Bioorganic & Medicinal Chemistry 22 (2014) 834–841

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Structurally diverse low molecular weight activators of the mammalian pre-mRNA 3′ cleavage reaction

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ARTICLE INFO

Article history:
Received 20 September 2013
Revised 23 November 2013
Accepted 3 December 2013
Available online 15 December 2013

Keywords:
RNA
RNA processing
Small molecule activators
Polyadenylation
3′ end formation
Histone code
Naphthalene ring

ABSTRACT

The 3′ end formation of mammalian pre-mRNA contributes to gene expression regulation by setting the downstream boundary of the 3′ untranslated region, which in many genes carries regulatory sequences. A large number of protein cleavage factors participate in this pre-mRNA processing step, but chemical tools to manipulate this process are lacking. Guided by a hypothesis that a PPM1 family phosphatase negatively regulates the 3′ cleavage reaction, we have found a variety of new small molecule activators of the in vitro reconstituted pre-mRNA 3′ cleavage reaction. New activators include a cyclic peptide PPM1D inhibitor, a dipeptide with modifications common to histone tails, abscisic acid and an improved L-arginine β-naphthylamide analog. The minimal concentration required for in vitro cleavage has been improved from 200 μM to the 200 nM–100 μM range. These compounds provide unexpected leads in the search for small molecule tools able to affect pre-mRNA 3′ end formation.

1. Introduction

Eukaryotic pre-messenger RNA (pre-mRNA) undergoes multiple processing steps prior to translation, and each of these steps provides the cell with an opportunity for gene expression regulation.[1] The pre-mRNA 3′ cleavage step, a site-specific RNA hydrolysis reaction that specifies where the polyadenylate (poly(A)) tail will be added to the mRNA, can take place at different locations near the end of a nascent mRNA, downstream of the translation stop codon. This variability leads in many genes to alternative mRNA isoforms with different 3′ untranslated regions (3′UTRs) between the stop codon and the poly(A) tail.[2,3] The pre-mRNA 3′ cleavage step can influence the stability of the mature mRNA because many 3′UTRs contain destabilizing sequences and miRNA binding sites, the loss of which, when 3′ cleavage occurs relatively close to the stop codon, may slow turnover and allow escape from repression.[2,4–6] Shorter 3′UTRs resulting from alternative polyadenylation in some oncogenes have been correlated with cellular proliferation and may contribute to oncogenesis.[7,8] The 3′ cleavage and polyadenylation reactions are coupled in vivo but can be studied separately in vitro. Natural product inhibitors of the pre-mRNA polyadenylation step have recently been found.[9,10] However, low molecular weight compounds that influence the 3′ cleavage step in cells are not yet available, but would enable new experimental inquiries into the multi-protein complex that carries out 3′ cleavage, and might provide a chemical tool to influence alternative polyadenylation.

We previously found in vitro evidence that a kinase–phosphatase pair may exert influence over the 3′ cleavage reaction.[11] Considering this possibility led us to propose that creatine phosphate, long known to stimulate the in vitro reaction at high concentration,[12,13] might do so by acting as a serendipitous inhibitor of an unknown 3′ cleavage-suppressing protein phosphatase. This model led to experiments identifying two protein phosphatase 2C (PP2C, a.k.a. PPM1) family inhibitors that stimulated 3′ cleavage in place of creatine phosphate.[14] In humans, the PPM1 superfamily consists of at least eighteen different Mg^{2+}- or Mn^{2+}-dependent Ser/Thr phosphatases defined by shared sequence homology.[15–18] In plants, the PPM1 family is greatly expanded, with at least 80 family members in *Arabidopsis*.[19] The in vitro 3′ cleavage reaction is carried out in the presence of excess EDTA, where free Mg^{2+} and Mn^{2+} concentrations are very low. Thus, if a PPM1 family member is involved in vitro, it would likely act in a manner that does not require its metal-dependent enzymatic activity, perhaps acting allosterically or by influencing the multi-protein 3′ cleavage factor complexes.

While progress in other major protein phosphatase families has benefited from the identification of natural product inhibitors,[20] inhibitor discovery for the PPM1 family has been slower. The inhibitors we previously identified as 3′ cleavage activators had been

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http://dx.doi.org/10.1016/j.bmc.2013.12.006

discovered using the PPM1A family member.[21] They had micromolar potency against PPM1A and only modest selectivity for the PPM1 family over the other major protein phosphatase families.[21] By analogy with the toxin inhibitors of the other protein phosphatase families, we expect PPM1 inhibitors to show a measure of family-specific activity, enabling 3′ cleavage activation even if PPM1A is not the actual family member involved in pre-mRNA 3′ cleavage. Here, we have tested more potent PPM1 inhibitors for pre-mRNA 3′ cleavage activity, and carried out structure–activity relationships for two previously identified 3′ cleavage activators. Though we are not yet able to identify the protein targeted by these small molecules, our results strengthen a model in which a PPM1 family member or related protein acts to suppress 3′ cleavage in vitro.

