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Non-viral cellular substrates for human immunodeficiency virus type 1 protease

Robert L. Shoeman, Cornelia Kesselmeier, Elfriede Mothes, Bernd Höner and Peter Traub

Max-Planck-Institut für Zellbiologie, Rosenhof, D-6802 Ladenburg/Heidelberg, Germany

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A computer search revealed 10 proteins with homology to the sequence we originally identified in vimentin as the site of cleavage by human immunodeficiency virus type 1 (HIV-1) protease. Of these 10 proteins (actin, γ-actinin, spectrin, tropomyosins, vinculin, dystrophin, MAP-2, villin, TRK-1 and Ig μ-chain), we show that 4 of the first 5 were cleaved in vitro by this protease, as are MAP-1 and -2 [(1990) J. Gen. Virol. 71, 1985–1991]. In these proteins, cleavage is not restricted to a single motif, but occurs at many sites. However, cleavage is not random, since 9 other proteins including the cytoskeletal proteins filamin and band 4.1 are not cleaved in the in vitro assay. Thus, the ability of HIV-1 protease to cleave specific components of the cytoskeleton may be an important, although as yet unevaluated aspect of the life cycle of this retrovirus and/or may directly contribute to the pathogenesis observed during infection.

HIV-1 protease; Cytoskeletal protein; Initiation factor 2 (IF 2)

1. INTRODUCTION

Retrovirally encoded proteases are aspartyl proteases responsible and required for the cleavage of viral precursor polyproteins into the functional proteins of the mature virus particle (reviewed in ref. 1 and 2). This processing is required for viral infectivity and no host proteases have been identified that can substitute for this enzyme. Since no specific cleavage of host cell proteins has been directly demonstrated in retrovirus infected cells, it has generally been accepted that the retroviral proteases are specific for viral proteins. We have recently shown that purified HIV-1 protease can cleave intermediate filament proteins in vitro and have identified the primary cleavage site of vimentin to be SLNL/RE [3]. This site resembles a hybrid between two classes of viral protein cleavage sites [4], with the novel presence of an R at the sissile bond. Since it was also observed that microinjected HIV-1 protease not only alters vimentin intermediate filament distribution in human fibroblasts, but also causes changes in cell shape [3], we have searched for other cytoskeletal components that can be cleaved by this enzyme. In the present study, candidate proteins were identified by computer search using consensus sequences of known cleavage sites. We report here that 4 cytoskeletal proteins can be specifically cleaved by HIV-1 protease in vitro and predict that additional proteins may also be substrates. Cleavage of one of these predicted substrate proteins, MAP-2, has been independently reported by Wallin et al. [5].

Correspondence address R. L. Shoeman, Max-Planck-Institut für Zellbiologie, Rosenhof, D-6802 Ladenburg/Heidelberg, Germany

Abbreviations. HIV-1, human immunodeficiency virus type 1; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DTT, dithiothreitol; MAP-1, MAP-2, microtubule associated proteins 1 and 2; TRK-1, transforming tyrosine kinase protein; IF2, initiation factor 2; AMV, avian myeloblastosis virus; Ig, immunoglobulin

2. MATERIALS AND METHODS

The SWISSPROT data base of the EMBL data bank (European Molecular Biology Laboratory, Heidelberg, Germany) was searched using the FASTSCAN program for those proteins with the best homology to the vimentin primary cleavage site SLNLRE [3]. A similar search of this data base was made for cytoskeletal proteins using the various cleavage sites tabulated by Kay and Dunn [2] for retroviral proteases.

Human cytoplasmic actin from HeLa cells was purified through 2 rounds of polymerization/depolymerization according to standard protocols [6,7]. Human vimentin was prepared from HeLa cells [3]. Total erythrocyte ghost proteins and spectrin (α- and β-forms) were isolated [8] from normal human erythrocytes obtained from a volunteered donation at a local blood bank. The remaining substrate proteins were obtained from commercial sources: bovine and chicken tropomyosins, chicken α-actinin, and chicken filamin were from Sigma (Deisenhofen, Germany) and chicken vinculin was from Amersham-Buchler (Braunschweig, Germany). HIV-1 protease (more than 90% pure) was kindly provided by S. Roy (Hoffmann-La Roche, Nutley, NJ, USA). Protein standards for M_r determination (LMW calibration kit) were purchased from Pharmacia-LKB (Freiburg, Germany). Bio-Rad SDS-PAGE standards, low range (Cat. No. 161-0304, Bio-Rad, Munich, Germany) were employed as a source of native proteins for control reactions (see below). This preparation consists of a mixture of rabbit muscle phosphorylase B (MW 97 400), bovine serum albumin (MW 66 200), hen egg white ovalbumin (MW 45 000), bovine carbonic anhydrase (MW 31 000), soybean trypsin in-

hibitor (MW 21 500) and hen egg white lysozyme (MW 14400), each at a concentration of ~2 mg/ml in 50% glycerol, 300 mM NaCl, 3 mM NaN_3 and 100 mM DTT. This solution was diluted 1:20 with the standard buffer for the in vitro HIV-1 protease assay (see below)

