

Actin Can Act as a Cofactor for a Viral Proteinase in the Cleavage of the Cytoskeleton*

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Cytoskeletal proteins are exploited by many viruses during infection. We report a novel finding that actin can act as a cofactor for the adenovirus proteinase (AVP) in the degradation of cytoskeletal proteins. Transfection studies in HeLa cells revealed AVP localized with cytokeratin 18, and this was followed by destruction of the cytokeratin network. For AVP to cleave cytokeratin 18, a cellular cofactor was shown to be required, consistent with AVP being synthesized as an inactive proteinase. Actin was considered a cellular cofactor for AVP, because the C terminus of actin is homologous to a viral cofactor for AVP. AVP was shown to bind to the C terminus of actin, and in doing so AVP exhibited full enzymatic activity. *In vitro*, actin was a cofactor in the cleavage of cytokeratin 18 by AVP. The proteinase alone could not cleave cytokeratin 18, but in the presence of actin, AVP cleaved cytokeratin 18. Indeed, actin itself was shown to be a cofactor and a substrate for its own destruction in that it was cleaved by AVP *in vitro*. Cleavage of cytoskeletal proteins weakens the structure of the cell, and therefore, actin as a cofactor may play a role in cell lysis and release of nascent virions.

During viral infections, different properties of actin are exploited (1). Actin has been shown to play a role in the transcription of several paramyxoviridae genomes. Actin stimulates human parainfluenza virus type 3 transcription; depletion of actin abolishes viral mRNA synthesis (2). A hallmark of oncogenic transformation by RNA tumor viruses is the loss of cytoskeletal integrity resulting from the disappearance of actin stress fibers, perturbation of focal adhesions, and aggregation of actin near the ventral surface of the transformed cell (3). In the case of human immunodeficiency virus, the Gag protein, which is both necessary and sufficient for viral budding, is associated with the actin cytoskeleton *in vitro* (4), and their association at the plasma membrane may play a role in the budding of retroviruses. During baculovirus infection by *Autographa californica* M nuclear polyhedrosis virus, there is a

dramatic rearrangement and eventual destruction of the actin cytoskeleton (5). The virus encodes a proteinase that specifically degrades actin. Here we reveal another property of actin that is exploited by a virus; actin can act as a cofactor to stimulate a virus-coded proteinase.

Throughout an adenovirus infection, the actin, cytoke-
ratin, tubulin, and vimentin networks that make up the cytoskeleton of the cell undergo dramatic changes (6). Chen *et al.* (7) have shown that late in an adenovirus infection, cytokeratin 18 is cleaved at two contiguous adenovirus proteinase (AVP)¹ consensus cleavage sequences, leading to the destruction of the cytokeratin network. In cells infected by a temperature-sensitive mutant of adenovirus that lacks proteinase activity at the non-permissive temperature, cytokeratin 18 is not cleaved, and the cytokeratin network remains intact.

This observation raises a conundrum. Cleavage of cytokeratin 18 by AVP takes place in the cytoplasm, yet the proteinase is synthesized in an inactive form and is activated in the nucleus by two viral cofactors within immature virions. One cofactor is pVIc, an 11-amino acid peptide that originates from the C terminus of the precursor to protein VI, pVI (8–10), and the other cofactor is adenovirus DNA (8, 11). Once AVP becomes activated, it cleaves the virion precursor proteins used in the assembly of virus particles, thereby rendering the virus particles infectious (12). The two cofactors activate AVP by increasing the specificity constant, k_{cat}/K_m , for substrate hydrolysis (11). Compared with AVP alone, the k_{cat}/K_m increases 1,130-fold with an AVP-pVIc complex, 110-fold with an AVP-viral DNA complex, and 34,100-fold in the presence of both pVIc and viral DNA. Presumably, if AVP were synthesized in an active form, it would cleave virion precursor proteins before virion assembly thereby aborting the infection (10, 13).

In this study the conundrum of how AVP may be activated in the cytoplasm is resolved; a new, cellular cofactor for AVP is described, actin. Cytokeratin 18 could not be cleaved by AVP *in vitro*. However, cytokeratin 18 could be cleaved by AVP in the cytoplasm of HeLa cells in the absence of other viral proteins. This prompted a search for a cellular cofactor. Actin was considered as a cofactor, because its C terminus shares homology with pVIc. *In vitro*, upon the binding of AVP to the C terminus of actin, the activity of AVP was greatly stimulated. *In vitro*, cytokeratin 18 could not be cleaved by AVP alone. Most important, in the presence of actin, cytokeratin 18 could be cleaved by AVP.

