Cleavage of recombinant and cell derived human immunodeficiency virus 1 (HIV-1) Nef protein by HIV-1 protease

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Abstract Recombinant purified Nef protein of HIV-1, as well as Nef protein derived from extracts of permanently HIV-1 infected glioblastoma cells and monocytes, are specifically cleaved by the HIV-1 protease. Nef cleavage products in cellular extracts treated with protease showed identical molecular weights as those obtained by digestion of purified Nef with recombinant HIV-1 protease. Since cellular extracts were prepared by detergent and mechanical lysis it cannot be excluded that physiological cytoplasmic conditions were altered. The lack of Nef cleavage by endogenous HIV-1 protease in infected cells might be due to low concentrations of viral protease and the presence of Gag precursor molecules as natural substrate. Using a panel of monoclonal antibodies two cleavage fragments of 19 kDa and 8 kDa were defined. The cleavage site was located by microsequencing between amino acid 57 and 58 (AW*LEAQEEEEVGF). The conserved cleavage motif within HIV-1 Nef suggests a potential biological function of Nef processing.

Key words: Nef protein; HIV-1 protease; Proteolytic cleavage

1. Introduction

The Nef protein is an accessory protein of the immunodeficiency viruses (HIV-1, HIV-2 and SIV). The functional role of HIV-1 Nef for the viral life cycle is still unclear, as it is neither essential for virus replication nor for the induction of cytopathic effects in vitro in permanent cell lines [1,2]. Contradictory observations were described concerning the putative negative regulatory function of HIV-1 Nef and its properties as an GTP binding protein [1–4]. The involvement of Nef protein in CD4 down-regulation emphasizes its role in the infection and replication process [5]. Immunofluorescence studies showed that Nef is localized in the nucleus [6,7] associated with cellular membranes [8], and found in the cytoplasm [9,10] concentrated in the perinuclear region [6,7,11]. Therefore it could be possible that Nef exerts several different regulatory functions during different stages of infection. This might indicate that Nef has to be post-translationally modified to exert its potential functions. An important modification is the myristoylation at the N-terminus of Nef [6,12]. Furthermore, phosphorylation [12,13] could contribute to multiple functional properties of Nef. As for the Gag precursor, a processing of Nef by the HIV-1 protease has been predicted [14] and was shown for recombinant Nef protein [15].

From these studies the question arises as to whether the cleavage event might occur during natural infection in eukaryotic cells. Such a kind of Nef processing could also alter or activate as yet unknown functions in vivo. Here we confirm this observation in more detail and also show that this is true for the Nef processing in cellular extracts of persistently HIV-1-infected cells in the same manner as with recombinant purified Nef protein.

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2. Materials and methods

2.1. HIV-1 Nef expression and purification

The HIV LAV-1 Bru nef was cloned into the prokaryotic expression vector pTG959 (kindly provided by B. Guy from Transgène, Strasbourg, France) and expressed in *E. coli*. Nef was solubilized from the bacterial lysate by addition of 8 M urea and 1% β-mercaptoethanol. Purification of Nef protein was performed by affinity chromatography on heparin-Sepharose (Pharmacia, Freiburg, Germany) in a one-step purification procedure as described by Kohleisen et al. (submitted). Nef was eluted with 200 mM NaCl in a 10 mM sodium phosphate buffer, pH 7.5, and was directly used for cleavage experiments.

2.2. Proteolysis of HIV-1 Nef using recombinant HIV-1 protease

Purified recombinant Nef protein was soluble in 10 mM sodium phosphate buffer, pH 7.5, with 200 mM NaCl. For enzymatic digestion, 12 mM purified Nef and 2 mM recombinant purified HIV-1 protease (Bachem Bioscience Inc., Philadelphia, USA) were incubated at 37°C for 1 h. The inhibitor for HIV-1 protease, RO31-5989, was kindly provided by Roche Products Ltd., Welwyn Garden City, UK.

For competition experiments equimolar amounts of p55 Gag protein (kindly provided by F. Bex, Department of Molecular Biology, University of Brussels, Belgium) were added to Nef protein prior to enzymatic digestion.

2.3. Cell lines and cell extraction

Cellular extracts were prepared from persistently HIV-1 infected (TH4-7-5) and non-infected glioblastoma cells (85HG66) as described by Brack-Werner et al. [16]. A monocytic cell line (RC-2A) infected with HIV-1 derived from TH4-7-5 cells (RC-2A_{TH}) was established as described by Kleinschmidt et al. (submitted). Cellular extracts were obtained by detergent lysis and high speed centrifugation as described previously [7].