Reaction conditions and SDS-PAGE were as described [3]. In brief, 10 μg of the individual proteins were incubated at 37°C for 30 min in the presence of 20 pmol (for vimentin) or 40 pmol (all other proteins) of HIV-1 protease in a final volume of 100 μl containing 20 mM sodium phosphate, pH 6.5, 5 mM EGTA, 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride. Control reactions were performed by adding pepstatin A, an inhibitor of aspartyl proteases [2], to the reaction mixtures at a final concentration of 0.7 mM from a 20 mM stock solution prepared in dimethyl sulfoxide. Equal aliquots of reaction mixtures were applied to 9-15% acrylamide gels and proteins were visualized by staining with Coomassie blue

3. RESULTS AND DISCUSSION

Ten eukaryotic proteins were identified by a computer search for homology to the vimentin cleavage sequence SLNLRE (Table I). When these 10 proteins and other cytoskeletal proteins such as actin, tubulin, and erythrocyte band 4.1 proteins were additionally searched for homology to the known viral protein cleavage site [2], only actin was found to possess significant homology to these sequences (Table I). Given the relatively low absolute sequence requirements of HIV-1 protease [1-4], these less-than-perfect matches (Table I) are of questionable significance Nonetheless, we chose to test a number of these potential substrates and control proteins in an in vitro assay for HIV-1 protease. Of the 5 predicted substrate proteins tested, 4 were found to be cleaved by HIV-1 protease in vitro: actin, α-actinin, α- and β-spectrin and tropomyosins (both chicken (Fig. 1) and bovine (data not shown)). Chicken vinculin was not cleaved in our assay (Fig. 1) even though it was a candidate based on the computer search The other cytoskeletal proteins tested, erythrocyte band 4.1 proteins and filamin, were not identified as candidate substrates by the computer search and thus served as controls; indeed these proteins were not cleaved (Fig. 1 and data not shown). Furthermore, additional proteins tested, phosphorylase B, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme (all in Fig. 1) and tubulin [5] were not cleaved by HIV-1 protease in vitro. The partial cleavage of bovine serum albumin observed (Fig. 1B) indicates that this protein, although electrophoretically pure, was partially denatured. We have previously shown that bovine serum albumin is cleaved by HIV-1 protease only when denatured [3] and have since observed that this is a sensitive assay of the quality of a bovine serum albumin preparation (data not shown). The cleavage of vimentin, actin, α-actinin, α- and β-spectrins and the various tropomyosins was dependent on the added HIV-1 protease (Fig. 1) and could be inhibited by the aspartyl protease inhibitor pepstatin A (data not shown). The likelihood of contaminating proteases contributing to the observed effect was reduced by routinely carrying out the reactions in the presence of EGTA and phenylmethylsulfonyl fluoride; however, no effect was noted in the presence or absence of these reagents.

The change in M_r of these proteins is in good agreement with at least one of the indicated sites in Table I for α-actinin as well as the tropomyosins and actin

Table I

Actual and predicted HIV-1 protease cleavage sites in mammalian cell proteins (see section 2) The vimentin cleavage site determined by microsequencing is printed **bold**, the predicted cleavage sites are in normal letters and lower case letters are used to indicate deviation from combinations of the consensus sequences The positions of the first amino acids (P4) of these sites were obtained from the data bank entries A "+" in the cleavage column indicates detectable cleavage by HIV-1 protease of the purified protein in an in vitro assay (see Fig 1) and a second "+" indicates good agreement of the M_r of the observed and predicted cleavage product(s) NT, not tested

