Sulphation of hirudin in BHK cells

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Hirudin, a thrombin inhibitor of the leech, was expressed in BHK cells; the α_1-antitrypsin signal peptide was used to direct secretion into the culture medium. The recombinant hirudin so produced inhibited thrombin and was shown by labelling experiments with [^{35}S]sulphate to have been post-translationally modified.

Hirudin; Sulphation; BHK cell; Vaccinia virus vector

1. INTRODUCTION

Control of haemostasis is of vital medical and pharmaceutical importance; the use of genetic engineering techniques to create new molecules useful in this regard is becoming well established [1,2]. Post-translational modifications have been shown to confer advantages on recombinant proteins intended for pharmaceutical use. In the case of hirudin, a small protein isolated from the blood leech, *Hirudo medicinalis*, sulphation at Tyr^{63} has been shown to increase the efficacy of thrombin inhibition [3–6]. Thus, systems for the production of post-translationally modified hirudin to investigate the biochemistry involved are of major interest.

The object of the work described here was the establishment of such a system; hirudin was shown to be secreted and sulphated by BHK cells, when expressed from a recombinant vaccinia virus using the α_1-antitrypsin (α_1AT) signal peptide.

2. MATERIALS AND METHODS

Restriction and DNA modifying enzymes were from New England Biolabs (Beverly, MA, USA) or Promega Biotech (Madison, WI, USA). Purified human thrombin was from Sigma (La Verpilliere, France). Chromozyme TH, a chromogenic substrate for thrombin was from Boehringer Mannheim (FRG).

^{35}S-labelled Na_2SO_4 (spec. act. 0.9–1.5 TBq/mg) and ^{35}S-labelled L-cysteine (spec. act. 22 TBq/nmol) were purchased from Amersham (Les Ulis, France).

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Monoclonal antibodies (mAbs) directed against different regions of recombinant hirudin variant 2 (rHV2) purified on Protein A Sepharose were a gift from Dr C. Koch (Statens Serum Institut, Copenhagen, Denmark). Polyclonal antiserum (raised in goats) against rHV2 with a Lys residue in position 47 (rHV2-Lys47) were from Neosystems (Strasbourg, France). Protein A Sepharose was purchased from Pharmacia (Uppsala, Sweden). Streptavidin-peroxidase complex was purchased from Amersham (Les Ulis, France) and orthophenylene diamine (OPD) was obtained through Sigma.

2.1. *Methods*

DNA was manipulated according to standard techniques [7]. The hirudin cDNA used here codes for rHV2-Lys47 [8]. Oligonucleotides were synthesized using a solid-phase synthesizer (Applied Biosystems, Foster City, CA, USA) and 5'-phosphorylated before use. Mutagenesis of DNA was carried out as previously described [8].

2.2. *Production of recombinant vaccinia virus (VV)*

Generation of thymidine kinase negative recombinant VV and the expression of proteins from them were done as previously described [9]. For protein purification, supernatants of BHK-21 cells infected with VV were UV-irradiated for 25 min prior to centrifugation at 20 000 × *g* for 2 h (Beckman SW60) to remove infectious virus.

2.3. *^{35}S-Labelling of rHV2-Lys47 in VV-infected cells*

Monolayers of 1.5×10^6 BHK-21 cells were infected with wild-type or recombinant VV at a multiplicity of 0.2 pfu/cell for 16 h in G-MEM + 10% fetal calf serum. The medium was then replaced with 1 ml of cysteine free MEM plus 1% bovine serum albumin supplemented with 250 μCi [^{35}S]cysteine. Incubation was for 8 h at 37°C. Labelling with $^{35}SO_4^{2-}$ was done similarly, except that monolayers of 7×10^6 cells were infected and the medium was replaced with 6 ml of SO_4^{2-} free MEM plus 1% BSA supplemented with 50 mCi $^{35}SO_4^{2-}$.

