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Fibronectin is a non-viral substrate for the HIV proteinase

M. Oswald and K. von der Helm

Pettenkofer Institute, University of Munich, D-8000-Munich, Germany

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The retrovirus encoded proteinase (PR) is required for the proper maturation of viral particles into infectious virus. The PR had been considered highly substrate specific, cleaving exclusively the viral gag and gag-pol protein precursor. It has recently been reported, however, that cytoskeleton and other cellular filament proteins can be cleaved by the HIV-1 PR. Here we have evidence that a cell-associated protein, the fibronectin (A-chain), is also cleaved in vitro specifically by this PR. The possibility of a cytotoxic role of the PR is conceivable.

Fibronectin; HIV proteinase

1. INTRODUCTION

Retroviruses encode a proteinase necessary for the maturation process of infectious retrovirus particles [1]. This enzyme is exogenous to the cell; it is transferred into the host cell by retroviral infection and processes retroviral protein precursors. As many cellular proteases are capable of acting catabolically, the question arose as to whether or not this viral enzyme might attack host cell proteins – as a side-effect and in a cytotoxic way. Based on the fact that infection by oncorna viruses generally does not cause cytotoxic effects but rather immortalizes the host cells, it was thought that the viral proteinase is prevented from cleaving cellular proteins by a high substrate specificity [2–4]. Recently it has, however, been reported that cellular proteins can be cleaved, at least in vitro, by purified HIV-proteinase [5–8]. This tempted us to determine whether or not cell-associated proteins like fibronectin [9] can serve in vitro as substrates for the purified HIV-1 proteinase. In this manuscript we demonstrate evidence that plasma fibronectin (A-chain) is cleaved in vitro by the HIV-1 proteinase at a site 39 kDa from the C-terminal end. This cleavage site is within a domain responsible for fibronectin binding to lymphocytes and the transamidase reactive site.

2. MATERIALS AND METHODS

2.1. *Recombinant proteinase*

Recombinant expression and preparation of recombinant HIV-1 proteinase was as described in [10,11]. The bacterial lysates were used partly purified as the source of proteolytic activity. In key experiments the recombinant HIV-1-proteinase was highly purified.

Correspondence address: K. von der Helm, Pettenkofer Institute, Pettenkoferstr. 9A, University of Munich, D-8000-Munich, Germany

2.2. *Fibronectin (FN)*

The human plasma fibronectin and the antiserum to it used was purchased from Sigma (Munich) or highly purified fibronectin used in the preparation of cleavage fragments for microsequencing was provided by H. Hörmann and H. Richter (Max Planck Institute of Biochemistry, Munich).

Microsequencing was done by Appligene (Straßbourg).

2.3. *In vitro proteolytic assay of fibronectin*

An equivalent of about 175 ng (Fig. 1A) and 50 ng (Fig. 1B and C) of the recombinant HIV-1 proteinase was incubated with about 65 μg (Fig. 1A), 4 μg (Fig. 1B) and 1 μg (Fig. 1C) of fibronectin at 37°C in a PNTE lysate buffer (50 mM MES, pH 6.5/150 mM NaCl/0.05% Tween/1 mM EDTA) for the periods of time indicated in the figure legends. To exclude proteolytic activities by non-aspartic proteases the incubation mixture contained 1 μM leupeptin, 0.1 mM EDTA, 0.2 mM PMSF. In a particular experiment the specific inhibitor of the HIV-1 proteinase, EMD 57464, (E. Merck, Darmstadt) [12] was used to verify the specific cleavage by the viral enzyme. The reaction was stopped by the addition of 4× concentrated electrophoresis buffer (7% SDS/30% glycerol/20% β-mercaptoethanol/0.01% Bromphenol blue in 90 mM Tris-HCl, pH 6.8) and boiling for 15 min. The reaction products were separated by 10% or 7% SDS/PAGE and stained with Coomassie blue or, in part, immunoblotted on nitrocellulose filters and incubated with antiserum to human fibronectin from Sigma. In kinetic studies (Fig. 2) the quantity of resulting cleavage product of the 39 kDa fragment was determined by densitometry (Elscript 400/Hirschmann, Unterhaching).

3. RESULTS AND DISCUSSION

The in vitro incubation of plasma fibronectin with the HIV-1 proteinase resulted in the cleavage of the 240 kDa fibronectin, yielding a polypeptide of about 39 kDa and 200 kDa analysed by SDS-PAGE and Coomassie blue or the Western blot technique (Fig. 1A, B and C).

