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Serine 71 of the glycoprotein HEF is located at the active site of the acetylesterase of influenza C virus

Brief Report

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Summary. The acetylesterase of influenza C virus has been reported recently to be inhibited by diisopropylfluorophosphate (DFP) [Muchmore EA, Varki A (1987) Science 236: 1293–1295]. As this inhibitor is known to bind covalently to the serine in the active site of serine esterases, we attempted to determine the serine in the active site of the influenza C acetylesterase. Incubation of purified influenza C virus with ^{3}H-DFP resulted in the selective labelling of the influenza C glycoprotein HEF. The labelled glycoprotein was isolated from a SDS-polyacrylamide gel. Following reduction and carboxymethylation, tryptic peptides of HEF were prepared and analyzed by reversed phase HPLC. The peptide containing the ^{3}H-DFP was subjected to sequence analysis. The amino acids determined from the NH_2-terminus were used to locate the peptide on the HEF polypeptide. Radiosequencing revealed that ^{3}H-DFP is attached to amino acid 17 of the tryptic peptide. These results indicate that serine 71 is the active-site serine of the acetylesterase of influenza C virus.

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Influenza C viruses contain only one surface glycoprotein (HEF) [4]. This protein is responsible for the receptor-binding (haemagglutination), the receptor-destroying, and the fusion activity of influenza C virus [3, 9, 10, 15, 18]. Receptor-binding and haemagglutination, respectively, involve attachment of virus to cell surface receptors containing N-acetyl-9-O-acetylneuraminic acid (Neu5, $9Ac_2$) [8, 17]. The receptor-destroying enzyme is an acetylesterase releasing O-acetyl residues from position C-9 of Neu5, $9Ac_2$ thus inactivating the virus receptors [7]. The fusion activity is dependent on a low pH and on the proteolytic cleavage of HEF into the cleavage products HEF_1 and HEF_2

[10, 15]. This proteolytic activation of the glycoprotein exposes a hydrophobic amino acid sequence at the NH_2-terminus of HEF_2 which in analogy to other influenza viruses is assumed to be responsible for the fusion activity [5, 12]. No information has been presented on the location of both the receptor-binding site and the active site of the acetylesterase.

The acetylesterase activity of influenza C virus has been reported recently to be inhibited by diisopropylfluorophosphate (DFP) [13]. This inhibitor binds covalently to serine in the active site of serine esterases. In the case of influenza C virus DFP binds to HEF_1, the larger of the two cleavage products obtained after proteolytic activation of the influenza C glycoprotein [13]. We attempted to localize the active site of the influenza C acetylesterase by labelling of the glycoprotein HEF with ^{3}H-DFP and sequencing of the tryptic peptide containing the covalently bound inhibitor.

MDCK I cells were maintained as described recently [9]. Influenza C virus (strain Johannesburg/1/66) has been grown in embryonated chicken eggs as described [8]. MDCK I cells grown to confluency in 135 mm plastic petri dishes were infected with influenza C virus at a multiplicity of 5 $TCID_{50}$/cell. After incubation at 33 °C for 2 days, virus was harvested from the medium and purified as described previously [6]. Purified virus obtained from 10 petri dishes was suspended in 0.5 ml of phosphate-buffered saline (PBS). After addition of 50 µl of propylene glycol containing 50 µCi of ^{3}H-labelled DFP (3.5 Curies/mmol, Amersham) the mixture was kept on ice. After incubation for 30 min, virus was layered onto a sucrose cushion (15% sucrose in 600 µl PBS) in a 1.5 ml centrifuge tube. After centrifugation at 30,000 rpm for 30 min in a Beckman TLA 100.3 rotor, the virus sediment was suspended in PBS.

Purified influenza C virus labelled with ^{3}H-DFP was subjected to SDS-polyacrylamide gel electrophoresis under non-reducing conditions as described previously [9]. After the run, small strips were cut from both sides of the gel and stained with Coomassie brilliant blue. The stained gel strips were used to localize the influenza C glycoprotein in the unstained gel. The portion of the gel containing the influenza C glycoprotein was excised. The glycoprotein was isolated from the polyacrylamide gel by electroelution at 200 V for 4 h using the Biotrap (Schleicher and Schüll) and the same running buffer as for electrophoresis. The DFP-labelled glycoprotein obtained by electroelution was mixed with a solution to obtain a final concentration of 8 M urea, 5 mM EDTA, 0.1 M Tris-HCl (pH 8.0), and 2 mM dithiotreitol. After incubation for 4 h at room temperature, iodoacetamide was added to a final concentration of 5 mM. The solution was kept under nitrogen and incubated at 37 °C for 30 min in the dark. Following the addition of dithiotreitol to a final concentration of 4 mM, the sample was dialysed against 10 mM NH_4HCO_3 for two days. In order to remove residual SDS, the sample was dried and extracted three times with 400 µl of a solution containing aceton, triethylamin, acetic acid, and water (87/5/5/3). After a final extraction with aceton, the sample remaining in the tube was dried.