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¹ The abbreviations used are: AVP, adenovirus proteinase; pVIc, 11-amino acid peptide, GVQSLKRRRCF, that originates from the C terminus of the viral precursor protein pVI; TBS, Tris-buffered saline; GFP, green fluorescent protein.

EXPERIMENTAL PROCEDURES

Materials—Purified G-actin (14) was a gift from Dr. Clarence Schutt. It was stored in G-buffer that contained 0.2 mM ATP, 0.5 mM dithiothreitol, 0.2 mM CaCl₂, 2 mM Tris (pH 8.0) at 4 °C. The concentration of actin was determined using a molar extinction coefficient of 26,600 M⁻¹ cm⁻¹ at 290 nm (15). AVP was purified as described previously (16). Its concentration was determined using a molar extinction of 26,510 M⁻¹ cm⁻¹ at 280 nm (17). pVlc was purchased from Research Genetics. Its concentration was determined by titration of its cysteine residue with Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoate). A molar extinction coefficient of 14,150 M⁻¹ cm⁻¹ at 412 nm was used to calculate the concentration of thionitrobenzoate (18). The fluorogenic substrate (Leu-Arg-Gly-Gly-NH)₂-rhodamine was synthesized and purified as described previously (16). PRODAN-labeled G-actin was synthesized according to published procedures (19, 20) where the PRODAN moiety (21) was covalently attached to Cys-374 of G-actin (19, 20).

Construction of the AVP-GFP Fusion Gene—The gene for AVP was amplified from the pT7AD23K8 plasmid (22) by PCR and then inserted into the CT-GFP fusion TOPO vector purchased from Invitrogen. Plasmid DNA was prepared using the High Pure Plasmid Isolation Kit from Roche Molecular Biochemicals according to the manufacturer's instructions. The AVP portion of the AVP-GFP fusion gene was sequenced to ensure there were no PCR errors.

Transfection and Immunofluorescence Microscopy—HeLa cells were plated on glass coverslips 24 h prior to transfection. The cells were transfected with AVP-GFP or GFP vectors using FuGENE 6 from Roche Molecular Biochemicals. Twenty hours after transfection, cells were fixed and immunostained. Where indicated, cells were treated with 50 µg/ml cycloheximide from Sigma for 4 h prior to fixing and staining. Cells were fixed with 4% formaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 10 min. The cells were blocked in 10% goat serum and incubated with a 1:100 dilution of anti-cytokeratin 18 antibody from Sigma for 1 h. The coverslips were washed in phosphate-buffered saline and incubated for 1 h with a 1:200 dilution of rhodamine-conjugated goat anti-rabbit antibody from The Jackson Laboratories. Coverslips were sealed in the presence of Slowfade antifade solution, from Molecular Probes, with nail polish. Cell staining was visualized using a Zeiss Axioskop microscope equipped for epifluorescence. The 100× Neo-Plan Fluor objective using a rhodamine or GFP filter from Chroma Technology was used. Images were captured using the Spot 2 cooled CCD camera from Diagnostic Instruments and presented using Adobe Photoshop.

Assay for Proteinase Activity—Standard assays in 1 ml contained 10 mM Tris-HCl (pH 8.0) and 5 mM octyl glucoside. Proteinase and cofactors were incubated for 5 min at 37 °C after which 3 µM (Leu-Arg-Gly-Gly-NH)₂-rhodamine was added. The increase in fluorescence was monitored as a function of time in an ISS PC-1 Spectrofluorometer. The excitation wavelength was 492 nm and the emission wavelength 523 nm, both set with a bandpass of 8 nm.

