METABOLIC FATE OF THE MAJOR CELL SURFACE PROTEIN OF NORMAL HUMAN FIBROBLASTS

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SUMMARY: The major cell surface protein of a strain of human fetal lung fibroblasts is a 220,000 dalton glycoprotein termed fibronectin. Fibronectin is released from fibroblasts into the culture medium with a half-time of about 25 hours. The release occurs with an initial (0-3 hours) rapid phase followed by a second (3-48 hours) slower phase. Release of fibronectin occurs in a stoichiometric fashion. All of the fibronectin released from the cells is quantitatively recovered from the culture media as a similar sized soluble glycoprotein. Thus, release of fibronectin from normal human fibroblasts results from mechanisms other than extensive degradation of the protein.

INTRODUCTION

The major cell surface protein of cultured fibroblasts is a large (220,000 dalton) glycoprotein. This macromolecule, termed fibronectin (1), cell surface protein (2), or large external transformation sensitive protein (3), is in part responsible for orderly cell-cell and cell-substratum interaction and for the maintenance of normal cell morphology (4-7). Transformed cells, aberrant in these biological characteristics, are deficient in either the quantity or quality of fibronectin on their external surfaces (2,7).

In normal cells, the metabolic fate of fibronectin is unclear. Several lines of evidence have suggested that fibronectin is continually shed from the cell surface (8-11), although the structural relationship between the cell-associated and released fibronectin is not established (12). It has been suggested that release of fibronectin may be mediated by

Abbreviations used: DMEM - Dulbecco's modification of Eagle's medium: PBS - phosphate buffered saline; SDS - sodium dodecyl sulfate

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proteolysis (13). However, a role for proteolysis in release, or subsequent metabolic fate of released fibronectin, is not well understood (11,12).

The present study demonstrates that all fibronectin released by normal human fibroblasts into culture media can be recovered in a form indistinguishable from cell-associated fibronectin. This observation suggests that in this normal cell strain extensive proteolysis neither mediates release of fibronectin nor affects its subsequent fate in the extracellular milieu.

MATERIALS AND METHODS

Cell Cultures. All studies were done with subcultivations 8 through 15 of a diploid fibroblast strain (HFL-1) derived from the parenchyma of a 16-week-old human fetal lung. HFL-1 has a doubling time of 25 hrs, exhibits contact inhibited growth, has a normal karyotype and has no evidence of infection with either mycoplasma or virus. Cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 µg/ml) and glutamine (0.06%) in a humidified atmosphere of 90% air, 10% CO_2.

Metabolic Labeling. Four to eight days after subcultivation, confluent cells were washed twice with phosphate buffered saline (PBS) and then re-incubated for 18 to 24 hrs with medium (minus fetal calf serum) containing [^{14}C]proline (5.8 µCi/ml, 260 mCi/mmole, Schwarz-Mann), [^{14}C]glucosamine (25 µCi/ml, 229 mCi/mmole, New England Nuclear) or [^{3}H]fucose (100 µCi/ml, 2780 Ci/mmole, New England Nuclear). During this period, the rate of incorporation of [^{14}C]proline into protein by HFL-1 cells was linear with time (data not shown). Following incubation, the medium and cell layer were separated. Labeled proteins in the medium were collected by precipitation with ethanol (80% v/v,-20°, 16 hrs) and centrifugation (10,000 g, 30 min) and then lyophilized. Labeled fibronectin in the cell layer was partially purified by extraction with 1 M urea using the method of Yamada et al. (4). The fibronectin preparation was precipitated with ethanol, collected and lyophilized as above.

Sodium dodecyl sulfate polyacrylamide (5% acrylamide-0.17% bis-acrylamide) slab gel electrophoresis was performed as previously described (14). To prepare the proteins for electrophoresis, the lyophilized medium or fibronectin preparation was solubilized in 60 mM Tris-HCl, pH 6.7, 1% SDS, 1% β-mercaptoethanol, and heated to 100° for 2 min. Molecular weight references included bovine serum albumin, phosphorylase A, β-galactosidase and cold insoluble globulin; the latter was prepared as described by Mosher (15). Following electrophoresis, the slabs were stained and destained (16) and autofluorograms were prepared as described (17).

Iodination of Cell Surface Proteins. HFL-1 cell surface proteins were labeled with ^{125}I (carrier free; New England Nuclear) using the lactoperoxidase catalyzed method as described by Hynes and Bye (3). Following labeling, the cell layer was washed (x3) with 10 mM NaI in PBS. Cells were

collected by scraping with rubber policeman and centrifugation (7,000 g, 15 min). The cell pellet was solubilized and electrophoresed as above. Following electrophoresis, the gels were stained, destained and dried and autoradiograms were prepared. The density dependence of HFL-1 cell surface proteins was examined by iodination at days 1 through 8 after subcultivation. Following labeling, the proteins were electrophoresed and autoradiograms prepared as above. The relative amounts of specific proteins were quantitated by densitometry.