Often minor amounts slightly smaller than the 39 kDa band could also be detected. The rate of cleavage appeared to be fast. The majority of the cleavage product was detectable after 10–15 min of the reaction (Figs. 1B and 2). In control experiments (not shown) we excluded the possibility that nonspecific proteinase con-

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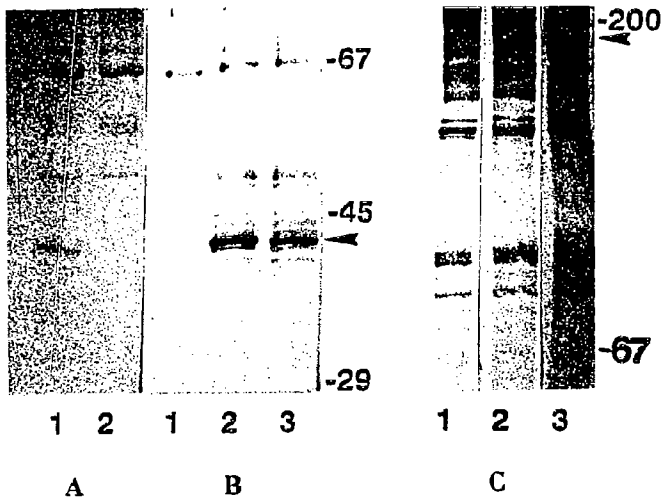

Fig. 1. Cleavage of plasma-fibronectin by HIV-1 proteinase yielding a polypeptide of about 39 kDa. Incubation of recombinant HIV-1 proteinase (A: 175 ng; B and C: 50 ng) with plasma-fibronectin (A: 65 μg; B: 4 μg; C: 1 μg) at 37°C in PNTE buffer containing 1 μM leupeptin, 0.1 mM EDTA, 0.2 mM PMSF to exclude proteolytic contaminations by non-aspartic proteases. The reaction products were analysed by SDS-PAGE (A and B: 10%; C: 7%) and Coomassie blue (A), and in addition (B and C), by immunoblot technique using polyclonal anti-fibronectin serum. (A) Coomassie blue staining: lane 1, incubation for 4 h; lane 2, no incubation. (B) Immunoblot: lane 1, no incubation; lane 2, incubation for 10 min; lane 3, incubation for 4 h. (C) Immunoblot: lane 1, no incubation; lane 2, incubation for 10 min; lane 3, incubation for 1 h. The arrows mark the cleaved polypeptide bands.

taminations from the bacterial lysate or fibronectin-inherent proteolytic activities were responsible for the cleavage observed: we incubated fibronectin with bacterial lysate carrying no PR construct or incubated fibronectin alone without lysate and did not detect any cleavage. Further, the specific HIV-1 proteinase inhibitor, EMD 57464, [12] did block the HIV proteinase cleavage of fibronectin indicating its specificity (not shown).

The cleavage of the viral gag and gag-pol protein precursor by retroviral proteinase is independent of Ca^{2+} or Mg^{2+} ions (von der Helm, unpublished). Also the fibronectin-cleavage by HIV-PR is – in contrast to Ca^{2+}-dependent fibronectin-inherent proteolytic activities [13,14] – independent of the presence of Ca^{2+}.

The results did not allow us to decide if the A- or B-chain of the fibronectin or both of them were cleaved by the HIV proteinase. Thus, the cleaved fragment of 39 kDa was purified and the N-terminal amino acids microsequenced. The resulting sequence of EEHGFRRTTP (Fig. 3) is unique for the amino acid sequence of the fibronectin A-chain because the complementary region in the B-chain is spliced out (Fig. 3) [15,16]. The resulting cleavage site in the fibronectin, ..QMIF/EEHG.., is not only different from the sequence pattern of the HIV-1 gag-pol precursor cleavage sites [1], but also from that of possible non-viral, cellular substrates [5–8]. Only the ...IF motif at the P2/P1 position resembles a putative consensus sequence of the cleavage site; the EE at the new N-terminus is apparently different from known HIV-1 proteinase cleavage sites and computer predictions thereof which would tolerate one E in the P'2 *or* P3 position but not in P'1 *and* P'2.

The observed fibronectin cleavage has been carried out with highly purified, intact fibronectin [21]. It is unlikely (see Fig. 1C) but not excluded that a fibronectin fragment rather than the full length A-chain was cleaved. This is, however, not essential in the context of the identification of non-viral sequences for a HIV-1 proteinase cleavage site hitherto unknown.

The cleavage site is located within the V segment of fibronectin (also called type III connecting segment; III CS), a region of alternative splicing (Fig. 3) [15,16]. Interestingly, the V segment contains – about 35 amino acids upstream of the HIV-1 proteinase cleavage site – a cell-binding domain (CS-1) mediating cell attachment of B and T lymphocytes via the integrin receptor $\alpha 4\beta 1$ [17–19]. The adhesion of B lymphocytes to fibronectin is mediated exclusively by the interaction of $\alpha 4\beta 1$ with this cell-adhesion site (CS-1) [19].