2. Results and discussion

2.1. Pre-mRNA 3′ cleavage activation by a potent PPM1D cyclopeptide inhibitor

Cyclic phosphopeptides based on substrates of the PPM1D family member, also known as Wip1, are among the most potent inhibitors of any PPM1 family phosphatase.[22,23] We used one of these, cyclic peptide 38 (cp38, Fig. 1A),[22] in place of 50 mM creatine phosphate in the in vitro pre-mRNA 3′ cleavage reaction. This peptide inhibits PPM1D with a K_i of 150 nM.[22] In the 3′ cleavage assay, a standard in vitro pre-mRNA 3′ cleavage substrate adapted from the simian virus 40 (SV40) late poly(A) signal[24] is exposed to partially purified 3′ cleavage factors from HeLa cell nuclear extract.[11,13,25,26] Cleavage of the radiolabeled substrate into its 5′ and 3′ fragments can be observed and quantitated following resolution on a denaturing polyacrylamide gel. In the absence of creatine phosphate, or other activator, this reaction is inefficient. In all experiments presented here, the amount of cleavage brought about by an activator (5′ fragment/(5′ fragment + uncleaved SV40L RNA)) is normalized to that brought about by 50 mM creatine phosphate (R.C., or relative cleavage in figures). As shown in Figure 1B, at 200 nM, cp38 activated a level of RNA cleavage comparable to that produced by 50 mM creatine phosphate. Removal of cp38's serine and tyrosine phosphate groups with calf intestinal alkaline phosphatase (CIP) prior to cleavage, led to complete loss of activation, while removal of only the tyrosine phosphate, using a tyrosine-specific phosphatase, did not reduce activity. (Residual CIP activity was inhibited[11] before cp38 was added to the in vitro reaction to prevent possible dephosphorylation of the cleavage factor proteins.) A repeat of this experiment is shown in Supplementary Figure S1. Cyclopeptide cp38 is therefore ~1000-fold more potent than the most potent 3′ cleavage activator previously identified,[14] and phosphorylation of its serine, which is required for PPM1 inhibition,[23] is required to activate pre-mRNA 3′ cleavage. PPM1D is present at very low levels in HeLa cells,[27] an observation that leads us to suppose that cp38 is likely not working through this PPM1 family member, but may instead be a family-specific inhibitor that activates 3′ cleavage through another family member, or homologous protein.

2.2. Pre-mRNA 3′ cleavage activation by abscisic acid

To further test the hypothesis that a PPM1 family member acts to suppress 3′ cleavage, we used the isoprenoid plant hormone (*S*)-(+)-abscisic acid (**ABA**). This natural product is a potent inhibitor of the group A plant PPM1 enzymes.[28] It works by forming a ternary complex with the PPM1 enzyme and a co-receptor from the PYR/PYL/RCAR family of **ABA** receptors.[28,29] For example, **ABA** inhibits the plant PPM1s ABI1 and ABI2 with IC_{50} values of 60 and 70 nM, respectively, when RCAR1 is present.[29] **ABA** is bound mainly by the PYR/PYL/RCAR receptor, but also makes contact with the PPM1.[30,31] In the absence of a PYR/PYL/RCAR co-receptor, **ABA** can directly inhibit some plant PP2C/PPM1 enzymes in vitro, though with much lower potency. For example, **ABA** reduces ABI2 phosphatase activity in vitro by about 15% at **ABA** concentrations above 3 μM.[29] We found no pre-mRNA 3′ cleavage activation when nanomolar concentrations of **ABA** were used in place of creatine phosphate (not shown). However, at and above 100 μM **ABA**, approximately 10–15% of the normal 3′ cleavage activity was observed (cf. 50 mM creatine phosphate) (Fig. 1C). Two repeats of this experiment are shown in Supplemental Figure S2. We consider two possible interpretations of this result. First, the absence of activation at nanomolar **ABA** concentration may indicate that there are no conserved mammalian proteins that can act in the manner of the plant PYR/PYL/RCAR co-receptors, though **ABA** is found in animal cells.[32] A BLAST search did not reveal any human RCAR homologs. Second, homology between the plant and human PPM1 proteins may be sufficient to conserve the direct but much less

