Active site mutants of human herpesvirus-6 proteinase

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Received 18 November 1998

Abstract Amino acid residues thought to comprise the catalytic triad of HHV-6 proteinase were changed by site-directed mutagenesis in the precursor form of the proteinase. By monitoring the ability of each mutant proteinase precursor to undergo autoprocessing, Ser116, His46 and His135 were identified as catalytically crucial. An attempt was made to mimic the catalytic triad arrangement of archetypal serine proteinases by replacement of the second histidine, His135, by an Asp. Instead of increasing the autoprocessing ability of the His135Asp mutant HHV-6 proteinase precursor, this mutation had a detrimental effect since the precursor persisted predominantly in its unprocessed form.

Key words: Human herpesvirus-6; Proteinase; Catalytic site; Site-directed; Mutagenesis; Precursor; Autoprocessing

1. Introduction

Human herpesvirus-6 (HHV-6) is one of eight human herpesviruses discovered to date. It is the causal agent of exanthema subitum [1] and has been implicated in a number of human diseases, especially those affecting immunocompromised individuals [2–4]. The genome of each herpesvirus encodes a proteinase which plays a crucial role in the viral life cycle [5]. The virally encoded assembly protein forms a scaffold onto which the viral capsid is built [6,7] and cleavage near the C-terminus of this substrate by the proteinase releases the processed assembly protein from the capsid allowing viral DNA to enter and replication to proceed.

The HHV-6 proteinase is synthesised as a polyprotein precursor consisting of 528 amino acids [8]. The N-terminal region constitutes the mature proteinase, whereas the C-terminal part encodes an extended form of the assembly protein (Fig. 1). Autoprocessing of the precursor occurs at two locations (Fig. 1): at the release (R) site, thereby generating the mature form of the proteinase (residues 1–230); and at the maturation (M) site, located towards the C-terminus of the assembly protein [9].

In the present study, we identify by site-directed mutagenesis the catalytic residues that enable this proteolytic activity to fulfil its important function in the viral life cycle.

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Abbreviations: HHV-6, human herpesvirus 6; HCMV, human cytomegalovirus

2. Materials and methods

2.1. Mutagenesis by overlapping extension PCR

The full-length proteinase precursor gene cloned into the expression vector pDS56,6His(2)Xa served as template DNA for the PCR mutagenesis reactions [8,9]. Mutations were introduced by two initial and one subsequent PCR reaction. Pairs of forward and reverse oligonucleotide primers (Pharmacia Biotech., St. Albans, UK) encoding the required mutation (and, where possible, a unique restriction site to aid screening) were used with partner wild-type reverse or forward oligonucleotides to amplify two separate DNA fragments. The purified fragments were subsequently combined and used as template DNA in a final PCR employing the two wild-type flanking primers. The nucleotide sequences of primers used and restriction sites introduced are shown in Table 1. Following the verification of its nucleotide sequence, each mutated DNA fragment was sub-cloned into the original wild-type construct.

2.2. Expression and immunodetection

Wild-type and mutant constructs were transformed into the *E. coli* strain M15, pDM1.1. Cells harbouring the plasmids were grown at 28°C until an $A_{600} = 0.8$ was reached. Induction was with 1 mM β-D-isopropyl-thiogalactopyranoside and samples were removed at various times post-induction for analysis by SDS-PAGE followed by Western blotting using primary antibodies recognising either the mature HHV-6 proteinase (residues 1–230) or the assembly protein (residues 285–528) [8]. Alkaline phosphatase conjugated anti-rabbit IgG antiserum was used as the second antibody to detect immunoreactive bands [9].

3. Results

Alignment of the sequence of HHV-6 proteinase [8] with those of the counterpart proteinases from other herpesviruses identified three conserved residues (Ser116, His46 and His135) which therefore had the potential to operate as the catalytic triad in the enzyme's active site. Accordingly, these three residues were replaced (individually) within the context of the precursor form of HHV-6 proteinase and the ability of the mutated precursor proteins to undergo autoprocessing in *E. coli* was investigated. Samples were removed at different time points and analysed by SDS-PAGE followed by Western blotting using antisera specific for the proteinase (Fig. 2, upper panels) and assembly protein regions (Fig. 2, lower panels), respectively. Processing of the wild-type precursor at both R and M cleavage sites (Fig. 1) to release the assembly protein and generate the mature form of HHV-6 proteinase was accomplished within about 30–60 min (Fig. 2, i). In contrast, when Ser116 was mutated to an Ala and the resultant Ser116-Ala mutant proteinase precursor gene was expressed, no processing whatsoever was detected with either antibody (Fig. 2, ii). Instead, a single immunoreactive band of ~65 kDa persisted throughout the entire time course. Since this is consistent in size with that of the full-length, unprocessed proteinase precursor, it would seem that the simple replacement of the -CH_2OH side-chain of Ser116 by -CH_3 in the mutant Ala variant completely removed the intrinsic ability of the protein-

