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Adenovirus Endopeptidase Hydrolyses Human Squamous Cell Carcinoma Antigens *in Vitro* but not *ex Vivo*

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The serpins SCCA1 and SCCA2 are highly expressed in the epithelium of the conducting airways, a common site of infection by group C adenoviruses, such as human adenovirus type 2 (Ad2). Based on the common location we examined a possible interaction between them. *In vitro* experiments with recombinant proteins showed that SCCA1 inhibited the viral protease in a dose-dependent manner. Both serpins were cleaved in a manner consistent with hydrolysis within their reactive site loop, without the formation of an SDS-resistant complex, as in the case of papain. Infection of SCCA1-expressing cells did not result in the cleavage of SCCA1, nor was the yield of infectious virus affected as compared to SCCA1-negative parental cells. This may be due to differential localization, the serpin being cytoplasmic and viral protease being nuclear. Surprisingly, however, virus infection, which tends to inhibit host protein synthesis, caused a significant increase in SCCA1 expression well into the late phase of infection.

Key Words: adenovirus type 2; protease; cysteine; serpins.

INTRODUCTION

Adenoviruses encode a cysteine endopeptidase which cleaves several viral proteins in the course of virus assembly and maturation (Weber, 1995). The enzyme is specific for substrate sequences containing a Gly in the P1 or P1′ position, a second Gly in the P2 position and Met, Ile, or Leu in the P4 position. Other than viral proteins the enzyme also cleaves cytokeratins K7 and K18, resulting in depolymerization and accelerating cell lysis (Chen *et al.,* 1993). Although the crystal structure of the enzyme is unique, the architecture of the active site closely resembles that of papain (Ding *et al.,* 1996).

High-molecular-weight serine proteinase inhibitors (serpins) regulate a diverse set of intracellular and extracellular processes such as complement activation, fibrinolysis, coagulation, cellular differentiation, tumor suppression, apoptosis, and cell migration (Gettins *et al.,* 1996; Wright, 1996). The ov-serpins are a subset of the serpin superfamily and are characterized by their high degree of homology to chicken ovalbumin, the lack of N- and C-terminal extensions, the absence of a signal peptide, and a Ser rather than an Asn residue at the penultimate position. Recently two new ov-serpins, SCCA1 and SCCA2, have been identified (Schneider *et al.,* 1995). Although no target proteases have been identified for these serpins, SCCA1 binds with a 1:1 stoichiometry and at second-order rate constants of $>10^5$/M/s with several papain-like cysteine proteinases (cathepsins S, K, L, and papain itself). SCCA1 and SCCA2 are 92% identical, but differ significantly at their reactive site loops. SCCA1 inhibits papain-like cysteine proteases and SCCA2 inhibits chymotrypsin-like serine proteases.

SCCA1 was found to be highly expressed in the epithelium of the conducting airways, a common site of infection by the group C adenoviruses, particularly human adenovirus type 2 (Ad2). Based on the common location and the target specificity is it possible that this serpin is an endogenous inhibitor of the Ad2 protease (AVP)? Here we report the results of *in vitro* experiments using recombinant proteins, which suggest that rAVP may cleave these serpins in their reactive site loop, resulting in reduced enzyme activity. In *ex vivo* experiments, SCCA1 expression was increased by virus infection but the serpin was not cleaved nor was virus production affected.

RESULTS AND DISCUSSION

Effect of serpins on adenovirus protease

In preliminary experiments purified, recombinant fusion proteins of serpins rGST-SCCA1 (1 μg) or rGST-SCCA2 (1 μg) were added to the adenovirus protease (0.5 μg rAVP) assay reaction using the fluorescent peptide substrate R110. No significant change in enzyme activity was observed. The experiments were repeated using the viral protein pVII substrate assay, and again enzyme activity remained essentially unaffected (results not shown). Similar results were obtained with ovalbumin, a noninhibitory serpin. Because ovalbumin contains

