinsed two times with 2 ml of 37 °C glass distilled water and 2 ml of 37 °C absolute ethanol. The samples were then dried with heat under vacuum for 60 min and examined with SEM and microprobe analysis. Calcification occurred in all experiments regardless of autoclaving.

In the second set of experiments, the polypentapeptide was incubated at 37 °C with serum alone, in one experiment for 15 days with a total of 7 ml of sterile bovine serum, in another for 7 days with a total of 35 ml of serum, and in another for 14 days with 70 ml of serum. For the last two experiments, the serum was changed daily in 5-ml aliquots. Calcification proceeded in both autoclaved and nonautoclaved polypentapeptide samples.

In the third set of experiments, the synthetic peptide was placed inside dialysis tubing, autoclaved, and incubated at 37 °C against 15 ml of sterile bovine serum changed daily for 7 days. The total volume of serum was 105 ml. As in the first two sets of experiments, controls without polypeptide were also run.

Scanning Electron Microscopy. Microanalysis of Calcified, Cross-Linked Polypentapeptides. Following calcification, the specimens were examined at 30× with a light microscope, dried in a vacuum oven at 60 °C, and then coated with several hundred Angstroms of evaporated aluminum at less than 5×10^{-5} mmHg. Examination in a JEOL JSM-U3 scanning electron microscope was used to assess the extent of calcification, by means of an EDAX Model 707A solid state x-ray detector and analyzer, and to view the surface features of the calcified material. After completing these preliminary checks, the sample was removed from the support used during calcification by lifting small portions loose with a metal probe. These portions were placed in "Beem" capsules, oriented to yield the desired cross-section upon sectioning, and embedded with Araldite 502 supplied by Ladd Research Laboratories, Inc. The epoxy was cured at 50–60 °C overnight in a vacuum oven. After curing, the samples were sectioned to the desired thickness (usually 0.5–1 μm) with an LKB 8800 ultratome using glass knives. The sections were floated on distilled water that was maintained above 40 °C, and collected on 180-mesh carbon-coated nylon grids supplied by E. F. Fullam, Inc. These were immediately air dried (by a heat lamp), mounted on 1-in. diameter carbon stubs, and coated with several hundred Angstroms of aluminum, as described previously. Scanning electron microscopy and x-ray microanalysis of the sections were performed at 25 kV. Elemental mapping and x-ray line profiles were used to determine the distribution of calcification throughout the entire sample cross section, and to define the

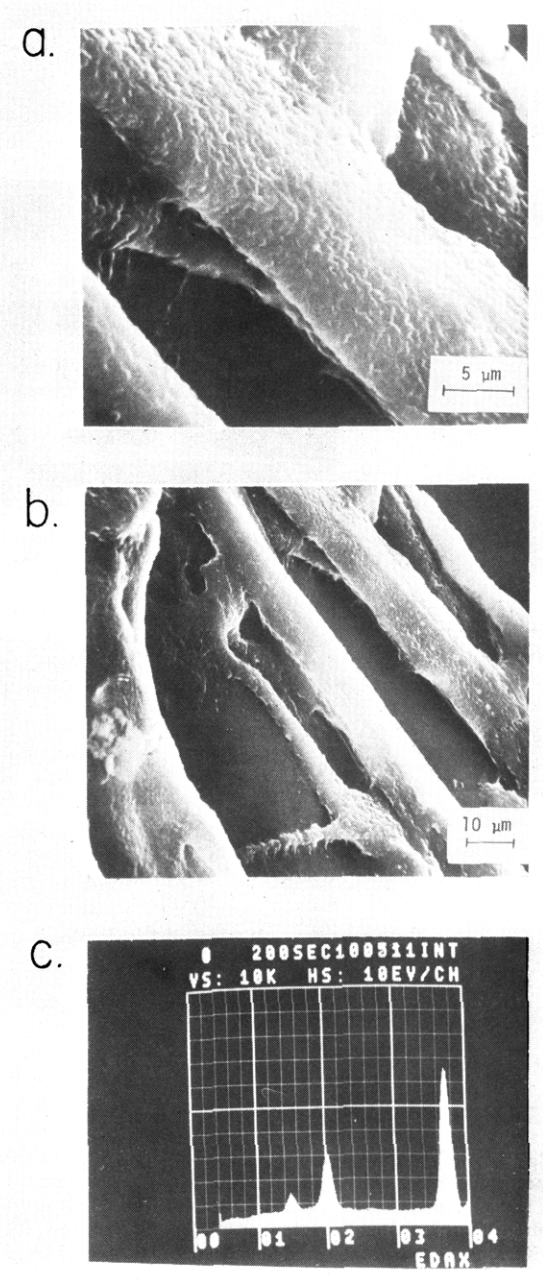

FIGURE 2: SEM and x-ray spectrum of the material in Figure 1, photographed at higher magnification to show the fibrous appearance of the X-PPP when calcified (25 kV, 45° tilt).