Protein	Actual and predicted cleavage sites						Position (first aa)	Cleaved
	P4	P3	P2	P1	P1'	P2		
Vimentin [24]	**S**	**L**	**N**	**L**	**R**	**E**	418	+ +
Tropomyosin [25]	S	L	N	r	R	I	30,51,87	+ +
Vinculin [26]	S	g	N	k	R	a	941	–
α-Actinin [27]	S	v	N	a	R	c	471	+
	i	Q	N	F	h	I	166	+ +
α-Spectrin [28]	k	f	y	L	F	L	1352	+
	S	Q	e	L	a	E	1376	+
	A	R	d	L	A	s	1697	+
Actin [29]	T	L	k	Y	P	I	66	+ +
	I	R	V	a	P	E	94	+ +
	t	F	N	t	P	a	126	+ +
Dystrophin [16]	S	L	N	L	R	w	2187	NT
	t	R	N	Y	P	q	148	NT
Ig, μ-chain [30]	S	L	N	L	R	s	81	NT
MAP-2 [31]	S	I	N	L	P	m	78	NT
TRK-1 [32]	S	L	N	r	R	I	51	NT
Villin [33]	S	G	N	L	R	d	732	NT

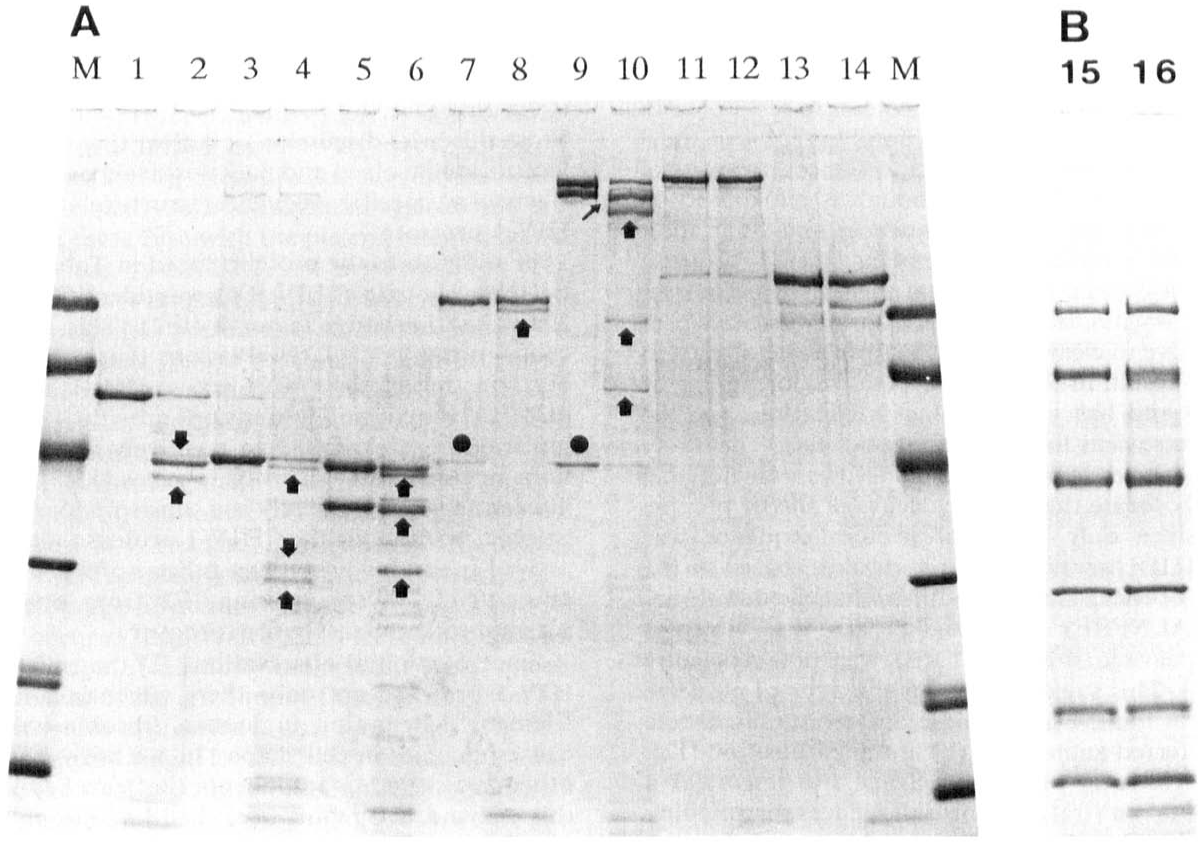

Fig 1. SDS-PAGE analysis of in vitro incubation assays for cleavage of various proteins by HIV-1 protease. Reaction conditions and SDS-PAGE were as described [3] The indicated proteins were incubated in the absence (odd numbered lanes) or presence (even numbered lanes) of HIV-1 protease (see section 2) For panel A, samples were applied as follows human vimentin, lanes 1 and 2, human actin, lanes 3 and 4, chicken tropomyosins, lanes 5 and 6, chicken α actinin, lanes 7 and 8; human spectrin (α- and β-), lanes 9 and 10, chicken filamin, lanes 11 and 12, chicken vinculin, lanes 13 and 14; and protein standards, lanes M The cleavage products of the respective proteins are indicated by arrows and an actin contaminant of the α-actinin and spectrin preparations is indicated by a dot, note that this actin is also readily cleaved The protein standards (Pharmacia-LKB, LMW kit) have M_r values (from top to bottom) of 94 000, 68 000, 43 000, 30 000, 20 000 and 14 000 Vimentin and actin are cleaved almost equally well under these conditions, the turnover number for the other proteins are lower by factors 10-100 For panel B, the proteins in the Bio-Rad standard (see section 2) (a glycerol suspension of, from top to bottom, rabbit phosphorylase B, bovine serum albumin, chicken ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor and chicken lysozyme) were incubated under standard conditions in the absence (lane 15) or presence (lane 16) of HIV-1 protease. The partial cleavage of bovine serum albumin is reminiscent of the cleavage of heat-denatured bovine serum albumin [3] The HIV-1 protease monomer has a M_r ~ 11 000 and gives rise to the two bands with the lowest M_r seen in lane 16

(which are multiply cleaved), but not for the highest M_r peptides derived from the spectrins, even though they are clearly cleaved. The tropomyosins are a family of closely related cytoskeletal proteins possessing amino acid sequences highly conserved not only among the various cellular isoforms, but also between species. The sequence presented in Table I is found in all of the various tropomyosins listed in the SWISSPROT data bank, hence the multiple listings for sequence location (Table I). The HIV-1 protease, like other retroviral proteases [9], can cleave heat-denatured, but not native BSA, making it doubly necessary to exercise caution in the interpretation of the relevance of the cleavages demonstrated in Fig. 1. We believe our actin and spectrin preparations to be in a native state since these proteins were isolated without the use of denaturing agents and these proteins retained the ability to form typical microfilaments and to bind actin, respectively (data not shown). Additionally, α- and β-spectrin were the only proteins in an erythrocyte ghost preparation that were detectably cleaved, supporting the notion that this cleavage is specific and not due to wholesale protein denaturation. Although we have no data on the state of the commercially obtained tropomyosins, it is known that this family of proteins is extremely robust and resistant to denaturation; indeed, these properties are used to isolate and purify these proteins [10].