Determining the Apparent Equilibrium Dissociation Constant for the Binding of AVP to PRODAN-Actin—Different concentrations of AVP, [AVP]_i, were added to 65 nM PRODAN-actin, [P-actin]₀, in 2 mM Tris-HCl (pH 8.0), 0.2 mM ATP, 0.2 mM CaCl₂, and 0.2 mM dithiothreitol. After 5 min at 25 °C, the fluorescence intensity, F_i, was measured with an excitation wavelength of 380 nm and an emission wavelength at 492 nm, both monochromators set with a bandpass of 8 nm. The concentration of bound AVP, [AVP]_b, was obtained as shown in Equation 1,

$$[\text{AVP}]_b = [\text{P-actin}]_0 \left(\frac{F_i - F_{\min}}{F_0 - F_{\min}} \right) \quad (\text{Eq. 1})$$

where F₀ is the amount of fluorescence in the absence of AVP, and F_{min} is the minimal amount of fluorescence, i.e. the amount of fluorescence when PRODAN-actin is saturated with AVP. The concentration of free AVP, [AVP]_f, is shown in Equation 2.

$$[\text{AVP}]_f = [\text{AVP}]_i - [\text{AVP}]_b \quad (\text{Eq. 2})$$

From a plot of [AVP]_b versus [AVP]_f, the apparent K_d can be obtained by standard techniques.

Cleavage of Cytokeratin 18 by AVP—A HeLa cell fraction enriched for cytokeratins was prepared as described previously (7). That fraction was incubated with AVP in the presence or absence of cofactors in 10 mM Tris (pH 8.0) and 5 mM octyl glucoside for 1 h at 25 °C. SDS sample buffer was added, and the reactions were incubated in a boiling water bath for 5 min. After fractionation by SDS-PAGE on an 8–16% polyacrylamide gradient gel, the proteins were electrophoretically transferred to a polyvinylidene difluoride membrane using the NOVEX X Cell Surelock Mini-Cell II Blot Module. The membrane was incubated

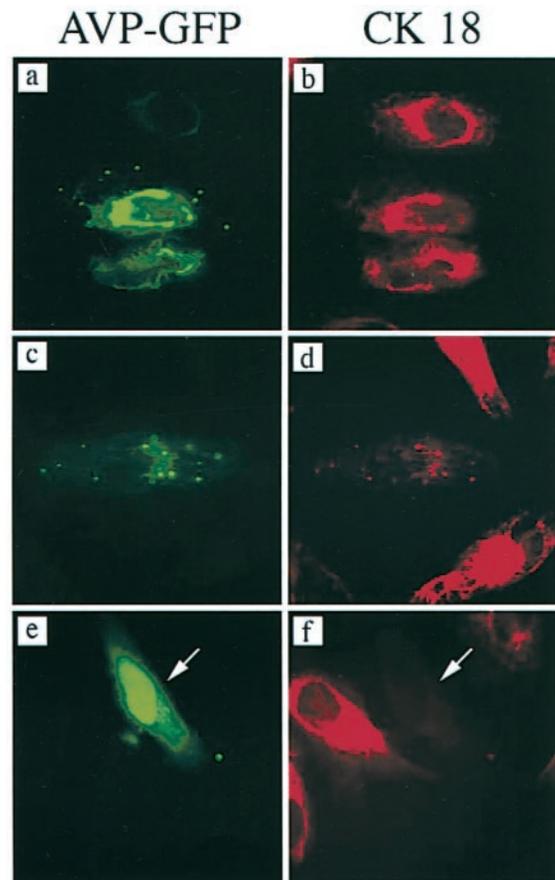


FIG. 1. AVP co-localizes with cytokeratin 18 and eventually destroys the cytokeratin 18 (CK 18) network in the absence of viral cofactors. An AVP-GFP chimeric gene was transfected into HeLa cells, and localization was visualized 20 h later. Localization of AVP was detected by green fluorescence (a, c, and e), and cytokeratin 18 by a specific antibody and a rhodamine-conjugated secondary antibody (b, d, and f). The effect of cycloheximide addition from 16 to 20 h post-transfection is shown in the lower panels (e and f); arrowhead indicates a cell expressing AVP (e) that has no detectable endogenous cytokeratin 18 (f).

overnight at 25 °C in blocking solution (TBS containing 0.1% Triton X-100 and 3% bovine serum albumin). TBS contained 10 mM Tris-HCl (pH 7.5) and 150 mM NaCl. The membrane was probed with a monoclonal anti-cytokeratin 18 antibody (Sigma clone KS-B17.2) in blocking solution for 1 h at 25 °C, washed repeatedly with TBSXS buffer (TBS containing 0.1% Triton X-100, 0.05% SDS, and 0.1% bovine serum albumin), and then placed in blocking solution for 5 min at 25 °C. The membrane was probed with a goat anti-mouse alkaline phosphatase-conjugated antibody (Bio-Rad) for 1 h at 25 °C. The membrane was washed three times in TBSXS followed by a wash with TBS. The blot was developed using the Alkaline Phosphatase-conjugated Substrate kit (Bio-Rad) according to the manufacturer's instructions.