Another interesting region, the transamidase reactive site [15,20,21], is also located closely to the HIV-1 proteinase cleavage site. The sequence GQQ is likely to be a target site on the fibronectin molecule for the action of the transamidase or the transglutaminidase factor XIIIa mediating covalent crosslinking of fibronectin molecules via Gln–Lys [15,22]. Presently we do not know whether or not the described in vitro cleavage will affect one or both domains because it is unknown whether such a cleavage occurs in vivo. If so, this would be further evidence for a possible cytotoxic role of the HIV proteinase: although fibronectin is not an integral cellular or membrane protein, it offers specific binding

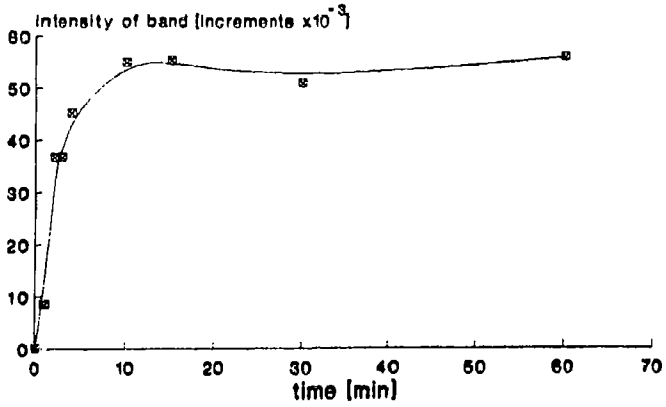

Fig. 2. Kinetics of the plasma-fibronectin cleavage by the HIV-1 proteinase. Recombinant HIV-1 proteinase (100 ng) was incubated with plasma-fibronectin (2 μg) for increasing periods of time (1–60 min) under the conditions described in Fig. 1. The reaction products were analysed by Western blot. The quantity of resulting cleavage product was determined by densitometry of the 39 kDa fragment in each lane (Elscript 400/Hirschmann, Unterhaching). The intensity of the 39 kDa band is plotted as a function of incubation time.

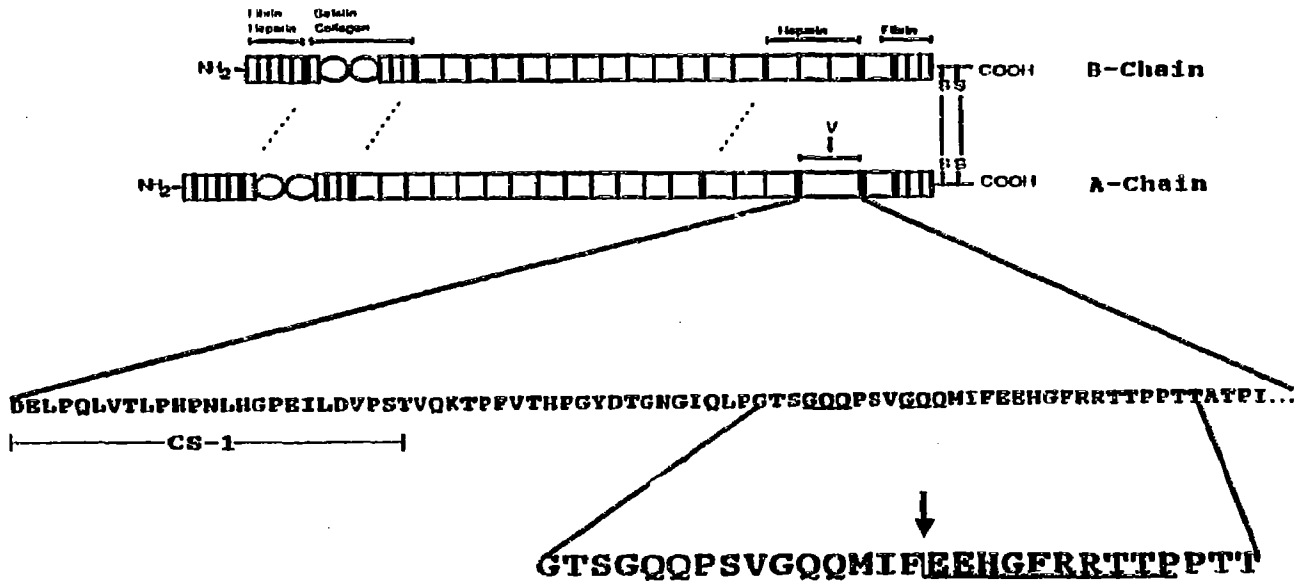

Fig. 3. HIV-1 proteinase cleavage site within the plasma-fibronectin molecule A-chain. The two chains of plasma-fibronectin are depicted. The N-terminal amino acid sequence EEHGFRRTTP (underlined) of the 39 kDa peptide arising after cleavage (arrow) is in the fibronectin A-chain. This sequence is located within the V segment (V), a region of alternative splicing, which is spliced out in the B-chain. Upstream of the HIV-1 cleavage site: a cell binding domain (CS-1) for B- and T-lymphocytes; the sequence GQQ (underlined), a target site for the action of a cell-associated transamidase of the transglutaminidase factor XIIIa mediating covalent crosslinking of fibronectin molecules via Gln–Lys.

sites for the cell surface mediating attachment to extracellular structure or cell-to-cell adhesion essential for cellular differentiation, growth and cell/tissue organization. Cleavage of the fibronectin at the indicated site upon HIV-1 infection might impair some or all of the mentioned functions.

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