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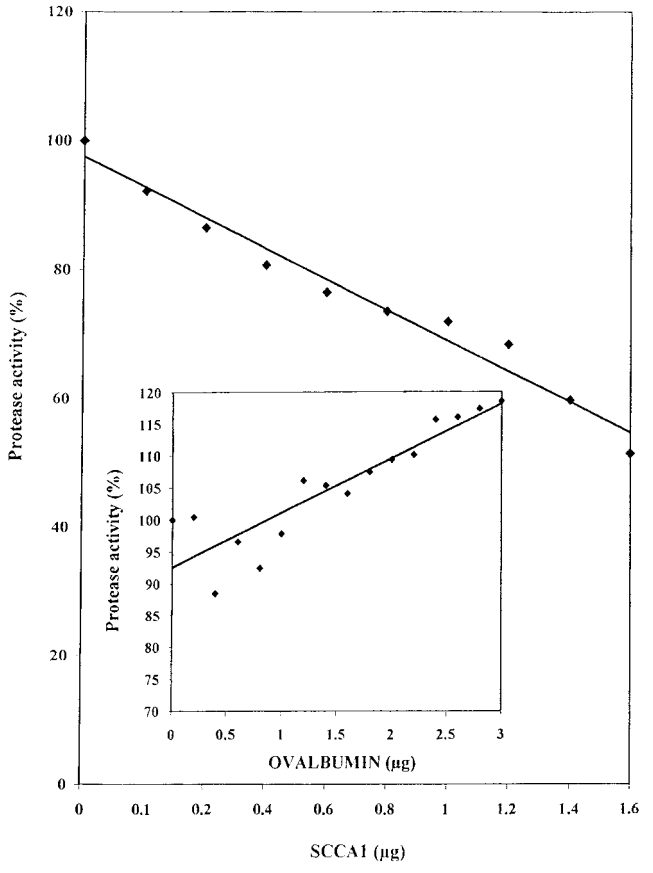

FIG. 1. Effect of rSCCA1 on protease activity. rAVP (0.5 μg) was preincubated for 30 min with different amounts of rSCCA1 before the addition of R110 substrate and further incubation for 2 h. The fluorescence units of the cleaved substrate is expressed in percentage as the mean of six independent determinations. (Inset) A similar experiment with ovalbumin as control.

an AVP consensus cleavage site, denatured ovalbumin can, however, act as a competitive inhibitor at ovalbumin/substrate ratios of greater than 10. It is possible that the GST fusion partner interferes with the activity of the serpins or that the protease has a much greater affinity for the R110 protease substrate than the serpins. For these reasons the experiments were repeated with rSCCA1 using a prior incubation with protease for 30 min before the addition of the R110 substrate. Under these conditions we observed a dose dependent decrease in enzyme activity as the ratio of serpin to enzyme was increased (Fig. 1). A ratio of 1 mol of serpin to 1 mol of enzyme gave 30% inhibition. This is comparable to the inhibition of papain by egg white cystatin, an industry standard for efficient enzyme inhibition by a protein inhibitor (Barrett, 1981). The observed inhibition is unlikely to be nonspecific because in a similar experiment ovalbumin did not show any inhibitory activity, but instead moderately stimulated protease activity, possibly by stabilizing or buffering the enzyme (Fig. 1, inset).

Serpins may be cleaved by adenovirus protease

So far only two cellular proteins, cytokeratins K7 and K18, have been shown to be digested by AVP in the course of infection (Chen *et al.,* 1993). Digestion occurred at the single AVP site present in both cytokeratins. Neither SCCA serpins contain AVP concensus cleavage sites. Could the SCCA serpins be substrates for AVP within their reactive site loop (RSL)? The results summarized in Fig. 2 show that, indeed, both rGST-SCCA1 and rGST-SCCA2 were digested by AVP. Although a variety of digestion conditions were explored, the degree of digestion was variable in extent, but not site, and never complete. rGST contains an AVP site (MLGG) at residue 84. This site was digested relatively inefficiently in rGST alone (Fig. 2, lanes j, k) when compared to the rapid appearance of the largest and dominant digestion product in rGST-SCCA1 and rGST-SCCA2 (band "a" in lanes d–e and g–h). An analysis of all the digestion products suggests that the primary cleavage takes place at the serpin RSL generating band "a" consisting of approximately 578 residues. When serpins act as substrates, limited proteolysis of the RSL occurs, with the primary sites of cleavage being within a domain of approximately 11 residues, from P_{10} to P'_1, where the P_1 position represents the cleavage site for the normal target protease (Gettins *et al.,* 1996). Consequently, in this case the exact size of band "a" is not known.