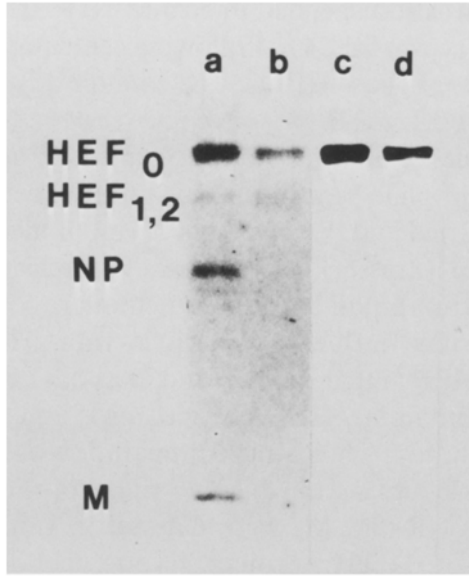

Fig. 1. SDS-Polyacrylamide gel electrophoresis of influenza C virus labelled with ^{3}H-DFP. Electrophoresis was performed under non-reducing conditions. The gel was first stained with Coomassie brilliant blue (*a* and *b*) and then processed for fluorography (*c* and *d*). *a* and *c* All virus proteins; *b* and *d* HEF obtained by preparative gel electrophoresis

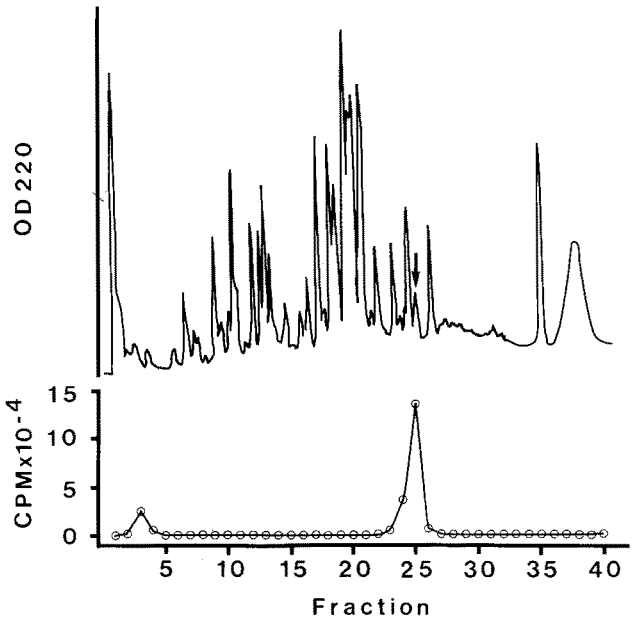

Fig. 2. Analysis by reversed phase HPLC of the peptides obtained by trypsin digestion of HEF labelled with ^{3}H-DFP. Upper panel: OD 220 of the eluate; lower panel: radioactivity of the column fractions. The peak containing the ^{3}H-DFP labelled peptide is marked by an arrow in the upper panel

The glycoprotein was resuspended in 50 mM NH_4HCO_3, 0.05% NP 40 and digested with 3 μg of trypsin for 24 h. Following centrifugation, the supernatant was analyzed by reversed phase HPLC. The column was eluted with a gradient of CH_3CN (0–50% in 0.1% TFA).

NH_2-terminal sequence analysis was performed on an Applied Biosystems model 470 A gas/liquid phae sequencer. For radiosequencing the PTH amino acids were collected, dried and dissolved in 0.15 ml of methanol. After addition of 10 ml of Quickszint (Zinsser), the radioactivity released at each cycle was determined in a Kontron liquid scintillation counter.

Incubation of purified influenza C virions with ^{3}H-DFP resulted in the selective labelling of HEF (Fig. 1, lanes a and c) as has been reported [13]. The portion of the gel containing HEF was cut out, and the glycoprotein was recovered by electroelution. Thus a pure preparation of HEF was obtained as shown in Fig. 1 (lanes b and d), in which no traces of the other major proteins of influenza C virus, NP and M, were detectable. Following reduction and carboxy-methylation of the glycoprotein, tryptic peptides were prepared and analyzed by HPLC. The result is shown in Fig. 2. The majorty of the radioactivity was recovered in fraction 25 indicating that only one of the peptides resolved contained covalently bound ^{3}H-DFP. The corresponding peptide was subjected to amino acid sequencing. The sequence at the N-terminus of the peptide was determined as follows: Ala-Gly-Ala-Ser-Val-Leu-X-Gln-. At cycle 7 no PTH-amino acid could be identified (Fig. 3). By comparing this sequence with the published sequence of HEF [14, 16] it was possible to identify amino acid 55 as the N-terminal residue of the tryptic peptide. At position 61 HEF

