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The Chloromethylketone Protease Inhibitor $AAPF_{CMK}$ Also Targets ATP-Dependent Helicases and SAP-Domain Proteins

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Abstract We have been studying a nuclear protease, which appears to be involved in cellular transformation, as well as in infections with high-risk human papillomaviruses (HPVs). This protease has a chymotrypsin-like substrate specificity and the chloromethylketone inhibitor $AAPF_{CMK}$ is a potent (and relatively selective) inhibitor of it. Recently, we have observed that $AAPF_{CMK}$ has potent effects in some model systems which appear not to be mediated by decreases in the nuclear protease. Here we show that $AAPF_{CMK}$ selectively reacts with ATP-dependent helicases as well as a limited spectrum of proteins in other DNA repair/chromatin remodeling nuclear complexes, including for example Cohesin complex components and proteins containing SAP-domains. In vitro, $AAPF_{CMK}$ selectively reacts with SV40 large T antigen, and inhibits its helicase activity. J. Cell. Biochem. 100: 716–726, 2007. © 2006 Wiley-Liss, Inc.

Key words: protease inhibitor; nucleus; carcinogenesis; HtrA protease; HPV; helicase; SAP domain

Over the last several years, we have been interested in a nuclear chymotrypsin-like protease activity (CLPA), which appears to be involved in carcinogenesis in multiple experimental systems [Clawson et al., 1993, 1995; Drubin and Clawson, 2004]. Early work showed that this activity represented a protease with an Mr~30,000 (actually a closely spaced doublet), showed a chymotrypsin-like substrate specificity, and was regulated by Ca^{2+} in vitro and in cells [Madsen et al., 1990; Clawson et al., 1992]. We identified a preferred substrate as AAPF, and found that the corresponding chloromethylketone version, $AAPF_{CMK}$, preferentially inhibited the nuclear CLPA. Using a biotinylated version of $AAPF_{CMK}$ ($bAAPF_{CMK}$), we were also able to localize the nuclear CLPA to the nuclear envelope in hepatocytes [Clawson et al., 1995]. Our working hypothesis is that the nuclear CLPA is somehow involved in induction of genomic instability, following the paradigms of the SOS DNA repair system in bacteria [d'Ari, 1985; Frank et al., 1996; Goodman, 2002; Beaber et al., 2004] and yeast [Wintersberger, 1984; Goodman, 2002].

Recently, we reported on the spontaneous transformation of an immortalized hepatocyte cell line [Drubin and Clawson, 2004]. With continued passage in culture, this cell line showed a major increase in nuclear CLPA, which was concurrent with development of aneuploidy and acquisition of the ability to grow in soft agar. Further, $AAPF_{CMK}$ blocked this spontaneous transformation. Surprisingly, however, this anti-transformation activity was without discernable effect on the NCLPA.

More recently, we examined $AAPF_{CMK}$ effects on human raft cell cultures infected with high-risk Human Papillomavirus (HPV) types, since

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high-risk HPV types are known to induce genomic instability [Munger and Duensing, 2004; Bellanger et al., 2005]. We found that major increases in nuclear CLPA were associated with infection with all six high-risk HPV types tested [Drubin et al., 2006], and further that $AAPF_{CMK}$ almost completely blocked high-risk HPV replication in raft cultures. $AAPF_{CMK}$ showed no signs of toxicity toward uninfected keratinocytes or cervical cancer cells not associated with HPV, and a chloromethylketone inhibitor for trypsin-like activity was also without affect [Drubin et al., 2006]. These effects were seen in raft cell cultures with a high-grade cervical cancer cell line containing integrated HPV16 (which makes only E6/E7), as well as with a low-grade cervical cancer cell line which stably maintains episomal HPV 31b (which undergoes the complete viral life cycle). Again, however, $AAPF_{CMK}$ had no apparent effect on the nuclear CLPA. While we have now identified a candidate protease (the HtrA protease Prss11) and are actively pursuing confirmation/characterization of it, given these aforementioned findings we also wished to identify non-protease targets of $AAPF_{CMK}$. Here, we report that $AAPF_{CMK}$ selectively targets complexes containing ATP-dependent DNA and RNA helicases, as well as a limited spectrum of other complexes involved in DNA repair and chromatin remodeling (such as Cohesin and SAP-domain containing proteins). Using SV40 Large T antigen as a model, we confirm that $AAPF_{CMK}$ interacts with SV40 large T antigen, and that $AAPF_{CMK}$ selectively inhibits T antigen helicase activity in in vitro assays.

MATERIALS AND METHODS

Nuclei were purified from rat liver (from Pelfreeze) as described [Clawson et al., 1992], generally using six livers/preparation. Nuclear pellets (approximately 1 mg/g liver) were resuspended in STKM2 (0.25 M sucrose, 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol), rinsed, resuspended in TMPI (50 mM Tris-HCl, pH 7.4, 0.5 mM $MgCl_2$, with a 1:100 dilution of protease inhibitor cocktail (consisting of 50 mM benzamidine, 1 mg/ml pepstatin, 20 μg/ml leupeptin, and 25 μg/ml aprotonin).

