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Post-translational modification of the β-subunit of the human fibronectin receptor

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Monoclonal antibody DH12, directed against the β-subunit of the fibronectin receptor recognizes a doublet of proteins (100 and 110 kDa) in Western blots of solubilized whole fibroblasts. Pulse-chase experiments with [^{35}S]methionine in human skin fibroblasts suggested that the two proteins might be metabolically related as precursor (100 kDa) and product (110 kDa). Endo H digestion and [^{3}H]fucose labeling suggested that maturation converted the high-mannose oligosaccharides (100 kDa) to the endoglycosidase H resistant complex type (110 kDa). This was supported by *N*-glycanase digestion and by chemical deglycosylation which showed a single polypeptide. Surface iodination of intact cells labeled only the presumed mature β-subunit.

Post-translational modification; Fibronectin receptor; (Human)

1. INTRODUCTION

The nature of the cell surface molecules involved in the adhesive interactions of various cells is gradually being unraveled. Among these, the cell adhesion molecules (CAMs) [1], as well as a family of transmembrane glycoproteins, the integrin family [2] or cytoadhesin family [3]. The fibronectin and vitronectin receptors, platelet glycoprotein IIb/IIIa as well as the very late T-cell antigens (VLA) and the LFA-I and C3bi receptors belong to this family. All are heterodimers, composed of non-covalently bound α- and β-subunits, which participate in the interaction of the cells with a particular substrate [2]. The family itself can be subdivided in three major subfamilies, each sharing one of three different β-subunits [2]. Each β-subunit in turn can form a heterodimer with a family of α-subunits [4]. The β-subunit of the human fibronectin receptor [5] crossreacts immunochemically with the chicken integrin and is a member of the VLA family of proteins [2,4,6].

Monoclonal antibody, DH12 (Mab DH12), specifically recognizes an epitope on the β-subunit of the human fibronectin receptor [7,8]. This Mab systematically reveals two proteins of slightly different molecular mass (100 and 110 kDa) in Western blots and in double immunoprecipitations of whole normal human fibroblast (NHF) preparations.

The present study will show that the 100 kDa form is an intracellular precursor of the mature 110 kDa β-subunit and that the difference between the two antigenically related proteins is due to post-translational glycosylation.

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Abbreviations: Mab, monoclonal antibody; SA, specific activity; DME, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Endo H, endoglycosidase H; PA-bead, protein A-Sepharose beads; RaM, rabbit anti-mouse IgG; NHF, normal human skin fibroblasts

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2. MATERIALS AND METHODS

2.1. *Radioactive labeling of the cells*

3×10^6 NHF cells, grown in 75 cm^2 plastic flasks [9], were washed once with methionine-free Dulbecco's modified Eagle's medium (DME, Gibco) and preincubated for 30 min in this medium. The cells were labeled for 1 h or for 24 h with 50 μCi [^{35}S]methionine (S.A. 1128 Ci/mmol, NEN Research Products) per ml methionine-free medium.

For pulse-chase experiments, the labeled medium was removed after 1 h, the cell layers washed twice with fresh unlabeled culture medium and further incubated for the periods indicated. Lactoperoxidase catalyzed surface iodination of intact NHF cells was done as described by Hynes [10].

Confluent monolayers of NHF cells were incubated for 24 h with 100 μCi of D-[6-^{3}H]glucosamine (S.A. 19.7 Ci/mmol, New England Nuclear), 50 μCi of $H_2{}^{35}SO_4$ (carrier free) or 100 μCi L-[6-^{3}H]fucose (S.A. 86.3 Ci/mmol, New England Nuclear) per ml serum-free DME containing 0.81 mM $MgCl_2$ instead of 0.81 mM $MgSO_4$ and 1 g of glucose/l (Gibco).