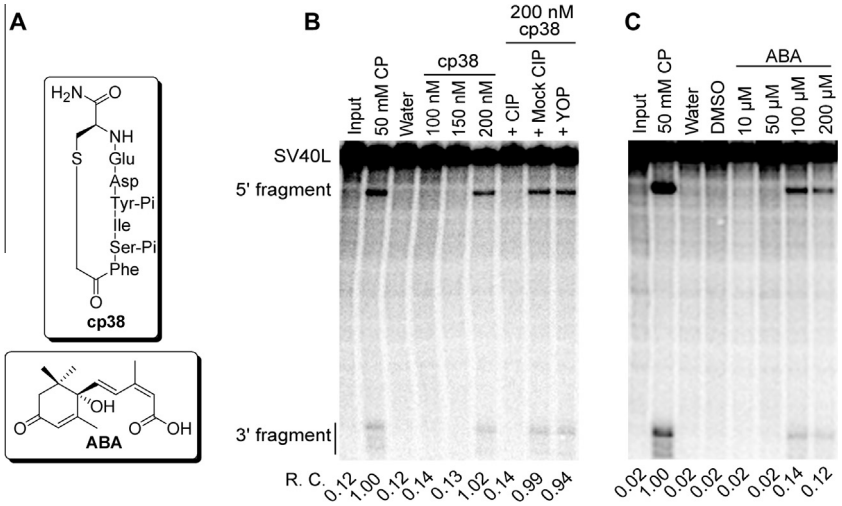


Figure 1. Pre-mRNA in vitro 3′ cleavage activation by PP2C inhibitors. (A) Structure of cyclic peptide cp38 and (*S*)-(+)-abscisic acid (**ABA**). (B) Denaturing gel analysis of SV40L pre-mRNA 3′ in vitro cleavage reactions activated by cp38. Creatine phosphate (**CP**) or cp38 was added, with or without pre-treatment with CIP (calf intestinal alkaline phosphatase), mock CIP (CIP buffer only) or tyrosine phosphatase (YOP). (Residual CIP activity was completely removed before mixing cp38 with cleavage factor proteins.[11]) Relative cleavage, R.C. = [5′ fragment/(5′ fragment + uncleaved RNA)] × 100, was normalized to **CP**, which is set to R.C. = 1.00. (C) Denaturing gel analysis of in vitro cleavage using **ABA** in place of **CP**. DMSO lane corresponds to reaction without **ABA**.

potent inhibition of PPM1 family members. In either case, though its cleavage activation efficiency was low, compared to our first generation compounds, **ABA** is a relatively potent 3′ cleavage activator. This result adds to the accumulating though circumstantial evidence that a PPM1 family member can influence pre-mRNA processing.

2.3. Structure–activity study of arginine β-naphthylamide: aryl group and sidechain

Prior to the present work, compound **1** (Fig. 2A) was identified as a 3′ cleavage activator through a limited structure–activity relationship study undertaken using commercially available compounds related to our initial lead, leucine β-naphthylamide, a weak PPM1A inhibitor.[14] To search for a more potent activator than **1**, we synthesized a series of analogs in which the naphthalene ring was altered (Fig. 2A, **2**–**4**) or the guanidino group was alkylated or replaced by another positively charged group (Fig. 2A, **5**–**6**). The synthesis of compounds **2**–**6** (Scheme 1A and B) was accomplished by coupling the relevant aryl amine to the suitably protected arginine, ornithine or lysine, followed by guanidinylation where necessary, and finally deprotection. We evaluated each of the compounds shown in Figure 2A for their ability to activate in vitro 3′ cleavage at 1 mM in place of, and compared to, 50 mM creatine phosphate. The results of duplicate reactions are shown in four independent 3′ cleavage reactions in Figure 2B–E. Changes to the naphthalene ring and guanidino group of **1** led to loss of activity (compounds **2**, **3**, **4**, **5**), and the guanidino group could not be replaced by trimethylammonium (compound **1** vs **6**), even though the two groups have a positive charge.

2.4. Arginine β-naphthylamide modified at the α-position retains potency

In previous work we found that acetylation of the α-amino group of leucine β-naphthylamide led to some loss of that compound's 3′ cleavage activity, as well as a significant decrease in solubility, a point of practical importance due to the high activator concentrations needed for activity. In view of the poor results changing the naphthyl and guanidino groups of lead compound **1**, we decided to modify the α-amino group. To minimize a reduction in solubility we chose the morpholine group for its high water solubility (compound **7**, Fig. 3, and Scheme 1C). Unlike the other changes, this modification retained activity. A representative in vitro 3′ cleavage comparison of **7** with **1** over 25–200 μM (as usual, in place of, and compared to, creatine phosphate) is shown in Figure 3B. Corroborating experiments are shown in Supplementary Figure S3. Compound **7** proved to be slightly more potent than lead compound **1**, and to have a similar maximum relative cleavage (Fig. 3B). While only slightly more potent than **1**, the activity of compound **7** indicates that modifications of the alpha amine can be tolerated, and that this part of the molecule merits a larger SAR study beyond the scope of the present work.