A criticism of the present study is the lack of demonstrated correlation between the results presented in Table I and Fig. 1. Corroboration of the original hypothesis, that the potential sites identified by homology comparisons are actually utilized by HIV-1 protease, requires that the sequence of all the cleavages observed in Fig. 1 be determined by peptide sequen-

cing. While this is a desirable approach, it was neither our original intention nor ultimate goal; rather, we chose to use the predictions presented in Table I as the basis for a rational selection of proteins to test in our in vitro assay. In this respect, it is notable that 4 of the 5 candidates tested were cleaved and the several control proteins were not cleaved. Although it remains to be demonstrated exactly which sequences and what additional target structure requirements HIV-1 protease possesses, it is quite obvious that there are, in contrast to current dogma, a broad spectrum of host cell proteins sensitive to cleavage by HIV-1 protease, at least in vitro. This result, in our opinion, is the major finding of this study and has wide-ranging implications for the study of retrovirus-host cell interactions.

During our original experiments [3], we were not able to precisely locate the primary cleavage site of porcine desmin (since only a partial protein sequence was available [11]); however, it was quite clear, based on the M_r values of the cleavage products, that the desmin sequence (ALNFRE) homologous to the vimentin primary cleavage site (SLNLRE) was not efficiently cleaved [3]. This suggests that for this type of cleavage site the S at the P4 position plays a more critical role than a preferred amino acid (F) at the P1 position [12]. Since the vimentin primary cleavage site resembles a hybrid of known viral consensus sequences surrounding the sissile bond, it is not unreasonable to predict that other similar hybrids of the known types of cleavage sites [1,2,4] may also be recognized by the enzyme While the presence of R at the P′ position of the vimentin primary cleavage site was originally surprising [3], the prediction of an R-R dipeptide as the sissile bond for tropomyosins (Table I) borders on the heretical. In systematic studies on peptide substrates of HIV-1 protease, Konvalinka et al. [12] found that no hydrolysis was observed when an R or E was present at position P1; however, their model peptides had sequences differing in additional positions from that of the vimentin primary cleavage site or those others proposed in Table I. In this respect, it is important to note that the active form of the HIV-1 protease is a mirror image dimer and that nonpeptide, symmetrical compounds can function as inhibitors of this enzyme [13]. It is thus conceivable that if one R can be well-tolerated within the active site, an R-R dipeptide may also (since it, too, presents a symmetrical conformation). Konvalinka et al. [12] also note that the ability of the substrate peptide to adopt an appropriate configuration within the active site is an important determinant for cleavage. It is thus conceivable that the configuration of the sites cleaved by HIV-1 protease in the various proteins (Fig. 1) may likewise play an important role in their utilization, as has been demonstrated for vimentin in native and heat-denatured forms [3]. A study by Margolin et al. [14] suggests that preferred residues at position P2′ should possess medium-sized hydrophobic side chains (i.e. I or L, but not W or F); for that case where a charged residue (E) is found, they suggest that the local microenvironment plays a crucial role in determining cleavability (in this case, by keeping the E residue protonated). From this brief discussion, it is clear that a multitude of factors are involved and may positively or negatively influence a specific sequence/structure's cleavage by HIV-1 protease.