2.2. *Double immunoprecipitation*

Confluent [^{35}S]methionine-labeled NHF monolayers were washed three times with 10 ml PBS, pH 7.2, containing proteinase inhibitors (1 mM EDTA, 1 mM iodoacetamide, 1 μg/ml pepstatin A and 100 U/ml aprotinin) and were scraped in the same buffer.

The cells were solubilized in 0.5 ml of double immunoprecipitation (DIPPA) buffer (0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, containing 1% sodium desoxycholate, 1% Triton X-100 and 0.1% SDS). The cell lysate was briefly sonicated and centrifuged in a Beckman Airfuge for 6 min.

Per 3×10^6 cells, 12.5 mg protein A-Sepharose beads (PA, Pharmacia) in 300 μl PBS were incubated with 80 μl rabbit anti-mouse IgG (RaM, Dako immunoglobulins) overnight at 4°C. The RaM-PA-beads were washed in DIPPA buffer and incubated overnight at 4°C with 250 μg DH12 monoclonal antibody. The beads were washed three times with 1 ml DIPPA buffer and incubated overnight at 4°C with the labeled cell lysate, which was pre-cleared by incubation overnight with RaM-PA-beads. The resulting immune complex was washed four times, with 1 ml DIPPA buffer and subsequently solubil-

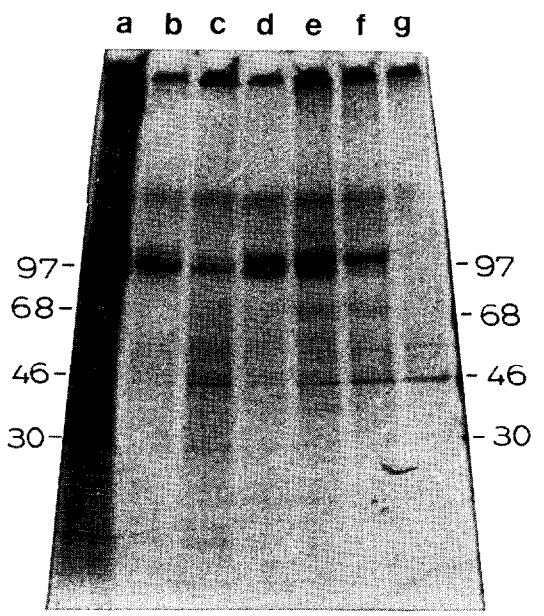

Fig.1. Immunoprecipitation of the fibronectin receptor β-subunit during pulse chase of NHF cells. NHF cells were pulsed for 1 h with [^{35}S]methionine and chased for the periods of time indicated. Cell lysates were immunoprecipitated with Mab DH12. Lanes: a–f are immunoprecipitates at 0, 1, 3, 6, 12 and 24 h of chase; g, an immunoprecipitate without DH12 Mab as a negative control of lane f.

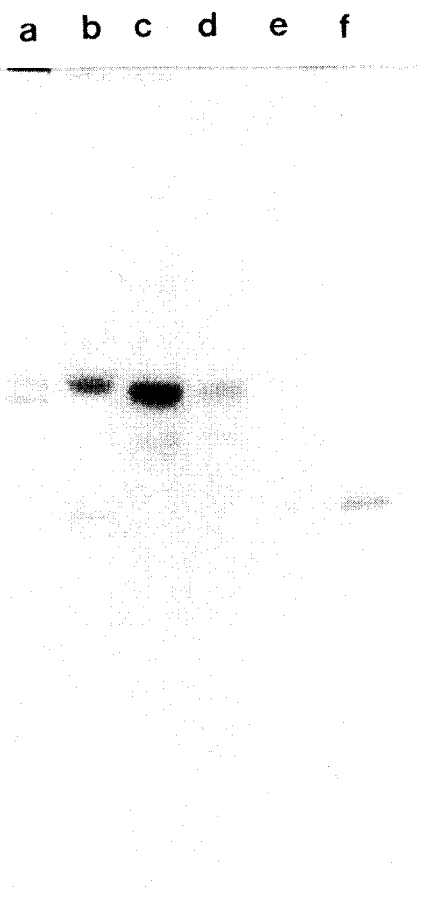

Fig.2. Immunoprecipitation of radioiodinated NHF and effect of trypsin treatment before and after radioiodination. Lanes: a, [^{35}S]methionine labeled NHF; b, radioiodinated cells without further treatment; c and d, cells treated with trypsin (0.05% 2 × crystalline (Sigma) in 0.52 mM EDTA before (c) or after (d) iodination; e, trypsinate of trypsin-treated cells; f, negative control of lane b (without DH12 Mab).