2.4. SDS-PAGE and Western blot analysis

Proteins were separated on linear 10–20% gels by SDS-PAGE and electroblotted onto nitrocellulose (NC BA83, Schleicher & Schuell, Dassel, Germany) according to [7].

Briefly, antibody dilutions were performed in 1% BSA in 50 mM Tris, pH 7.5, with 150 mM NaCl and 0.05% Tween 20. For detection of Nef, mouse monoclonal antibodies (mAbs) and a rabbit polyclonal Nef antiserum (27/89 4' [7]) were used.

Mouse mAbs with mapped epitope specificities D141 (aa 33–35), D138 (aa 68–72), D212 (aa 51–71), D286 (aa 151–170), D5 (aa 198–204) were kindly provided by P. Wernet and L. Shi, Institut für Transfusionsmedizin, Universität Düsseldorf, Germany. mAbs nef21 (aa 81–90) and nef1 (aa 170–190) were a gift from R. v. Baehr, Charité, Humboldt-Universität Berlin, Germany. Mab 3E6 (aa 168–175) and 2H12 (aa 171–190) described by Ovod et al. [17] were kindly provided by K. Krohn, Department of Biomedical Science, University of Tampere, Finland. The purified mouse mAb NCRD 10/11 (Behring AG, Marburg, Germany) was used as first antibody for p55 Gag detection.

As second antibody, an IgG-alkaline phosphatase conjugate

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(Dianova, Hamburg, Germany) and as substrate Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate [18] were used. The more sensitive ECL system was applied for detection of low protein concentrations with a horseradish peroxidase (HRPO)-labelled IgG as second antibody according to the instructions of the manufacturer (Amersham-Buchler, Braunschweig, Germany). The nitrocellulose was exposed to an X-ray film (ECL hyperfilm, Amersham-Buchler, Braunschweig, Germany) at −70°C.

2.5. Microsequencing of the cleavage fragment

Amino acid sequencing was independently performed by M. Eulitz, GSF, Institut für klinische Molekularbiologie und Tumorgenetik, and F. Lottspeich, TopLab, both Munich, Germany, using eleven cycles of Edman degradation after transfer of protein onto PVDF membrane (Bio-Rad, Munich, Germany).

2.6. Sequence similarities

HIV sequences are compared according to Myer's database [19]. It contains 24 members of the HIV-1 group (premature stopped sequences were rejected). Alignments and comparisons are performed with programs from the GCG software package [20]: Pileup and Plotsimilarity. Plotsimilarity calculates the distances between the different sequences in a window (win), which has to be defined. win = 1 was chosen to compare the single amino acids at the cleavage site and win = 7 to determine the global structure of the *nef* gene.

3. Results and discussion

3.1. Cleavage of Nef protein by HIV protease

Cleavage of Nef by recombinant HIV-1 protease is shown in Fig. 1. Nef protein is rapidly and specifically digested into two fragments. Since mAb 2H12 was mapped to react with a peptide encompassing aa 171–190 of Nef, only the larger Nef fragment with a molecular weight of approximately 19 kDa is detected. Eleven cycles of Edman degradation of this cleavage fragment revealed the amino acid sequence LEAQEEEEVGF, corresponding to the cleavage site between Trp^{57} and Leu^{58}.

First cleavage products are detected 5 min after incubation at 37°C at pH 7.5. The specificity of the cleavage was demonstrated by using 100 nM protease inhibitor RO31-5989. No cleavage products were observed in the presence of RO31-5989,

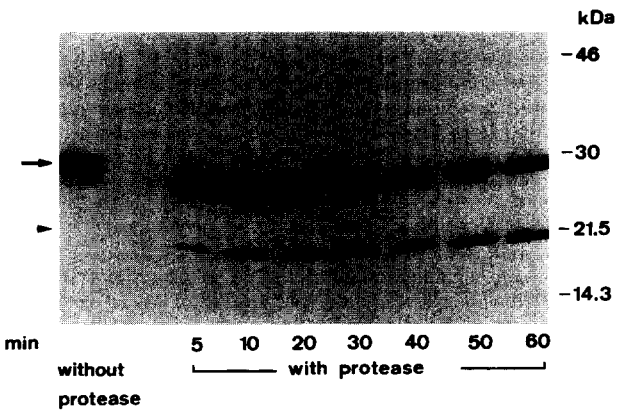

Fig. 1. Digestion of recombinant (rec) HIV-1 Nef protein with rec HIV-1 protease. *E. coli* expressed and purified Nef was digested with protease for various incubation times as indicated. As a control, Nef without protease was incubated for 60 min at 37°C. Proteins were separated on 10–20% SDS-polyacrylamide gels and electroblotted onto NC. For detection of the 19 kDa Nef fragment (arrow head), NC was probed with mAb 2H12, anti-mouse IgG-HRPO and ECL as substrate. The smaller 8 kDa fragment is not recognized since the epitope specificity of 2H12 is located in the C-terminal part of Nef. Molecular weights (MW) of marker proteins are indicated on the right and Nef protein with an arrow.