To verify the cleavage in the serpin RSL we digested the rGST-SCCA1 fusion protein with thrombin followed by digestion with rAVP. Thrombin digestion yielded the expected fragments of 390 (band "d") and 226 (band "f") residues and AVP digestion generated the expected subfragment of approximately 352 (band "e") residues (Fig. 2, lanes l, m) due to presumed digestion in the RSL. Finally, we purified rSCCA1 after thrombin digestion of the fusion protein and subjected this pure rSCCA1 to rAVP digestion. The conversion of band "d" to band "e" (lanes o, p) supports the contention of digestion of rSCCA1 in the RSL region by rAVP.

As evident from Fig. 2, no higher molecular weight bands, representing SDS and heat-stable complexes such as the SCCA1–chymotrypsin complex, were detected after hydrolysis of the rSCCA serpins (Nawata *et al.,* 1995). Papain apparently also fails to form such a complex after cleaving SCCA1 (Nawata *et al.,* 1997).

Although rAVP was relatively pure, it remains possible that some or all of the observed cleavages were due to a contaminating protease which copurified with rAVP. Consequently a series of control experiments were carried out to examine this possibility. The following control experiments argue against this possibility: (a) most of the visible contaminants were 10 kDa or less, whereas proteases tend to be at least 20 kDa or larger in size, (b) rAVP only cleaved the known substrate proteins in a

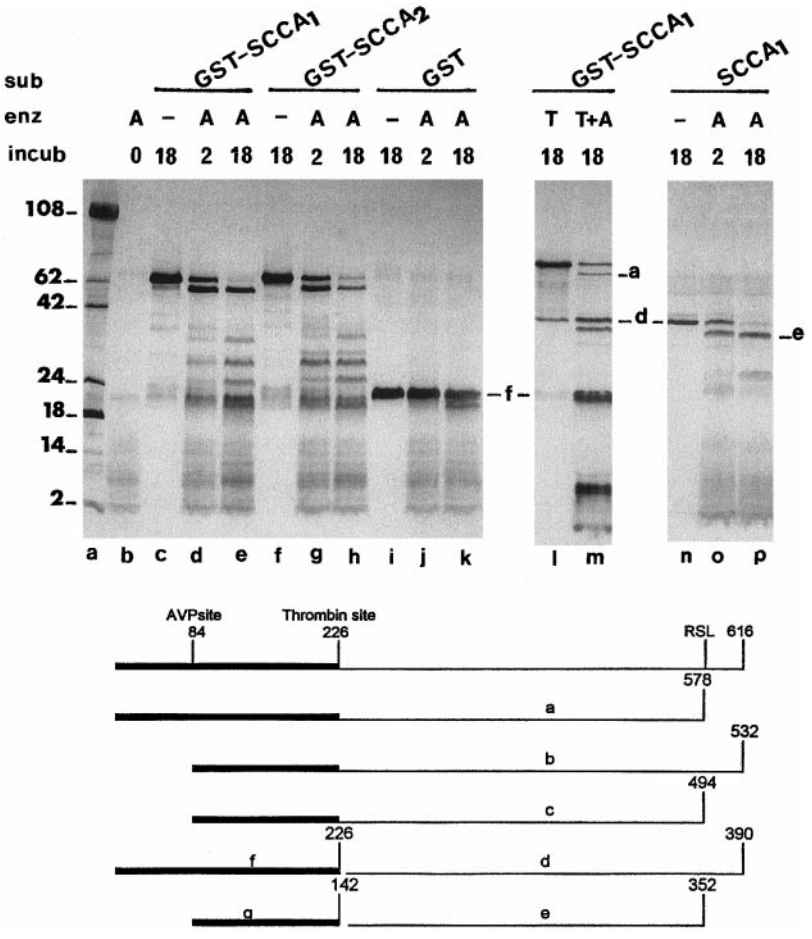

FIG. 2. Specific cleavage of recombinant serpins SCCA1 and SCCA2 by adenovirus protease. A 12% polyacrylamide–SDS gel stained with silver. GST–SCCA fusion proteins, GST, and purified SCCA1 substrates (sub; 1 μg) were incubated (incub) with AVP enzyme (A; 0.02 μg) or thrombin (T) for 0, 2, or 18 h as indicated. The reactions were stopped by boiling in SDS–lysing solution. Lane a contains adenovirus type 2 as marker proteins labeled in kDa. The cleavages are interpreted schematically in the lower figure as fragments a–g. The thick line designates the GST portion of the fusion proteins and the thin line the serpin portion. The numbers indicate native or postcleavage C-terminal residues.

mixture of viral and cellular proteins, (c) cleavage activity was only inhibited by cysteine protease-specific inhibitors and not by serine protease-specific inhibitors, (d) a mutant rAVP (C104G/C122G with 0 activity; Rancourt *et al.,* 1996) purified identically did not show any cleavage activity (Fig. 3). It might also be argued that a contaminating proteolytic activity present in the recombinant serpins is responsible for some of the observed cleavages. Overstained gels of self-incubated rSCCA1 showed no changes, thus arguing against this possibility. We therefore suggest that rAVP is responsible for the digestion of rSCCA1 and rGST-SCCA2 in their RSL.