ized in 100 μl of 50 mM Tris buffer, pH 6.8, containing 5% SDS and boiled for 5 min. The solubilized precipitates were analysed by SDS-PAGE (6–20% linear polyacrylamide gels) [11] and the dried gel was autoradiographed. Tritium-labeled material was processed for fluorography [12].

2.3. *Enzymatic treatments*

Antigen loaded immunobeads, obtained as described above, were boiled for 5 min in 0.1 M sodium phosphate buffer, pH 6, 20 mM EDTA and 1% SDS.

After adding Triton X-100 (to a final concentration of 5%) and proteinase inhibitors (100 mM 6-aminohexanoic acid, 200 U/ml aprotinin, 5 μg/ml pepstatin, 40 μg/ml leupeptin), the supernatant was divided into two equal parts, about 40 μl each, and incubated overnight at 37°C with or without 20 mU of endoglycosidase H (Endo H, Boehringer). After enzymatic digestion an excess of SDS (to about 4%) was added to the samples, which were boiled for 5 min and analysed by SDS-PAGE (6–20%).

2.4. *Trifluoromethane sulfonic acid treatment*

The immunoprecipitated proteins were eluted from the protein A-Sepharose beads by boiling in SDS, were lyophilized and treated with trifluoromethane sulfonic acid as described by Edge et al. [13].

3. RESULTS AND DISCUSSION

Monoclonal antibody DH12 (DH12 Mab) reacted consistently with two proteins, of 100 kDa and of 110 kDa (unreduced) in Western blots and in double immunoprecipitations of [^{35}S]methionine-labeled NHF homogenates [7]. These two proteins could either be two independent but immunochemically related proteins or be a precursor and the mature form of a single β-subunit of the fibronectin receptor.