Sensitivity of Fibronectin to Trypsin. Confluent cells, iodinated as above, were incubated with trypsin (1 to 2.5 μg/ml in PBS, North America Biologicals) for 30 min at 37°. The cells were scraped, collected by centrifugation, solubilized and electrophoresed as above. Autoradiograms were prepared and quantitated as above. To evaluate the effect of trypsin on the capacity of the fibronectin preparation to agglutinate formalin treated sheep red blood cells, fibronectin from unlabeled HFL-1 cells was prepared and dialyzed against 0.2 M NaCl, 5 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.4. Aliquots were incubated with trypsin (1 μg/ml, 37°, 60 min) and tested for passive hemagglutination activity at 23° (4).

Evaluation of the Relationship Between Cell Surface Fibronectin and Medium Proteins. Confluent HFL-1 cells were labeled with [^{14}C]proline for 60 min. The medium was decanted and the cell layer rinsed (x4) with PBS containing 10 mM unlabeled proline. The cells were then re-incubated with "chase" medium (DMEM with 10 mM unlabeled proline, 0.06% glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin) for periods up to 48 hrs. At specified times, the incubation was stopped and the medium and cell layer separated. Labeled medium proteins were then precipitated with ethanol and collected as above. The cell layer was scraped with a rubber policeman and harvested by centrifugation. Medium and cell layer proteins were then subjected to electrophoresis on SDS-acrylamide gels. The relative concentrations of protein bands in the resulting autofluorograms were quantitated by densitometry.

RESULTS

Several criteria were used to identify the major cell surface protein of HFL-1 fibroblasts as fibronectin: (1) the major protein labeled by lactoperoxidase catalyzed iodination of HFL-1 cells is a 220,000 dalton molecule (Figure 1A); (2) when HFL-1 cells were incubated with [^{14}C]proline, [^{14}C]glucosamine or [^{3}H]fucose, these precursors were actively incorporated into a protein which co-migrated on SDS-acrylamide gels with the 220,000 dalton ^{125}I-labeled cell surface protein (Figure 1 B-D); (3) the quantity of this protein on the surface of HFL-1 cells was dependent on the density of the fibroblast cultures; when HFL-1 cells were subcultured, the amount of 220,000 dalton cell surface protein/cell steadily increased with time in passage such that by day 5, when the cells were confluent, the 220,000 dalton protein/cell was 5 times that present on day 1; and (4) the 220,000

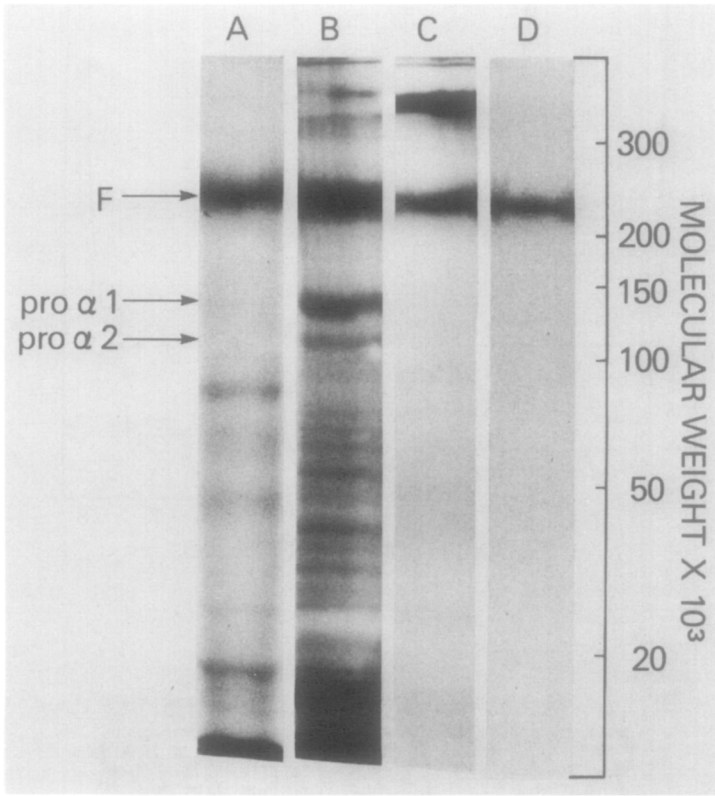
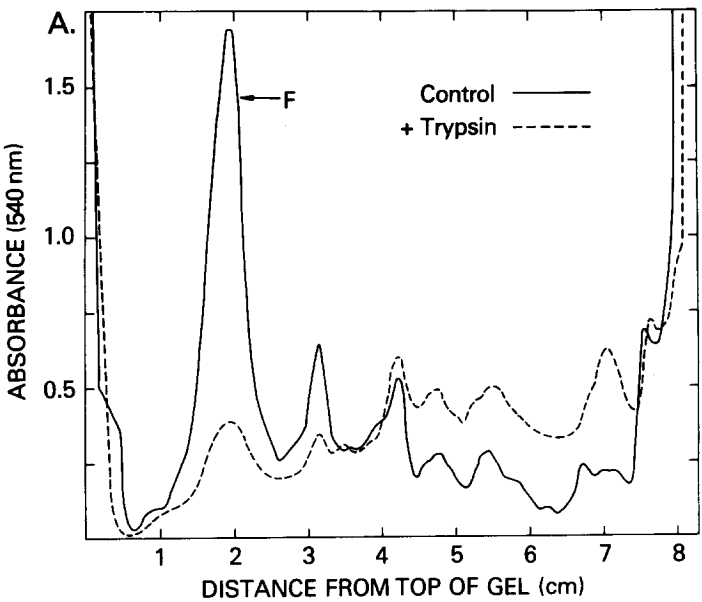