In addition to the proteins listed in Table I, a potential cleavage site (SLDLRY) was identified in *Escherichia coli* initiation factor 2 (IF2). This is of significance in the light of observations that in recombinant bacteria the expression of active HIV-1 protease appears to be toxic and inhibits cell growth. This property has even been exploited to positively select for mutations in cloned HIV-1 protease genes [15]. It will be of interest to see if IF2 really is a substrate for HIV-1 protease; if so, the yield of HIV-1 protease might be improved in strains possessing other isoforms of IF2 or, alternatively, overexpressing IF2 from a compatible plasmid to compensate for turnover.

Since our initial observations [3] that microinjected HIV-1 protease not only alters vimentin intermediate filament distribution in human fibroblasts, but also causes changes in cell shape [3], we have searched for other cytoskeletal components that can be cleaved by this enzyme. We report here that 4 additional proteins can be specifically cleaved by HIV-1 protease in vitro and predict that additional cytoskeletal proteins may also be substrates (Table I). Of these, dystrophin, an important muscle cell cytoskeletal protein modified or absent in muscular dystrophy [16], is of special interest in light of the unexplained myopathy often observed in many HIV-1 infected individuals [17]. Of course, cleavage of other cytoskeletal proteins, such as actin, may equally likely play a role in this, although if actin is involved it must be in a cytoskeletal form rather than part of an actin-myosin complex to fit with the pathological findings. After completion of this manuscript, Wallin et al. [5] reported that HIV-1 protease cleaves both MAP-1 and MAP-2, a finding consistent with our hypothesis (Table I).

Given the conservation of structure and similarities of cleavage sites among retroviral proteases [1,2], we would predict that other retroviral proteases probably also cleave host cell proteins; indeed, these are reports [18] of alterations in cell shape in Rous sarcoma virus (RSV) infected cells that could easily be accounted for if the RSV protease has similar properties. Wallin et al. [5] have shown that the protease encoded by both HIV-1 and AMV (avian myeloblastosis virus) can cleave MAP-1 and MAP-2 in vitro, thereby interfering with microtubule assembly. We have previously noted [3] the cleavage of vimentin observed [19] in Moloney murine sarcoma virus infected cells is likely due to the viral protease. The ability to disrupt the host cell cytoskeleton, which among other functions anchors to

and stabilizes the plasma membrane, may be an obligate requirement for an enveloped virus for both entry into the cell and especially for an orderly budding from the cell surface during virus proliferation. Protease-defective HIV-1 strains do not bud in a regular fashion [20,21]; these studies suggest that such viruses bud aberrantly into intracellular vesicles and are released when these fuse with the plasma membrane. A murine retrovirus, A-2/Cl.2.2, has been observed [22] to occasionally have long actin microfilaments attached to free viral particles, presumably as a consequence of the budding process. It has previously been proposed that actin microfilaments are actively involved in the budding process of budding viruses [23]. Preliminary experiments (data not shown) indicate that, as with vimentin [3], polymeric actin (F-actin) is much more resistant than actin monomers (G-actin) to cleavage by HIV-1 protease in vitro. The significance of this observation is not yet completely understood, but clearly suggests that cleavage of actin, if it indeed takes place in vivo, must occur at specific structural loci rather than as random cleavage of all actin molecules. We propose that disturbance of the crosslinks of the cytoskeleton by cleavage of sensitive proteins (Table I and Fig. 1) by the retroviral protease might be necessary before the bud, containing the viral precursor proteins, may be pushed out and released from the cell.

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