FIBRONECTIN FROM CHICKEN EMBRYO FIBROBLASTS CONTAINS COVALENTLY BOUND PHOSPHATE

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ABSTRACT

Fibronectin isolated from cultures of chicken embryo fibroblasts (CEF) contains phosphorus linked to serine and threonine by monoester bonds. Normal and Rous sarcoma virus (RSV)-transformed cells were incubated with [^{32}P]orthophosphate, and fibronectin was isolated from the cell surfaces and conditioned media. ^{32}P was stably associated with fibronectin during immunoprecipitation, SDS-polyacrylamide gel electrophoresis, phospholipid solvent extraction, and hot acid but not alkaline treatment. After a limited acid hydrolysis of fibronectin, both phosphoserine and phosphothreonine were found. The specific radioactivity of the ^{32}P-labeled fibronectin from the conditioned medium of normal CEF was higher than that from the cultures of transformed CEF.

KEY WORDS phosphoprotein · fibronectin · conditioned medium · cell surface · RSV transformation · chicken embryo fibroblast

Fibronectins are a class of glycoproteins present in the plasma of vertebrates and on the surfaces of their fibroblasts both in vivo and in culture. The plasma form was originally called cold-insoluble globulin (CIg) because of its precipitation with fibrinogen at low temperature (33). More recently, it has also been called plasma fibronectin (29). The molecule found in cell cultures has a variety of names: CAP, CSP, gap a, LETS, SFA, Zeta, and cell fibronectin (see references 20 and 52 for review). It has been isolated from both cell surfaces and conditioned media of cultured fibroblasts, endothelial cells, and glial cells (20, 52). Immunofluorescence studies have demonstrated that the basement membranes of many organs and embryos, as well as connective tissues, are rich in this glycoprotein (31, 42, 58).

The detailed structure of fibronectin is only partially understood. Some studies have shown that fibroblast-derived and plasma fibronectins are similar with respect to their electrophoretic mobilities in urea or SDS polyacrylamide gels, their sugar and amino acid compositions, and their immunoreactivities (46). The fibronectin molecule consists primarily of two disulfide-linked subunits each with a molecular weight of ~240,000. Cell surface fibronectin also exists in higher molecular weight forms (9, 22, 27, 54). The interchain disulfide bridges of both plasma and fibroblast-derived fibronectins are confined to one end of the molecule, probably near the carboxyl termini (8, 21, 23, 25, 53). The amino termini of both forms are blocked (23, 34, 54).

When fibroblasts are transformed by viruses or carcinogens, the synthesis of fibronectin is reduced several-fold, its secretion into the medium is decreased slightly, its attachment to the cell surface is decreased at least five-fold, and the rate of its turnover is increased (39, 52, 44, 46). The addition of fibronectin purified from normal fibroblasts to cultures of transformed fibroblasts has been reported to restore partially a normal fibroblastic appearance and parallel alignment to the cells (3, 9, 53). The addition of fibronectin also increased the speed of cell migration, and the rate of attachment of transformed cells to plastic tissue culture substrata (2, 3, 53, 57). The possibility that fibronectin plays a role in cell-to-cell and cell-to-substratum adhesiveness is further demonstrated by data showing that purified fibronectin aggregates erythrocytes (4, 56), and increases the aggregation of dissociated chicken embryo and

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baby hamster kidney cells (53), as well as their attachment and spreading on collagen (53). Plasma fibronectin is also reported to mediate the adhesion of cells to plastic dishes (19).

The cause(s) for the changes in fibronectin metabolism and properties after virus or chemical transformation of cells remains unknown. Nor is it clear what relationship the adhesion properties of fibronectin have to its biological function(s) in vivo. Knowledge concerning the functional group(s) responsible for the biological activity of fibronectin is only rudimentary. The half-life of fibronectin in culture medium seems to be longer when it is glycosylated (38). It has been reported that reduction of the disulfide bonds inhibits the effects of cellular fibronectin on cell-to-substratum adhesion (1). Reports from several laboratories indicate that plasma and cell surface fibronectin can serve as substrates for plasma transglutaminase (Factor $XIII_a$) (5, 24, 26, 35, 36, 37). The site of the transamidation seems to be near one end of the fibronectin subunits (24). It has been proposed that the incorporation of plasma fibronectin into a blood clot or cellular fibronectin into cell matrices involves this enzymatic reaction (5, 24, 26, 35, 36, 37).