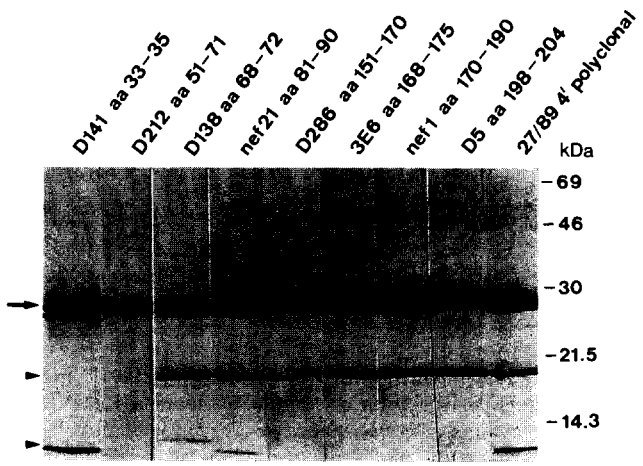

Fig. 2. Analysis of cleavage fragments by mAbs. Digested recombinant Nef fragments were separated by SDS-PAGE. In Western blot analysis each lane was probed with a different mouse monoclonal antibody. Epitopes recognized by mAbs are indicated. Arrow, Nef protein; arrowheads, 19 kDa and 8 kDa Nef fragments.

an inhibitor designed to block the viral protease (data not shown).

After 10 min of incubation a clear band representing the larger cleavage Nef fragment was observed. A longer incubation time up to 60 min only led to a small increase in the amount of the cleaved product. This might be due to degradation of the viral protease during incubation or conformational inaccessibility of the cleavage site in multimer forms of Nef. No degradation of Nef is observed after 60 min at 37°C without protease (Fig. 1).

3.2. Analysis of cleavage products by monoclonal antibodies

To demonstrate the presence of the smaller cleavage product and to analyze the reactivity of the two fragments, a panel of eight mAbs covering the Nef protein from aa 33 to 204 was applied. Mab D141 (aa 33–35) reacted exclusively with a smaller fragment of about 8 kDa whereas all other mAbs, except mAb D212, recognized the 19 kDa fragment. Mab D212 reacted only with the uncleaved Nef protein since the epitope located at aa 51–71, which is recognized by this antibody, is destroyed by the cleavage event (Fig. 2).

3.3. Competition experiments with Gag protein p55

To compare the efficiency of Nef cleavage with the cleavage of the natural substrate p55 Gag, competition experiments were performed. In the presence of equimolar amounts of purified Nef and p55 Gag proteins both substrates were cleaved by the protease (Fig. 3). Although the cleavage of Nef is less efficient in the presence of the Gag competitor, the 19 kDa Nef cleavage product is clearly detectable. This indicates that Nef might also be specifically processed in HIV-1-infected cells where large amounts of Gag and Gag-Pol precursor proteins are present. Furthermore, proteolytic fragmentation of cellular proteins [21], and especially components of the cytoskeleton [22–24], is described, demonstrating a broad spectrum of intracellular enzymatic activity of the HIV-1 protease.

3.4. Proteolytic fragmentation of Nef protein in cellular extracts

To elucidate possible inhibitory effects and to assay the

cleavage efficiency of in vivo Nef synthesized in eukaryotic cells in its viral context we analyzed Nef cleavage in cellular extracts of persistently HIV-1-infected cells. We used the monocytic cell line RC-2A$_{TH}$ which supports active replication of the virus, and thus reasonable amounts of Nef and Gag-Pol proteins are present; as well as the astrocytic cell line TH4-7-5, repressing HIV-1 replication to a great extent in favor for an excess of Nef production. Addition of recombinant protease to cellular extracts of TH4-7-5 and RC-2A$_{TH}$ led to a Nef cleavage pattern identical to digestion of recombinant Nef (Fig. 4). In extracts of non-infected cells no specific 19 kDa fragment appeared after protease treatment. Correct folding and post-translational modifications like phosphorylation and glycosylation of the Nef protein translated and processed during a natural cycle of infection can be assumed.

The physiological environment seems to permit cleavage of Nef synthesized and modified by the cell machinery. Such a truncated Nef fragment might exhibit modified or additional biological functions which could be important for viral replication in vivo.