$AAPF_{CMK}$ and GR_{CMK} were obtained from MP Biochemicals. We find that "protease-free" DNase I preparations are nearly always badly contaminated with chymotrypsin (although recombinant DNAse is now available which is protease-free) and RNase A preparations also contain significant CLPA. They were therefore routinely pretreated with diisopropylfluorophosphate (DFP) as follows. DNase I and RNase A were resuspended at 10 mg/ml in water. DFP was added to 1 mM and incubation was overnight at 4°C. DFP was then inactivated by incubation for 1 h at 37°C, and the preparations were then assayed for CLPA using 1 mM or 100 μM $AAPF_{AMC}$ as substrate. This process was repeated one or two more times, until proteolytic activity was eliminated or reduced to less than 1%. DNase I preparations were then tested for DNase activity, and concentrations were adjusted as necessary to compensate for any decreases in DNase activity caused by the DFP protocol.

Nuclei were treated with 50 μg/ml of DFP-treated DNase I (or the activity-adjusted equivalent) for 10 min at 4°C. The suspension was centrifuged at 2,500*g* for 10 min at 4°C. The supernate was saved, and pellets were resuspended in TMPI, and centrifuged as before. The pellet was resuspended in 2 M NaCl in TMPI, incubated for 10 min at 0°C, and the suspension was centrifuged at 5,000*g* for 30 min at 4°C. The supernate (the high-salt extract) was saved, and the pellet was rinsed in TMPI and centrifuged at 5,000*g* for 30 min at 4°C. The pellet was resuspended in TMPI also containing Triton X-100 at 1%, and the mixture was incubated on ice for 10 min, and then centrifuged at 5,000*g* for 30 min at 4°C. The supernate was again saved. The pellet was resuspended in TMPI, and DNAse I and RNase A were added to 200 μg/ml. The suspension was incubated for 1 h at 20°C, and the suspension was centrifuged at 5,500*g* for 30 min at 4°C. The supernate was saved, and the pellet was rinsed in TMPI, recentrifuged, and then rinsed in TM. The resulting nuclear scaffold preparation was then resuspended (by gentle sonication) in TKMC (TKM + 10 mM $CaCl_2$). The various supernatant fractions and the nuclear scaffold fraction were then tested for CLPA in TKMC + 10% dimethylsulfoxide (DMSO) using $AAFP_{AMC}$ substrate (AMC is 7-amino-5-methylcoumarin) at 100 μM at 37°C for various periods. Reactions generally contained 5 μg protein, as determined using the BCA protein assay (Pierce, Rockford, IL), with bovine serum albumin as standard. Measurements were made using a Hoefer DNA

Fluorometer (excitation at 365 nm, emission at 460 nm), and fluorescence production was generally linear for a number of hours. In later experiments, measurements were taken with a Synergy HT plate reader.

To identify proteins interacting with $AAPF_{CMK}$, we utilized the supernates obtained from the high-salt (2 M NaCl) extraction step, which contained the major share of nuclear CLPA. A biotinylated version of $AAPF_{CMK}$ ($bAAPF_{CMK}$, from Enzyme Systems Products) was added to a final concentration of 250 μM, and the suspension (containing approximately 60 mg protein) was incubated for 1 h at 37°C. This incubation reduced CLPA to background levels, when assessed as above. Controls were incubated with 10% DMSO.

We then utilized UltraLink™ Immobilized Monomeric Avidin kits (Pierce) to isolate proteins covalently bound to the $bAAPF_{CMK}$, following the manufacturer's instructions (this included a pretreatment step designed to eliminate irreversible biotin binding sites). After thorough rinsing (with A_{280} of the effluent returning to baseline), bound proteins were eluted using the biotin blocking and elution buffer supplied. Protein was then quantitated by A_{280}, the pertinent fractions were combined and concentrated using Centricon YM10 or 100 columns. Samples were generally prebound to columns to eliminate non-specifically binding proteins.

2-D PAGE was performed using a Zoom IPGRunner (Invitrogen), according to manufacturer's instructions. Samples were dissolved in rehydrating buffer (containing 8 M urea, 2% CHAPS, 20 mM dithiothreitol) for isoelectric focusing, and electrophoresis was performed using the step voltage protocol, with 2 mA/1 Watt settings, except that the maximum voltage step was 1250 V. The strips were then run in the second dimension using the NuPage system and Zoom 4%–12% Bis-Tris gels (Invitrogen) with MOPS running buffer for 50 min, as described (Mark12 unstained markers, also from Invitrogen, were also run). Sample preparation is not fully denaturing, which allows some tightly complexed proteins to remain associated (e.g., Claudin 7 dimers and complexes identified here). Gels were then generally stained with Coomassie blue and destained in 50% methanol, 10% acetic acid. In some cases, gels were silver stained. In some additional repeat experiments, biotinylated proteins were analyzed after 1-D PAGE using the Nu-PAGE system (Invitrogen) according to manufacturer's instructions. Gels were photographed in an Eagle Eye II (Stratagene). The hydrated gels were subsequently sent to the Wistar Institute for in-gel tyrptic digestion and analysis (see Results and Discussion). For preservation, individual gels were soaked in E-Z Breeze overnight, and then placed in biogel membranes, clipped, and allowed to dry overnight at room temperature.