Since phosphorylation of certain proteins is known to play an important role in regulating their conformation and their function, we have examined purified fibronectin for the presence of covalently bound phosphate. We present data indicating that the fibronectin from the cell surface and the conditioned medium of cultured chicken embryo fibroblasts contains phosphate covalently linked to amino acids.

MATERIALS AND METHODS

Cell Culture

Primary chicken embryo fibroblasts (CEF) were prepared from virus antigen-free, 11-d-old chicken embryos (SPAFAS, Norwich, Conn.) (18). The secondary cultures were grown in 100-mm dishes (Corning Glass Works, Corning, N. Y.), in Sherer's medium containing 2% tryptose phosphate broth (TPB) and 5% calf serum. Virus-infected cells were obtained by infecting secondary CEF with either the Schmidt-Ruppin strain of Rous sarcoma virus, subgroup A (SR-A RSV), or its transformation-defective variant (td SR-A RSV) (50). Tertiary cultures of normal and virus-infected CEF were prepared in flasks (150 cm^2) (Corning Glass Works) in the same medium.

Preparation of FCS(−PLG, −CIg, $+P_w$)

Fetal calf serum (FCS) was first depleted of plasminogen (PLG) by chromatography through a column of lysine-Sepharose CL-4B (13). The effluent (FCS-[−PLG]) was passed through a column of gelatin-Sepharose CL-4B to remove plasma fibronectin (CIg) (16). Successful removal of plasma fibronectin was demonstrated by the absence of any protein band visible in the region of plasma fibronectin on gradient SDS polyacrylamide gel electrophoresis of the preparation. A growth-promoting factor(s) (P_w) which is adsorbed to lysine-Sepharose CL-4B was eluted with phosphate buffer (0.3 M potassium phosphate/2 mM EDTA, pH 7.4), further dialyzed against phosphate-buffered saline (PBS), and added back to the above material to give final preparation of FCS(−PLG, −CIg, $+P_w$).

[^{32}P]Orthophosphate Labeling of Cell Cultures

Radioactive labeling was carried out essentially by the method of Wang et al. (49). To insure that the CEF fibronectin was not contaminated with fibronectin from calf serum and to protect against hydrolysis of the fibronectin by plasmin, the normal growth medium of confluent tertiary cultures was replaced by Sherer's medium containing 2% TPB, 2% FCS(−PLG, −CIg, $+P_w$), 3.8 mM ε-aminocaproic acid (ε-ACA) (Aldrich Chemical Co., Milwaukee, Wis.), and 5 μM soybean trypsin inhibitor (STI) (Miles Laboratories, Inc., Elkhart, Ind.) for 24 h to 48 h before labeling with $^{32}P_i$. All of the supplements described above plus 1 mM benzamidine·HCl (Aldrich Chemical Co.) were present in the media used in subsequent steps (49). Cells were incubated in medium containing $^{32}P_i$ (3.57 Ci/mmol, 5 mCi/20 ml/flask) (ICN Pharmaceuticals, Inc., Irvine, Calif.) for 6 h. Samples were taken from the medium of the cultures at 0 and 6 h and measured for radioactivity. The total uptake of $^{32}P_i$ per flask was then calculated. At the end of 6 h, the medium was removed and discarded. The cultures were further incubated in fresh Sherer's medium containing 3.63 mM nonradioactive phosphate (7.26 μmol/20 ml/flask). The medium was collected at the end of 36 h thereafter, and cell surface proteins were extracted.

