DIMERIC CHARACTER OF FIBRONECTIN, A MAJOR CELL SURFACE-ASSOCIATED GLYCOPROTEIN

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Received December 7,1976

SUMMARY: Exposed proteins of cultured chick and human fibroblasts were labeled by lactoperoxidase-catalyzed iodination and analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Extracts from both cell types contained the characteristic, heavily labeled band of fibronectin (molecular weight = 2.2×10^5) when analyzed after reduction with 2-mercaptoethanol. Without prior reduction, however, the 2.2×10^5 molecular weight band was missing and replaced by labeled bands of 4.4×10^5 and of very high molecular weight. This finding indicates that fibroblast cell-surface fibronectin, like the fibronectin purified from plasma, is composed of two high molecular weight polypeptides held together by disulfide bonds, and suggests that the dimer may in addition form disulfide-bonded multimers.

Fibronectin is a glycoprotein composed of high molecular weight subunits and exists in vertebrates in several immunologically cross-reactive forms (1). The form present in human plasma, also known as "cold-insoluble globulin", has the following properties: electrophoretic mobility of a β-globulin (2), sedimentation coefficient of 12-14S (2,3), and molecular weight, determined by sedimentation equilibrium, of 4.5×10^5 (4). Gel electrophoresis in SDS with and without prior reduction indicated that plasma fibronectin is composed of two polypeptide chains, molecular weight = 2.2×10^5, held together by disulfide bonds (4,5). Cell surface-associated fibronectin, also known as the "large external transformation sensitive protein" (6), has been characterized as a polypeptide of a molecular weight = $2.1\text{-}2.5 \times 10^5$ that is missing from surfaces of malignantly transformed fibroblasts in culture (7 - 12). This loss represents a major quantitative difference in protein composition between normal and virus-transformed fibroblastic cells (1). We now report experiments indicating that fibronectin associated with surfaces of normal fibroblasts is a disulfide-bonded dimer that has a tendency to form disulfide-bonded multimers. The probable bivalent nature of fibronectin may be important in its proposed roles as a structural glycoprotein and as a determinant of cell adhesion and morphology.

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Abbreviation: SDS, sodium dodecyl sulfate

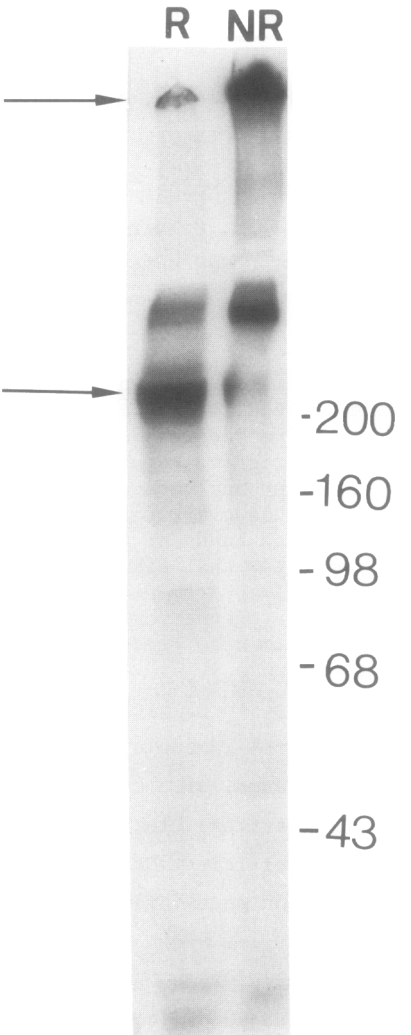

Figure 1. Autoradiograms of SDS-gel electrophoretograms of surface-iodinated chick fibroblasts. R: cell extracts reduced by 2-mercaptoethanol, NR: cell extracts without 2-mercaptoethanol. Electrophoresis was performed for 1.2 x the time needed for bromphenol blue to migrate through the gel. For molecular weights see Fig. 3. Arrows indicate the positions of fibronectin monomer and the very high molecular weight region.

MATERIALS AND METHODS

Reagents. Reagents were purchased from the following suppliers: Glucose oxidase, Worthington Biochem. Corp., Freehold, N.J., USA; lactoperoxidase, Sigma, St. Louis, Mo., USA; carrier-free Na(^{125}I), Radiochemical Centre, Amersham, England; RP Royal X'Omat X-ray film, Kodak, Rochester, N.Y., USA.
Cell cultures. Subcultures of an established line (ES) of human adult skin fibroblasts (13) and secondary cultures of chick embryo fibroblasts (12) were prepared as described earlier. The stock cultures were tested weekly for mycoplasma by staining for cytoplasmic DNA (14) with negative results.