Figure 1. Sodium dodecyl sulfate acrylamide gels of fibronectin prepared from human lung fibroblasts after labeling by: (A) lactoperoxidase catalyzed labeling of external cell surface proteins with ^{125}I; (B) [^{14}C]proline; (C) [^{14}C]glucosamine; and (D) [^{3}H]fucose. The positions of fibronectin (F) and the pro α chains of collagen are indicated.

dalton cell surface glycoprotein was sensitive to low concentrations of trypsin; limited exposure to trypsin removed more than 80% of I^{125}-labeled 220,000 dalton protein (Figure 2A) and also destroyed the ability of fibronectin preparations from HFL-1 cells to agglutinate formalin treated sheep red blood cells (Figure 2B).

Further study demonstrated that fibronectin synthesized by HFL-1 cells was released into the culture medium. Gel electrophoresis of culture medium from cells labeled with [^{14}C]proline, [^{14}C]glucosamine and [^{3}H]fucose demonstrated the presence of a glycoprotein similar to



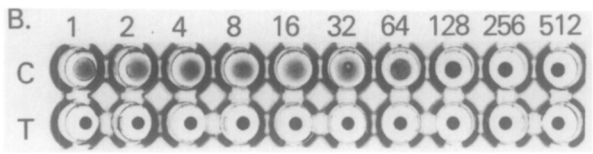


Figure 2. Sensitivity of HFL-1 fibronectin to mild exposure to trypsin. (A) Densitometric scans of sodium dodecyl sulfate acrylamide gels of an ^{125}I-labeled fibronectin preparation before (———) and after exposure to trypsin (2.5 µg/ml, 37°, 30 min) (------). (B) Ability of HFL-1 fibronectin to agglutinate formalin treated sheep red blood cells (labeled "C"); when the fibronectin preparation was exposed to trypsin (1 µg/ml, 37°, 30 min), it lost the ability to agglutinate formalin treated sheep red blood cells (labeled "T"). Reciprocal of dilution is indicated above corresponding wells.

fibronectin (Fig. 3A-C). When the cell surface proteins of HFL-1 fibroblasts were labeled with ^{125}I, and cells re-incubated with fresh medium, a 220,000 dalton ^{125}I-labeled protein appeared in the medium (Figure 3D). This could be prevented by removing cell surface fibronectin with trypsin prior to re-incubation with fresh medium (Figure 3E).