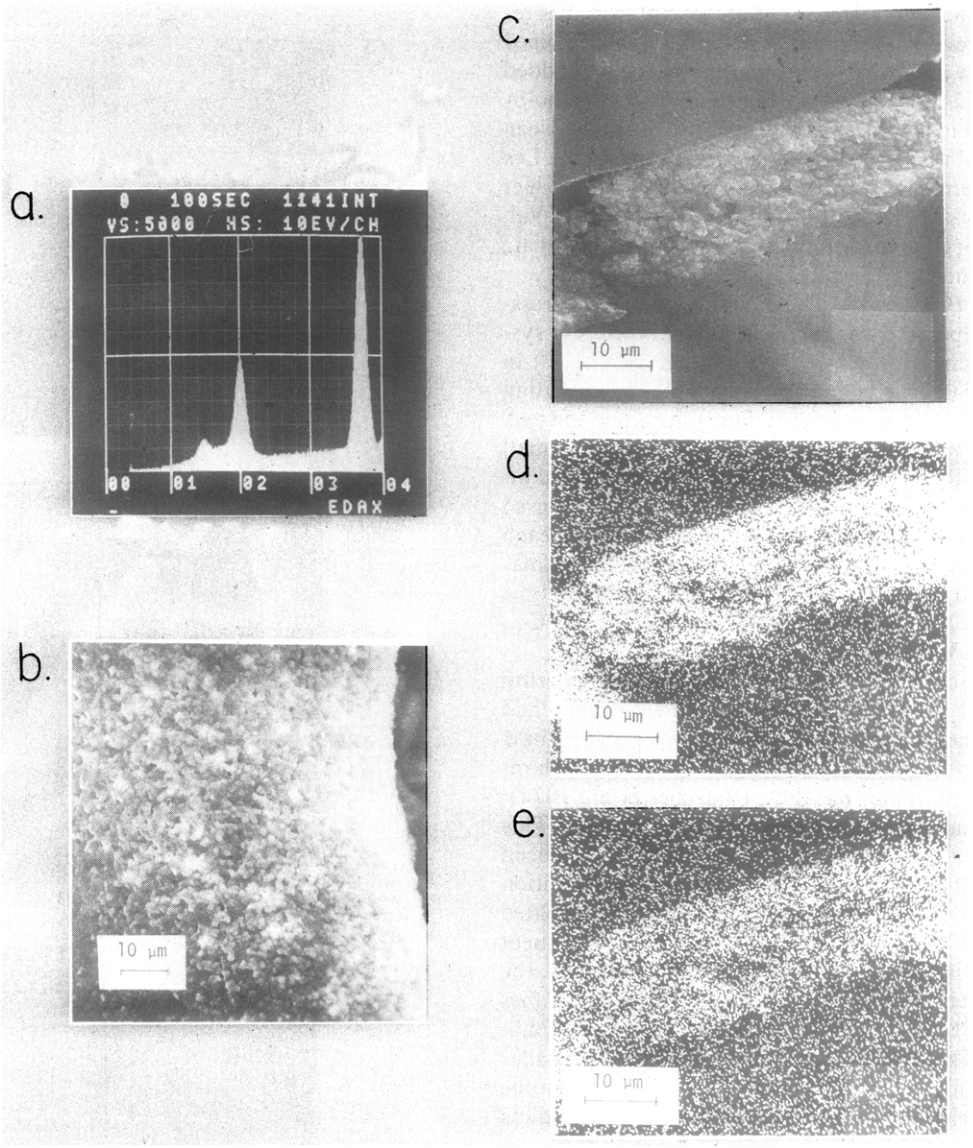

FIGURE 3: SEM, x-ray spectrum, and elemental maps of the calcified X-PPP. The sample was separated from the serum (to which no $CaCl_2$ or KH_2PO_4 had been added) by a dialysis membrane. The x-ray spectrum (a) gives no evidence of any other elements than calcium (3.69 keV), phosphorus (2.01 keV), and aluminum (1.4 keV, from the coating). The 1000× secondary electron image (b), shows the rough surface of the calcified matrix. This granular appearance is throughout the specimen as seen in c, the cross section cut from b. Figures d and e are the calcium and phosphorus elemental maps, respectively, of the thin section seen in c. All data taken at 25 kV, 0° tilt.

occurrence and distribution of other elements heavier than fluorine in the samples.

