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Mutation of the *Myxoma virus* SERP2 P1-site to prevent proteinase inhibition causes apoptosis in cultured RK-13 cells and attenuates disease in rabbits, but mutation to alter specificity causes apoptosis without reducing virulence

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We would like to dedicate this work in memory of Frederique Messud-Petit. Her studies helped to inspire this project.

Abstract

Myxoma virus (MYX) prevents apoptosis in RK-13 cells and forms thick dermal lesions with 100% mortality in rabbits. MYX encodes the virulence factor SERP2, a serine proteinase inhibitor (serpin). SERP2 was mutated to evaluate SERP2 function during MYX infection. MYXΔSERP2::lacZ (deleted for SERP2) did not inhibit apoptosis in RK-13 cells; infected rabbits had thin dermal lesions and <10% mortality. MYX-SERP2-D294A, a P1-site aspartate to alanine mutant, inactivated the serpin; infection was indistinguishable from MYXΔSERP2::lacZ. SERP2-D294E prevented inhibition of caspase-8, caspase-10 and granzyme-B; and MYX-SERP2-D294E failed to block apoptosis in RK-13 cells, but was fully virulent in rabbits. MYXΔSERP2::crmA expressed crmA instead of SERP2 and inhibited apoptosis in cell culture, but caused thin lesions and only 70% mortality in rabbits, hence crmA cannot fully substitute for SERP2. Control of apoptosis in culture does not correlate with virulence in rabbits. Virulence may instead depend on inhibition of proinflammatory proteinases by SERP2.
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Keywords: Myxoma virus; Poxvirus; Serpin; Proteinase; Protease; Caspase; Apoptosis; Inflammation; Pathogenesis; Virulence

Introduction

Myxoma virus (MYX) is a poxvirus within the genus *Leporipoxvirus*. The Lausanne strain of MYX causes lethal infection in European rabbits (*Oryctolagus cuniculus*) and is transmitted mechanically by biting insects (Fenner and Woodroffe, 1953). Poxviruses encode a variety of proteins that can be considered virulence factors that are of interest clinically because of their anti-inflammatory and anti-apoptotic properties (McFadden and Murphy, 2000). One class of poxvirus virulence factors are serine proteinase inhibitors (serpins). MYX encodes three serpins designated SERP1, SERP2 and SERP3 (Cameron et al., 1999).

Serpins are found in all higher organisms, but poxviruses are the only viruses that encode serpins that function as proteinase inhibitors (Silverman et al., 2001). Inhibition of proteinase activity occurs when proteinases recognize and cleave a scissile bond (designated P1–P′) in the reactive center loop (RCL) of a target serpin leading to a conformational change and formation of a stable serpin/proteinase complex which renders the proteinase inactive (Huntington et al., 2000). In addition to acting as proteinase inhibitors, some serpins interact with cellular components through regions of the serpin other than the RCL. For example, antithrombin is a serpin that contains a heparin binding site (Chang et al., 1996) located on the serpin backbone rather than the RCL. The specificity of some serpin/proteinase interactions is modified by secondary binding sites in

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the backbone of the serpin (Djie et al., 1997). It is well established that directed mutation of serpins can lead to greater understanding of serpin function. For example, mutation of the P1-site of a serpin can alter the profile of proteinases inhibited by the serpin (Holmes et al., 1987).

SERP2 from MYX and the cytokine response modifier A (crmA) gene from the *Orthopoxvirus Cowpox virus* (CPV) are poxvirus serpins that contain an aspartate at the P1-site in the RCL. Overall, SERP2 and crmA have 34.9% identity at the amino acid level, but 57.3% similarity (Petit et al., 1996). This level of similarity is typical of that found across random members of the serpin superfamily. However, because both poxvirus serpins encode a P1-aspartate, they might be expected to inhibit similar proteinases in vitro. In fact, SERP2 and crmA are both cross-class inhibitors that inhibit proinflammatory and pro-apoptotic cysteine aspartyl-specific proteinases (caspases) (Petit et al., 1996; Garcia-Calvo et al., 1998) as well as the serine proteinase granzyme B (Turner et al., 1999; Quan et al., 1995).

Consistent with the ability of SERP2 to inhibit proinflammatory and pro-apoptotic proteinases in vitro, the presence of SERP2 is reported to decrease inflammation and apoptosis during intradermal MYX infection of New Zealand White (NZW) rabbits (Messud-Petit et al., 1998). This group showed that MYX deleted for SERP2 (MYXΔSERP2) caused dermal lesions with increased mononuclear inflammation compared to infection with MYX. Unlike lesions caused by MYX, the lesions caused by MYXΔSERP2 were non-myxomatous. Extensive apoptosis was observed in parotid lymph node sections from rabbits infected with MYXΔSERP2 but not in rabbits infected with MYX. Increased inflammation and apoptosis did not affect the relative virus load in tissues infected with MYXΔSERP2. Hence, SERP2 was thought to control and inhibit inflammation and apoptosis in infected rabbits.