2.5. Structure–activity study on phosphocholine

We previously identified phosphocholine as a pre-mRNA 3′ cleavage activator. Like creatine phosphate, it activates 3′ cleavage over the 10–50 mM range.[14] Its ability to inhibit PPM1 family proteins is unknown. Phosphocholine is an intermediate in the biosynthesis of phosphatidylcholines, and it is unlikely to be a physiological cofactor in 3′ cleavage.[14] However, understanding the determinants of 3′ cleavage activity of this very simple structure might help us to design more potent activators. To learn whether all three methyl groups are necessary for activity, we synthesized the mono- and dimethyl phosphocholine analogs, **9** and **10** (Scheme 2A). Their synthesis is shown in Scheme 2B. We also made an analog of phosphocholine with an extra carbon between the charged groups (compound **11**, Scheme 2A, C). None of these compounds showed detectable in vitro 3′ pre-mRNA cleavage activity (not shown). The lack of activity in these analogs showed that all three methyls of the trimethylammonium group are required, and the two-carbon spacing between this group and the phosphate is critical.

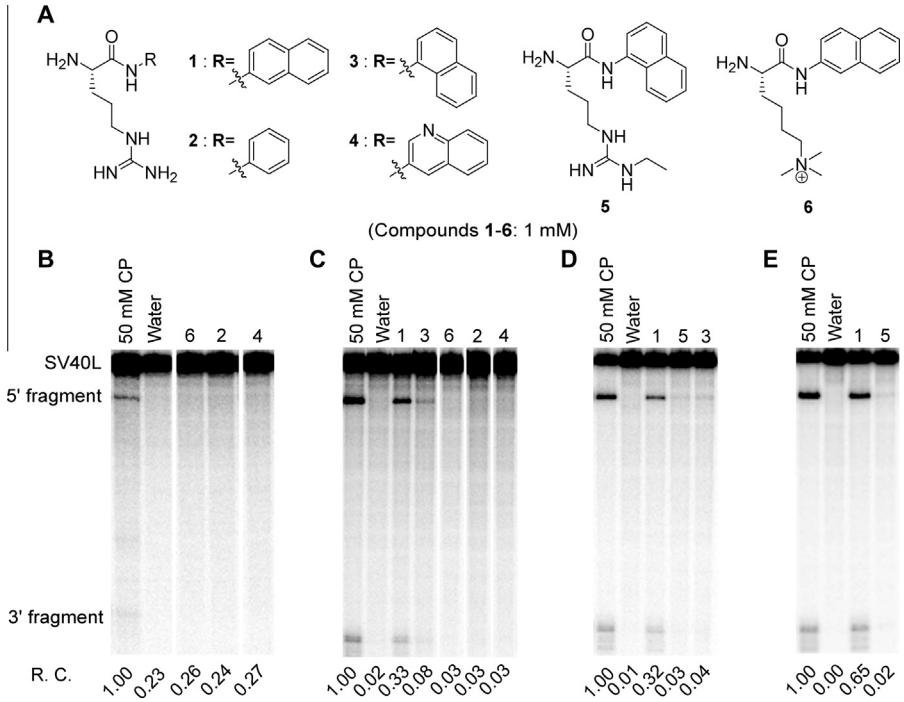
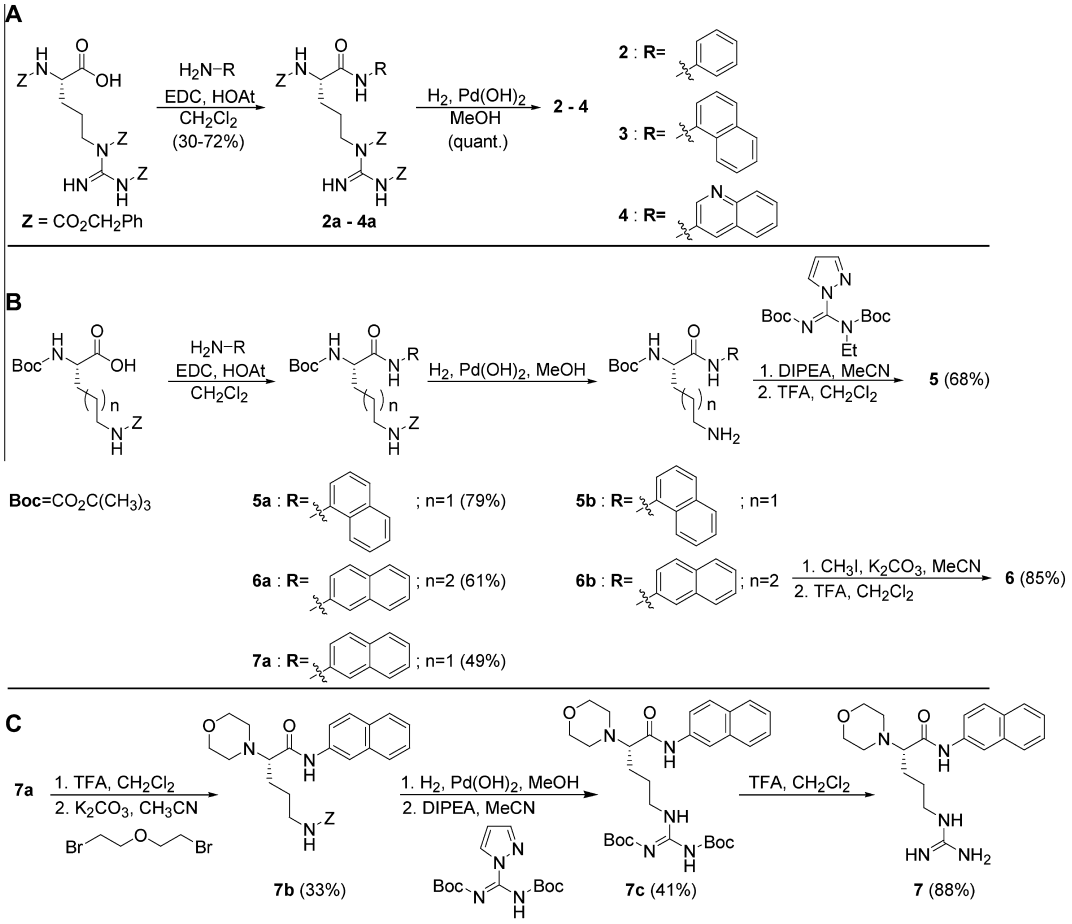


