Post-translational modification of the myxoma-virus anti-inflammatory serpin SERP-1 by a virally encoded sialyltransferase

Piers NASH^{*1}, Michele BARRY^{*}, Bruce T. SEET[†], Kirstin VEUGELERS^{*}, Susy HOTA[†], Jody HEGER[†], Carley HODGKINSON[†], Kathryn GRAHAM^{*}, Ronald J. JACKSON[‡] and Grant McFADDEN^{†2}

^{*}Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7, [†]The John P. Roberts Research Institute and the Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada N6G 2V4, and

[‡]Pest Animal Control CRC, CSIRO Wildlife and Ecology, Canberra, A.C.T. 2601, Australia

SERP-1 is a secreted serpin (**serine-proteinase inhibitor**) encoded by myxoma virus, a poxvirus pathogen of rabbits. SERP-1 is required for myxoma-virus virulence, and the purified protein has been shown to possess independent anti-inflammatory activity in animal models of restenosis and antigen-induced arthritis. As an inhibitor of serine proteinases, SERP-1 acts against tissue-type plasminogen activator, urokinase-type plasminogen activator, plasmin, thrombin and Factor Xa. In the present study, examination of SERP-1 glycosylation-site mutants showed that the N-linked glycosylation of Asn¹⁷² was essential for SERP-1 secretion, whereas mutation of Asn⁹⁹ decreased secretion efficiency, indicating that N-linked glycosylation plays an essential role in the processing and trafficking of SERP-1. Furthermore, comparison of SERP-1 from wild-type myxoma virus

and a virus containing a targeted disruption of the *MST3N* sialyltransferase locus demonstrated that SERP-1 is specifically modified by this myxoma-virus-encoded sialyltransferase, and is thus the first reported viral protein shown to be modified by a virally encoded glycosyltransferase. Sialylation of SERP-1 by the *MST3N* gene product creates a uniquely charged species of secreted SERP-1 that is distinct from SERP-1 produced from other eukaryotic expression systems, though this has no apparent effect upon the kinetics of *in vitro* proteinase inhibition. Rather, the role of viral sialylation of SERP-1 likely relates to masking antigenicity or targeting SERP-1 to specific sites of action *in vivo*.

Key words: glycosylation, poxvirus, proteinase, sialylation, virulence factor.

INTRODUCTION

Glycosylation is a key determinant in immunological recognition and signalling events involved in the immune response to an invading parasite. Sialylated structures in particular are critical as the ligands of the immunological signalling molecules of the CD62 class of E-, P-, and L-selectins, and CD22 [1–3]. It is not surprising, therefore, that a variety of pathogenic organisms employ molecular mimicry of glycosylated moieties to assist in the evasion of the immune system of the infected host. For example, the sialic acid-containing capsules of *Neisseria meningitidis*, *Escherichia coli* K1 and group-B streptococci are important virulence factors [4,5]. Although the marked effects of bacterially and parasite-encoded sialyltransferase genes in conferring increased virulence has been well documented [6,7], it has only been recently that a *bona fide* sialyltransferase has been identified from a virus [8].

Myxoma virus, a leporipoxvirus member of the poxvirus family of double-stranded-DNA viruses, has recently been reported to encode and express a functional sialyltransferase showing sequence similarity to cellular $\alpha 2,3$ -sialyltransferases [8]. Myxoma virus evolved in New World rabbits in which it causes a relatively benign and non-lethal disease characterized by persistent dermal lesions [9]. However, when myxoma virus

infects Old World, or European, rabbits (*Oryctolagus cuniculus*) a highly lethal disease state known as myxomatosis is produced [9]. The disease of myxomatosis is characterized by fulminating primary and secondary lesions, and extensive immunosuppression resulting in death of infected rabbits within 2 weeks caused by supervening Gram-negative-bacterial infections. One of the most intriguing aspects of myxoma virus is its striking ability to systematically disable the immune and inflammatory responses of the infected host. The study of this virus has identified a wide range of immunomodulatory proteins capable of subverting specific aspects of the host's immune system [10], one of which is the serpin (**serine-proteinase inhibitor**) SERP-1.

SERP-1 is expressed as a secreted glycoprotein possessing three predicted sites for N-linked glycosylation that belongs to the serpin family of serine-proteinase inhibitors [11,12]. Serpins are involved in maintaining homeostasis in a variety of systems including the modulation of inflammatory responses, complement activation, fibrinolysis, thrombosis and development [13]. Given the critical role of proteinases in diverse activities, it is not surprising that some viruses have utilized serpins in order to disrupt proteinase-regulated processes that would otherwise be antagonistic to the virus [14]. Numerous serpins have been described from a number of members of the poxvirus family, but SERP-1 remains the only example of a virus-encoded serpin that

Abbreviations: tPA, tissue-type plasminogen activator; CHO, Chinese-hamster ovary; BGMK, baby-green-monkey kidney; pfu, plaque forming unit; moi, multiplicity of infection; TBS, Tris-buffered saline; VV-S1, vaccinia virus expressing SERP-1; vMyxWT, wild-type myxoma virus strain Lausanne; mAb, monoclonal antibody; ORF, open reading frame; serpin, **serine-proteinase inhibitor**; Endo-H, endoglycosidase-H; the mutant N28A is Asn²⁸ → Ala (etc.); tmNA, triple mutant N28A/N99A/N172A; IEF, isoelectric focusing; pNA, p-nitroaniline; ER, endoplasmic reticulum.