PII: S0014-5793(98)01605-6

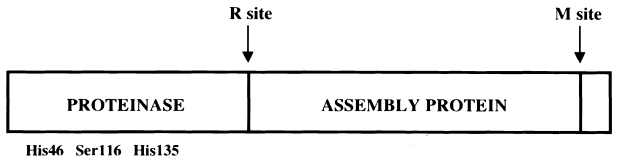

Fig. 1. Schematic representation of the HHV-56 proteinase precursor. Autoprocessing at the R and M sites releases the mature proteinase from the N-terminus and generates a (C-terminal) form of the viral assembly protein. The locations of the putative active site residues that were mutated are indicated.

ase precursor to undergo autoprocessing at both R and M sites.

A second potential residue to contribute to the catalytic triad of the HHV-6 proteinase was identified by the same token to be His46. Expression of the His46Ala mutant proteinase precursor gene in *E. coli* resulted in the detection of several immunoreactive bands, the largest and most predominant of which migrated at 65 kDa (Fig. 2, iii). A band, migrating slightly faster than the ~65-kDa intact precursor was also revealed and, at ~61 kDa, this corresponds in size to the full-length precursor that had undergone cleavage at its M site (Fig. 1). The two further bands that were detected had apparent molecular weights (~56 kDa and 52 kDa, respectively) which cannot be accounted for by cleavage at either or both of the R and M cleavage sites. It has been suggested [9] that these bands arise as a result of the non-specific action of *E. coli* proteinases on the HHV-6 proteinase precursor.

The third potential catalytic site residue, His135, was mutated to an Asp and the expression profile for this mutant proteinase precursor is shown in Fig. 2, iv. The pattern of immunoreactive bands detected is closely similar to that obtained for the His46Ala mutant precursor. In both cases, it is readily apparent that the majority of the protein produced persisted as the ~65-kDa proteinase precursor throughout the length of the time course examined; this is in total contrast to the rapid autoprocessing observed for the wild-type proteinase precursor (Fig. 2, i).

4. Discussion

These data suggest that the catalytic triad in HHV-6 proteinase consists of Ser116, His46 and His135, with Ser116 likely to act as the active site nucleophile.

The three-dimensional structure of HHV-6 proteinase is not yet known but structures have been solved recently by X-ray crystallography for the proteinases from the closely related human cytomegalovirus [10–13], herpes simplex viruses-types 1 and 2 [14] and varicella zoster virus [15]. The equivalent residues to Ser116, His46 and His135 in human cytomegalovirus, for example, are Ser132, His63 and His157 and the crystal structure indicates that these three residues do indeed lie spatially adjacent, in close juxtaposition to one another [10–13]. Indeed, superposition of the Ser/His/His catalytic triad of mature HCMV proteinase with the corresponding Ser195/His57/Asp102 catalytic triad residues of chymotrypsin reveals that, despite the distinct overall folds, the three residues can be overlaid to within an rms deviation of 0.8 Å [11].

The somewhat unusual nature of the catalytic triad in the serine proteinases of the herpesviruses is reflected in the proteolytic efficiency of these enzymes. Measurement of kinetic parameters for the hydrolysis of peptide substrates by purified HHV-6 proteinase [9] revealed that this enzyme, in common with the proteinases from other herpesviruses, exhibited substantially lower values for the turnover number (k_{cat}) relative to those measured for archetypal serine proteinases such as chymotrypsin. Since the viral proteinases have the serine nucleophile and His46 (equivalent to His57 in chymotrypsin) in common with their archetypal counterparts, it was thus con-