Sequence analysis of peptide digests was performed at the Wistar Institute Proteomics Facility using microcapillary reverse phase HPLC nano-spray tandem mass spectrometry on a ThermoFinnigan LCQ quadrupole ion trap mass spectrometer, after in-gel digestion with trypsin. A portion of the peptide digest was injected onto the nanocapillary reverse phase HPLC coupled to a nano-electrospray ionization source of the ion trap mass spectrometer (in early experiments, samples were digested and individual peaks were isolated via HPLC and then analyzed). This mass spectrometer measures peptide masses and then fragments individual peptides to produce MS/MS spectra of fragments that reflect the peptide sequence. The MS/MS spectra were run against a sequence database using the SEQUEST program developed by Yates and co-workers [Sadygov et al., 2004]. Further data-mining was performed on the NCBI website.

To determine if $AAPF_{CMK}$ interacted with SV40 large T antigen (from CHIMERx), 17.5 nmol (~1.7 μg) of large T antigen was incubated with 100 μM $AAPF_{CMK}$ or with 100 μM GR_{CMK} (or the equivalent amount of DMSO alone) for 30 min at 37°C. After incubations, the proteins were examined by standard PAGE on 7% gels.

Helicase assays were performed according to the manufacturer's (Perkin Elmer Life Sciences Wallac Oy, Turku, Finland) instructions in white 384 Optiplates with 2.4 pmol purified SV40 large T-Antigen (from CHIMERx) and TruPoint Helicase assay kits, in final volumes of 30 μl/well. Basically, this assay contains a helicase substrate, consisting of two complementary oligodeoxynucleotide strands. One (a 44-mer) is labeled with a europium chelate, and the other (a 26-mer) is labeled with the QSY7 quencher. When the helicase unwinds the double-stranded DNA, the quencher strand reanneals to excess unlabeled 26-mer present in the incubation mixture, preventing loss of

fluorescence. One hundred micromolar of $AAPF_{CMK}$ or GR_{CMK} (or equivalent DMSO) was added to helicase in reaction buffer, and the mixture was incubated for 1 min at room temperature. The plates were then placed back on ice, the working solution was added in, the plate was shaken for 2 min at room temperature, and incubations were then performed for 90 min at 35°C. Plates were read on a Synergy HT plate reader using KC4 and time-resolved fluorescence.

RESULTS AND DISCUSSION

Nuclei were purified from rat liver and subnuclear fractions were prepared as described. Subnuclear fractions were analyzed for CLPA using $AAPF_{AMC}$ as substrate. The major share of nuclear CLPA was found in the high-salt extract, and so this fraction was selected for further analysis. The high-salt extract was prebound to monomeric avidin columns, to remove avidin-binding proteins, and then incubated with $bAAPF_{CMK}$ for 2 h at 37°C. The $bAAPF_{CMK}$-modified proteins were then bound to the monomeric avidin columns, and then eluted and concentrated using Centricon YM10 spin columns. The $bAAPF_{CMK}$-modified proteins were then analyzed either by 2-D PAGE or standard 1-D PAGE (Fig. 1). On 2-D gels, a single, unique new spot with an ~Mr 36,000 (pI ~7.5) was consistently found in samples incubated with $bAAPF_{CMK}$ (Fig. 1, upper left panel), and the presence of $AAPF_{CMK}$ in this spot was verified using a fluorescently labeled version of (Fig. 1, lower panel). There was a notable loss of larger molecular weight proteins/complexes in the samples incubated without $bAAPF_{CMK}$ as compared with samples incubated with it, which presumably reflects proteolytic degradation during the protocol (since we had to omit inhibitors effective against CLPA). To obtain larger Mr $bAAPF_{CMK}$-modified proteins/complexes in sufficient quantities for analysis, some samples were therefore run on 1-D gels, under the conditions which allow tightly complexed proteins to remain associated. There were a number of bands which consistently showed