¹ Present address: Molecular Biology of Cancer, Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto, Ontario, Canada M5G 1X5.

² To whom correspondence should be addressed (e-mail mcfadden@rri.on.ca).

is secreted as a processed soluble glycoprotein from infected cells [14]. SERP-1 serves to dampen the *in vivo* inflammatory response to virus infection, and previous studies have shown that a targeted disruption of the SERP-1 gene results in greater inflammation of the primary lesion, more rapid resolution of the infection, and attenuation of virus virulence upon infection of immunocompetent hosts [11,15]. SERP-1 has also been shown to inhibit a number of serine proteinases *in vitro*, including urokinase, tissue-type plasminogen activator (tPA), plasmin and thrombin [16,17]. Purified SERP-1 protein has also proved effective as an anti-inflammatory agent in models of coronary restenosis following balloon angioplasty [18], and in antigen-induced arthritis [19].

The role of post-translational modification of virus-encoded immunomodulatory proteins in the complex processes of infection, immune evasion and antigenicity remains poorly understood. Previous studies have shown that SERP-1 is N-glycosylated, and that secretion is blocked by tunicamycin [15], a drug that selectively blocks the addition of N-linked oligosaccharides, suggesting that SERP-1 secretion is dependent upon correct and complete glycosylation. Furthermore, the discovery of a functional $\alpha 2,3$ -sialyltransferase encoded by myxoma virus that is capable of modifying N-linked oligosaccharide chains by the addition of sialic acid moieties [8] suggests that secreted viral proteins such as SERP-1 may be targets for terminal sialylation of N-linked glycans. Knock-out analysis of the myxoma virus sialyltransferase gene revealed that it is required for enhanced viral virulence, though the actual role of sialylation by the *MST3N* gene product during infection has not yet been characterized [8].

We have investigated the nature of the post-translational glycosylation of the myxoma-virus virulence factor SERP-1. We show that SERP-1 secretion is dependent upon glycosylation, specifically at Asn¹⁷², and to a lesser extent at Asn⁹⁹. Furthermore, we demonstrate that SERP-1 is the first example of a secreted virulence factor that is post-translationally modified by a virus-encoded glycosyltransferase, though this does not affect the ability of SERP-1 to inhibit proteinases *in vitro*.

EXPERIMENTAL

Materials

The human enzyme tPA (two-chain form) was obtained from Sigma. Proteinase substrate Chromozym-TPA was purchased from Boehringer-Mannheim. Anti-SERP-1 monoclonal antibodies (mAbs) AQ.H9 and AG.F11 were kindly provided by Leona Ling (Biogen, Cambridge, MA, U.S.A.). Neuraminidase (sialidase) was obtained from Boehringer-Mannheim and used as per the manufacturer's instructions.

Viruses and cell lines

The complete SERP-1 gene, including the signal sequence, was inserted into the thymidine kinase locus of vaccinia-virus strain Western Reserve under the control of a synthetic late promoter utilizing the pMJ-601 plasmid by homologous recombination as described [15], in order to produce the recombinant virus termed VV-S1 (vaccinia virus expressing SERP-1). SERP-1 from Chinese-hamster ovary (CHO) cells expressing SERP-1 was a gift from Leonna Ling (Biogen). Myxoma viruses Lu169, L14Lz, MSTN⁻ and MSTNRev were described previously [10].

Purification of SERP-1 from virus infections

SERP-1 was purified from viral supernatants by FPLC as previously described [22]. SERP-1 was resolved by SDS/PAGE,

and proteins were revealed by either silver staining or immunoblotting using anti-SERP-1 mAb AQ.H9 (Biogen).

[³⁵S]Met/Cys pulse-chase

Baby-green-monkey kidney (BGMK) cells were grown in six-well tissue-culture plates to 80% confluency (approx. 10⁶ cells/well). Cells were infected by myxoma virus (Lausanne) or VV-S1 at a multiplicity of infection (moi) of 10 in an adsorption volume of 0.5 ml for 1 h at 37 °C. Following virus adsorption, the cells were washed with PBS and allowed to recover in 1.0 ml of Dulbecco's modified Eagle's medium supplemented with 10% NBCS. At 8 h post-infection the cells were washed three times with sterile PBS and starved in 1.0 ml of serum-free Met-/Cys-media (Gibco BRL) for 30 min at 37 °C. The cells were then pulsed with 500 μ Ci of [³⁵S]Met/Cys (Dupont-NEN) and incubated for 15 min to allow protein labelling. The cells were then washed three times with sterile PBS, and 1.0 ml of serum-free medium was added. Cells were harvested at appropriate times post-pulse by incubating with SSC (150 mM NaCl/15 mM sodium citrate, pH 7.0), pipetting repeatedly, and transferred to microcentrifuge tubes. Cells were pelleted by centrifuging at 14000 g in a microcentrifuge for 3 min and then resuspended in 0.5 ml of lysis buffer (1% Nonidet P40/150 mM NaCl/50 mM Tris/HCl, pH 8.0). Cell lysis was allowed to proceed for 60 min at 4 °C with constant agitation. Cell nuclei were removed by centrifugation at 14000 g in a microcentrifuge for 15 min. The supernatant containing the soluble portion of the cell lysates was transferred to a clean tube and the pellet containing the nuclei and cell debris was discarded.