Results

In studying calcification of the α-elastin and tropoelastin coacervates there is a limitation due to the solubility of the α-elastin in the serum, that is, it is difficult to use large volumes of serum or to repeatedly change the serum for the purpose of providing an adequate calcium phosphate pool because the coacervate is partially solubilized and removed with each serum change. For this reason, only 1 ml of serum was used to which $CaCl_2$ and KH_2PO_4 were added. In the initial studies on the cross-linked polypentapeptide the same conditions were used and calcification proceeded as seen in Figures 1 and 2.

Because the cross-linked polypentapeptide is an insoluble matrix, it is possible to use larger volumes, to change the serum at regular intervals, and thereby to carry out the experiment without added calcium phosphate. All such experiments showed calcification of the polypentapeptide, and, with sufficient time, calcification was observed through the entire depth of the matrix from the serum side to the Plexiglas support side. Electron microprobe analysis commonly showed a weak sulfur peak as in Figures 1 and 2. Presumably, the sulfur peak derived from adhering sulfur containing serum proteins or sulfated mucopolysaccharides.

In order to limit the accessibility of serum macromolecules to the calcifying matrix, the dialysis experiments were initiated. Figure 3 shows the x-ray spectrum of the calcified matrix to be devoid of any detectable elements other than the aluminum coating and the serum-derived calcium and phosphate. Thin sections show the polypentapeptide matrix to calcify throughout its bulk.

Discussion

In 1971 this laboratory proposed a neutral site mechanism for the initiation of calcification, the essential feature of which is not the presence of negatively charged moieties at the Ca^{2+} binding site, but rather is a peptide sequence or combination of sequences wherein the peptide carbonyls supply the primary requirements of the Ca^{2+} binding site by providing the basis

for cation affinity and selectivity (Urry, 1971). By this mechanism, as the matrix becomes charged with positive Ca^{2+} ions, the polyvalent counterions are drawn from solution to neutralize the charging matrix, to allow additional Ca^{2+} binding followed by more anions and thereby to initiate calcification particularly when the stereochemistry of binding sites is favorable. The proposed mechanism was placed on a credible basis when α-elastin coacervates, with chemically blocked amino and carboxyl groups, were shown to calcify from a barbital buffer medium in which the calcium and phosphate ions concentrations were 1.25 and 1.50 mM, respectively (Starcher and Urry, 1973). Subsequently blocked α-elastin and free and blocked tropoelastin coacervates were shown to calcify from a serum medium (Starcher et al., 1974; Cox et al., 1975). Then the recent demonstration, by scanning electron microscopy and microprobe analysis of calcified coacervate thin sections, that calcification occurs throughout the depth of the coacervate and not simply at the aqueous solution–coacervate interface shows that it is indeed a bulk property of the coacervate that is responsible for calcification (Urry et al., 1976a).

All studies on natural products suffer from the possibility, however remote, that trace contaminants or hard to remove components could be responsible for the initiation of calcification of the coacervates. Furthermore, if indeed the mechanism is becoming understood, it should be possible to make a synthetic matrix that would initiate calcification. It was in this vein that the present effort was begun.

With the demonstration by Gray and Sandberg and their colleagues of the presence of glycine containing repeat peptides in the sequence of tropoelastin (Foster et al., 1973)—glycine-containing sequences that had been previously proposed as probable sequences with calcium-binding capacity (Urry, 1971)—it becomes an obvious step to synthesize the repeat peptides and to assess their Ca^{2+} affinity and selectivity. This was done and the peptides were found to be Ca^{2+} selective and to have substantial affinity (Urry et al., 1973; Urry, 1974a; Urry and Ohnishi, 1974a; Long et al., 1974, 1975). The next step is one of obtaining a synthetic product that would be suitable for the in vitro calcification studies. As reported above, the synthetic cross-linked polypentapeptide readily calcifies from a serum medium (see Figures 1 and 2) and does so when the serum is separated from the synthetic matrix by a dialysis membrane with a 12 000 mol wt cutoff (see Figure 3). In addition, Figure 3 shows that the matrix calcifies throughout its bulk and not simply at its interface with the aqueous medium. Studies are in progress to determine whether the calcific deposits are crystalline or amorphous. The x-ray microanalysis data, however, indicate Ca:P ratios which are greater than that of hydroxylapatite.

A detailed description of the serum calcification is in the process of being obtained. The x-ray microanalysis curve in Figure 3 is sensitive to all elements heavier than fluorine and, therefore, demonstrates all elements other than the aluminum coating and the serum-derived calcium and phosphorus to be below detectable limits. Thus, one can reasonably consider only Ca and P as involved in the mechanism. With regard to the insoluble matrix and the ion binding site, while there may be traces of free γ-carboxylates and ϵ- and α-amino groups that could be involved in a binding site, the greater weight of evidence is that the pentapeptide repeat, Val_1-Pro_2-Gly_3-Val_4-Gly_5, provides the basic elements for the binding site. Regardless of the details of the initiating mechanism what has been achieved is the synthesis of a relatively simple calcifiable matrix which may have potential medical applications. Efforts are under way to evaluate the in vivo calcifiability of the synthetic matrix.

