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The Kaposi's sarcoma-associated herpesvirus complement control protein (KCP) binds to heparin and cell surfaces via positively charged amino acids in CCP1–2

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Abstract

The Kaposi's sarcoma-associated herpesvirus (KSHV) complement control protein (KCP) inhibits the human complement system, and is similar in structure and function to endogenous complement inhibitors. Other inhibitors such as C4b-binding protein and factor H, as well as the viral homologue vaccinia virus complement control protein are known to bind heparin and, for the two latter, also to glycosaminoglycans at the surface of cells. We report here that KCP also binds to heparin at physiological ionic strength. With help of site directed mutagenesis, positively charged amino acids in the two N-terminal complement control protein (CCP) domains 1–2 were found to be necessary for heparin binding. In silico molecular docking of heparin to KCP confirmed the experimental data, and further explored the heparin binding site, enabling us to present a model of the KCP–heparin interaction. Furthermore, the docking analysis also yielded insights of the KCP structure, by indicating that the angle between CCP domains 1–2 during the initial binding of heparin is more extended than in the model we have previously presented. We also found that KCP binds to heparan sulfate and weakly to glycosaminoglycans at the surface of cells. This might indicate that KCP at the surface of viral particles aids in the primary attachment to the target cells, which is known to involve binding to heparan sulfate. Therefore, the present study contributes to the knowledge of heparin–protein interactions in general as well as to the understanding of the biology of KSHV.

1. Introduction

KSHV is the most recently discovered human herpesvirus. It has been found to cause Kaposi's sarcoma (Chang et al., 1994) primary effusion lymphoma (Cesarman et al., 1995) and Castleman's disease (Soulier et al., 1995). KSHV has a broad cellular tropism. In vivo, KSHV DNA has been found in B-cells (Cesarman et al., 1995), endothelial cells (Boshoff et al., 1995), keratinocytes (Foreman et al., 1997) and monocytes (Monini et al., 1999). In vitro, KSHV can infect a variety of human cells; e.g. endothelial cells (Panyutich et al., 1998), fibroblasts (Akula et al., 2003; Vieira et al., 2001) and keratinocytes (Cerimele et al., 2001) as well as several cell lines from different species, e.g. CHO cells (Bechtel et al., 2003).

KSHV attachment to its target cells involves binding to heparan sulfate (Akula et al., 2001b; Birkmann et al., 2001), in accordance with the observation that binding to heparan sulfate is a common initial step of cell entry for many of the human herpes viruses (reviewed in Shukla and Spear, 2001; Spear and Longnecker, 2003). Heparan sulfate is a ubiquitously expressed glycosaminoglycan, present at the surface of cells via covalent binding to a cell membrane attached core protein. Heparan sulfate is similar in structure to the soluble glycosaminoglycan heparin, which is why heparin binding often is interpreted as an indication of heparan sulfate binding. The KSHV encoded

Abbreviations: KSHV, Kaposi's sarcoma-associated herpesvirus; KCP, KSHV complement control protein; RCA, regulator of complement activation; CCP, complement control protein domain; C4BP, C4b-binding protein; VCP, vaccinia virus complement control protein; DAF, decay accelerating factor; CR1, complement receptor 1

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glycoprotein gB and gpK8.1 have been identified as heparan sulfate binding proteins on the KSHV lipid envelope (Akula et al., 2001a; Birkmann et al., 2001; Wang et al., 2001). gB also binds to the host cell integrin α3β1 via its RGD sequence, which induces intracellular signalling (Akula et al., 2002; Naranatt et al., 2003). Endocytosis following the initial attachment has been observed as entry mechanism for the KSHV particle (Akula et al., 2001b, 2003).

The focus of this study is the protein encoded by the fourth open reading frame of KSHV; KCP. KCP is a homologue to the proteins of the human gene family called RCA. The RCA proteins are soluble or membrane bound and they inhibit the complement system by disrupting the C3 convertases (either the classical or the alternative C3 convertase), which are the key enzyme complexes formed during the complement activation cascade. KCP inhibits the C3 convertases in two distinct manners: (i) decay acceleration of the classical C3 convertase and (ii) cofactor activity for factor I, that in the presence of KCP can cleave C3b and C4b, which are parts of the alternative and the classical C3 convertase, respectively (Mullick et al., 2003; Spiller, 2003; Spiller et al., 2003).

KCP is expressed during the lytic phase (Jenner et al., 2001), and has been detected at the surface of KSHV infected pleural effusion lymphoma cells upon induction of lytic replication (Spiller et al., 2003). It is thus likely that KCP is present on the subsequently formed KSHV particles. Hence, it is probable that KCP protects KSHV particles and infected cells from eradication by the complement system.