Figure 2. Structure–activity study of compound **1**, previously found to activate pre-mRNA 3′ cleavage in vitro. (A) Analogs included changes to the naphthyl group and the arginine side-chain of **1**. (B) In vitro 3′ cleavage results for duplicate cleavage reactions using the indicated analogs in place of **1**, all at 1 mM. R.C., relative cleavage, defined in Figure 1, normalizes all compounds to activation by 50 mM **CP**.



Scheme 1. (A and B) Synthesis of the arginine β-naphthylamide analogs used in Figure 2. (C) Synthesis of morpholine analog **7** used in Figure 3.

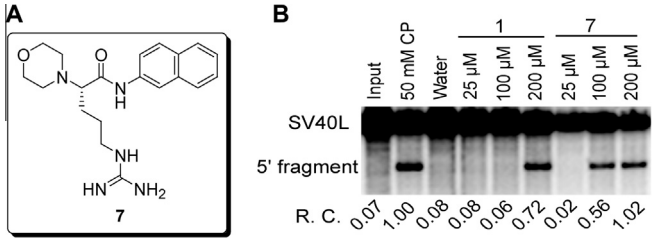
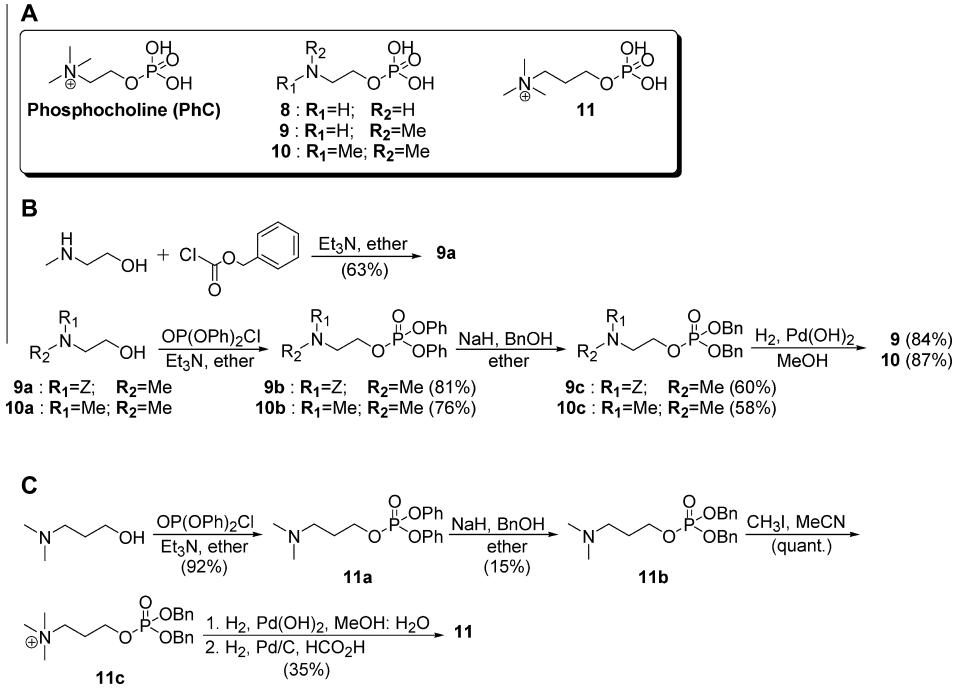


Figure 3. Structure and 3′ cleavage activity of a morpholine-modified arginine β-naphthylamide analog. (A) Structure of **7**. (B) Side by side comparison of **1** and **7** in an SV40L pre-mRNA 3′ in vitro cleavage reaction. R.C., relative cleavage as defined in Figure 1, with activation by 50 mM creatine phosphate (**CP**) set to 1.00.