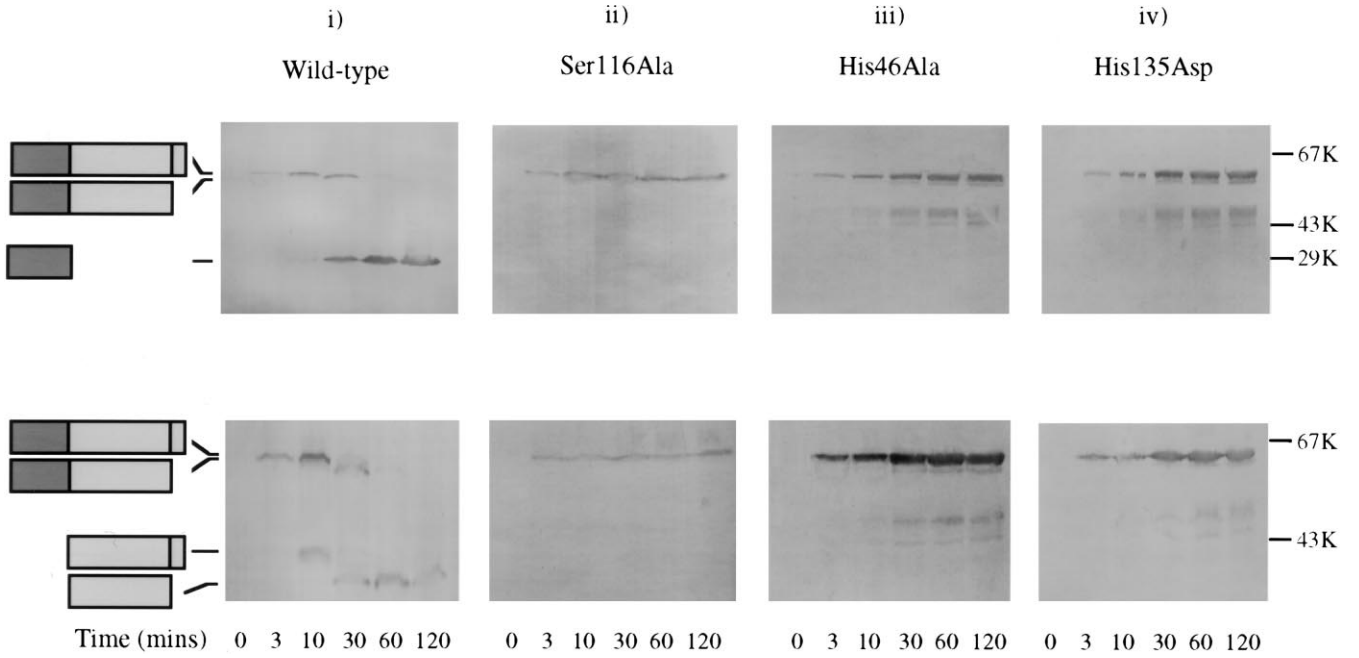

Fig. 2. Autoprocessing of wild-type and mutant HHV-6 proteinase precursors. *E. coli* harbouring plasmids encoding (i) wild-type, (ii) S116A, (iii) H46A and (iv) H135D mutant proteinase precursors were induced with isopropyl-β-D-thiogalactopyranoside. Aliquots were removed at the indicated times and analysed by SDS-PAGE followed by Western blotting using antisera specific for the mature proteinase (upper panels) and assembly protein (lower panels), respectively. Markers of M_r, approximately 67 000, 43 000 and 29 000, migrated as indicated.

Table 1
Mutagenesis oligonucleotides

Mutation created	Direction	Mutagenesis oligonucleotide sequence (5′–3′)	Partner oligonucleotide	Restriction site introduced
S116A	F	GGTCTAGCGCTGTCTAGCAAAC	R1	*Eco*47III
	R	GACAGCGCTAGACCAGGAAAAC	F1	
H46A	F	GTTGAATATTAACGCGAACGAAAAGGCCAC	R1	None
	R	GCCTTTTCGTTCGCGTTAATATTCAACGGT	F1	
H135D	F	CCGTTTTTTCATGACGTCTCTGTATGTGGA	R1	*Aat*II
	R	TCCACATACAGAGACGTCATGAAAAAACGG	F2	

The sequences of the partner oligonucleotides were: F1: 5′-GCTGCCGCTAGCAAATTTGGGTAGGTGGATTC-3′; F2: 5′-TCGATTTAGAG-CATGGGC-3′; R1: 5′-GCCCTCGTCGACTCAAGCTTTAATATACGTACATTTAG-3′.