Immunoprecipitation of SERP-1

Protein A-Sepharose beads (Sigma) were pre-swollen in lysis buffer and washed three times in lysis buffer prior to preparing a slurry of 50% (v/v) Protein A beads in lysis buffer. Anti-SERP-1 mAb AQ.H9 was added to the soluble portion of the cell lysate or supernatant (see above) to a final concentration of 3 μ g/ml and incubated at 4 °C for 60 min with constant agitation. Protein A slurry (30 μ l) was then added to each sample and incubated for 30 min at 4 °C with constant agitation. The beads were then recovered by briefly centrifuging in a microcentrifuge. The supernatant was discarded and beads washed three times with 100 μ l of lysis buffer.

Endoglycosidase-H (Endo-H) treatment

Following immunoprecipitation of SERP-1, the Protein A beads with SERP-1 bound was subjected to Endo-H treatment. A portion of the beads were resuspended in 50 μ l of denaturing buffer (New England Biolabs, Beverly, MA, U.S.A.) and boiled for 10 min. A 5 μ l portion of 10 $\times$ G5 buffer (New England Biolabs) was added, followed by 5 μ l of Endo H_t (New England Biolabs), and the samples incubated at 37 °C for 60 min. Samples were then boiled in denaturing/reducing SDS/PAGE gel-loading buffer [100 mM Tris/HCl (pH 6.8)/200 mM dithiothreitol/4% SDS/0.2% Bromophenol Blue/20% glycerol] and separated by SDS/PAGE. SERP-1 was revealed by drying the gels on filter paper and exposing them to X-ray film (Kodak) at -80 °C.

Preparation of N-glycosylation site mutants

Mutagenesis of SERP-1 was achieved with the aid of the Altered Sites II mutagenesis system (Promega). Briefly, a *Bam*HI/*Hind*III fragment containing the complete SERP-1 open reading frame

(ORF) was cloned into the pAlter-1 vector (Promega). Purified pAlter-SERP-1 was denatured and annealed to mutagenic oligonucleotides: Nmut1 (CCGATACGTCTACGCCGAAAGCGACAACG) resulted in an Asn²⁸ → Ala mutation; Nmut2 (GAGTTCTCCTCGCGTTTCGCTACCTCCGTGCAAC) destroyed an *Nru*I site and caused an Asn⁹⁹ → Ala change; Nmut3 (CCTTTTATTCGGAGCGGCTACATACAAGG) introduced a *Bsr*B1 site and caused an Asn¹⁷² → Ala mutation. These oligonucleotides were used to produce SERP-1 mutants designated N28A (Asn²⁸ → Ala), N99A, N172A and tmNA (triple mutant). Mutants were then sub-cloned into the pMJ-601 vector to allow expression in vaccinia virus (see above).

Western-blot analysis of SERP-1

Samples were separated on Tris/glycine/SDS/10% polyacrylamide gels using the Laemmli buffer system [20] and transferred to Hybond C-extra (Amersham) nitrocellulose by semi-dry electroblotting. Blots were blocked in Tris-buffered saline (TBS; 150 mM NaCl/2.5 mM KCl/25 mM Tris/HCl, pH 7.4) containing 5% (w/v) skimmed-milk powder and 0.1% (v/v) Tween-20 for more than 2 h, and SERP-1 was detected by incubating for 1 h with 0.005% (v/v) monoclonal anti-SERP-1 antiserum AQ.H9 (Biogen) in blocking buffer. After washing with TBS containing 0.1% (v/v) Tween-20, a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (Bio-Rad) was applied for 1 h. Immunoreactive SERP-1 species were revealed using an ECL[®] (enhanced chemiluminescence) detection system (Amersham) on Kodak X-ray detection type film.

Isoelectric-focusing (IEF) gels

IEF was accomplished on IEF Ready-Gels (Bio-Rad). The sample loading buffer consisted of TBS/50% glycerol and gels were run with a 7 mM phosphoric acid anode buffer (which acts to create a pH gradient) and a cathode buffer consisting of 20 mM lysine free base (Sigma) and 20 mM arginine free base (Sigma). IEF gels were run for 3 h at 200 V in Mini-Protean II Gel Units (Bio-Rad). The relative pI positions were determined with IEF marker proteins (Bio-Rad). Following IEF separation, the gels were soaked in 1% SDS for 30 min, followed by 10 min in transfer buffer, and transferred by semi-dry electrophoretic transfer as described above.