The specific radioactivity of $^{32}P_i$ for the cultures was defined as the amount of $^{32}P_i$ taken up per culture flask during the 6-h labeling period relative to the amount of phosphate present in the medium thereafter (7.26 μmol/flask). This specific radioactivity was then used for the calculation of the number of phosphate molecules per molecule of fibronectin after the ^{32}P content of isolated fibronectin, cpm/μmol of fibronectin, was determined.

Purification of Fibronectin from Conditioned Medium

The conditioned medium was centrifuged at 10,000 *g* for 10 min. Ultrapure guanidine·HCl (Heico, Inc., Delaware Water Gap, Penn.) was added to the supernate to a final concentration of 0.5 M. This solution was passed through a column containing 4 ml of gelatin-

Sepharose CL-4B per 200 ml of conditioned medium (15). The column was preequilibrated with 0.5 M guanidine·HCl in 50 mM HEPES (Research Organics, Inc., Cleveland, Ohio), pH 7.5, 5 mM EDTA, and 3 mM benzamidine·HCl. The column was then washed extensively with the equilibration buffer. Adsorbed fibronectin was eluted with 1.5 M guanidine·HCl in the same buffer. Fractions of 0.5 ml were collected and checked for absorbancy at 280 nm and for radioactivity.

Gelatin-Sepharose CL-4B chromatography as described above was repeated once to decrease the level of nonspecific radioactive contamination. The eluted fibronectin was dialyzed either against 0.2 M sodium phosphate, pH 7.0, containing 5 mM EDTA and 3 mM benzamidine·HCl, or against PBS containing 0.5 M NaCl, 5 mM EDTA, and 3 mM benzamidine·HCl. The dialyzed samples were stored on ice in the presence of 0.02% sodium azide.

Extraction and Purification of Fibronectin from the Cell Surface

Cell surface fibronectin was extracted and purified according to the procedure of Yamada and Weston (55) with the following modifications: (*a*) the extracting solution contained 2% β-mercaptoethanol, (*b*) the ammonium sulfate fractionation was carried out at 35% saturation at room temperature in the presence of 2% β-mercaptoethanol and 0.02% sodium azide for 4 h, and (*c*) the precipitated fraction was dissolved in and dialyzed against 0.2 M sodium phosphate, pH 7.0 containing 0.3 M NaCl, 5 mM EDTA, and 3 mM benzamidine·HCl. Fibronectin was further purified by preparative SDS polyacrylamide gel electrophoresis (51).

Immunoprecipitation

Purified chicken plasma fibronectin or fibronectin from the conditioned medium of normal CEF (4 μg/10 μl in PBS) was diluted to 140 μl with radioimmune precipitation (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2) (10), and incubated with 5 μl of rabbit anti-chicken plasma fibronectin serum at 4°C for 1 h. 10 μl of swollen Protein A-Sepharose CL-4B (Sigma Chemical Co., St. Louis, Mo.) was then added to the mixture and the mixture was slowly agitated at 4°C for another 60 min (10, 28). The mixture was centrifuged in an Eppendorf Centrifuge for 30 s. The pellet was washed and centrifuged four times with RIPA buffer and one time with H_2O. The antigen-antibody complexes were separated from the Protein A-Sepharose CL-4B by boiling in 30 μl of sample buffer (10% β-mercaptoethanol, 20% glycerol, 5% SDS, 0.004% bromophenol blue, 0.083 M Tris HCl, pH 6.3) (30, 43) for 3 min, and followed by centrifugation. The supernate was analyzed by SDS polyacrylamide gel electrophoresis and autoradiography.

Hot TCA Extraction

Samples of fibronectin were spotted onto glass fiber filters (Whatman GF/C, Whatman, Inc., Clifton, N. J.) and air dried. Hot TCA extraction was performed according to the method of Bitte and Kabat (6). The radioactivity remaining on the filters was counted in the presence of scintillation fluid (see below).