Structurally, KCP resembles the human RCAs. It contains four extracellular CCP domains and it is attached to the cell surface by a transmembrane spanning area. There are three splice variants of KCP, which differ in the region between the CCP domains and the transmembrane region (Spiller et al., 2003). We have recently engineered a wide range of site-directed KCP mutants and analysis of their function confirmed that the molecular mechanisms used by KCP strongly resemble those used by human complement inhibitors (Mark et al., 2004). Some of the human RCAs; factor H and C4BP, bind to heparin, which is essential for their biological function. Another viral RCA homologue, VCP, has also been shown to bind to heparin and to the surface of cells (Smith et al., 2000, 2003).

The aim of this study was to investigate whether KCP is capable of binding to proteoglycans as heparin, given its structural similarity to factor H and C4BP. Such an interaction could be of biological significance: it would indicate that KCP aids in the attachment of viral particles to its target cells.

2. Materials and methods

2.1. Cells

CHO-K1 cells were purchased from American Type Culture Collection (Manassas, USA) or received as a kind gift from Dr Dick Heinegård (Lund University, Sweden). CHO pgsA-745 cells, which are deficient in xylosyltransferase and do not produce glycosaminoglycans (Esko et al., 1985) were received from Dr Dick Heinegård. The cells were propagated in F-12K Nutrient Mixture (Kaighn's Modification, GIBCO-Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum and 50 U/ml penicillin and 50 μg/ml streptavidin.

2.2. Proteins

Recombinant wild type KCP and the KCP point mutants were produced as described previously (Mark et al., 2004). Briefly, the DNA encoding wild type or mutated KCP (the signal peptide, the four CCP domains and the following 48 amino acids) was cloned into a eukaryotic expression vector, which yields a fusion protein with the addition of an in-frame C-terminal human IgG1 Fc region (described in Harris et al. (2000)). The resulting soluble and dimeric proteins were expressed in CHO cells and purified from the cell supernatant by affinity chromatography using protein A-sepharose (GE Health care, Fairfield, USA). The dimeric form of KCP is used for all experiments, unless otherwise indicated. A KCP-Fc dimeric construct without CCP4 (called KCP delCCP4) was produced in a similar manner, using forward primer 5′-**TCTAGA**GCTAGCATGGCCTTTTTAAGACAAAC and reverse primer 5′-**GCGGCCGC**AGCTGCTGTATGGGTGTCTTCA to amplify the DNA encoding CCP1 to CCP3 of wild type KCP. The DNA was cleaved by *Xba*I and *Not*I (in bold) prior to ligation into the same expression vector as for the wild type and point mutants.

Monomeric KCP without the Fc-tail, was generated by cleavage with fXa. Cleavage was performed at room temperature for 12 h, using 0.5 units of factor Xa (R&D systems, Minneapolis, MN, USA) per μg of KCP. Human plasma factor I (Crossley and Porter, 1980), C3 (Andersson et al., 1991) and C4 (Andersson et al., 1991) were purified from plasma as previously described. To create C4met and C3met, C4 and C3 were treated with methylamine to hydrolyze the internal thioester bond; proteins were incubated with 100 mM methylamine pH 8–8.5, at 37 °C for 1 h, followed by dialysis in 50 mM Tris–HCl, pH 7.5 and 150 mM NaCl or 10 mM Hepes–KOH, pH 7.4 supplemented with 50 mM NaCl and 0.005% Tween 20. For simplicity, C4met and C3met are referred to as C4b and C3b in the text. For radioactive C3b and C4b, proteins purchased from Advanced Research Technologies (San Diego, USA) were used. They were iodinated with ^{125}I using the chloramine T method (Greenwood et al., 1963).

2.3. C3b/C4b degradation assay

For cleavage of C3b and C4b by factor I, KCP (0.3 μM) was incubated with 375 nM C3b or 125 nM C4b, 60 nM factor I and trace amounts of ^{125}I labelled C3b/C4b in 50 μl of 50 mM Tris–HCl, 150 mM NaCl, pH 7.4. A sample without factor I was used as a negative control. The samples were incubated at 37 °C for 1.5 h prior to separation by SDS-PAGE (10–15% gradient) under reducing conditions. The radiolabelled C3b or C4b was visualized by autoradiography using Phosphoimaging analysis (GE Health care, Fairfield, USA). Cleavage products were quantified by densitometry. Heparin blocking of the reaction was achieved by preincubation of KCP with low molecular weight heparin Fragmin (stock 25000 IU/ml (156.2 mg/ml) Pfizer, New York, USA) for 30 min at room temperature before starting the