Determination of SERP-1 concentration by ELISA

Flat-bottomed 96-well ELISA plates (Nunc) were coated with 100 µl of anti-SERP-1 mAb AG.F11 at 1 µg/ml in 50 mM carbonate buffer, pH 9.6, overnight at 4 °C. Prior to use, plates were washed three times with wash buffer (PBS/0.1% Tween-20) and incubated with 200 µl of blocking buffer [PBS/2% (w/v) BSA/0.05% Tween-20] for 2 h. SERP-1 standards and samples were diluted in assay buffer [PBS/1% (w/v) BSA/0.01% Tween-20] and 50 µl added to triplicate wells. SERP-1 standards consisted of highly purified SERP-1 with the concentration as determined using A_{280} and a molar absorption coefficient of 32 700 M⁻¹·cm⁻¹ [17]. Samples and standards were incubated in the plate for 1 h, washed three times with wash buffer and 50 µl of 1 mg/ml biotinylated AQ.H9 (Biogen) in assay buffer was added and incubated for 45 min. The plate was washed again and 50 µl of 1 µg/ml streptavidin-horseradish peroxidase in assay buffer was added and incubated for 45 min. The plate was then washed three times and 100 µl of chromogenic substrate [0.5 mg/ml *o*-phenylenediamine (Sigma)/0.003% H₂O₂/0.05 M

citric acid/0.05 M sodium phosphate buffer, pH 5.0] was added. When colour development was complete, the reaction was stopped with the addition of 100 µl of 2 M H₂SO₄ and the A_{490} determined using a Bio-Rad ELISA plate reader.

Slow binding inhibition kinetics

Inhibition progress curves were obtained by incubating a limiting concentration of tPA (125 pM) with various concentrations of SERP-1 (0.125–100 nM) in the presence of Chromozym-tPA chromogenic substrate as previously described [22]. Reactions were allowed to proceed at 37 °C in a total volume of 800 µl of 100 mM Tris/HCl/100 mM NaCl/2 mM CaCl₂/0.005% Triton X-100, pH 7.5). The time-dependent inhibition of each enzyme by SERP-1 was monitored by monitoring the rate of production of *p*-nitroaniline (pNA) at 405 nm using a CARY 3 spectrophotometer equipped with a six-cell cuvette holder. The reactions were allowed to proceed until the steady-state velocity of pNA formation was attained, and the progress curves for each reaction were then fitted to the integrated rate equation for slow binding inhibition (eqn. 1):

$$A_t = v_s t + [(v_i - v_s)/k_{\text{obs}}](1 - e^{-k_{\text{obs}} t}) + A_0 \quad (1)$$

[21,22], in which A_t is the absorbance at 405 nm at time t , due to the evolution of pNA; k_{obs} is the apparent first-order rate constant for the inhibition of enzyme by SERP-1; v_i and v_s are the initial and steady-state velocities for reaction of the chromogenic substrate; and A_0 is the initial absorbance at 405 nm. Values for v_i , v_s , and k_{obs} were obtained for the progress curves measured at each SERP-1 concentration using non-linear regression analysis (DeltaGraph 4.0).

K_m determination for chromogenic proteinase substrate

The K_m for the reaction of tPA with chromogenic substrate Chromozym-tPA was determined directly under the conditions used for the slow-binding-inhibition experiments. A K_m value for the reactions of tPA with Chromozym-tPA was found to be 160 µM.

RESULTS

The intracellular trafficking of secreted proteins begins with the co-translational translocation of the nascent polypeptide chain into the lumen of the endoplasmic reticulum (ER). This step requires a signal sequence that is cleaved by signal peptidase in the lumen of the ER and is therefore not present on the mature protein. We confirmed the predicted cleavage of the signal sequence from the mature SERP-1 polypeptide by sequencing the N-terminus of the purified secreted protein. A sequence of RDIGLWTR was determined, which corresponds to encoded residues Arg¹⁴–Arg²¹ of the SERP-1 sequence and is consistent with the predicted signal peptidase cleavage site at Cys¹³–↓–Arg¹⁴.

SERP-1 secretion occurs only after modification of glycosylation

To examine the rate of SERP-1 processing, virus-infected cells were pulse-labelled with [³⁵S]Met/Cys and the levels of intracellular and secreted ³⁵S-labelled SERP-1 monitored by immunoprecipitation and SDS/PAGE autoradiography. In order to monitor the movement of SERP-1 from the ER to the Golgi, immunoprecipitated SERP-1 samples were subjected to Endo-H treatment. This deglycosylates only the immature ER or *cis*-Golgi form of SERP-1, while leaving mature SERP-1 that has

Figure 1 Endo-H-resistance of intracellular and secreted SERP-1

Pulse-chase time course for the processing and secretion of SERP-1 from BGMK cells infected with VV-S1 (**A** and **C**) and myxoma virus (**B** and **D**). At 10 h post-infection, cells were pulse-labelled with 500 μ Ci of [35 S]Met/Cys and incubated for 15 min. The cells were washed and then both cells (**A** and **B**) and secreted proteins (**C** and **D**) were harvested at times of 0 h (lanes 1 and 2), 1 h (lanes 3 and 4), 2 h (lanes 5 and 6), 4 h (lanes 7 and 8), 6 h (lanes 9 and 10), and 8 h (lanes 11 and 12) post-labelling (times are indicated above each panel). Samples were either treated with Endo-H (+) or incubated in the absence of Endo-H (—). Mature glycosylated SERP-1 appears as a 55 kDa species ('M'), immature glycosylated SERP-1 appears as a 48 kDa species ('I'), whereas SERP-1 deglycosylated following Endo-H treatment appears as a 42 kDa species ('P').