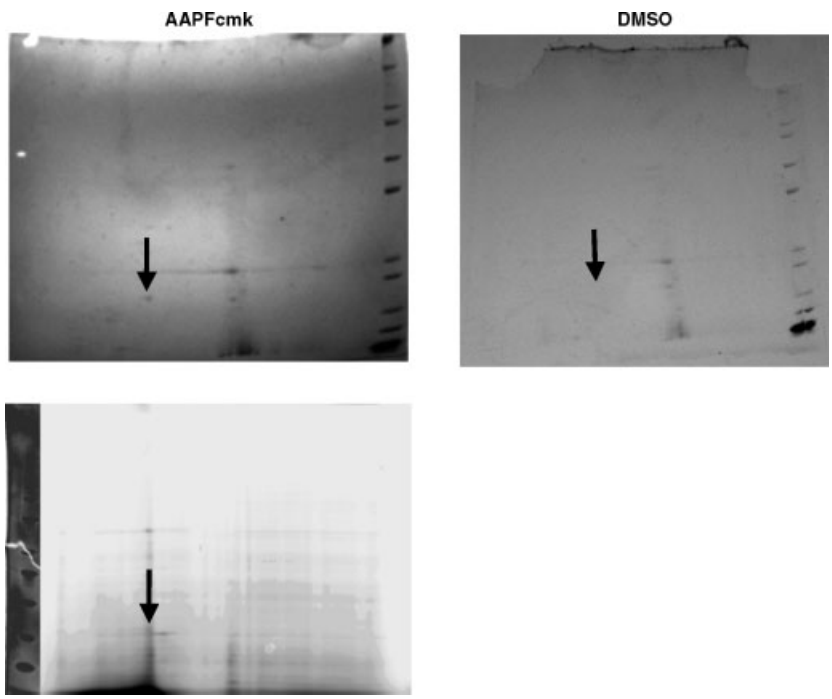

Fig. 1. 2-D PAGE analysis of high-salt nuclear extracts after incubation with $bAAPF_{CMK}$. Extracts were prepared, incubated with $bAAPF_{CMK}$, and biotinylated proteins were isolated on immobilized monomeric avidin columns. Proteins were analyzed on 2-D gels (see Materials and Methods for details). A novel spot was routinely visualized after the incubation with $bAAPF_{CMK}$ (compared with extract incubated with DMSO alone). An arrow denotes the spot, on the left panel, which had an ~Mr 36,000 and a pI ~7.5. An arrow shows the equivalent position on a gel with a preparation incubated with DMSO solvent control. The presence of $AAPF_{CMK}$ in the spot was verified using a fluorescently labeled version of it (lower panel). The Mr 36,000 spot (from multiple gels) was then analyzed by mass spectrometry after in-gel tryptic digestion.

altered migration in such gels, with approximate Mr's of 200,000, 160,000, 116,000, 55,000, and 36,000 (not shown).

The novel Mr 36,000 spot from the 2-D gels (6–7 different gels were analyzed in all), or the five bands showing altered migration in 1-D gels, were then subjected to in-gel tryptic digestion and analyzed by tandem mass spectrometry. In most cases, fragments from multiple proteins were found in the spots and bands (often with $\sim$ 8–10 high-confidence identifications) and the proteins were repeatedly found in independent preparations/gels. In many cases, fragments from the same proteins were present in multiple, progressively smaller bands on the standard gels (see Table I).

First, a single protease homolog was identified in the Mr 36,000 spot from the 2-D gels. The protease is known as Prss11 (Gene ID #65164, #56213 for mouse), which is also referred to as IGF binding protein 5. It is a member of the high-temperature requirement factor A (HtrA) family, and is widely expressed in normal human tissues [De Luca et al., 2003]. Prss11 is a serine protease with chymotrypsin-like substrate specificity. It has previously been characterized as a secreted form (although the HtrA2 family member is nuclear), which functions as an inhibitor of TGFβ receptor signaling [Oka et al., 2004]. This is the first description of it (or a portion thereof) in the nucleus, although such a relocalization is perhaps not unexpected [Ehrmann and Clausen, 2004].

Identification of Prss11 is of considerable interest. It contains a PDZ domain, which functions as a reversible switch in activity [Clausen et al., 2002; Ehrmann and Clausen, 2004], and which has been implicated in chaperone, heat shock response, and apoptotic functions. Overexpression of Prss11 has been identified in human ductal carcinomas in situ [Allinen et al., 2004] compared with normal breast epithelium. Curiously, although Prss11 is highly expressed in malignant melanomas [Baldi et al., 2002], its expression is downregulated during human melanoma progression, and in fact its expression strongly inhibits growth of metastatic melanoma cells [Baldi et al., 2002]. Further characterizations of this putative nuclear form of Prss11 are underway.

In addition to the identification of Prss11, it quickly became apparent that the balance of the bAAPF$_{CMK}$-modified proteins reflected a surprisingly restricted spectrum. First and foremost, the vast majority of the modified proteins were either the rat homologs of ATP-dependent (DEAD-box, DEXH-box, or DEAH-box) helicases, or had previously been identified as helicase binding partners (Table I). This seems to also include the two RNA pol II subunits identified, polR2a/b. These co-precipitate with the helicase activity of the RNA pol II complex, again suggesting that these represent a helicase and/or helicase-associated proteins.