The effect of crmA deletion from CPV is also consistent with the ability of crmA to inhibit proinflammatory and pro-apoptotic proteinases. CrmA inhibits inflammation (Pickup et al., 1986) and apoptosis (Nathaniel et al., 2004) in chicken chorioallantoic membranes (CAMs) during CPV infection. In this model, deletion of crmA from CPV causes a decrease in virus titer (Palumbo et al., 1989). Virus yield correlates to the ability to control apoptosis during infection of CAMs and is independent of the presence or absence of inflammation (Nathaniel et al., 2004).

To date, SERP2 and crmA inhibit the same proteinases in vitro and both are thought to control inflammation and apoptosis in animal models, however they do not fully substitute for each other during infection. When crmA is replaced by SERP2 during CPV infection of CAMs, SERP2 is able to prevent apoptosis and restore virus yields, but it cannot block inflammation (Nathaniel et al., 2004). Likewise, when SERP2 is replaced by crmA during MYX infection of NZW rabbits, crmA is able to partially restore virus virulence (with a mortality rate of 70%), but it does not cause the myxomatous lesions that are characteristic of MYX infection (Nathaniel et al., 2004). This suggests that these two poxvirus serpins (1) show species specificity in their ability to inhibit proteinases, (2) inhibit different, currently unidentified proteinases or (3) have additional functions in vivo that do not involve proteinase inhibition.

The goal of this project was to determine how SERP2 functions as a virulence factor during MYX infection of NZW rabbits. Specific, single amino acid alterations were made at the P1-site of SERP2 to determine the mechanism by which SERP2 functions as a virulence factor. SERP2 D294A was created with an alanine in place of aspartate at P1 and was expected to retain the serpin structure but lack the ability to inhibit proteinases. This mutation allowed us to assess the role of proteinase inhibition mediated by SERP2 in virulence and disease. SERP2 D294E replaced the SERP2 aspartate at P1 with glutamate. This mutation was chosen based upon the human serpin PI-9 which contains a P1-glutamate. PI-9 inhibits granzyme B at a physiologically significant rate (Bird et al., 1998); but PI-9 does not effectively inhibit caspase-1, caspase-3, caspase-4 or caspase-8 (Annand et al., 1999). We had hoped that the SERP2 D294E mutation would allow us to assess the relative effect of inhibiting granzyme B alone (without concurrent caspase inhibition) during MYX infection of NZW rabbits. However, we show that, rather than converting SERP2 into a selective inhibitor of granzyme B, SERP2 D294E inhibited caspase-1 but no longer inhibited granzyme B, caspase-8 or caspase-10. Although SERP2 D294E did not behave as expected, MYX SERP2 D294E allowed us to examine how controlling inflammation without controlling apoptosis affected the pathogenesis of MYX infection of rabbits.

Results

Complex formation between serpins produced in vitro and human granzyme B

Altered SERP2 proteins with P1-site mutations were tested for the ability to form a stable complex with granzyme B in vitro using a gel-shift assay. ^{35}S-methionine-labeled proteins produced by in vitro transcription and translation were incubated with varying amounts of human granzyme B then analyzed on an SDS–10% polyacrylamide gel (Fig. 1). For reference, crmA is a 38 kDa protein (Palumbo et al., 1989) and SERP2 is a

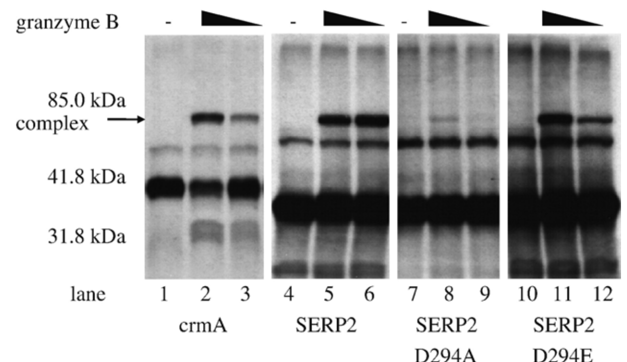

Fig. 1. Detection of serpin/proteinase inhibitory complexes using a gel-shift assay. ^{35}S-labeled proteins were expressed in vitro, incubated with varying amounts of human granzyme B and evaluated by SDS–PAGE.

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