RESULTS

AVP Cleaves Cytokeratin 18 in the Cytoplasm in the Absence of Any Viral Cofactor—A possible explanation for the activity of AVP in the cytoplasm during an adenovirus infection was that a viral cofactor stimulates AVP to cleave cytokeratin 18. To determine whether this is the case or whether AVP interacts and cleaves cytokeratin 18 in the absence of other virus-coded components, an expression vector for an AVP-green fluorescent protein (GFP) chimeric gene was transfected into HeLa cells. AVP was localized by visualizing the GFP moiety, and cytokeratin 18 was visualized with antibodies, using fluorescence microscopy. AVP was found in the cytoplasm where it co-localized with cytokeratin 18 in a network-like pattern (Fig. 1, a and b). Transfection with the parent GFP vector yielded diffuse fluorescence, evenly distributed throughout the cytoplasm and

nucleus (data not shown). In cells expressing relatively higher AVP-GFP levels, AVP and cytokeratin 18 co-localized in bleb-like structures that have been described previously (7, 23) in adenovirus-infected cells as aggregates of degraded cytokeratin filaments (Fig. 1, *c* and *d*). Thus, AVP appeared to interact with and cleave cytokeratin 18 in the cytoplasm in the absence of any other virus-coded components.

AVP Can Destroy the Cytokeratin Network in the Absence of Other Viral Proteins—For the complete degradation of the cytokeratin network by AVP during an adenovirus infection, shutdown of host cell protein synthesis is required (23). To determine whether AVP, in the absence of other viral components, was capable of completely degrading the cytokeratin network, HeLa cells, at 16 h post-transfection with the AVP-GFP vector, were treated with cycloheximide for 4 h. Then AVP-GFP and cytokeratin 18 were visualized. Under these conditions, AVP was found in both the cytoplasm and nucleus (Fig. 1*e*). In those cells expressing AVP-GFP, cytokeratin 18 was no longer detectable, suggesting that AVP had destroyed the cytokeratin network (Fig. 1*f*). Thus AVP, in the absence of other viral proteins and in the absence of protein synthesis, appeared to degrade completely the cytokeratin network.

Actin Is a Potential Cellular Cofactor for AVP because Its C-terminal Sequence Is Homologous to pVlc—Another possible explanation for the activity of AVP in the cytoplasm was that a cellular component was acting as a cofactor for AVP in its cleavage of cytokeratin 18. Actin was considered a potential cofactor for AVP, because the C-terminal amino acid sequence of actin is highly homologous to the amino acid sequence of pVlc (Fig. 2*a*). Of the last 8 amino acid residues of actin, 4 are identical and 3 homologous to the last 8 amino acid residues in pVlc. Comparisons of the 10 C-terminal amino acid residues in the α -, β -, and γ -actin isomers revealed that these residues are strictly conserved. There are numerous actin-related proteins, but their C termini are not homologous to the C terminus of actin. The penultimate amino acid in actin is Cys-374. The penultimate amino acid in pVlc, Cys-10, is a major determinant in the reversible binding of pVlc to AVP (24). Furthermore, a disulfide bond forms between Cys-10 of pVlc and Cys-104 of AVP, both *in vitro* (24, 25) and *in vivo* (24) in the virus particle. For actin, in particular its C terminus, to be a cofactor for AVP, the C terminus must be accessible to interact with AVP. Inspection of the crystal structure of actin (26) or an actin-profilin complex (27) shows that the C terminus of actin is on the surface and therefore could be accessible to interact with AVP.

Actin Acts as a Cofactor for AVP in Vitro—To determine whether actin could act as a cofactor for AVP, increasing concentrations of monomeric actin (G-actin) were incubated with a constant amount of AVP, and proteinase activity was measured as a function of time with the fluorogenic substrate (Leu-Arg-Gly-Gly-NH)₂-rhodamine (Fig. 2*b*). In the absence of actin, there was little or no enzyme activity; in the absence of AVP, there was no enzyme activity. In the presence of actin, the amount of substrate hydrolyzed to fluorescent product Leu-Arg-Gly-Gly-NH-rhodamine increased linearly with time. Furthermore, the rate of substrate hydrolysis was proportional to the actin concentration. Thus, actin could indeed act as a cofactor for AVP.