Two other proteins are of particular interest, a DNA-binding protein and Mybbp1a, since they seem to tie together the various other identified groups. One is the putative DNA-binding (bihelical) motif protein (GenomeLOC309197). This contains an SP1a and RYanodine (SPRY) domain (Smart00449.1), which is related to the DEAD-box ATP-dependent RNA helicase Ddx-1 from Danio. However, it also contains a SAP (SMART00513) domain: this is a DNA-binding domain, which binds preferentially to matrix attachment regions (MARs). The SAP domain is named for its occurrence in SAF-A/B, ACINUS, and PIAS proteins. This domain has also now been found in a number of other DNA repair enzymes and ATP-dependent DNA helicases (e.g., ATP-dependent DNA helicase II). Also identified in these 2-D gels was Splicing Factor 3b, subunit 2, a SAP-containing protein which interacts with p68 helicase as well as Smad3 [Warner et al., 2004].

The PIAS protein is Protein Inhibitor of Activated Stat protein, which is related to DEAD/H-box binding protein 1 [Ungureanu et al., 2003]. It is known to interact with RNA helicase II (among others), and is found in nuclear "speckles" [Ishiai et al., 2004] which overlap those formed by the MRN (Mre11a, Rad50, and Nbs1) complex. In this regard, the rat homolog of Rad50 was also identified in the Mr 200,000 band (Table I). The ACINUS protein [Sahara et al., 1999] is an established interactor with the Myb-binding protein 1A (Mybbp1a; Table I). Mybbp1a interacts with an HMG-1 homolog [Zelenaya-Troitskaya et al., 1998], as well as with ACINUS (predicted), the ash2l proteins of the SETS/ash2 histone methyltransferase complex of the ash2 protein [Ikegawa et al., 1999], and ATP-dependent chromatin assembly factor large subunit ACF1 [Lusser and Kadonaga, 2003]. Additional interactors with Mybbp1a include nucleophosmin [Npm, Colombo et al., 2002], RAN [Kierszenbaum et al., 2002], RANbp17 [Koch et al., 2000],

TABLE I. bAAPF$_{CMK}$-Modified Protein Complexes in Nuclear Extracts

Group	Name	Genid# or locus#	Functions & interactions	Found in
Splicing factors	SF3b, subunit 1	81898	Complex with Prp8, a DEAD-box helicase	2D, 200, 160, 116, 55
	SF3b, subunit 2	293671	N-terminal RRM domains, Smad 3 interactor which interacts with p68 RNA helicase, contains SAP domain	2D
	SF3b, subunit 4	107701	RRM domains, homologs complex with cdc28/ Prp8, Ddx-39, Ddx16, Dbr1	2D
	SF, similar to U2AF65	XP141947.2	Contains RRM domains, homologs complex with; (a) DEAD-box helicase required for snRNP branchpoint interaction; and (b) tightly associated with RNA pol II largest two subunits, co-precipitate with helicase activity; (c) modulates efficient transcript elongation	2D
	SFU2AF large chain	P26368	RRM domains, binds to RNA polypyrimidine	2D
U5-Specific splicing factors	hPrp8	10594	DEAD-box helicase	200, 116
	U5 200 kD helicase	O75643	DEXH-box helicase	200, 160
	U-5 specific hPrp8 binding protein (40 kD)	AAC69625	Homologs complex with hPrp8	116, 55
	Rat homolog of hPrp8	287530	ATP-dependent RNA helicase	160, 116
	U5 elongation factor Tu	20624	Homologs complex with: (a) hPrp8, Hel25E, Dxh8, Dbp1, and Dhx16 ATP-dependent D-box helicases; (b) brr2; (c) cdc5 and Cef1; and (d) 4Ebp1, a binding protein for eukaryotic initiation factor 4E	116
Other helicases	HMGB-2	P17741	ATP-dependent helicase, SET complex	2D
	RNA helicase A	304859	ATP-dependent DEAH-box helicase	200, 160, 116
	Smarca5	307766	DEXH-box helicase; matrix-associated, actin-dependent helicase, also involved with regulation of polII transcription	55
	RRP5 homolog	309458	Homolog to DEAD-box helicase DBP1 from *S. Cervisiae*, DEAH-box polypeptide 16 from mouse, poly(rC) binding protein, and DBR1 debranching enzyme homolog 1	200
	Ortholog of Aqr, Aquarius	9716	DEAD-box helicase, Superfamilly I DNA & RNA helicase	200
RNA Polymerase II Complex	polR2a	P24928	Co-precipitates with helicase activity of polII complex: SAF-B couples transcription/ splicing to matrix attachment regions	200, 160
	polR2b	231329		160
Structural Maintenance of Chromosomes Cohesin Complex	SMC1	63996	Cohesin complex is target for proteolytic cleavage, which releases complexes and allows meiotic chromosome separation	200
	SMC3	P97690	Tethrs chromosomes to spindle pole	200, 160
	Cohesin subunit SA-1		Homologous to Stag1 in Xenopus complex	160, 116
NOPP140 Complex	Nol5	60373	Box C/D class of small nucleolar RNP particles, which catalyze methylation/modification of rRNAs	116, 55, 36
	Nol5a	362214		55
	Nol6	313167		116
	Nop56 homolog	BAC37015	Co-localizes with putative methyltransferase fibrillarin	55, 36
Nuclear Scaffold/ Pore/Export	Mad1l1	17120	Mitotic arrest deficient 1-like 1, meiotic spindle checkpoint	2D
	Lamin C2	BAA03578		2D, 160, 55
	Rcc1, regulator of chromosome condensation 1	100088	Guanyl-nucleotide exchange factor	2D
	Lamin B1	16906		2D
	Exportin 1	85252	Rat homolog of CRM1, mediates nucleophosmin transport, mitotic spindle assembly	116
	Exportin 7/ RANbp16	Q9EPK7	Interacts with Mybbp1a	116
	Importin Beta	16211	Interacts with RAN and Mybbp1a, Centrosome	116
	Nucleoporin 133	292085		160