2.4. *Immunoprecipitation*

Culture supernatant was harvested and supplemented with the same volume of Buffer I (1 M KCl, 2% Triton X-100, 50 mM Tris-HCl, pH 8.8). 5 μl of anti-rHV2-Lys47 antiserum per reaction were added and incubation performed for 1 h at 20°C. 4 mg of equilibrated Protein A Sepharose was then added and incubation continued for 1 h. For the competititon assays, an excess of unlabelled rHV2-Lys47 (50 μg/ml) was added to the supernatant prior to immunoprecipitation. After washing the Sepharose 5 times with Buffer II (0.55 M KCl, 0.8% Triton X-100, 10 mM Tris-HCl, pH 8.8) and twice with 10 mM Tris-HCl, pH 8.8, 50 μl/mg Laemmli sample buffer were added, the

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samples boiled for 5 min and then examined by polyacrylamide gel electrophoresis, according to Laemmli [10]. The sample buffer contained 10 mM DTT and the electrophoresis buffer 8 M urea. Detection was by fluorography [11].

2.4. *Determination of rHV2-Lys47 in cell culture supernatants by ELISA*

rHV2-Lys47 in cell supernatants was determined using two mAbs recognizing different epitopes of the molecule, one of which was biotinylated. The ELISA was carried out essentially as described in the Amersham handbook supplied with the streptavidin-peroxidase complex, the substrate being *o*-phenylenediamine. The absorbance at 492 nm was read in an automated ELISA reader (Molecular Devices, Palo Alto, CA, USA). Supernatants from cells infected with wild-type vaccinia virus served as controls.

2.4. *Assay of anti-protease activity*

Anti-thrombin activity was measured as follows. Aliquots of culture medium were mixed with human thrombin in a buffer containing 0.15 M KCl, 0.1% PEG 6000 and 0.1 M Tris-HCl, pH 8.0 in a 1 ml cuvette. After 20 min at room temperature, residual activity was determined by the addition of 100 nmol of chromozym TH and monitoring the absorbance change at 410 nm over a period of 4 min. Uninhibited human thrombin had an activity of 0.04 optical density units/min under these conditions.

3. RESULTS AND DISCUSSION

3.1. *Expression and secretion of hirudin using the α_1 at signal sequence*

To examine whether the processing and modification of a heterologous protein was possible, two DNA constructions were made to allow the expression of the hirudin variant rHV2-Lys47. This protein is normally sulphated at the Tyr^{63} residue [3,12]. As other natural hirudin variants have been shown to be sulphated at the C-terminal Tyr residue, rHV2-Lys47 can be considered as being representative of this protein family.

The constructions used to express rHV2-Lys47 are shown diagrammatically in Fig. 1. One construction ($Hir(Ile^1)$, Fig. 1a) contains the cDNA of rHV2-Lys47 fused directly to the α_1AT signal peptide; proteolytic cleavage of the signal peptide must therefore take place at an amino acid pair not normally recognised by the signal peptidase (Ala-Ile instead of Ala-Glu). However, mature rHV2-Lys47 will be generated upon cleavage.

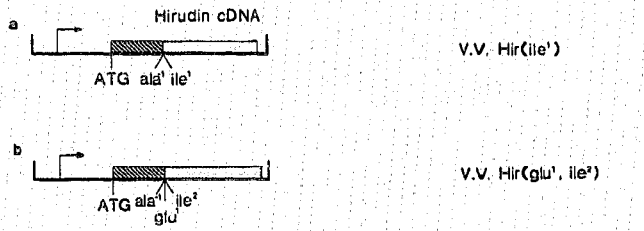

Fig. 1. Schematic representations of constructions used to express the rHV2-Lys47 derivatives outlined in the text. Hatched blocks represent the α_1AT signal peptide and speckled blocks the rHV2-Lys47 cDNA. The constructions are contained on plasmids carrying viral thymidine kinase sequences, enabling recombination with vaccinia virus. The amino acids at the join of the α_1AT signal peptide and the rHV2-Lys47 cDNA are indicated; the arrow indicates the viral 7.5 K promoter.

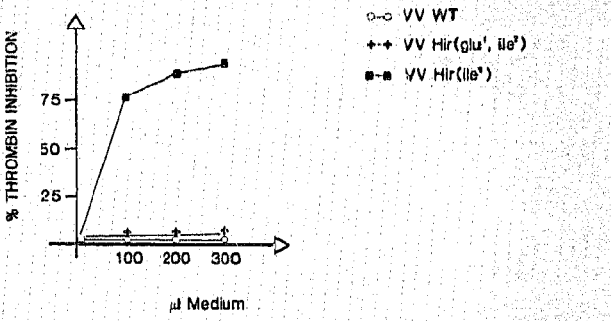

Fig. 2. Detection of anti-thrombin activity in the growth medium of BHK cells infected with recombinant vaccinia viruses bearing the cDNA for rHV2-Lys47. VV WT = wild-type vaccinia virus.