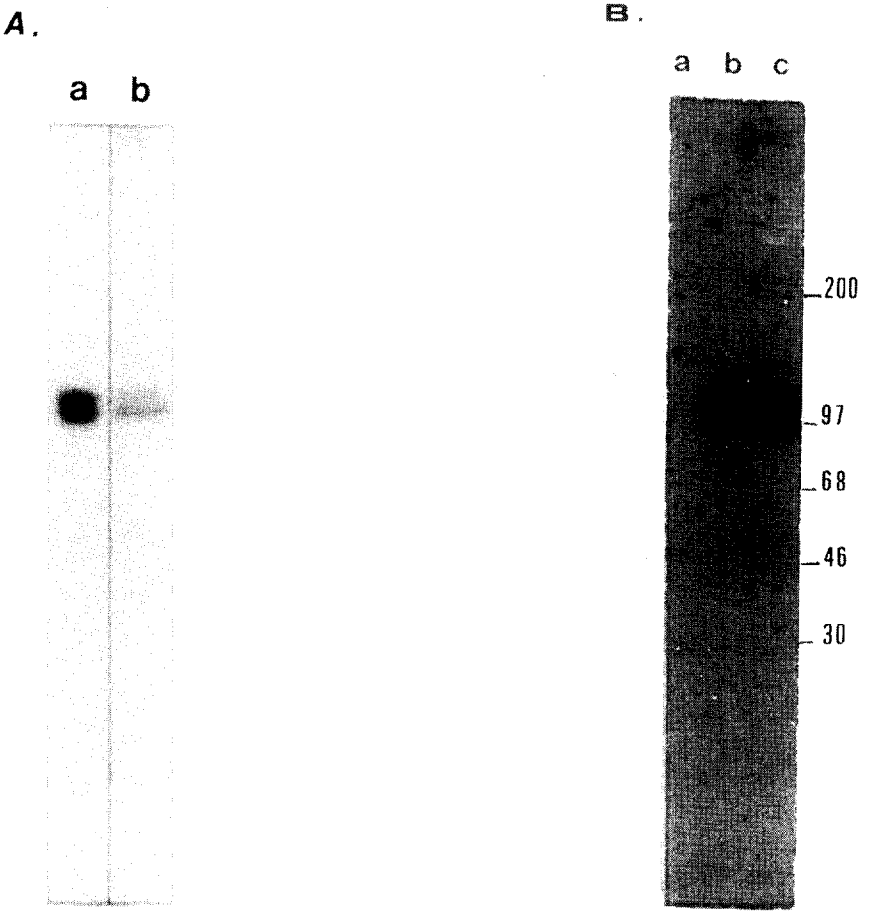

Fig.3. Glucosamine, sulfate and fucose labeling. NHF were labeled for 24 h and cell lysates were immunoprecipitated with DH12 Mab as described in section 2. (A) Lanes: a, labeling with [^{3}H]glucosamine; b, control with [^{35}S]methionine. (B) Lanes: a, negative control of lane b (no Mab DH12); b, labeling with L-[6-^{3}H]fucose; c, control with [^{35}S]methionine.

Data consistent with the second possibility were obtained in a pulse-chase experiment in which NHF cells were labeled for 1 h and chased for 1, 3, 6, 12 and 24 h. The cell lysates were immunoprecipitated with DH12 Mab as described in section 2, analysed by SDS-PAGE and autoradiographed.

As shown in fig.1 only the 100 kDa labeled protein was immunoprecipitated after 1 h of chase, while after 6 h and beyond the 110 kDa subunit also appeared concomitant with an apparent decrease in the amount of the 100 kDa protein. After 24 h of chase only the 110 kDa protein could be visualized. Therefore this form is presumed to be the mature β-subunit. A similar finding was observed in mouse 3T3 cells [14].

The apparent transition time (3–6 h) from precursor to mature form is rather long compared to most other glycoproteins (review Rothman [15]). For Mac-1, it was demonstrated that association between the α- and β-precursors is required for their processing and surface expression [16,17] and a similar mechanism may be operative for the vitronectin receptor [18,19] and for the fibronectin receptor (this study). The existence of a large intracellular pool, which is only slowly repleted by newly synthesized β-subunits might lead to similar apparently slow turnover kinetics.

Surface iodination of intact cells, with lactoperoxidase and glucose-oxidase, labeled only the 110 kDa protein (fig.2, lane b). This was not due to extraneous differences of the two proteins of the doublet, since similar labeling of crude cell-membrane preparations visualized both the 100 kDa and the 110 kDa proteins [7].

The differential radioiodination of the proteins

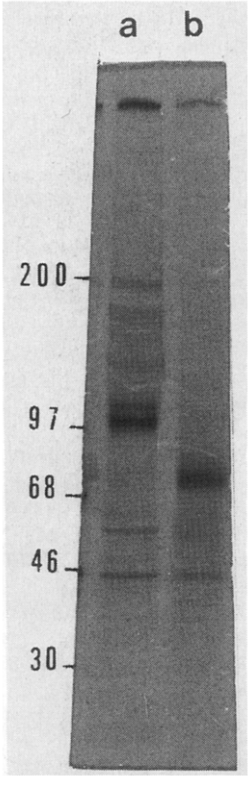

Fig.4. Effect of TFMS treatment. Immunoprecipitates of [^{35}S]methionine-labeled NHF cells (for 24 h) were applied to SDS-PAGE before and after TFMS treatment. Lanes: a, control without TFMS treatment; b, deglycosylation by TFMS.