(*Continued*)

TABLE I. (*Continued*)

Group	Name	Genid# or locus#	Functions & interactions	Found in
	Nucleoporin 188	227699		160
	TPR/nuclear pore complex-associated	DAA00370		
Miscellaneous	Putative DNA-binding protein with SPRY and SAP domains	LOC309197	SPRY domain related to DEAD-box helicase Ddx1 from Danio rerio. SAP binds to MARs, named from occurrence in SAF-A/B, ACINUS, and PIAS proteins. Domain is found in a number of DNA repair enzymes and ATP-dependent DNA helicases such as ATP-dependent DNA helicase II	2D
	RAD50	64012	MRN complex (containing Rad50) overlaps PIAS and RNA helicase II nuclear speckles	200
	Myb-binding protein 1A	60571	PAR-interacting protein. Interacts with HMG-1 homolog, ACINUS, ash2l of the SETS/ash2 histone methyltransferase complex, and ATP-dependent chromatin assembly factor large subunit ACF1.SAF-A/B proteins with SAP domains include hnRNP methyltransferases	200, 160, 116
	DNA methyltransferase 3A	AAP75901		55
	Poly (rC) binding protein	5093		2D, 36
	Similar to polybromo-1D	306254	Contains HMG-box domain	160
	Tardbp	230908	Similar to U5 elongation factor Tu, U5 components, including Dhx16, Ddx39, dbr1, Prp16, U2AF large subunit	2D

RANGAP [Kusano et al., 2003], SET protein isoform 2 [Beresford et al., 2001], and Exportin 7 [Koch et al., 2000].

The third group of SAP-domain proteins is the SAF-A/B proteins. These include, for example, hnRNP methyltransferase Hmt1 (GENE ID #3276), and in this regard a rat ortholog of DNA Methyltransferase 3A from Bos taurus was also identified in the Mr 55,000 band. SAF-B protein also provides an additional link with the identified RNA Pol II proteins, as it has been shown to couple transcription and pre-mRNA splicing to MAR regions [Nayler et al., 1998], and it is also linked to DNA/chromatin modification and RNA processing [Carty and Greenleaf, 2002].

A small group of nuclear scaffold/pore/transport proteins was also identified. Lamin C2 was repeatedly found in multiple different bands, lamin B1 was identified, and two nucleoporins were found in the Mr 160,000 band. Mad1l1 was identified, which functions as a spindle checkpoint during meiosis [Zhang et al., 2005]. Importin beta, exportin 1, and exportin 7 were identified on our gels, along with Rcc1. Rcc1 is a guanyl-nucleotide exchange factor. Importin beta interacts with RAN [Plafker and Macara, 2002], which is a Ras-related GTPase. RAN in turn has been implicated in microtubule assembly and centrosome function [Kierszenbaum et al., 2002], and RAN also interacts with Mybbyp1a. Exportin 7, also known as Ranbp16, has also been identified as a Mybbp1a interactor, as have a whole spectrum of nucleoporins.

Many of these identified proteins are clearly involved in genomic instability. For example, a mutant RanGAP protein is responsible for Segregation Distorter phenotype in *Drosophila* [Kusano et al., 2003]. Npm is involved in several tumor-associated chromosome translocations [Colombo et al., 2002]. Npm trafficking is mediated by the RAN/Crm1 pathway, and is another checkpoint factor maintaining proper centrosome duplication [Budhu and Wang, 2005]. Nucleoporin 133 appears to have involvement in microtubule function, and an importin beta family member may also have a spindle checkpoint function [Chen et al., 2004]. In yeast, the nuclear pore complex is functionally related to the spindle assembly checkpoint [Iouk et al., 2002], particularly Mad1l1.