The effect of virus infection on SCCA1 expression and vice versa

Virus infection generally suppresses host protein synthesis. Here we wish to test if this holds true for SCCA1 and more particularly whether SCCA1 is potentially cleaved by the viral protease during infection. The results clearly show that SCCA1 is not suppressed in the recombinant cell line, but increased significantly during infection under a variety of conditions (Figs. 4A–4C). SCCA1 expression remained unchanged in cells mock-infected for 2 or 3 days (results not shown). There was no evi-

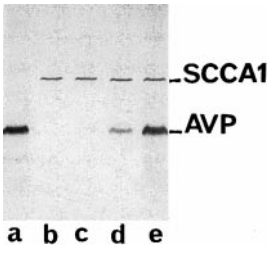

FIG. 3. Mutant protease does not cleave SCCA1. rSCCA1 (0.5 μg) was incubated for 18 h with 0, 0.02, 0.2, and 1 μg (lanes b–e, respectively) of a mutant rAVP purified identically to the wild type enzyme and the reaction mixtures separated by SDS–PAGE and stained with silver. Lane "a" contained 1 μg of rAVP only.

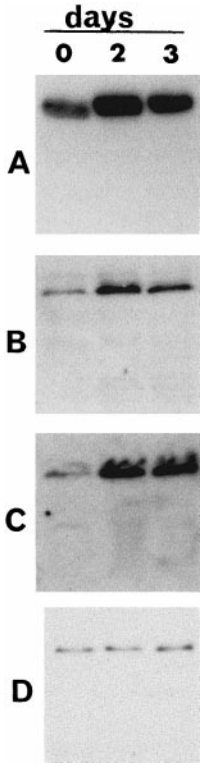

FIG. 4. Immunoblot of SCCA1 expression in adenovirus infected cells. MDA-435/S1-3 cells were infected with 5 (A) or 50 (B and C) PFU/cell for 0, 2, or 3 days at 37°C (A and B) or 39°C (C). SKGIIIa cells were infected for 0, 2, or 3 days at 37°C with 50 PFU/cell at 37°C (D). Cell lysates were subjected to SDS–PAGE, blotted, and reacted with anti-SCCA1 serum followed by ^{125}I-labeled protein A.

dence of SCCA1 cleavage under any of these conditions. Lack of cleavage may be due to compartmentalization in that SCCA1 is in the cytoplasm and active protease within virus particles. Increased SCCA1 expression during virus infection could be due to several mechanisms including an increased half-life or increased gene expression or both. In an attempt to study this problem we also looked at the effect of infection on the expression of the endogenous SCCA1 gene in SKGIIIa cells. The results did not show any change in the expression of SCCA1 during the course of virus infection (Fig. 4D). The increased SCCA1 expression subsequent to infection of the MDA-435/S1-3 cell line may therefore be due to stimulation of the artificial promoter (EF-1α) in this transformed cell line.

A possible effect of the serpins on the course of virus infection was also examined. SCCA1 expression did not have any noticeable effect on the synthesis of infectious virus (Table 1). Virus titers of the parental MDA-MB-435 cell line, which does not express SCCA1, and the SCCA1-expressing transformed derivative cell line MDA-435/S1-3 were the same. The absence of any correlation between SCCA1 expression and ability to support virus replication also extended to such other human cancer cell lines as Hep2, MCF7, and SKGIIIa. The low virus titers in these cell lines, relative to the Hep2 cell line, prompted us to verify the efficiency of infection by estimating the production of the major capsid protein hexon. This showed some variability among the cell lines, but did not affect the conclusion with regard to the indepen-