The second construction is similar; in this case, the signal peptide extends to include the first amino acid of the mature α_1AT molecule. Thus, this construction has a native cleavage site, which will give rise to a rHV2-Lys47 molecule containing an extra Glu residue at the N-terminus ($Hir(Glu^1, Ile^2)$, Fig. 1b).

BHK cells were infected with recombinant viruses bearing these constructions and the semi-thrombin activity in the culture medium was measured. The results are shown in Fig. 2; although rHV2-Lys47 was expressed to the same level in both constructions (46 ng/ml as determined by ELISA), anti-thrombin activity was only detected with the $Hir(Ile^1)$ construction. This indicates that rHV2-Lys47 with an additional Glu residue at the N-terminus has a significantly reduced anti-thrombin activity. This can be explained by the 3-dimensional structure of the thrombin-hirudin complex in which the N-terminus of hirudin extends into the active site of thrombin [13,14]. In addition, the presence of an additional Met residue has been shown to diminish drastically its inhibitory activity [15]. N-Terminal protein sequencing of the recombinant hirudin secreted from BKH cells will be required to show unequivocally the presence of the Glu residue.

Immunoprecipitation of secreted rHV2-Lys47 after expression in $^{35}SO_4^{2-}$ containing medium revealed a 7 kDa protein co-migrating with rHV2-Lys47 labelled in the presence of [^{35}S]cysteine (Fig. 3). The effective competition of an excess of unlabelled hirudin with the $^{35}SO_4^{2-}$ labelled protein during immunoprecipitation confirmed its identity to rHV2-Lys47. Tyr^{63} in rHV2-Lys47 is located in a region with a cluster of negatively charged amino acids (E-E-I-P-E-E-Y-L-Q) in the highly accessible C-terminal region of the molecule. These features, in addition to a turn inducing amino acid such as proline near Tyr^{63}, have been proposed to be important parts of the recognition sequence for tyrosine sulphation [16]. Furthermore, no disulfide bonds have been found between amino acids −7 to +7 of a sulphated Tyr residue in other proteins thus making sulphation of the other Tyr residue in rHV2-Lys47 (Tyr^3) unlikely [16]. In addition, it was recently shown

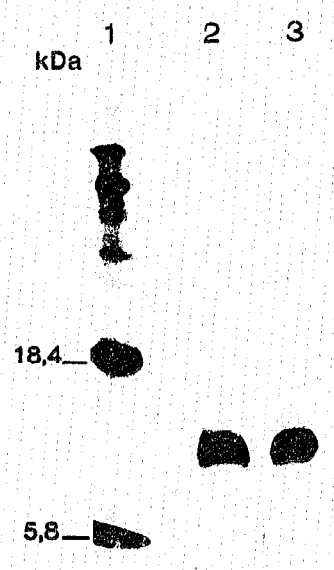

Fig. 3. Autoradiography of ^{35}S-labelled rHV2-Lys47 after immunoprecipitation and polyacrylamide gel-electrophoresis in the presence of SDS. Lane 1 = ^{14}C labelled molecular weight markers; lane 2 = ^{35}S-labelled rHV2-Lys47 from BHK-cells grown in the presence of [^{35}S]sodium sulfate; lane 3 = ^{35}S-labelled rHV2-Lys47 from BHK-cells grown in the presence of [^{35}S]cysteine; lane 4 = same as lane 2 but the immunoprecipitation was done in the presence of an excess of unlabelled rHV2-Lys47.

that rHV2-Lys47 can be successfully sulphated at Tyr^{63} in vitro using preparations of partially purified tyrosylprotein sulfotransferases [17]. While unambiguous assignment of Tyr^{63} as the site of sulphation awaits further analysis, there is good circumstantial evidence that this is the site for sulphation in BHK cells.

The results presented here show that rHV2-Lys47 has been successfully sulphated in BHK cells and that this system will be useful in studying the processes involved. Furthermore, the vaccinia system coupled with the α_1AT signal peptide should allow the secretion and post-translational modification of other proteins to be examined.

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