Extraction with Phospholipid Solvents

Samples of fibronectin spotted on glass fiber filters were treated with hot TCA. The dried filters were incubated sequentially at room temperature for 20 min with occasional agitation in the following solvents: once each in acetone, ethanol, and chloroform, twice in ethanol/ethyl ether (3:1), and once in ethyl ether (11). The filters were air dried and measured for radioactivities.

pH Stability

Samples of radioactive fibronectin, 30 μl in PBS, 0.35 M NaCl, 5 mM EDTA, were mixed in glass tubes with 30 μl of each of the following solutions: 0.1 N HCl, PBS, 0.2 N KOH, or 1.0 N KOH. The final pH values were 1.0, 7.4, 12.8, and 13.3, respectively. The tubes were placed in a 55°C water bath for 120 min (6). The pH of the mixtures was then adjusted to 7.4 with 30 μl of 0.1 N KOH, PBS, 0.2 N HCl, and 1.0 N HCl, respectively. Proteins were precipitated by cold TCA (20%) in the presence of 125 μg of bovine serum albumin (BSA). The precipitates were collected on glass fiber filters, washed three times with 5% TCA at room temperature, dried under a lamp, and the radioactivity was determined.

Detection of O-Phosphoserine and O-Phosphothreonine

^{32}P-labeled fibronectin (1,900 cpm) was precipitated with cold TCA (20%) in the presence of 125 μg of BSA as carrier and washed once with 75% ethanol. The pellet was dried, redissolved in 200 μl of 6 N HCl, and incubated at 109°C for 6¼ h under vacuum. The hydrolysates were evaporated to dryness, dissolved in 20 μl of 2.5% formic acid, 7.8% acetic acid, pH 1.9, and analyzed for phosphoamino acids using high voltage paper electrophoresis in the same buffer (14). Phosphoamino acids were detected by ninhydrin spray (14) and autoradiography. Radioactive spots were cut out and measured for radioactivity. O-phosphoserine and O-phosphothreonine (10 μg of each) and $^{32}P_i$ (300 cpm) were used as standards.

Preparation of Lysine- and Gelatin-Sepharose CL-4B

Lysine and gelatin (Sigma Chemical Co.) were coupled to cyanogen bromide-activated Sepharose CL-4B

(Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) according to published methods (40).

SDS Polyacrylamide Gel Electrophoresis

Gradient slab gels of 5–16% acrylamide were prepared using the methods of Laemmli (30) and Studier (43). The standards for molecular weight estimation were: rabbit skeletal muscle myosin (M_r = 200,000), β-galactosidase (M_r = 130,000), bovine serum albumin (M_r = 69,000), ovalbumin (M_r = 45,000), chymotrypsinogen (M_r = 26,000), and ribonuclease (M_r = 13,000). After electrophoresis, gels with radioactive samples were boiled in 20% TCA for 20 min to remove nucleic acids and inorganic phosphate. Gels were then stained for protein with Coomassie brilliant blue (Sigma Chemical Co.) (17) and dried. Dried gels were exposed to X-ray films (Eastman Kodak Co., Rochester, N. Y.) using intensifying screen (DuPont Instruments, Wilmington, Del.). In some cases, the autoradiograms and corresponding gels were photographed to make positive films. The positive films were cut into strips and scanned at 511 nm with a Gilford Spectrophotometer 240 (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and plotted on a chart recorder.

Measurement of Radioactivity

Samples were placed in vials, and scintillation fluid composed of toluene, Triton X-100 and Omnifluor (2 1: 1 1:10 g) was added. The samples were counted in a Packard scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Measurement of Protein Concentration

Protein concentrations were measured by the methods of Lowry et al. (32) and Bohlen et al. using fluorescamine (Hoffman-La Roche, Inc., Nutley, N. J.) (7).

RESULTS

Fibronectin from the conditioned medium of CEF was purified by adsorption to gelatin-Sepharose CL-4B in the presence of 0.5 M guanidine·HCl. The bound proteins could be eluted with 1.5 M guanidine·HCl and consisted primarily of fibronectin and minor contaminants of lower molecular weights (Fig. 1). Fibronectin (FN_m) had a molecular weight of ~240,000 after reduction with β-mercaptoethanol.