AVP Binds to the C Terminus of Actin—The hypothesis that actin could act as a cofactor for AVP was based upon the sequence homology between pVlc and the C terminus of actin. To determine whether the C terminus of actin binds to AVP, binding studies were performed with PRODAN-labeled G-actin (PRODAN-actin) (19, 20), where the PRODAN moiety (21) was

a.

Amino Acid Sequence of β -actin

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MDDIIAALVVDNDSGSMCKAGFAGDDAPRAVFPISVGRPRHQGVMVGMGQKDSYVGDQAQS
KRGILTLLKYP IIEHGI VTNWDDMEK IWHHTFYNELRVAP EHPVLLTEAPLNFKANREKMT
QIMFETNTFAMYVAIQAVLSLYASGRRTTGIVMDSGDGVHTVTPYIEGYALPHAILRLDL
AGRLDLDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKSY
ELPDGQVITIGNERFRCPEALFQPSFLGMESCGIHTTFNSIMKCDVDIRKDL YANTVLS
GGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSIILASLSTFQQMWISKQ
EYDESGPSIVHRKCF

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	pVlc	G V Q S L K R R R C F
α -actin C-terminus	A G P S I V H R K C F	
β -actin C-terminus	S G P S I V H R K C F	
γ -actin C-terminus	S G P S I V H R K C F	

C-terminus
Proteinase consensus cleavage sequences
Identity or homology in amino acid residues

b.

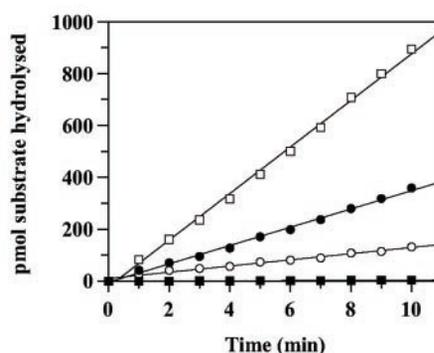


FIG. 2. C terminus of actin is homologous to viral cofactor pVlc, and actin can function as a cofactor in stimulating AVP activity. *a*, the amino acid sequence of β -actin and comparison of the amino acid sequences of pVlc to the C termini of actin isomers. The C-terminal 11 amino acid residues of β -actin are colored orange. AVP consensus cleavage sites are colored green. Amino acid residues are colored blue for identity and red for homology. *b*, stimulation of AVP activity by actin. Increasing concentrations of G-actin at 0 (■), 10 (○), 20 (●), and 50 nM (□) were incubated with 50 nM recombinant AVP for 5 min at 37 °C, after which 3 μ M (Leu-Arg-Gly-Gly-NH)₂-rhodamine was added, and the increase in fluorescence (pmol of substrate hydrolyzed) was measured as a function of time.

covalently attached to the penultimate amino acid Cys-374 (19, 20). Binding of a ligand to the C terminus of PRODAN-actin decreases the fluorescence intensity of the fluorophore. When increasing concentrations of AVP were added to a constant amount of PRODAN-actin, the fluorescence intensity decreased, indicating that AVP was binding to actin, more specifically to the C terminus of actin (Fig. 3*a*). The decrease in fluorescence intensity eventually reached a plateau, implying that binding to the C terminus of actin could be saturated by AVP.

DNase I was used in a control experiment. DNase I binds to subdomain 2 on actin, a region that does not contain the C terminus. Thus, DNase I will bind to PRODAN-actin (20), but there should be no decrease in fluorescence. Increasing amounts of DNase I, well above the equilibrium dissociation constant, K_d , for binding to actin, were incubated with PRODAN-actin. There was a minimal decrease in fluorescence intensity in the presence of DNase I (Fig. 3*b*).

Apparent Equilibrium Dissociation Constant for the Binding of AVP to PRODAN-Actin—From the data in Fig. 3*a*, an apparent equilibrium dissociation constant, $K_{d(\text{app})}$, for the binding of AVP to PRODAN-actin can be calculated. The concentration of bound AVP, $(\text{AVP})_{\text{bound}}$, at any initial AVP