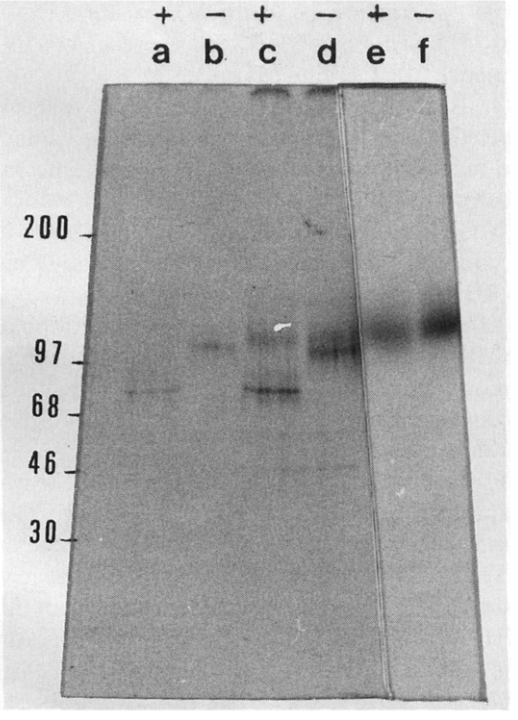

Fig.5. Deglycosylation of the β-subunit precursor with endoglycosidase H. NHF cells were labeled for 1 h with [^{35}S]methionine to obtain only the β-precursor form or were labeled for 24 h to obtain both the precursor as well as the mature form. The mature β-subunit was obtained separately by surface iodination of intact NHF cells. The immunoprecipitates of the precursor form (lane a and b), the mature form (lane e and f) or both forms (lane c and d) were analysed as such (lane b, d and f) or after treatment with endo H (lane a, c and e).

in intact cells therefore indicates that only the higher molecular mass protein was expressed at the cell surface.

Circumstantial evidence for the surface location of the iodinated 110 kDa protein was obtained by trypsinization of skin fibroblast cell layers.

Fig.2 (lane c and d) shows the result of trypsin treatment before and after radioiodination. In both cases the same pattern was obtained, with a small shift in the mobility of the 110 kDa protein (cf. lanes b and c, fig.2), which could be explained by the cleavage of a small fragment from the surface-expressed β-subunit.

Double immunoprecipitation of the trypsinate of iodinated cells after immunoprecipitation showed negligible amounts of label (fig.2, lane e), suggesting that the β-subunit remained associated with the cell surface after trypsinization or that the epitope recognized by DH12 was destroyed.

To characterize further the 100 kDa and 110 kDa proteins, NHF cells were grown in the presence of [^{3}H]glucosamine or [^{3}H]fucose for 24 h. Both the mature and precursor β-forms were labeled with [^{3}H]glucosamine (fig.3A), while only the mature form of the β-subunit became radiolabeled with [^{3}H]fucose (fig.3B).

N-Glycanase digestion of the individual glycoproteins reduced the molecular mass to nearly 80 kDa in both cases (not shown).

After deglycosylation of the protein doublet by TFMS (which cleaves *O*-glycosyl bonds, while *N*-glycosyl bonds between asparagine and *N*-acetylglucosamine remain intact [13]), only one protein band of 73 kDa remained (fig.4).

Endo H digestion of the precursor form also reduced the molecular mass of 100 kDa to 73 kDa (fig.5, lane a and b), suggesting the presence of *N*-linked high-mannose oligosaccharides. The mature β-subunit labeled by surface iodination of intact NHF cells, was not affected by Endo H treatment (fig.5, lane e and f).

Our findings suggest that the β-subunit of the human fibronectin receptor is rapidly (30–60 min pulse) synthesized in the endoplasmic reticulum as a precursor with a polypeptide core of 73 kDa and high-mannose oligosaccharide side chains. Maturation of this high-mannose oligosaccharide yields the Endo H resistant glycoprotein containing fucose.

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