This procedure was employed to isolate the fibronectin from the conditioned medium of [^{32}P]orthophosphate-labeled normal CEF cultures. The immunoreactivity of this purified fibronectin with rabbit anti-chicken plasma fibronectin serum was tested in comparison with chicken plasma fibronectin. Fig. 2 shows the protein pattern and the autoradiogram of immunoprecipitates after SDS polyacrylamide gel electrophoresis. Fibronectin isolated from the medium of ^{32}P-labeled fibroblast cultures and unlabeled plasma fibronectin were analyzed before and after immunoprecipitation with anti-chicken plasma fibronectin. It can be seen that medium-derived monomeric fibronectin (FN_m) (column E) had a mobility similar to that of plasma fibronectin (columns A and B), and was immunoprecipitated by the anti-serum (column D) but not by the preimmune serum (column C). Columns F and G are the autoradiograms of D and E, respectively, and

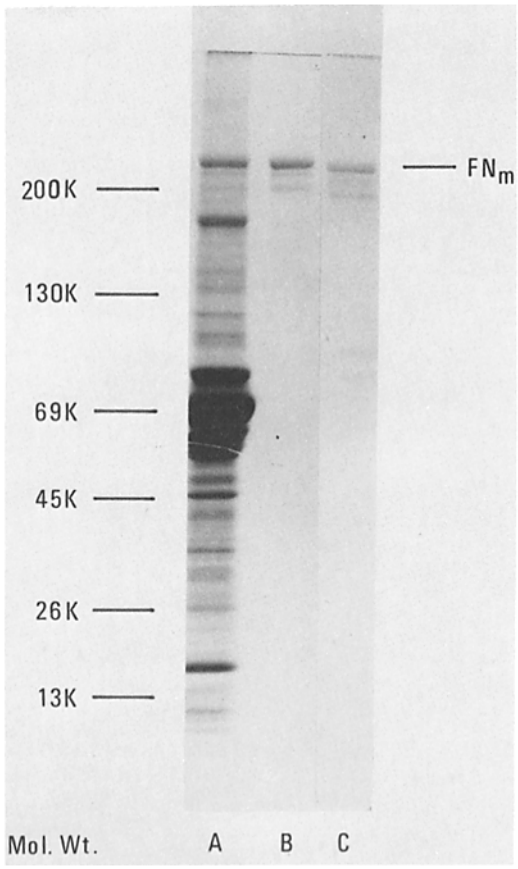

FIGURE 1 Purification of fibronectin from conditioned medium of normal CEF. Conditioned medium was passed through a gelatin-Sepharose CL-4B column, and adsorbed fibronectin was eluted with 1.5 M guanidine·HCl as described in Materials and Methods. Samples were reduced with β-mercaptoethanol, analyzed in a 5–16% polyacrylamide slab gel, and stained for protein. *A*, conditioned medium, 50 μg; *B*, guanidine·HCl eluent, 4 μg; and *C*, chicken plasma fibronectin, 3 μg. FN_m on the right panel indicates the monomeric form of chicken fibronectin.