sidered that the reduction in catalytic efficiency might result from the replacement of Asp102 in the catalytic triad of the herpesvirus proteinase by a second histidine (His135). It was on this basis that His135 was mutated to Asp in the HHV-6 proteinase precursor. However, the His135Asp mutation, far from increasing the intrinsic catalytic activity of the mutant HHV-6 proteinase precursor, appeared to have a detrimental effect since the precursor persisted predominantly in its ~65-kDa unprocessed form throughout the length of the time course studied (Fig. 2). In this context, it might also be of value to compare the kinetic parameters for substrate hydrolysis by an Asp102His mutant of chymotrypsin or trypsin [16] with those of a wild-type herpesvirus proteinase, such as that from HHV-6. The orientation of the catalytic Ser,His,His residues in the herpesvirus proteinases may require additional features to contribute to the stabilisation that is achieved so readily by the classical serine proteinases. However, in biological terms, a herpesvirus encoding a proteinase with 'too high' an intrinsic activity might be considered to be unfavourable since proteolytic cleavage must not take place until the temporal and spatial requirements for capsid assembly are met. Under such circumstances, the viruses may well have evolved to produce a proteinase that is capable of only a limited level of catalytic activity.

Acknowledgements: N.J.T. was sponsored by an MRC-Industrial Co-operative Studentship in collaboration with the Roche Research Centre, Welwyn Garden City, Herts, UK.

References

[1] Yamanishi, K., Okuno, T., Shiraki, K., Takahashi, M., Kondo, T., Asano, Y. and Kurata, T. (1988) Lancet 1, 1065–1067.
[2] Drobyski, W.R., Eberle, M., Majewski, D. and Baxter-Lowe, L.A. (1993) J. Clin. Microbiol. 31, 1515–1520.
[3] Luppi, M. and Torelli, G. (1996) Haematologica 81, 265–281.
[4] Soldan, S.S., Berti, R., Salem, N., Secchiero, P., Flamand, L., Calabresi, P.A., Brennan, M.B., Maloni, H.W., McFarland, H.F., Lin, H.C., Patnaik, M. and Jacobson, S. (1997) Nat. Med. 3, 1394–1397.
[5] Matusick-Kumar, L., McCann, P., Robertson, B.J., Newcomb, W.W., Brown, J.C. and Gao, M. (1995) J. Virol. 69, 7113–7121.
[6] Preston, V.G., Al-Kobaisi, M.F., McDougall, I.M. and Rixon, F.J. (1994) J. Gen. Virol. 75, 2355–2366.
[7] Nicholson, P., Addison, C., Cross, A.M., Kennard, J., Preston, V.G. and Rixon, F.J. (1994) J. Gen. Virol. 75, 1091–1099.
[8] Tigue, N.J., Matharu, P.J., Roberts, N.A., Mills, J.A., Jupp, R. and Kay, J. (1996) J. Virol. 70, 4136–4141.
[9] Tigue, N.J. and Kay, J. (1998) J. Biol. Chem. 273, 26441–26446.
[10] Chen, P., Tsuge, H., Almassy, R.J., Gribskov, C.L., Katoh, S., Vanderpool, D.L., Margosiak, S.A., Pinko, C. and Kan, C.-C. (1996) Cell 86, 835–843.
[11] Qui, X., Culp, J.S., DiLella, A.G., Hellmig, B., Hoog, S.S., Janson, C.A., Smith, W.W. and Abdel-Meguid, S.S. (1996) Nature 383, 275–279.
[12] Shieh, H.-S., Kurumbail, R.G., Stevens, A.M., Stegeman, R.A., Sturman, E.J., Pak, J.Y., Wittwer, A.J., Palmier, M.O., Wiegand, R.C., Holwerda, B.C. and Stallings, W.C. (1996) Nature 383, 279–282.
[13] Tong, L., Qian, C., Massariol, M.-J., Bonneau, P.R., Cordingley, M.G. and Legacé, L. (1996) Nature 383, 272–275.
[14] Qui, X.Y., Janson, C.A., Culp, J.S., Richardson, B., Debouck, C., Smith, W.W. and AbdelMeguid, S.S. (1997) Proc. Natl. Acad. Sci. USA 94, 2874–2879.
[15] Hoog, S.S., Smith, W.W., Qui, X.Y., Janson, C.A., Hellmig, B., McQueney, M.M., Odonell, K., Oshanessy, D., DiLella, A.G., Debouck, C. and AbdelMeguid, S.S. (1997) Biochemistry 36, 14023–14029.
[16] Unal, A., Pray, T.R., Lagunoff, M., Pennington, M.W., Ganem, D. and Craik, C.S. (1997) J. Virol. 71, 7030–7038.