2.6. Phosphocholine as a possible modified histone protein mimic

Trimethyllysine and phosphoserine residues are commonly found among the histone proteins of chromatin,[33] where in vivo transcription takes place. Since several of the 3′ cleavage factor proteins associate with transcribing RNA polymerase II[34,35] and the 3′ cleavage reaction is coupled to transcription and begins co-transcriptionally,[36,37] it is conceivable that a histone protein could, within the chromatin, locally interact with RNA polymerase II-associated cleavage factor proteins to play a role in 3′ cleavage. In trying to understand how phosphocholine activates 3′ cleavage in vitro, we have considered the possibility that it may mimic a modified histone protein having closely situated trimethyllysine and phosphoserine (or threonine) residues (Fig. 4A). In an initial test of this idea, we replaced 50 mM creatine phosphate in a 3′ cleavage reaction with dipeptide **12**, Ac-Lys(Me)$_3$-Ser(PO$_4$)-CO$_2$H (Fig. 4B). Peptide **12** may also be viewed as a derivative of phosphoserine which, like creatine phosphate, activates 3′ cleavage in the 10–50 mM range.[38] Peptide **12** activated in vitro 3′ cleavage at ~1 mM (Fig. 4C), while phosphoserine and phosphocholine[14] have no effect at this concentration. This result shows that placing the two functional groups of phosphocholine in the structural context of a dipeptide, where they can approach each other as closely as they do in phosphocholine, reduced the concentration necessary for them to activate 3′ cleavage. Viewed differently, placing phosphoserine in a dipeptide coupled to *N*-(acetyl)trimethyllysine (to create **12**) led to increased potency. While far from directly implicating a histone protein in the 3′ cleavage reaction, this result indirectly supports the possibility that phosphocholine works by mimicking such a cleavage-promoting modified histone variant. We note that the partially purified protein 3′ cleavage factors used in our assay are fractionated from soluble nuclear proteins, and not from the chromatin-rich insoluble nuclear pellet, so a cleavage-enhancing chromatin protein would be depleted or lost during cleavage factor preparation. Interestingly, the PPM1G family member plays a role in histone H2A–H2B exchange into chromatin.[39] PPM1G is also involved in splicesomal splicing,[40] a pre-mRNA processing step that can be coupled to 3′ cleavage.[41] In view of this context, this result justifies additional study of suitably modified lysine, serine and threonine-containing peptide sequences taken from the heavily modified histone protein tails.



Scheme 2. Phosphocholine (**PhC**) analogs. (A) Structures of the analogs. (B) Synthesis of analogs **9** and **10**. (C) Synthesis of analog **11**.

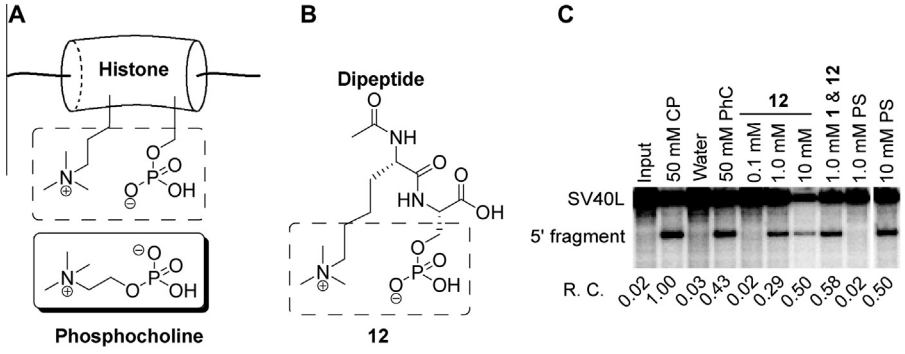


Figure 4. Phosphocholine (**PhC**) as a mimic of a modified histone protein tail. (A) Phosphocholine holds a trimethylammonium group close to a phosphate, as a histone protein can do with trimethylated lysine and phosphoserine (or phosphothreonine). (B) Structure of dipeptide **12**. (C) SV40L pre-mRNA 3′ cleavage activation by peptide **12** compared to phosphocholine (**PhC**), creatine phosphate (**CP**) and phosphoserine (**PS**). R.C., relative cleavage, as defined in Figure 1.