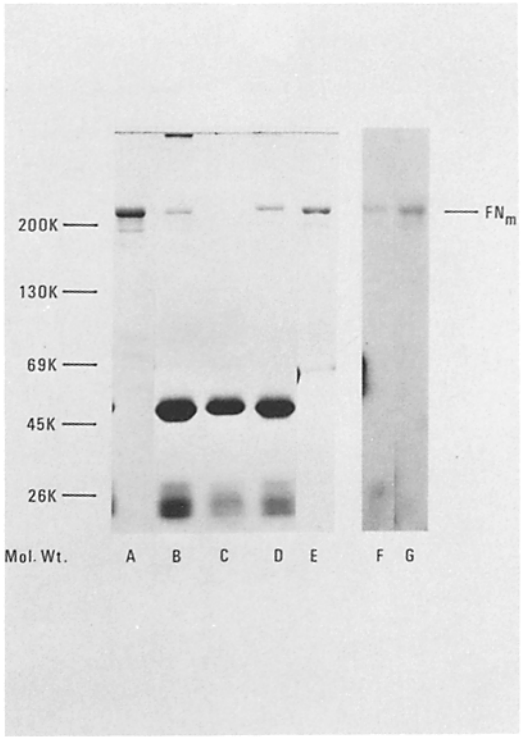

FIGURE 2 Immunoprecipitation of fibronectins from chicken plasma and conditioned medium of ^{32}P-labeled normal CEF. Experimental details are described in Materials and Methods. Antigen-antibody complexes were analyzed in a 5–16% SDS polyacrylamide slab gel. *A*, chicken plasma fibronectin, 6 μg; *B*, immunocomplex of plasma fibronectin and antiserum; *C*, control, fibronectin from conditioned medium and preimmune serum; *D*, immunocomplex of fibronectin from conditioned medium and antiserum; *E*, fibronectin from conditioned medium, 4 μg; and *F* and *G*, autoradiograms of *D* and *E*, respectively. Protein bands at the regions of 55,000 and 23,000 daltons correspond to the heavy and light chains of immunoglobulin.

demonstrate that ^{32}P was stably associated with fibronectin through immunoprecipitation and TCA extraction. In addition to the monomeric fibronectin band (FN_m), two groups of proteins were also observed in immunoprecipitated samples (columns B, C, and D), corresponding to the heavy and light chains of immunoglobulin (M_r = 55,000 and 23,000, respectively).

Fibronectins from conditioned media of ^{32}P-labeled normal and SR-A RSV-transformed CEF cultures were compared. ^{32}P comigrated with fibronectin under either reducing or nonreducing conditions in both samples (Fig. 3). The specific radioactivity of the ^{32}P-labeled sample from normal CEF was at least twice that of the material from transformed CEF (191 × 10^3 cpm/mg protein for the former and 90 × 10^3 cpm/mg protein for the latter before SDS polyacrylamide gel electrophoresis). The difference in ^{32}P content of fibronectin from normal and transformed cultures was apparent when the intensities of stained SDS polyacrylamide gels were compared to the autoradiograms of the same material (Fig. 3). Twice as much protein from the transformed cultures as from the normal cultures was placed on the gel. The intensity of the radioactive band of the former, however, was much less than that of the latter. This difference has been observed repeatedly. In addition, the specific radioactivity of ^{32}P-labeled fibronectin isolated from the conditioned medium of CEF infected by transformation-defective variant of SR-A RSV (td SR-A RSV) was similar to that of normal CEF (data not shown).

The content of phosphorus was estimated to be two phosphate groups per molecule of fibronectin dimer from normal CEF. This estimation was based on the assumption that the specific radioactivity of $^{32}P_i$ was the amount of $^{32}P_i$ taken up by

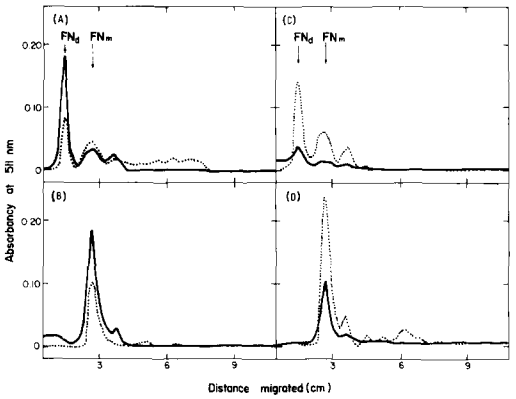

FIGURE 3 Nonreduced and reduced fibronectins from conditioned media of ^{32}P-labeled normal and SR-A RSV-transformed CEF. Purified fibronectin was either nonreduced or reduced with β-mercaptoethanol and analyzed in a 5–16% SDS polyacrylamide slab gel. The positive films obtained by photographing the stained gel and its autoradiogram were cut into strips and scanned. The figure represents the scan. (A), nonreduced fibronectin from normal CEF, 1.3 μg; (B), reduced fibronectin from normal CEF; (C), nonreduced fibronectin from transformed CEF, 2.6 μg; and (D), reduced fibronectin from transformed CEF. Plot of films from the protein stain (·····); from the autoradiogram (——). FN_m and FN_d designate the monomeric and dimeric forms of fibronectin, respectively.

cultures divided by the total amount of phosphate present in the medium after the 6-h-labeling as described in the Materials and Methods.