migrated as far as the *trans*-Golgi intact. Figure 1 shows immunoprecipitated SERP-1 at various times following a 30 min [35 S]Met/Cys pulse. The diffuse bands are the result of long exposure necessary to reveal SERP-1, which labels poorly. Intracellular SERP-1 from cells infected with either myxoma virus or a vaccinia vector that overexpresses SERP-1 (VV-S1) appears as an immature glycosylated band that is fully sensitive to Endo-H treatment (Figures 1A and 1B) and likely represents the initial ER-resident glycosylated form of SERP-1. This Endo-H-sensitive immature SERP-1 remains the predominant intracellular form of SERP-1 for the 4 h (myxoma virus; Figure 1B) and 6 h (VV-S1; Figure 1A) chase periods, at which point we detect the first appearance of an Endo-H-resistant species that migrates with an apparent molecular mass of 55 kDa on SDS/PAGE. This Endo-H-resistant intracellular form is the mature SERP-1 just prior to secretion, and is consistent with the appearance of mature Endo-H-resistant SERP-1 in the supernatant at around the same time for both VV-S1 (Figure 1B) and myxoma virus (Figure 1D). By 8 h post-pulse, virtually all SERP-1 labelled during the pulse has been secreted as a mature Endo-H-resistant glycoprotein, and no immature species remains in either the supernatant or cell lysates.

Treatment with Brefeldin A, a fungal metabolite that selectively blocks anterograde transport from the ER to Golgi apparatus,

blocked SERP-1 secretion (results not shown). This is consistent with SERP-1 following the default pathway for secretion rather than an alternative Golgi-independent pathway as has been reported for selected viral proteins [23] and at least one serpin plasminogen-activator inhibitor-2 ('PAI-2') [24]. Brefeldin A treatment resulted in quantitative intracellular accumulation of SERP-1 as an immature glycosylated species, presumably located within the collapsed ER (results not shown).

SERP-1 secretion requires glycosylation at Asn¹⁷⁸

In order to determine the sites at which SERP-1 is N-glycosylated, as well as to examine whether these sites are actually required for protein secretion, mutant SERP-1 constructs were engineered with each of the three putative N-glycosylation sites (Figure 2) disrupted by mutation of Asn to Ala (N28A, N99A and N172A). A fourth SERP-1 construct was engineered lacking all three putative N-glycosylation sites, referred to as triple mutant Asp $\rightarrow$ Ala (tmNA). The resulting SERP-1 mutants were produced in the recombinant vaccinia-virus expression system described in the Experimental section. Figure 2(B) shows a Western blot of SERP-1 secreted from each of the glycosylation-mutant constructs. The N28A mutation caused an increased mobility of SERP-1 on SDS/PAGE, indicating a partial decrease in the

Figure 2 Secretion and degradation of SERP-1 glycosylation mutants

(A) Schematic representation of SERP-1 indicating the three putative N-glycosylation sites at Asp²⁸, Asp⁹⁹ and Asp¹⁷². Hypothetical glycosylation structures are shown at these positions. The reactive-centre loop ('RCL') of SERP-1 is indicated as a grey box near the C-terminus, and the P1 (Arg, R) and P1' (Asn, N) sites are indicated. The N-terminal signal sequence is shaded in black with the cleavage site ('P1-P1') indicated. (B and C) SERP-1 mutants N28A (lane 2), N99A (lane 3), N172A (lane 4) and tmNA (lane 5) were expressed from recombinant vaccinia-virus constructs and secreted proteins (B), and cell lysates (C) were collected, separated by SDS/PAGE and detected by Western blotting with anti-SERP-1 mAb AQ.H9. WT, wild-type.

extent of glycosylation, with protein levels equivalent to the wild-type. In contrast, the N99A mutant, while migrating at a lower apparent molecular mass, also resulted in demonstrably decreased levels of SERP-1 secretion. The N172A mutant failed to be secreted at all, as did the tmNA construct. In order to confirm that the intracellular forms of these proteins were being produced, lysates were also examined by Western blotting (Figure 2C). In addition, β -galactosidase staining of infected cells confirmed that the levels of infection were equivalent and that late gene expression was occurring (results not shown). N28A and N99A proteins appeared as intracellular glycosylated species, presumably retained primarily within the ER or *cis*-Golgi. N172A and tmNA were present at much smaller steady-state levels and only in a non-glycosylated form. It appears that the absence of Asn¹⁷² results in a form of SERP-1 protein that is either unstable or rapidly targeted for degradation.