With regard to methyltransferases, the Nopp140 complex functions in methylation of rRNA, and the identified Nop56 homolog (Table I) actually co-localizes with the putative methyltransferase fibrillarin [Gonzales et al., 2005]. It is possible that $AAPF_{CMK}$ interacts with methyltransferases, although it also seems

likely that the Nopp140 complex would also contain helicases to unwind rRNA for modification.

Given our sequencing results, we wished to test $AAPF_{CMK}$ for potential effects on helicase activity. We utilized SV40 large T antigen as a model for ATP-dependent helicases, because it is immunologically related to the human DEAD-box protein p68 [Uhlmann-Schiffler et al., 2002] and was readily available in purified form.

We found that incubation of purified SV40 large T Ag with $AAPF_{CMK}$ resulted in a slight band-shift which was noticeable by standard PAGE analysis (Fig. 2A). In contrast, GR_{CMK}, a chloromethylketone inhibitor of trypsin-like proteolytic activity, had no effect on the electrophoretic migration of T Ag (Fig. 2A).

We also found that incubation of large T Ag with $AAPF_{CMK}$ produced a marked concentration-dependent decrease in large T Ag helicase activity (Fig. 2B). In contrast, GR_{CMK} produced little or no significant decrease in large T Ag helicase activity (Fig. 2B). Overall activity was decreased by $86 \pm 9\%$ by 100 μM $AAPF_{CMK}$ ($P < 0.01$). Helicase activity showed a slight decrease with GR_{CMK} ($19 \pm 6\%$), which was generally not significant. In all experiments, effects of $AAPF_{CMK}$ differed significantly from those of GR_{CMK} ($P < 0.03$ to < 0.001) and from DMSO ($P < 0.01$ to < 0.001).

These findings clearly indicate that $AAPF_{CMK}$ interacts with cellular ATP-dependent helicases, and that this interaction can result in inhibition of helicase activity. The mechanism by which $AAPF_{CMK}$ interacts with SV40 TAg is not clear. We were unable to identify the site of attachment using tandem MS, which could be due to any number of different reasons. Increasing the ATP concentration in the helicase assays had no effect on $AAPF_{CMK}$ inhibition, although ATP binding per se was not assessed. Since similar ATP binding sites are present in many other types of proteins, it seems unlikely that the ATP-binding site is targeted. $AAPF_{CMK}$ binding to other regions of T Ag could disrupt

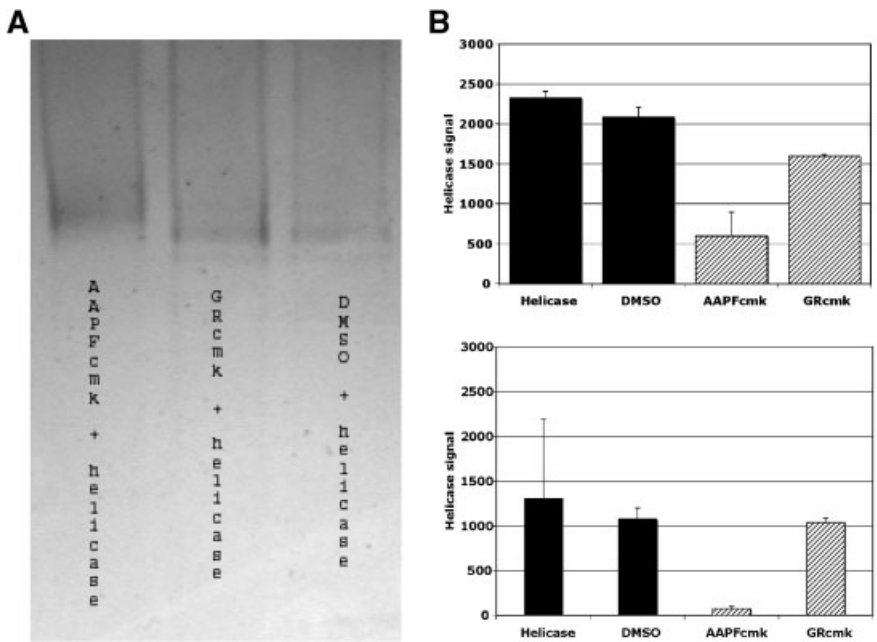

Fig. 2. Interaction of $AAPF_{CMK}$ with SV40 large T Antigen. **Panel A**: 17.5 nmol of purified large T Ag was incubated with 100 mM $AAPF_{CMK}$, 100 μM GR_{CMK}, or with DMSO as described. After incubation, preparations were examined by PAGE. A shift in migration was observed in preparations after incubation with $AAPF_{CMK}$ as compared with preparations incubated with GR_{CMK} or DMSO. Molecular weight markers were run in parallel (not shown), and the large T antigen migrated at ~Mr 97,000 as expected. **Panel B**: Helicase Assays—2.4 pmol of purified large T Ag was pre-incubated with 100 mM $AAPF_{CMK}$, 100 mM GR_{CMK}, DMSO (or buffer), at room temperature for 15 min, and then working solution was added and incubation was for 90 min at 35°C. After incubation, helicase assays were performed as described. The Panel shows two representative experiments. Overall inhibition was $86 \pm 9\%$ after incubation with $AAPF_{CMK}$. A slight decrease in activity was observed with GR_{CMK}, although it was not generally significant.

hexamerization [Gai et al., 2004; Valle et al., 2006], although this seems unlikely since it would presumably be unique to T Ag and not generally applicable to the wide spectrum of helicases with which $AAPF_{CMK}$ interacts (Table I). Perhaps the most reasonable speculation is that $AAPF_{CMK}$ interacts directly with the helicase domain, a possibility we are now testing using T Ag mutants lacking the helicase domain.