Fibronectin from the surface of ^{32}P-labeled normal CEF was isolated by urea extraction, ammonium sulfate fractionation, and preparative SDS polyacrylamide gel electrophoresis as described in Materials and Methods. This sample was then analyzed by SDS gradient polyacrylamide gel electrophoresis and autoradiography. This sample appears to be less homogeneous than that from the conditioned medium (Fig. 4). Nevertheless, it does contain radioactive phosphorus.

The nature of the association of radioactive phosphorus with fibronectin was examined first by testing the stability of the phosphate bond to several chemical and physical treatments. The association of ^{32}P and protein in the sample from the conditioned medium of normal CEF was stable in hot TCA and at acid pH, but not at alkaline pH (Table I). Furthermore, the radioactive phosphorus of labeled fibronectin adsorbed to glass fiber filters could not be extracted by various phospholipid solvents. These properties suggest that the association is an ester linkage involving the hydroxyl side chains of amino acids (6). The data for the fibronectin from transformed CEF show similar properties, although the association of phosphate was less stable for unknown reason(s).

TABLE I

Stability of the Association of ^{32}P with Fibronectin from the Conditioned Media of Normal and Transformed CEF

	Experimental conditions	% Radioactivity remaining N	T
(I)	Cold TCA (control)	100	100
	Hot TCA	91 ± 1	72 ± 6
	Hot TCA + Phospholipid Solvents	87 ± 4	58 ± 0
(II)	pH 1.0 (PBS:0.1 N HCl = 1:1)	90 ± 4	84 ± 6
	pH 7.4 (PBS only)	100	100
	pH 12.8 (PBS:0.2 N KOH = 1:1)	56 ± 9	21 ± 2
	pH 13.3 (PBS:1.0 N KOH = 1:1)	10 ± 2	4 ± 2

Purified fibronectins from the conditioned media of ^{32}P-labeled normal and RSV-transformed CEF were treated with TCA or with solutions of various pH values as described in Materials and Methods. N, samples from normal CEF (280 cpm/0.9 μg/30 μl). T, samples from transformed CEF (258 cpm/1.9 μg/30 μl). Duplicates of each sample were tested. Data show the average from the duplicates and the range of deviation.

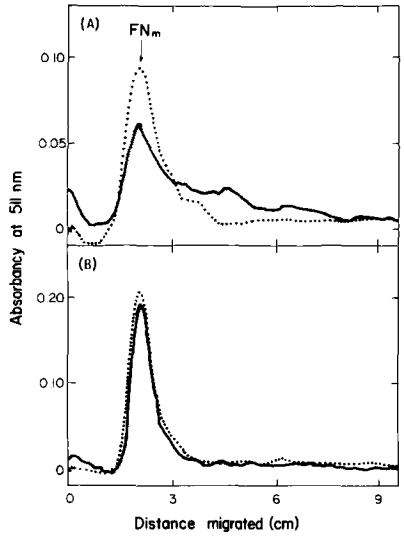

FIGURE 4 Fibronectin from the cell surface and conditioned medium of ^{32}P-labeled normal CEF. Purified samples were reduced and analyzed in a 5-16% SDS polyacrylamide slab gel. Data were obtained the same way as in Fig. 3. (A), fibronectin from the cell surface; and (B), fibronectin from conditioned medium. Plot of film from protein stain (·····); from autoradiogram (——).