SERP-1 is modified by the myxoma-virus sialyltransferase

Myxoma virus has been demonstrated to encode a functional sialyltransferase encoded by the *MST3N* gene, although the biological function of this glycosyltransferase enzyme is not yet known [8]. To investigate the effect of the *MST3N* gene product on the SERP-1, we compared SERP-1 secreted from cells infected with the parental Lausanne strain (Lu169), a control lacZ⁺ variant of the parental virus (L14Lz), *MST3N* knock-out virus (*MSTN*⁻) and a *MSTN*⁺ revertant virus (*MSTN*⁺ Rev). On reducing SDS/PAGE, secreted SERP-1 harvested from the *MST3N* knock-out virus exhibits increased mobility (Figure 3A, lane 3), suggesting that SERP-1 is directly modified by the *MST3N*-gene product in wild-type myxoma virus. To confirm that this alteration in mobility is due to glycosylation differences

and not other changes in the protein, SERP-1 samples from the lacZ⁺ parental virus (L14Lz) and the *MST3N*⁻ virus were digested with N-glycosidase F to remove all N-linked glycosylation (Figure 3B). The deglycosylated SERP-1 from both viruses exhibits an identical apparent molecular mass of 42 kDa, confirming that the mobility difference observed for *MSTN*⁻-derived SERP-1 was due to oligosaccharide variations.

Sialylation alters the negative charge of secreted SERP-1

To further investigate the effect of SERP-1 modification by the *MST3N*-gene product, we compared the pI distribution of SERP-1 species from parental, *MSTN*⁻, revertant viruses and recombinant SERP-1 produced from vaccinia-virus and CHO systems. The diffuse band on SDS/PAGE is evidence that SERP-1 glycosylation exhibits some heterogeneity, and the mature secreted SERP-1 appears as several distinct charged species when separated by IEF (Figure 3C, lanes 1, 2 and 4). Wild-type myxoma-virus SERP-1 resolves into two major distinct bands at pI $\approx$ 5.4 and $\approx$ 4.5. By contrast, SERP-1 from the *MSTN*⁻ virus appears as a predominant species at pI $\approx$ 4.5 with a ladder from pI $\approx$ 4.5 to pI $\approx$ 5.4 (Figure 3C, lane 3). This 'ladder' effect has been observed for other sialylated glycoproteins and is due to the progressive addition of neuraminic acid residues with each band varying by 0.05–0.5 pH unit [25]. Recombinant SERP-1 purified from either vaccinia virus or CHO cells exhibits a pI of $\approx$ 5.0 (Figure 3C, lanes 5 and 6). These data indicate that the *MST3N*-gene product modification of SERP-1 shifts the pI to $\approx$ 5.4. The lower-pI species (pI $\approx$ 4.5) observed from all of the myxoma-virus-derived products, as well as the pI $\approx$ 5.0 species observed for vaccinia-virus- or CHO-cell-produced SERP-1, are likely to be the result of cellular sialyltransferases, as the pI values are

Figure 3 SERP-1 secreted from myxoma-virus-infected cells is sialylated by the *MST3N*-gene product

(A) Culture supernatant from BGMK cells infected with wild-type (wt) myxoma-virus Lu169 (lane 1), a control myxoma virus construct with a LacZ cassette, L14Lz (lane 2), myxoma virus in which the sialyltransferase gene is disrupted by the lacZ cassette, MSTN⁻ (lane 3) or the MSTN⁺ revertant virus (lane 4) were collected and concentrated 10-fold. Following separation by SDS/PAGE, samples were revealed by Western blotting using anti-SERP-1 mAb AQ.H9. (B) Culture supernatants from L14Lz (lanes 1 and 3), or MSTN⁻ (lanes 2 and 4) were concentrated 10-fold and treated with N-glycosidase F (lanes 3 and 4). Samples were separated by SDS/PAGE and proteins revealed by Western blotting with anti-SERP-1 mAb AQ.H9. (C) SERP-1 produced from BGMK cells infected with myxoma virus strains Lu169 (lane 1), L14Lz (lane 2), MSTN⁻ (lane 3), the MSTN⁺ revertant virus (lane 4) or from VV-S1 (lane 5) and uninfected CHO cells engineered to overexpress SERP-1 (lane 6) were separated by IEF and detected by Western blotting with anti-SERP-1 mAb AQ.H9. The pI standards 4.5, 5.0 and 5.4 are indicated on the left. (D) Both cellular and viral sialyltransferases modify SERP-1. SERP-1 secreted from BGMK cells infected with myxoma virus (lanes 1 and 2), MSTN⁻ (lanes 3 and 4), and VV-S1 (lanes 5 and 6) were subjected to treatment with neuraminidase (lanes 2, 4 and 6). Samples were separated by SDS/PAGE and detected by Western blotting using mAb AQ.H9.

significantly lower than predicted for the mature SERP-1 protein on the basis of its amino acid sequence (pI 6.29).

Cellular sialyltransferases also act upon SERP-1

From the results reported above it was suspected that cellular sialyltransferases act on SERP-1 in addition to the *MST3N* modification. SERP-1 from myxoma parental, MSTN⁻, revertant viruses and recombinant protein from vaccinia virus and CHO systems was subjected to neuraminidase in order to cleave all sialic acid from the protein. The mobility shift shown by blot analysis (Figure 3D) indicates that SERP-1 from each source was susceptible to neuraminidase digestion. Thus SERP-1 is modified by both the *MST3N*-gene product and still-unidentified host sialyltransferases in myxoma-virus-infected cells and exclusively by the host enzyme(s) in VV-S1-infected cells.