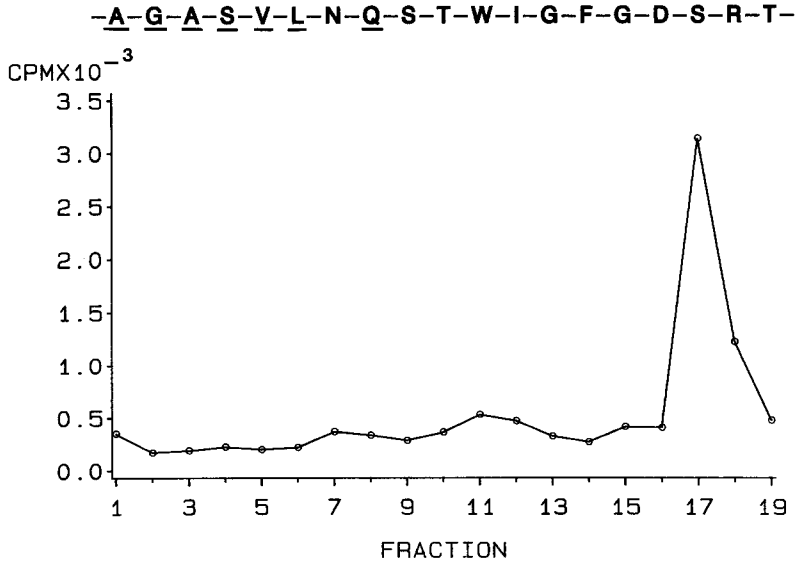

Fig. 3 Radiosequencing of the tryptic peptide of HEF labelled with ^{3}H-DFP. The underlined amino acids presented at the top of the panel were identified as PTH-amino acids and have been used to locate the peptide on the primary structure of HEF

Table 1. Sequences at the active-site serine of serine hydrolases[a]

acetylcholinesterase	-Gly-Glu-*Ser*-Ser-Glu-
alkaline phosphatase	-Thr-Asp-*Ser*-Ala-Ala-
trypsin	-Gly-Asp-*Ser*-Gly-Gly-
chymotrypsin	-Gly-Asp-*Ser*-Gly-Gly-
HEF-protein	-Gly-Asp-*Ser*-Arg-Thr-

[a] The data were taken from [1, 11]

contains an asparagine, which represents a potential glycosylation site. Thus, an oligosaccharide attached to asparagine 7 of the tryptic peptide is probably the reason for the failure to detect the corresponding PTH-amino acid. Radiosequencing revealed that the radioactivity was recovered with amino acid 17 of the tryptic peptide (Fig. 3). Therefore, this residue, which corresponds to amino acid 71 of HEF, represents the serine in the active site of the influenza C acetylesterase. By comparing the amino acids adjacent to amino acid 71 with the sequence at the active site of other serine hydrolases, some similarity can be detected (Table 1). The dipeptide Gly-Asp found at the NH_2-terminal side of serine 71 of the influenza C acetylesterase is also found in the corresponding position at the active site of trypsin and chymotrypsin. A unique feature of the influenza C enzyme is that serine 71 is followed by an arginine at the C-terminal side. Among the HEF-genes of 11 strains sequenced so far, the sequence — Phe(68)-Gly-Asp-Ser-Arg-Thr-Asp(74)— was found to be conserved [2, 14, 16] suggesting that this sequence is essential for the acetylesterase activity of HEF. However, other amino acids located at different positions of the protein are expected also to be important for the active site of the enzyme. By site-directed mutagenesis it will be possible to establish which of the amino acids of HEF are essential for the acetylesterase activity of influenza C virus.

The HEF-protein has some structural similarities to the haemagglutinin (HA) of influenza A and B viruses. HA has receptor-binding and fusion activity, but is lacking receptor-destroying activity. As HEF is larger than HA [14, 16], it has been speculated that the enzyme function of HEF may reside in the region of residues 230–380, which has no significant homology to HA [16]. Our data do not support this assumption, because the serine at the active site is not part of this region. As DFP has been reported to inhibit the esterase but not the haemagglutination activity of influenza C virus, it has been assumed that these activities are located on different epitopes [13]. Future studies will have to show, where the receptor-binding site is located on the glycoprotein of influenza C virus.

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