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Electrophoretic Analysis of Substrate-Attached Proteins from Normal and Virus-Transformed Cells†

Lloyd A. Culp

ABSTRACT: The proteins which have been left tightly bound to the tissue culture substrate after ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA)-mediated removal of normal, virus-transformed, and revertant mouse cells and which have been implicated in the substrate adhesion process have been analyzed by slab sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Three size classes of hyaluronate proteoglycans were resolved in the 5% well gel; approximately half of the protein in the substrate-attached material coelectrophoresed with these polysaccharides—so-called glycosaminoglycan-associated protein (GAP). A portion of the GAP was shown to be highly heterogeneous and displaced from the polysaccharide by preincubation with calf histone before electrophoresis. The relative proportions of the proteoglycans varied in material deposited during a variety of cellular attachment and growth conditions. The remainder of the cellular protein in substrate-attached material was resolved as several major and distinct protein bands in 8 or 20% separating gels (a limited number of distinct serum proteins have also been identified as substrate bound). Protein C_0 (molecular weight 220 000) was a prominent component in the material from a variety of normal and virus-transformed cells and resembled the so-called LETS or CSP glycoprotein in several respects; protein C_a was myosin-like in several respects; protein C_2 was shown to be actin; and protein C_1 (molecular weight 56 000) does not appear to be tubulin. Histones were also present in most preparations of substrate-attached material, particularly at high levels in transformed cell material, and may result from EGTA-mediated leakiness of the cell and subsequent binding to the negatively charged polysaccharide. These substrate-attached proteins were (a) prominent in substrate-attached material from many cell types in characteristic relative proportions, (b) deposited by EGTA-subcultured cells during the first hour of attachment to fresh substrate, (c) deposited by cells growing on plastic or glass substrates (three additional components were also prominent in glass-attached material), and (d) deposited during long-term growth on or initial attachment to substrates coated with 3T3 substrate-attached material. Pulse-chase analyses with radioactive leucine indicated that these proteins exhibit different turn-over behaviors. These results are discussed with regard to the possible involvement of these substrate-attached proteins in the substrate adhesion process, with particular interest in the interaction of cytoskeletal microfilaments with other surface membrane components and with regard to alteration of substrate adhesion by virus transformation.

A variety of experimental approaches have been used to demonstrate that the growth and motility properties of normal and virus-transformed mammalian cells are considerably different on artificial substrates such as glass and specially treated polystyrene plastic. An understanding of cell–substrate adhesion at the molecular level should provide insight into the basic alterations of the cell surface which may characterize a malignant cell (Taylor, 1961; Weiss, 1962; Curtis, 1973). Microexudates "deposited" by cells onto the culture substrate have been detected by ellipsometric (Rosenberg, 1960; Poste et al., 1973) and electron microscopic (Yaoi and Kanaseki, 1972; Revel and Wolken, 1973) techniques. Particular interest is now being focused on identification of substrate-bound serum and cell surface components which mediate these adhesions (Takeichi, 1971; Revel and Wolken, 1973; Grinnell, 1974; Grinnell, 1975; Culp and Buniel, 1976).

Removal of cells from the tissue culture substrate with the Ca^{2+}-specific chelating agent EGTA[1] results in the persistence of cell-synthesized protein and polysaccharide on the substrate—so-called substrate-attached material, requiring treatment with alkali or $NaDodSO_4$ for efficient removal (Culp and Black, 1972a; Terry and Culp, 1974; Culp et al., 1975). Several experimental approaches (Culp and Black, 1972a; Culp, 1974, 1975; Culp et al., 1975; Mapstone and Culp, 1976) have indicated that this material is deposited during direct contact between the cell and the substrate and may mediate adhesion of cells to the substrate, perhaps via the

† From the Department of Microbiology, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106. *Received February 13, 1976.* This investigation was supported in part by U.S. Public Health Service Research Grant 5-R01-CA-13513 and an American Cancer Society Research Grant BC-217 (with partial support from the Ohio Division). The author is a Harry H. Pinney Fellow in Cancer Research at Case Western Reserve University and a Career Development Awardee of the National Cancer Institute (1-K04-CA70709).

[1] Abbreviations used are: BB, bromphenol blue dye marker; Con A, concanavalin A; tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid; GAG, acidic glycosaminoglycans (formerly referred to as mucopolysaccharides); GAP, glycosaminoglycan-associated proteins; MEM X 4, Eagle's minimal essential medium supplemented with four times the concentration of vitamins and amino acids; MSV, murine sarcoma virus; mol wt, molecular weight; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; SV40, Simian virus 40; Temed, tetramethylenediamine.