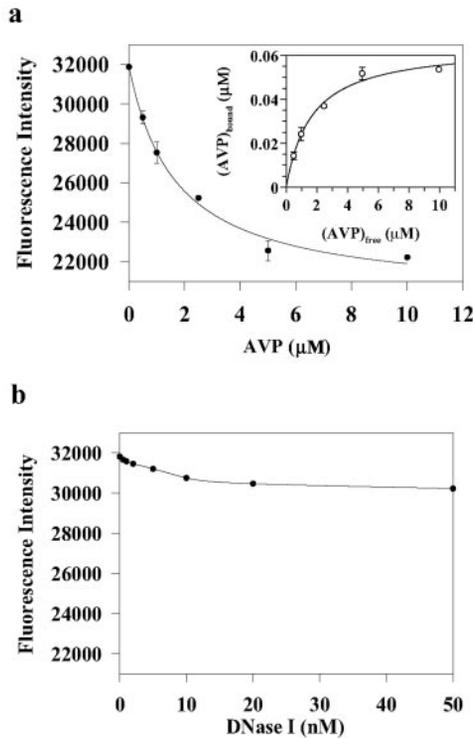


FIG. 3. AVP binds to the C terminus of actin. *a*, binding of AVP to PRODAN-labeled G-actin. Increasing concentrations of AVP in G buffer were incubated with 65 nM PRODAN-actin, and the fluorescence intensity was measured with excitation at 380 nm and emission at 492 nm. These data were then converted into (AVP)_{bound} and (AVP)_{free}, and the K_d value was obtained from the graph in the inset. *b*, binding of DNase I to PRODAN-actin. Increasing concentrations of DNase I were incubated with 65 nM PRODAN-actin, and the fluorescence intensity was measured.

concentration, (AVP)_i, is the ratio of the change in fluorescence due to the presence of AVP, divided by the maximal change in fluorescence, times the concentration of PRODAN-actin, as described under "Experimental Procedures." The concentration of free AVP, (AVP)_{free}, is equal to (AVP)_i - (AVP)_{bound}. From the graph of (AVP)_{bound} versus (AVP)_{free}, a K_d (app) of $1.7 \pm 0.3 \mu\text{M}$ was calculated. The K_d value is apparent until a major assumption in the analysis is verified, namely that one molecule of AVP is bound to the C terminus of one molecule of PRODAN-actin.

AVP Did Not Cleave Cytokeratin 18 in the Absence of Cofactors—In adenovirus-infected cells and in AVP-transfected cells, AVP appeared to have cleaved cytokeratin 18 in the cytoplasm. In transfected cells, it cleaved cytokeratin 18 in the absence of any viral cofactors. It is possible that AVP does not need a cofactor to cleave cytokeratin 18. To determine whether a cofactor is required, a cytokeratin 18-enriched HeLa cell fraction was prepared, in which the DNA, RNA, and soluble proteins, including G-actin, were removed (7). AVP was incubated with the cell fraction; the proteins were fractionated by SDS-PAGE and transferred to a membrane, and the cytokeratin 18 was visualized in an immunoblot with an anti-cytokeratin 18 antibody. The results showed that in the absence of AVP (Fig. 4, lane 2) no cleavage of cytokeratin 18 occurred. Most important, in the presence of AVP (Fig. 4, lane 3) no cleavage of cytokeratin 18 occurred. Thus, AVP needs a cofactor to cleave cytokeratin 18.

AVP Cleaves Cytokeratin 18 in the Presence of the Cofactor pVIc—The above results imply that AVP requires a cofactor to cleave cytokeratin 18 *in vitro*. That this was the case was

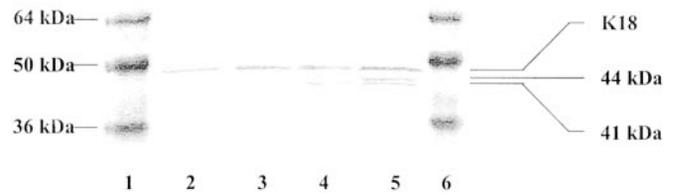


FIG. 4. Cleavage of cytokeratin 18 by AVP utilizing actin as a cofactor. A cytokeratin 18-enriched fraction from HeLa cells was suspended in 10 mM Tris-HCl (pH 8.0) and 2 mM octyl glucoside. Aliquots were incubated with the following: nothing added (lane 2), 1 μM AVP (lane 3), 1 μM AVP and 2 μM actin (lane 4), and 1 μM AVP and 1 μM pVIc (lane 5). After 1 h at 25 °C, the proteins were fractionated by SDS-PAGE, transferred to a membrane, and then visualized by using an anti-cytokeratin 18 antibody. Pre-stained molecular markers are in lanes 1 and 6. K18 signifies cytokeratin 18.