The existence of an amino acid-monophosphoester linkage was verified by the identification of phosphoserine and phosphothreonine after limited acid hydrolysis of fibronectin from the conditioned media of normal and RSV-infected cultures. After hydrolysis in 6 N HCl at 109°C for 6¼ h, 6-13% of the radioactive phosphorus comigrated with a phosphoserine standard and 0.3% with a phosphothreonine standard in high voltage paper electrophoresis (figure not shown). The remaining radioactivity was at the position of orthophosphate. To eliminate the possibility that these phosphoamino acids might be derived from proteins other than fibronectin, protein was extracted from the fibronectin band after preparative SDS polyacrylamide gel electrophoresis, hydrolyzed, and analyzed. Both phosphoserine and phosphothreonine were detected by this procedure (data not shown). We conclude that fibronectin contains phosphorus covalently linked to the hydroxyl side chains of serine and threonine.

DISCUSSION

It has been reported that fibronectin can be purified from conditioned media and from urea extracts of chicken and human fibroblasts using gelatin-Sepharose CL-4B affinity column chromatography (15, 16). We found that fibronectin from the conditioned medium of CEF could be adsorbed to gelatin-Sepharose CL-4B in the presence of 0.5 M guanidine·HCl. The purity of the fibronectin isolated from the conditioned medium after this procedure (Fig. 1) was comparable to that reported from another laboratory (16).

The conclusion that fibronectin from the conditioned media of normal and virus-transformed CEF contains ester-linked phosphorus is supported by the following facts: (*a*) ^{32}P coprecipitated with fibronectin in the presence of antiserum to chicken plasma fibronectin (Fig. 2), (*b*) ^{32}P comigrated with both nonreduced and reduced fibronectin in SDS polyacrylamide gel electrophoresis (Fig. 3), (*c*) ^{32}P remained stably associated with fibronectin in phospholipid solvents and hot acid, but not in alkali (Table I) (6), and (*d*) [^{32}P]phosphoserine and [^{32}P]phosphothreonine were found in the acid hydrolysates of fibronectin.[1] Purified fibronectin from the cell surface of normal CEF and from the conditioned medium of td SR-A RSV-infected CEF also contained phosphorus (Fig. 4 and unpublished result).

Filamin, a cellular protein with a molecular weight of 240,000, is present in avian and mammalian smooth muscle as well as in cultured fibroblasts (12, 47, 48). It can be phosphorylated by protein kinase in vitro (12, 47). Whether phosphorylation occurs in vivo remains unknown. Nevertheless, the possibility that the ^{32}P in our samples might be derived from contaminating filamin was ruled out by the fact that the major portion of the radioactivity comigrated with the nonreduced dimer (450,000 daltons, FN_d) or the reduced monomer of fibronectin (FN_m) in SDS polyacrylamide gels (Fig. 3). Furthermore, the radioactivity coprecipitated with fibronectin during immunoprecipitation (Fig. 2).

At present, the absolute amount of phosphorus in the fibronectins from the cell surface of normal CEF and conditioned media of normal and transformed CEF is uncertain. A preliminary estimate is that there are two molecules of phosphorus per dimeric molecule of fibronectin isolated from the conditioned medium of normal CEF. The fibronectin from transformed cultures contains less phosphorus under our culture and labeling conditions. The significance of these differences is complicated by the following factors concerning labeling in these two cultures: (*a*) the uptake of orthophosphate may differ, (*b*) the intracellular pool sizes of phosphate and its derivatives may differ, (*c*) the rates of synthesis and turnover of fibronectin are not equivalent, and (*d*) the growth rates of the two cell cultures may be different. Experiments designed to determine what effect these potential differences between normal and transformed cells may have on the specific radioactivity of phosphorus in fibronectin are in progress.

It is tempting to speculate about the possible functional significance of the phosphate groups in fibronectin. Our data clearly do not allow much speculation, but it may be worthwhile to call attention to the known association of particular phosphopeptides with collagen in bones and teeth (41, 45). Cellular fibronectin has an affinity for certain forms of collagen and may promote cellular adhesion to extracellular matrices (16). In this respect, it will be interesting to know whether the phosphogroups are involved in the interaction of fibronectin and collagen.

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[1] Because of the relatively low yields of phosphoserine and phosphothreonine, the existence of other phosphoamino acids which may be completely hydrolyzed during limited acid hydrolysis can not be ruled out.

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