Cells were studied upon reaching confluence, 3-4 days after subculture.
Plasma fibronectin (cold-insoluble globulin). The protein was isolated by a modification of a published method involving precipitations by ammonium sulfate precipitation, cold ethanol precipitation, and ion-exchange chromatography (5). Fibrinogen was removed prior to ion-exchange chromatography by heat precipitation (56°C, 3 min) rather than by controlled clotting by thrombin.
Surface -labeling. The cell cultures were washed quickly three times with warm serum-free medium. Lactoperoxidase-catalyzed iodination was performed in phosphate buffered saline containing glucose (5 mM), carrier-free $Na^{125}I$ (100 uCi/ml), glucose oxidase (0.1 unit/ml), and lactoperoxidase (10 μg/ml). The reaction was initiated by adding lactoperoxidase, allowed to proceed for 10 min at room temperature, and terminated by four washes with phosphate buffered saline. Cells were dissolved in a small volume (250 μl per $4x10^5$ cells) of Tris-buffered 4 % SDS, pH 6.8. Half of the sample was made 10 % in 2-mercaptoethanol. Both non-reduced and reduced samples were incubated at 37°C for 2 hours prior to electrophoresis.
Polyacrylamide gel electrophoresis in SDS. Vertical slab gels and a discontinuous buffer system were used (15). The acrylamide concentrations of the stacking and running gels were 3.3 % and 5 %, respectively. When applying samples, care was taken not to contaminate non-reduced samples with trace amounts of 2-mercaptoethanol. Following electrophoresis the gels were stained for protein (16), dried, and subjected to autoradiography. In two dimensional gel system the gel of the non-reduced sample was cut out, incubated with sample buffer containing 2-mercaptoethanol (15) for 2 hours and analyzed in a 5 % SDS-polyacrylamide gel without spacer.

RESULTS

Surface-iodinated fibroblasts were analyzed in polyacrylamide gels in the presence of SDS with and without prior reduction. In samples of chick cells analyzed after reduction, most of the label migrated in a band with apparent molecular weight of $2.2x10^5$ characteristic to fibronectin (Fig. 1). Without prior reduction the amount of label in this major band was decreased and replaced by larger amounts of label in a band of higher apparent molecular weight and also at the top of the stacking gel. A sample of chick cells (similar to sample NR in Fig. 1) was first electrophorezed under non-reducing conditions and then analyzed in second dimension under reducing conditions. This two-dimensional analysis showed that both the dimeric and multimeric regions (the top of the stacking gel) were composed of radioactive bands comigrating with reduced fibronectin monomer. The multimer region contained some radiolabeled protein that did not enter the gel even under reducing conditions (gel not shown).

Autoradiograms of gels of surface-iodinated human fibroblasts exhibited more complex patterns of radiolabeled polypeptides (Fig. 2B). Differences between the patterns of reduced and non-reduced samples are listed in Table 1. As in the chick cells, the major differences between reduced and non-reduced samples were in the amounts of radiolabel at the $2.2x10^5$ and $4.4x10^5$ mole-