3.5. Sequence analysis of the cleavage site

Sequencing of the cleavage site also demonstrated, that predictions of potential recognition sites for the viral protease based on the model of Poorman et al. [25] are not reliable. The two cleavage motifs in the Nef protein as hypothetically defined by this method are not processed by the viral protease. Chou [26] defined 21 possible cleavage sites for the HIV-1 protease. The motif selection JJJJJEJJ, where J stands for a random amino acid, appears rather simplified and it is unlikely that 19 out of 21 sites, as predicted by this model, might be cryptic and therefore are not accessible to the protease.

Sequence comparison of the cleavage motif by Plotsimilarity revealed a high conservation of the amino acids corrsponding to positions 56–65 in HIV-1 Bru (AW*LEAQEEEE) in most of the HIV-1 isolates in group A, B, D, and two out of four in group 0. The high conservation of this region in comparison to the N- and C-terminal parts of Nef indicates that it may have

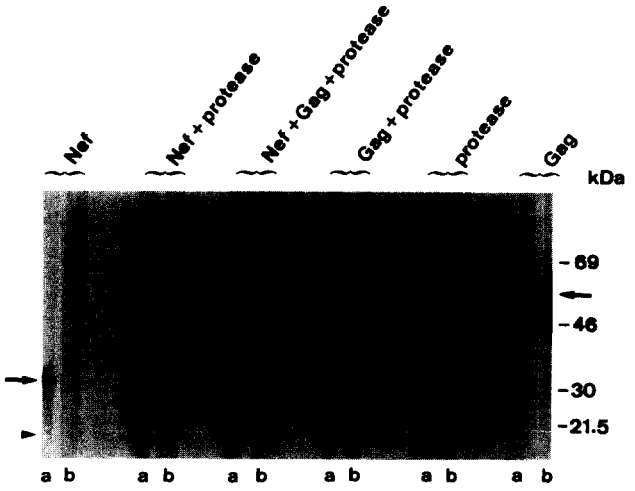

Fig. 3. Purified p55 Gag as competitor. The cleavage of Nef, p55 Gag and Nef/p55 Gag was performed at 37°C for 30 min. After SDS-PAGE and electroblotting onto NC, Western blot analysis was performed with mAb 2H12 for Nef (a) and NCRD10/11 for p55 Gag (b). ECL system was used for visualization. Arrow on the right, p55 Gag; arrow on the left, Nef protein; arrowhead, 19 kDa Nef fragment.

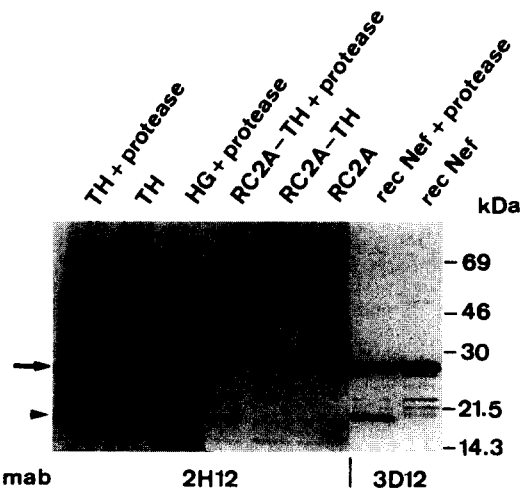

Fig. 4. Digestion of eukaryotic-expressed Nef in extracts of infected glioblastoma cells and monocytes. Infected and non-infected cellular extracts were treated with recombinant protease. Western blot analysis was performed with mAb 2H12 and 3D12 and the ECL system. Recombinant digested and undigested Nef were used as controls. Arrow, Nef protein; arrowhead, 19 kDa Nef fragment.

a crucial biological function for Nef. The cleavage site is situated to the left in a relatively constant part of the Nef protein, about 100 aa in length.

A negative hydrophilic motif around the cleavage site of HIV-1 protease similar to that in the Nef protein can also be found in non-viral substrates like the Alzheimer amyloid precursor (AAP). The similarity between the cleavage site described here and one of the cleavage sites [22] in the AAP is remarkable. In the region of the cleavage site six amino acids are identical: AW*LEAQEEEEVGF in Nef vs. KVVEVA*EEEEVAE in the extracellular region of AAP. A similar motif (VFFEEQEDEIIGF) is found in the thyrotropin receptor [27], but cleavage by HIV-1 protease is not yet tested.

Because of the presence of this sequence motif in cellular proteins one could assume that the cleavage of Nef by the HIV-1 protease is not only an event by chance but might have a biological function. The demonstration of the cleavage event within HIV-1-infected cells remains to be elucidated.

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