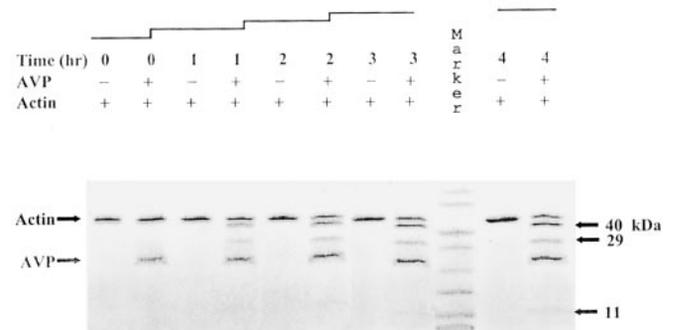


FIG. 5. Cleavage of actin by AVP as analyzed by SDS-PAGE. Actin (2.5 μM) was incubated with AVP (2.5 μM) for the indicated digestion times after which the proteins were fractionated by SDS-PAGE (lanes 4–6). As a control, actin (2.5 μM) was incubated under the same conditions. The markers had molecular masses of 94, 67, 43, 30, 20, and 14.4 kDa. The cleavage products of actin had molecular masses of 40 kDa, for cleavage at the N terminus of actin, and 29 and 11 kDa for cleavage at the C terminus of the 40-kDa fragment of actin.

shown directly by incubating AVP and pVIc with the cytokeratin 18-enriched HeLa cell fraction. The result (Fig. 4, lane 5) indicated that AVP with its virus-coded cofactor pVIc cleaved cytokeratin 18 into two fragments running at 44 and 41 kDa. These are the predicted sizes of fragments of cytokeratin 18 if cleavage occurred at the two contiguous AVP consensus cleavage sites (7). Thus, AVP can cleave cytokeratin 18 in the presence of the cofactor pVIc.

Actin Can Act as a Cofactor in the Cleavage of Cytokeratin 18 by AVP—To determine whether actin can serve as a cofactor for the cleavage of cytokeratin 18 by AVP, the proteinase and actin were incubated with the cytokeratin 18-enriched HeLa cell fraction. One cleavage product was detected, the 41-kDa fragment of cytokeratin 18 (Fig. 4, lane 4). The 41-kDa fragment is the major cytokeratin 18 cleavage product 36 h after an adenovirus infection (7). Thus, AVP can use actin as a cofactor for the cleavage of cytokeratin 18.

Actin Can Act as a Cofactor in the Cleavage of Actin by AVP—Analysis of the amino acid sequence of β -actin reveals two AVP consensus cleavage sequences, one at the N terminus MVGM ↓ G and one at the C terminus LSGG ↓ where cleavage occurs at the down arrow (Fig. 2a). This raised the possibility that actin is not only a cofactor for AVP but it also is a substrate for AVP. Cleavage at the N-terminal AVP consensus cleavage sequence should yield a polypeptide with a molecular weight of 40,000; the product of cleavage at the C terminus should have a molecular weight of 29,000. Accordingly, actin and AVP were incubated together, and as a function of time, aliquots were withdrawn and the proteins fractionated by SDS-PAGE. The concentration of actin was much higher than its K_d value for AVP to ensure that all the AVP was saturated with actin. The

results (Fig. 5) indicated that actin was indeed cleaved by AVP. After a 1-h incubation, a 40-kDa band appeared and increased in intensity as a function of time. A 29-kDa band appeared later than the 40-kDa band, and an 11-kDa band appeared even later. The simplest interpretation of these data is that AVP preferentially cleaved at the N terminus of actin yielding the 40-kDa fragment. Then the 40-kDa fragment was cleaved at its C terminus to yield bands of 29 and 11 kDa. As a control, actin was incubated under the same conditions but in the absence of AVP; no cleavage was observed. Thus, actin is indeed a substrate for AVP.

DISCUSSION

The experiments presented here resolve a conundrum. During an adenovirus infection, how is cytokeratin 18 cleaved by AVP in the cytoplasm since AVP is synthesized in an inactive form that is later activated in the nucleus within immature virions by two viral cofactors? Clearly, the cytokeratin network is destroyed *in vivo* during an adenovirus infection (23). The transfection experiments with an AVP-GFP chimeric gene showed that AVP destroyed the cytokeratin network in the absence of any other viral components. *In vitro*, AVP was not able to cleave cytokeratin 18; however, it was able to utilize actin as a cofactor to cleave cytokeratin 18. Thus, AVP can utilize a cellular protein as a cofactor in the cleavage of cytokeratin 18.