3. Conclusions

Guided by a model proposing that a PPM1 phosphatase acts to suppress or negatively regulate the pre-mRNA 3′ cleavage reaction, an obligatory step in the maturation of nearly all eukaryotic mRNAs, we have assayed a group of structurally diverse PPM1 inhibitors and new synthetic small molecules for their ability to activate this reaction in vitro. Our results lend further, though circumstantial, support to this model. The minimal concentration required for in vitro cleavage has been improved from 200 μM to the 200 nM-100 μM range. In addition, our results point to future experiments aiming to further the goal of developing membrane-permeable small molecule activators of 3′ cleavage sufficiently potent for use in tissue culture experiments.

4. Experimental section

4.1. Materials and methods

Unless otherwise stated, commercial reagents and solvents were used without additional purification. Solvents were purchased from VWR. Cyclic peptide 38 (cp38) was made as previously described.[22] (*S*)-(+)-Abscisic acid (**ABA**), phosphocholine (**PhC**), and phosphoserine (**PS**) were purchased from TCI America. Creatine phosphate (**CP**) was purchased from Calbiochem. Compound **1** was purchased from Bachem. Compound **8** was purchased from Fluka. Compound **12** was purchased from Shanghai Apeptide Co., Ltd. Unless otherwise stated, starting reagents were purchased from VWR. Analytical TLC was performed on silica gel 60 F_{254} plates. Flash chromatography was performed manually in glass columns on 230–400 mesh silica gel (Alfa Aesar). Analytical samples were dried overnight in vacuo over phosphorus pentoxide prior to testing. Melting points were measured on a Laboratory Devices Melt-Temp apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 300 spectrometer or Varian Inova 500 spectrometer. High-resolution mass spectra (HRMS) were measured using electrospray ionization (ESI) on a Waters LCT XE (TOF) mass spectrometer. Infrared (IR) spectra were recorded using a Thermo Nicolet 380 FT-IR spectrometer or Thermo Nicolet 6700 FT-IR spectrometer.

4.2. General procedure for the synthesis of compounds 2–4

Z_3-Arg-OH (Z, benzyloxycarbonyl) was dissolved in dichloromethane. 1.2 equiv of the amine was added followed by 2.5 equiv

of 1-hydroxy-7-azabenzotriazole (HOAt). 1.5 equiv of 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC) was dissolved in dichloromethane and slowly added to the reaction at room temperature. Reaction progress was monitored by TLC (ethyl acetate:dichloromethane). The solution was washed sequentially with 0.5 M HCl solution, water, brine, dried, and concentrated. The crude was purified by flash chromatography.[42] The resulting product was hydrogenated in methanol using a latex balloon with catalytic amount (approximately a spatula tip) of palladium hydroxide (Pd 20% on carbon, nominally 58% water) for 3 h. The solution was then filtered through celite and concentrated to yield the final product.

4.2.1. 2-Amino-5-guanidino-pentanoic acid phenylamide (2)

White solid (yield 72%, mp 110 °C dec). ^{1}H NMR (300 MHz, D_2O) δ: 7.17–7.32 (m, 4H), 7.10 (m, 1H), 3.89 (t, J = 6.33 Hz, 1H), 2.95–3.11 (m, 2H), 1.70–1.87 (m, 2H), 1.43–1.61 (m, 2H). ^{13}C NMR (75 MHz, D_2O) δ: 169.49, 156.78, 135.98, 129.51, 129.35, 126.24, 122.05, 121.83, 53.80, 53.51, 40.43, 28.65, 23.63. IR (neat) ν (cm^{-1}): 3175, 1655, 1596, 1546, 1442, 1360, 1309, 1253, 752, 691. HRMS (ESI) [M+H]$^+$: calcd for $C_{12}H_{19}N_5O$ m/z = 250.1668, found m/z = 250.1655.

4.2.2. 2-Amino-5-guanidino-pentanoic acid naphthalen-1-ylamide (3)

White solid (yield 62%, mp 166 °C dec). ^{1}H NMR (300 MHz, D_2O) δ: 7.57–7.75 (m, 3H), 7.21–7.41 (m, 4H), 4.18 (t, J = 6.33 Hz, 1H), 2.99 (t, J = 6.60 Hz, 2H), 1.79–1.99 (m, 2H), 1.47–1.73 (m, 2H). ^{13}C NMR (75 MHz, D_2O) δ: 169.52, 156.62, 133.93, 130.49, 128.71, 128.46, 128.05, 127.07, 125.63, 124.10, 121.95, 121.66, 53.35, 53.11, 40.34, 28.33, 23.86. IR (neat) ν (cm^{-1}): 3149, 1658, 1536, 1497, 1348, 793, 770. HRMS (ESI) [M+H]$^+$: calcd for $C_{16}H_{21}N_5O$ m/z = 300.1824, found m/z = 300.1816.