The appearance of fibronectin in the culture medium had a stoichio-

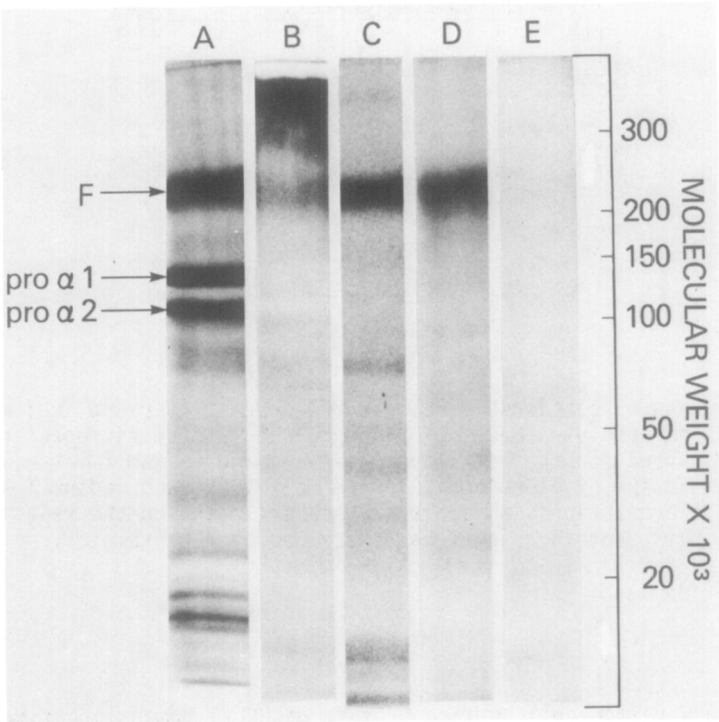


Figure 3. Sodium dodecyl sulfate acrylamide gels of medium proteins of HFL-1 fibroblasts labeled with: (A) [^{14}C]proline; (B) [^{14}C]glucosamine; (C) [^{3}H]fucose; (D) lactoperoxidase catalyzed labeling of external cell surface proteins with ^{125}I, 20 hrs prior to the collection of medium; and (E) same as (D) except that immediately after external labeling with ^{125}I, the cell layer was exposed to trypsin (2.5 μg/ml, 37°, 30 min) and then re-incubated with fresh medium. The positions of fibronectin and collagen proα chains are indicated.

metric relationship with loss of fibronectin from the cell layer (Figure 4). Using [^{14}C]proline as a label to follow the fate of newly synthesized fibronectin in pulse-chase experiments, the sum of the quantity of this protein in the cell layer plus medium was constant from 3 to 48 hrs after the chase period started. Fibronectin appeared in the medium in two phases: an initial (0 to 3 hrs) rapid phase followed by a second slower (3 to 48 hrs) phase. Fifty percent of the fibronectin on the surface of HFL-1 cells was shed within 25 hrs.

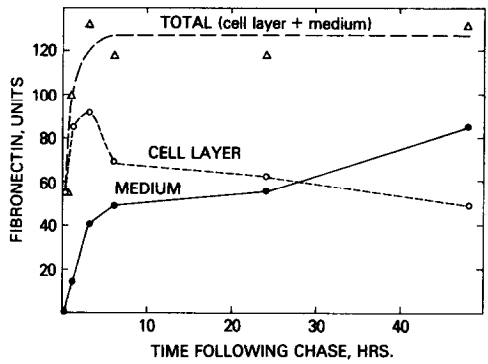


Figure 4. Temporal relationship between HFL-1 cell layer and medium fibronectin. The absolute amount of [^{14}C]proline labeled fibronectin on the gels is shown for the cell layer (------) and medium (———). Also shown is the sum of fibronectin in the cell layer and medium (—— ——). One "unit" of fibronectin is an arbitrary measure based on the relative quantities of this protein present as determined by densitometry.

DISCUSSION

The 220,000 dalton cell surface protein of HFL-1 human lung fibroblasts is defined as fibronectin by the following criteria (18): it has an identical molecular weight; it demonstrates density dependence; it is a glycoprotein synthesized by the cell; it is very labile to digestion by a neutral protease; and it functions as a hemagglutinin while fractions exposed to mild proteolytic digestion have lost this functional capacity. In addition, this protein cross-reacted with antisera to the major cell surface protein of chick embryo fibroblasts.*

Several studies have described fibronectin on normal cells and demonstrated that it is continually shed from the cell surface (8-11). The present study shows that fibronectin is progressively released from the cell layer of normal human fibroblasts in a form identical (within the limits of the analytical methods used) to the cell surface associated protein. Most importantly, quantitative analysis of the labeled proteins of the cell layer and medium of HFL-1 cells pulsed with [^{14}C]proline

*Kindly done by Dr. K. M. Yamada

demonstrated that all fibronectin released from the HFL-1 cell layer could be found as the medium protein. Thus, not only was there a precursor-product relationship between HFL-1 fibronectin and the medium 220,000 dalton protein, but the relationship was stoichiometric. In contrast to a recent study using chick embryo fibroblasts (19), the data presented here demonstrates that all of the fibronectin from normal human cells is conserved as it is released from the cell surface. Thus, at least for human fibroblasts, the shedding of fibronectin does not involve extensive proteolysis.

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