The rationale for actin being able to serve as a cofactor for AVP is that its C terminus is highly homologous to the viral cofactor pVlc; of the last 8 amino acid residues of actin, 4 are identical and 3 homologous to the last 8 amino acid residues in pVlc. This homology implied that AVP could bind to the C terminus of actin, and it did. Furthermore, this homology implied that actin, like pVlc, could stimulate the activity of AVP; it did so in a concentration-dependent manner.

Are other data consistent with the C terminus of actin behaving like pVlc? Of the last 11 amino acids at the C terminus of actin, the 3 at the N terminus, AGP, are not homologous to those in pVlc, GVQ, whereas the next 8 amino acids are homologous. It has been reported that deletion of GVQ in pVlc results in an inactive cofactor (28). However, we have observed that deletion of GVQ from pVlc yielded a peptide that binds to AVP with only a 3-fold higher K_d value and exhibits a 3-fold lower k_{cat} value than that of wild-type pVlc.² Alanine-scanning mutagenesis on pVlc indicates the GtoA mutant has a 13-fold higher K_d value for binding to AVP; the VtoA mutant has a 7-fold lower k_{cat} value for substrate hydrolysis, and the QtoA mutant behaves like wild-type pVlc (10). It is possible that PRODAN bound to Cys-374 enhanced the binding of AVP to the C terminus of actin. However, AVP binds to underivatized actin with an equilibrium dissociation constant of 4 nM as opposed to the equilibrium dissociation of 1.7 μ M with PRODAN-labeled actin.³ Thus, PRODAN bound to Cys-347 of actin actually interferes with the binding of AVP to actin. Additionally, there is direct evidence that AVP binds to the C terminus of actin. We have observed that in the presence of DNA a peptide containing the amino acid sequence of the last 11 amino acids of actin specifically behaves as a cofactor in stimulating AVP and that a peptide with the same amino acids but in a randomly chosen sequence does not stimulate AVP.³

There is no facile way to determine the relevance of our observation that actin can act as a cofactor for AVP *in vitro* to what occurs *in vivo* in an adenovirus-infected cell. Actin is an essential protein; therefore, a deletion mutant of actin will not be viable. However, given that cytokeratin 18 is cleaved by AVP

in vivo (7), that *in vitro* AVP will not cleave cytokeratin 18 in the absence of a cofactor, that *in vitro* actin can act as a cofactor for the cleavage of cytokeratin 18, and that the K_d value for the binding of actin to AVP is lower than the *in vivo* G-actin concentration, it seems very likely that in an adenovirus-infected cell, cytokeratin 18 is cleaved by an actin-AVP complex.

During an adenovirus infection AVP is exposed to actin in the cytoplasm. In infected cells, proteinase activity can be detected as early as 14 h post-infection with maximal activity beginning at 20 h (29). AVP can be detected in the cytoplasm and nucleus at 24 h post-infection by Western blot (30). This timing correlates with the disassembly of the cytokeratin system that begins to fall apart at about 16 h post-infection (6), disassembly being complete at 36 h (7).

In virus-infected cells, cleavage of cytoskeletal proteins weakens the mechanical structure of the cell, and this may promote cell lysis and release of nascent virions (7). AVP cleaves cytokeratin 18 within the N-terminal domain yielding a 41-kDa fragment that is incapable of participating in filament elongation. Such fragments significantly inhibit the elongation of cytokeratin filaments, even when the amount of cleaved cytokeratin comprises only 1% of the population. Inspection of the amino acid sequences of other cytoskeletal proteins reveals AVP consensus cleavage sequences in tubulin, vimentin, and even actin itself (Fig. 2a). The latter observation raised the possibility that actin may be a cofactor for its own destruction, and this was shown to occur.

Degradation of cytoskeletal proteins by virus-coded proteinases during lytic infections is not unusual. The rhinovirus 2A proteinase cleaves cytokeratin 8 (31) and other virus-coded proteinases cleave actin (5, 32) and vimentin (33). What is currently unique about AVP is that it uses actin as a cofactor.

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² M. L. Baniecki and W. F. Mangel, unpublished observations.

³ M. T. Brown and W. F. Mangel, unpublished observations.

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