Sialylation/glycosylation variations in SERP-1 do not affect *in vitro* proteinase inhibition

SERP-1 has been shown to function *in vitro* as an inhibitor of a number of plasma serine proteinases [16,17]. To test the effect of sialylation and glycosylation variations upon SERP-1 proteinase-

inhibition function, we attempted to quantify the effectiveness of various isolates of SERP-1 at inhibiting tPA *in vitro* using the techniques of slow binding inhibition kinetics [17,22,26]. The kinetics of tPA inhibition were measured using purified SERP-1 derived independently from vaccinia virus (VV-S1), CHO cells, myxoma virus (Lausanne) and the MSTN⁻ strain of myxoma. The proteinase was incubated with various concentrations of SERP-1 in the presence of a chromogenic substrate (Chromozym-tPA). The time-dependent inactivation of the enzyme through reaction with SERP-1 was monitored continuously by measuring the accompanying decrease in the rate of substrate turnover. Experiments were carried out at a low concentration of enzyme (125 pM) so that the reaction could be followed for many hours without significantly depleting the chromogenic substrate. SERP-1 was always present at concentrations that were in > 10-fold excess with respect to the proteinase, thus giving rise to pseudo-first-order conditions with respect to SERP-1. The progress curves were fitted to eqn. (1) (see above and [22]) to obtain values for the rate constant for the approach to the steady state, k_{obs} , and the velocity of substrate turnover at the steady state, v_s , as described in the Experimental section. Values for the apparent steady-state rate constant, K_i , and the apparent second-order rate constant, k_{app} , were determined from these data [17]. The

Table 1 Observed kinetic parameters for the reaction of SERP-1 glycosylation variants with tPA

Values for the apparent inhibition constant, $K_{i(\text{app})}$ and the apparent second-order rate constant for inhibition, k_{app} were determined in slow-binding-inhibition experiments (100 mM Tris/HCl/100 mM NaCl/2 mM CaCl_2 /0.005% Triton X-100, pH 7.5, 37 °C). Both $K_{i(\text{app})}$ and k_{app} are corrected for the competitive binding of chromogenic substrate in the slow-binding-inhibition assays as described in [22].

Source	$K_{i(\text{app})}$ (nM)	k_{app} ($\text{M}^{-1} \cdot \text{s}^{-1}$)
VV-S1	0.14 ± 0.02	$4.3 \pm 0.4 \times 10^4$
CHO cells	0.13 ± 0.03	$4.5 \pm 0.6 \times 10^4$
MyxWT	0.16 ± 0.04	$4.2 \pm 0.5 \times 10^4$
MyxMST3N ⁻	0.15 ± 0.04	$4.5 \pm 0.6 \times 10^4$

dependence of k_{obs} on the concentration of SERP-1 for the reaction of tPA with purified SERP-1 from VV-S1, CHO cells, wild-type myxoma virus strain Lausanne (vMyxWT) and myxoma virus strain MSTN⁻ (vMyxMSTN⁻) were plotted and the slopes of the 'best fits' used to determine values for the apparent second-order rate constants for the reaction of tPA with purified SERP-1 from VV-S1, CHO cells, vMyxWT, and vMyxMSTN⁻ [22]. The apparent second-order rate constants, steady-state inhibition constants and dissociation constants for the reaction of tPA with purified SERP-1 from VV-S1, CHO cells, vMyxWT and vMyxMSTN⁻ are presented in Table 1. No significant variation is apparent in the kinetics of tPA inhibition for any of the SERP-1 variants tested, indicating that alterations in the glycosylation and sialylation state of SERP-1 do not alter *in vitro* proteinase inhibition. In addition, purified SERP-1 from each of the viruses was used to form heat- and SDS-stable complexes that could be resolved by SDS/PAGE, indicating that SERP-1 variants exhibited comparable physical properties *in vitro*.

DISCUSSION

SERP-1 is the only known virally encoded secreted serpin, and thus acts in the extracellular milieu of infected tissue. In previous studies, SERP-1 has been shown to be an important virulence factor in the pathogenesis of myxomatosis [11,15]. Furthermore, highly purified SERP-1 protein has been shown to act as an anti-inflammatory agent in distinct models of inflammation [18,19]. The effects of SERP-1 are manifest even at extremely low doses, suggesting that it inhibits a rare serine proteinase essential for establishing an early inflammatory response in these model systems [17]. SERP-1 is a moderately potent inhibitor of the plasma serine proteinases urokinase-type plasminogen activator, tPA, plasmin, thrombin, Factor Xa and C1s, the inhibition kinetics for these reactions having been described in great detail [16,17]. There seems to be a further level of complexity in the action of SERP-1 in virus-infected tissues, since the protein is the target of the myxoma-virus-encoded sialyltransferase, and glycosylation at Asn¹⁷² is essential for secretion. The sialylation of SERP-1 by the myxoma-virus MST3N sialyltransferase, which has recently been shown to be a *bona fide* virus-encoded glycosyltransferase [10], is the first reported example of a virally encoded glycosyltransferase that acts on a secreted virus-encoded virulence factor.