TABLE 1

Effect of SCCA1 on Virus Infection

Cell line	Infection (days)	SCCA1 expression[a]	Titer × 10^5 (PFU/ml)	Hexon expression[b] (%)	Colony formation assay[c] (%)
HEP2	0	−	0	0	3.6
	2	−	8600	50	<0.03
	3	−	67000	100	<0.03
MDA-MB-435S	0	−	0	0	4.5
	2	−	8	0	<0.05
	3	−	16	8	
MDA-435/S1-3	0	+	0	0	9
	2	+++	4	2	<0.05
	3	++	16	40	<0.05
MCF 7	0	−	0		
	2	−	530		
	3	−	270		
SKGIIIa	0	+	0	0	5.8
	1	+	0.003		<0.05
	2	+	0.8		<0.05
	3	+	3	40	
	4	+	3	50	
	5	+	7	60	

[a] Relative expression estimated from Western blots.
[b] Hexon expression estimated from SDS–PAGE of cell lysates stained with Coomassie blue.
[c] Cells were infected with 10 PFU/cell for the indicated times, plated, and colonies counted 2 weeks later.

dence of virus production from serpin expression. The efficiency of infection was also verified by colony formation assay, based on the observation that infected cells fail to attach and divide (Table 1). In agreement with virus and hexon production, the data suggest that more than 99% of the cells of each cell line tested were infected by virus. In summary, these experiments are in agreement therefore, with the foregoing conclusion that the viral protease does not encounter and inhibit or cleave SCCA1.

MATERIALS AND METHODS

Enzyme and assays

Recombinant adenovirus type 2 protease (rAVP; EC3.4.22.–) was purified by chromatography from an *Escherichia coli* expression system (pLPV) as described before (Keyvani-Amineh *et al.,* 1995). This enzyme has 14 foreign amino acids fused to the N-terminus. This fusion peptide has no detectable effect on the properties of the enzyme, as determined by comparison with rAVP without this extension or enzyme isolated from virus particles. Enzyme activity was measured with a fluorescent peptide substrate, R110, purchased from Molecular Probes, Inc. (Eugene, OR), essentially as described before (Diouri *et al.,* 1995). The reaction mixture contained TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA), 40 μM pVIc (oxidized enzyme activating peptide GVQSLKRRRCF), 3.3 μM R110, and 1 μg of rAVP, unless otherwise indicated, in a total volume of 300 μl. Incubation was at 37°C for 18 h and the fluorescence was measured. In the case of protein substrates (SCCA1, etc.), unless otherwise indicated, the reaction mixture contained 1 μg substrate, 0.02 μg rAVP in a total volume of 60 μl. The latter reactions were stopped by boiling in lysing solution.

Qualitative assays were done as described before with substrates of ts1-infected cell lysates labeled with [^{35}S]methionine at the nonpermissive temperature (39°C) at 24 h p.i. as a source of viral precursor proteins, particularly PVII (Keyvani-Amineh *et al.,* 1995). Endogenous protease activity was inactivated by boiling this substrate for 2 min. Enzyme activity is assessed from the conversion of PVII to VII visualized on autoradiograms of SDS–PAGE separations.

Thrombin was from Sigma and used as per the manufacturer's instructions (0.01 Units/μg substrate).

Serpins

Recombinant human SCCA1 and SCCA2 were purified as GST-fusion proteins as described previously (Schick *et al.,* 1997). Recombinant SCCA1 was also purified from yeast (generously supplied by P. Pemberton). Purity of these proteins was determined by SDS–PAGE and visualized by silver staining. Protein concentration was determined by the Bradford assay (Bio-Rad). Ovalbumin (crystallized, grade VII) was purchased from Sigma.

Cells and virus

Hep2, a human epidermoid carcinoma of the larynx, MCF7, a human breast adenocarcinoma, and MDA-MB-435S, a human breast ductal adenocarcinoma, cell lines were obtained from ATCC (Rockville, MD). The MDA-435/S1-3 cell line is a recombinant cell line obtained by transforming the MDA-MB-435S line with a plasmid carrying the SCCA1 cDNA under the control of the EF-1α promoter and a hygromycin B resistance gene. SKGIIIa is a carcinoma cell line derived from the cervix which expresses both SCCA1 and SCCA2. All cell lines were cultured in DMEM medium and 10% fetal calf serum. The MDA-435/S1-3 cell line was grown in the presence of 500 μg/ml hygromycin B (Gibco). Human adenovirus type 2 (Ad2) was grown in HEp2 cells and titered by either plaque formation or end-point dilution in HEp2 cells. All experiments were done at an m.o.i. of 10 PFU/cell.

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