The reason that the inhibition is specific for $AAPF_{CMK}$ (vs. GR_{CMK}) is not clear, although a few references may be pertinent in this regard. First, RNA helicase A recruits RNA Pol II (or acts as a bridging factor) to the CREB-binding protein via the minimal transactivation domain [Aratani et al., 2003]. There are three conserved tryptophan residues in RNA helicase A, which are important in this function, and $AAPF_{CMK}$ may somehow be mimicking one of those residues. Analogously, p68 seems to perform a similar function with Smad 3 and CBP [Ueda et al., 2002] in transactivation of TGFβ-regulated responses. Along another line, The HMGB1/2 proteins are intimately involved in integration of exogenous DNA into the genome of HeLa cells, and it has been reported that a phenylalanine wedge at the DNA exit site in HMGB1 is essential for promoting RAG1/2 complex assembly during V(D)J recombination [Swanson, 2004; Dai et al., 2005]. Interestingly, HMGB2 has also been shown to partner in a nuclear complex associated with resistance to DNA conformation-altering chemotherapeutic drugs, and has been identified by gene array expression profiling as important in drug resistance in ovarian cancer [Bernardini et al., 2005] as well as an ovarian carcinoma cell line [Varma et al., 2005].

The findings here have implications for potential mechanisms involved in the inhibition of HPV replication by $AAPF_{CMK}$. High-risk HPV types are known induce genomic instability [Munger and Duensing, 2004; Bellanger et al., 2005]. Perhaps the most pertinent finding, potentially related to HPV pathogenesis, is the identification of SAP-domain containing proteins as targets of $AAPF_{CMK}$. In this regard, the DEK protein is upregulated by E7 proteins from high-risk HPV types [Wise-Draper et al., 2005]. Since DEK contains a SAP-domain [Bohm et al., 2005], it is tempting to speculate that $AAPF_{CMK}$ blocks the E7-induced upregulation of DEK, and that this may be the mechanism by which $AAPF_{CMK}$ selectively blocks growth of keratinocytes infected with high-risk HPV [Drubin et al., 2006]. While DEK is not a classical helicase, it preferentially binds to superhelical and cruciform DNA and induces positive supercoils, and is thought to function as an chromatin architectural protein analogously to the HMG family [Waldmann et al., 2004].

There are also some interesting possible connections between Prss11 and HPV E7 proteins. Firstly, high-risk E7 protein (which shares sequence and some functional properties with SV40 T Ag), forms oligomeric structures which show chaperone "holdase" activity [Alonso et al., 2006], and which involve transition from an intrinsically disordered state into an ordered tertiary structure [Alonso et al., 2004]. This raises the possibility that Prss11 might interact directly with E7 protein. We speculate that this interaction might occur through the C-terminal PDZ domain, activating Prss11 (which might perhaps not be readily accessible to $AAPF_{CMK}$), which could explain why infection with high-risk HPV types was associated with very large increases in nuclear CLPA [Drubin et al., 2006].

Given the direct interaction between AAPFcmk and DNA/RNA helicases, it would seem reasonable to suggest that AAPFcmk may inhibit HPV infections via inhibition of HPV E1 helicase activity. However, we found that $AAPF_{CMK}$ inhibited HPV replication by ~95% in raft cell cultures [Drubin et al., 2006] with high-grade cervical cancer cells containing integrated HPV 16; under these circumstances the only viral products made are the E6/E7 proteins. This level of inhibition was equivalent to the ~95% inhibition observed with a low-grade cervical cancer cell line containing episomal HPV 31b, which undergoes the complete life cycle, so the inhibition appears to occur independently of E1 expression.

Helicases are increasingly becoming of interest in the context of carcinogenesis [Abdelhaleem, 2004; Doxsey et al., 2005; Fukasawa, 2005; Sluder, 2005], with DNA helicases engendering interest as potential drug targets [Sharma et al., 2005]. As an example, Ddx10 is a putative ATP-dependent DEAD-box RNA helicase [Savitsky et al., 1996]. It has been implicated in development of various hematologic malignancies [Arai et al., 1997; Ikeda et al., 1999; Yamamoto et al., 2005] where its fusion with nucleoporin-98 occurs.

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