We have shown here that correct glycosylation of SERP-1 is essential for secretion of the mature protein. Mutation of the glycosylation site at Asn¹⁷⁸ caused both failure of SERP-1 to appear as a secreted protein and the rapid degradation of intracellular SERP-1 in the ER and secretory pathway. This is in keeping with the role of the ER as a site for conformation-based

'quality control' [27–30]. It is now well established that damaged or misfolded proteins are removed from the early secretory pathway and degraded by the proteasome [31–33]. Recent studies have implicated the chaperone/lectin calnexin in recognizing specific oligosaccharide motifs on misfolded proteins [27,30,34]. Our data suggest that the removal and degradation of proteins from the ER may be triggered by changes in glycosylation state alone, at least in certain circumstances.

Glycosylation, and in particular the addition of sialic acid residues to the oligosaccharide termini, has a significant role in a variety of cellular and immune responses. These include immunological recognition or masking of antigens, initiation of inflammatory responses [35] and cell-specific adhesion events [36]. A wide variety of viral stealth strategies have been described in the past few years [37,38], and poxviruses such as myxoma virus have served as paradigms in this emerging field [10]. Myxoma virus has already been documented to encode at least five distinct anti-apoptotic proteins [39], to down-regulate CD4 and the class I MHC [40,41] and produce a wide range of soluble cytokine receptor homologues [42].

The demonstrated attenuated virulence of the MST3N⁻ knock-out virus [8] could be due to an increased antigenic profile for secreted and cell-associated virus-encoded anti-immune molecules. Alternatively, since glycosylation has been shown in other systems to prolong the biological half-life of proteins, the effects of disrupting the MST3N locus may result from decreased half-life of virulence factors within the infected tissues [8]. Sialylation of SERP-1 by the MST3N gene product may also be required to localize the mature secreted protein to the surface of specific target cells. The low effective doses of SERP-1, both in animal models of inflammation and in virus-infected lesions, have led to speculation that it acts at low concentrations to block a proteinase-mediated pro-inflammatory cascade set up by rare sentinel cells of the immune system [17]. Since SERP-1 functions biochemically as a serpin, it acts in a concentration-dependent manner. Localization to the surface of target cells would raise the effective local concentration of SERP-1, resulting in a more effective inhibition of pro-inflammatory proteinase cascades. It would also sequester the serpin away from a plasma environment of competing proteinases, thereby avoiding interaction with inappropriate target enzymes that would either deplete, or irrevocably cleave, SERP-1.

There are a variety of potential ligand–receptor interactions that would facilitate localization of a sialylated viral protein to the surface of target immune cells. The sialoadhesin proteins are members of the immunoglobulin superfamily that act as sialylation-dependent adhesion molecules. Among the sialoadhesin family members are the macrophage/myeloid marker CD33, and sialoadhesin, which is expressed on activated macrophages at sites of infection [43–45]. Both of these receptors recognize and bind Neu2,3Gal β 1,3GlcNAc and Neu2,3Gal β 1,4GlcNAc (Neu is neuraminic or 'sialic' acid), which are among the products of the MST3N enzymic activity [46]. In addition, the MST3N-gene product could act in association with a cellular GlcNAc α 1,3/4-fucosyltransferase to form sialyl-Lewis a and sialyl-Lewis x, which act as ligands for the adhesion proteins E-, P- and L-selectins that are involved in inflammation and immune responses [47]. Interestingly, the MST3N-gene product does appear to be able to transfer sialic acid to the fucosylated acceptors from Lewis a and Lewis x [46], a unique activity that distinguishes the MST3N-gene product from all known mammalian sialyltransferases. Thus sialylation of SERP-1 by the myxoma-virus-encoded α 2,3-sialyltransferase has the potential to dramatically alter the actions of SERP-1 in complex biological tissues while not affecting the proteinase inhibition by SERP-1 *in vitro*.

Inactivation of the *MST3N* gene in myxoma virus results in a reduced virulence in immunocompetent hosts, but does not cause any defects in virus replication in tissue culture [8]. This is consistent with the *MST3N*-gene product acting on secreted myxoma virulence factors and regulating antigenicity, biological half-life or localization within complex tissue systems. Many bacteria and parasites express sialyltransferase activities in order to mask antigenic sites [48]. The observation that the *MST3N*-gene product is not the only sialyltransferase responsible for modifying SERP-1 is intriguing. The *MST3N* sialyltransferase creates a distinct sialylated species of SERP-1 with a consistent charge resulting in a protein with a pI of 5.4. This differs from that the more acidic (pI 4.5) species observed in the absence of the *MST3N*-gene product in either myxoma virus, vaccinia virus or CHO cells, that is produced through the compensating action of cellular sialyltransferases. This suggests that it is not simply the extent of charge imparted by sialylation that is important to myxoma virus, but rather that the particular linkage resulting from the *MST3N* sialyltransferase may be the critical factor. Further investigation of this area will be required before the effects of glycosylation and sialylation on SERP-1 *in vivo* function and myxoma virus virulence can be fully ascertained.

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