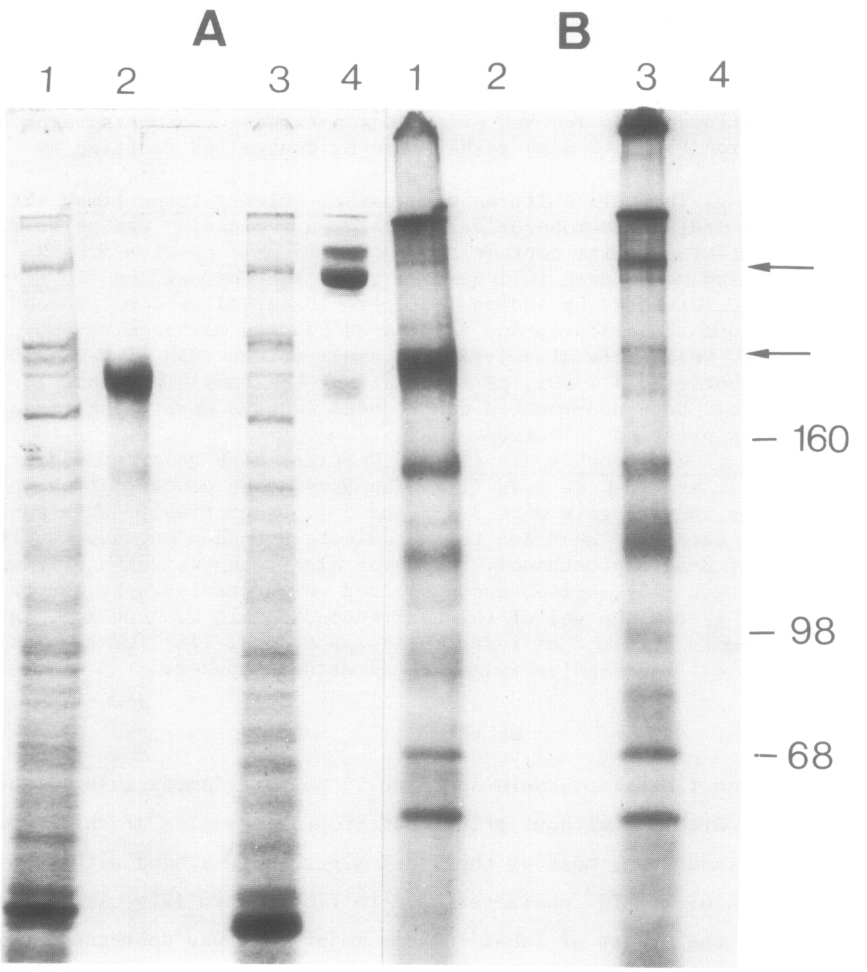

Figure 2. Gel electrophoresis in SDS of surface iodinated human fibroblasts and purified plasma fibronectin. Samples were: 1) fibroblast extract, reduced, 2) purified plasma fibronectin, reduced, 3) fibroblast extract, non-reduced, 4) purified plasma fibronectin, non-reduced; A: protein staining, B: autoradiogram. Arrows indicate the positions of cellular fibronectin monomer and dimer. Molecular weight markers are listed in the caption of Fig. 3.

cular weight positions (Fig. 3) and at the top of the stacking gel. The surface-labeled 2.2×10^5 molecular weight band in the reduced sample (Fig. 2B) corresponded to the middle band of the triplet found in the 2.0×10^5 to 2.5×10^5 molecular weight region of the gels stained for protein (Fig. 2A). (The bottom band, molecular weight = 2.0×10^5, appears to correspond to myosin (17) and the upper band, molecular weight = 2.5×10^5, to "filamin" (18)). The middle band (molecular weight = 2.2×10^5) was not present in the gel of non-

Table 1. Summary of major differences between reduced and non-reduced samples of cellular proteins observed in Fig. 2.

Description of band	Molecular weight	Intensity reduced	Intensity non-reduced
AUTORADIOGRAPHY			
top of stacking gel	$\gg 1.6\times10^6$	+	++++
within stacking gel	$> 1.6\times10^6$	-	+
interphase of the gels		++	++
(fibronectin dimer)	4.4×10^5	+	+++
	3.9×10^5	-	+
	2.8×10^5	+	-
(fibronectin subunit)	2.2×10^5	++++	+
	1.32×10^5	+	++
	1.05×10^5	+	-
	9.5×10^4	-	+
	9.0×10^4	+	-
	8.5×10^4	-	+
	6.5×10^4	+	-
PROTEIN STAINING			
	4.4×10^5	+	++
	2.7×10^5	-	+
	2.2×10^5	+	-

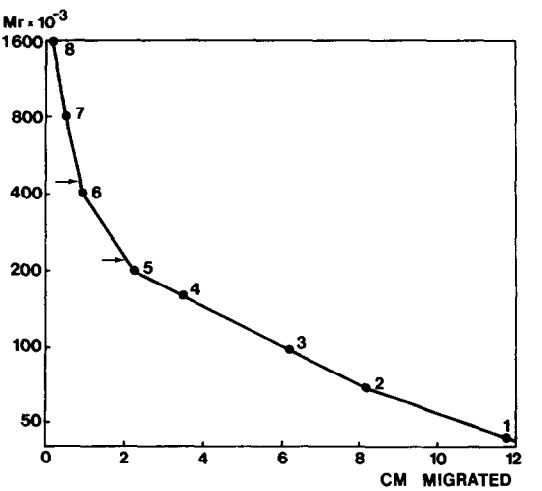

Figure 3. Proposed relationship of migrations of cellular and plasma fibronectin in 5 % running gel to molecular weights. The following proteins were used as molecular weight (M_r) markers: 1) ovalbumin (4.35×10^4), 2) human serum albumin (6.8×10^4), 3) phosphorylate A (9.8×10^4), 4) human alfa$_2$-macroglobulin (1.6×10^5; ref. 19), and 5) reduced human plasma fibronectin (2.0×10^5). We have assumed that the bands in non-reduced plasma fibronectin represent: 6) dimer (4.0×10^5), 7) tetramer (8.0×10^5), and 8) octamer (1.6×10^6); however, these bands could also represent dimer, trimer (6.0×10^5), and tetramer. Arrows indicate the positions of monomer and dimeric cellular and fibronectin.

reduced samples, while more stain was present in the band at the $4.4x10^5$ molecular weight position. Reduced cellular fibronectin migrated slightly behind corresponding sample of purified plasma fibronectin, while the $4.4x10^5$ molecular weight band in samples of non-reduced cellular proteins migrated slightly behind the dimer of non-reduced plasma fibronectin. Some minor differences were also seen (Table 1).

DISCUSSION

The present data indicate that both cell-surface fibronectin and fibronectin purified from plasma glycoproteins of molecular weight $4.0\text{-}4.4x10^5$ composed of two disulfide-bonded $2.0\text{-}2.2x10^5$ molecular weight subunits. In addition, under non-reducing conditions, fibronectin from either source is capable of forming higher multimers. Cell-surface fibronectin can be cross-linked to high molecular weight complexes by physiological concentrations of plasma transglutaminase (13). According to the present study the multimers formed in cell culture conditions represent fibronectin held together mainly by cystine disulfide bonds. The small amount of surface-labeled cellular protein at the dimeric ($4.4x10^5$) position found in reduced samples could represent fibronectin held together by transglutaminase catalyzed $\varepsilon(\gamma$-glutamyl)-lysine cross-links only.

Furthermore, these and other experiments we have reported (12,13), do not distinguish whether the presumed high molecular weight complexes of surface-associated fibronectin are composed of fibronectin alone or whether they contain some additional components. However, the higher multimers of purified plasma fibronectin must be held together solely by disulfide bonds, since only the monomer was observed after reduction.

Since all protein-staining material was missing from the $2.2x10^5$ molecular weight position in samples analyzed without prior reduction, all cellular fibronectin probably occurs as disulfide-bonded dimers or multimers. This finding is important because immunofluorescent studies of human skin fibroblasts indicate that fibronectin is present both as fibrils on the cell surface and in a patchy distribution within the cell (1). Electrophoretograms of both reduced and non-reduced samples demonstrated a slight but reproducible difference in migration between the cellular and plasma forms of fibronectin, indicating that the plasma form is slightly smaller. These data suggest that circulating fibronectin molecules may have arisen in vivo by limited proteolytic cleavage (20). However, fibronectin shed from cultured chick fibroblasts to growth medium comigrated with cellular fibronectin

(12), and thus a similar cleavage may not take place in cell culture. Recently, noncollagenous disulfide-bonded proteins composed of high molecular weight subunits were described in medium (21) and cell extracts (22) of cultured fibroblasts. It is extremely likely that these proteins are identical to fibronectin.

Plasma fibronectin has a single N-terminal sequence of pyroglutamyl-alanyl- (4) and the reduced and carboxymethylated subunits of plasma fibronectin migrate as a single hand at pH 8.6 in polyacrylamide gels containing 8 M urea (5). Purified plasma fibronectin often electrophoreses in SDS after reduction as a closely spaced doublet (4, 23). Pending further structural studies it seems that fibronectin is composed of two identical subunits and has two-fold symmetry.

Studies of two large globular proteins which are disulfide-bonded dimers with two-fold symmetry, IgG and fibrinogen/fibrin, have demonstrated functional bivalency: IgG had two antigen-binding Fab fragments (24,25), and fibrinogen/fibrin has two fibrin-binding D fragments (26-28). If fibronectin has a similar functional bivalency, this property may be important in understanding its biological role. By immunofluorescence, fibronectin on the surface of cultured fibroblasts is largely found in fibrils (29), and Muir et al. (30) have suggested that fibronectin may be the protamer of the 100-120 Å diameter microfibrillar component of connective tissue. In addition, several authors have proposed that fibronectin may play a role in cell adhesion and morphology in culture (31,32). Finally, it was found that fibronectin extracted from cultured chick fibroblasts agglutinated formalinized sheep red cells (33). A bivalent fibronectin would be able to form ordered bidirectional fibrils, like fibrin, or to link together membrane-bound ligands, like IgG.

ACKNOWLEDEMENTS. We thank Ms. Anja Virtanen and Ms. Päivi Lehtovuori for technical assistance. This work was supported by grant number CA 17373 awarded by the National Cancer Institute, DHEW, and by grants from the Finnish Medical Research Council, and the Finnish Cancer Foundation.

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