4.2.3. 2-Amino-5-guanidino-pentanoic acid quinolin-3-ylamide (4)

Yellow solid (yield 30%, mp 113 °C dec). ^{1}H NMR (300 MHz, D_2O) δ: 9.34 (s, 1H), 8.97 (s, 1H), 8.02 (d, J = 8.80 Hz, 1H), 8.07 (d, J = 8.53 Hz, 1H), 7.91 (t, J = 7.70 Hz, 1H), 7.72–7.84 (m, 1H), 4.19 (t, J = 6.46 Hz, 1H), 3.11 (t, J = 6.74 Hz, 2H), 1.97 (m, 2H), 1.51–1.73 (m, 2H). ^{13}C NMR (75 MHz, D_2O) δ: 168.80, 156.79, 138.45, 138.08, 135.98, 135.76, 135.28, 134.63, 131.59, 130.88, 129.07, 120.31, 119.94, 53.65, 53.39, 40.37, 28.01, 23.66. IR (neat) ν (cm^{-1}): 3159, 1663, 1558, 1491, 1466, 1365, 782, 747. HRMS (ESI) [M+H]$^+$: calcd for $C_{15}H_{20}N_6O$ m/z = 301.1777, found m/z = 301.1786.

4.2.4. 2-Amino-5-(*N*′-ethylguanidino)-pentanoic acid naphthalen-1-ylamide (5)[43]

Boc-ornithine(Z)-OH (500 mg, 1.36 mmol) was dissolved in dichloromethane (25 mL). 1-Naphthylamine (234 mg, 1.64 mmol) was added followed by HOAt (464 mg, 3.41 mmol) and EDC (392 mg, 2.05 mmol). Reaction progress was monitored by TLC. The resulting material was purified by flash chromatography, eluting with dichloromethane/ethyl acetate (9:1) to give **5a** (531 mg, 1.08 mmol, 79% yield). Compound **5a** (531 mg, 1.08 mmol) was hydrogenated for 2 h in methanol (50 mL) with a catalytic amount of palladium hydroxide as described in Section 4.2. The solution was filtered through celite and concentrated to give **5b**. Compound **5b** was dissolved in acetonitrile (7 mL) and ethylated *N*,*N*′-bis-tert-butoxycarbonylpyrazole-1-carboxamidine (364 mg, 1.08 mmol) was added followed by diisopropylethylamine (181 mg, 2.34 mmol). The solution was stirred overnight, concentrated and purified by flash chromatography, eluting with dichloromethane/ethyl acetate (4:1) to give 520 mg (0.83 mmol) of a white, sticky solid. The resulting compound (170 mg, 0.27 mmol) was dissolved in dichloromethane (2 mL) and deprotected with trifluoroacetic acid (2 mL) overnight. The solution was concentrated, suspended in fresh dichloromethane, and extracted with water. The aqueous layer was concentrated and dried overnight in a drying pistol with phosphorus pentoxide to give **5** as a sticky, white solid in 68% yield. ^{1}H NMR (300 MHz, D_2O) δ: 7.59–7.81 (m, 3H), 7.24–7.45 (m, 4H), 4.11–4.23 (m, 1H), 3.05 (m, 2H), 2.85 (q, J = 6.88 Hz, 2H), 1.85–2.02 (m, 2H), 1.60 (m, 2H), 0.81 (t, J = 7.01 Hz, 3H). ^{13}C NMR (75 MHz, D_2O) δ: 169.49, 163.23, 162.76, 155.38, 133.99, 130.53, 128.39, 127.09, 126.75, 125.64, 123.90, 122.11, 118.25, 114.38, 53.35, 53.13, 40.15, 36.20, 28.26, 23.84, 13.02. IR (neat) ν (cm^{-1}): 3208, 3051, 1659, 1542, 1181, 1131, 798, 721. HRMS (ESI) [M+H]$^+$: calcd for $C_{18}H_{25}N_5O$ m/z = 328.2137, found m/z = 328.2121.

4.2.5. [5-Amino-5-(naphthalen-2-ylcarbamoyl)-pentyl]-trimethyl-ammonium (6)

Boc-Lys(Z)-OH (1 g, 2.6 mmol) was dissolved in dichloromethane (50 mL). 2-Naphthylamine[44] (0.37 g, 2.6 mmol) was added followed by HOAt (0.89 g, 6.54 mmol) and EDC (0.76 g, 3.96 mmol). After 1.5 h, the solution was concentrated and purified by flash chromatography, eluting with chloroform/ethyl acetate (4:1) to give **6a** (800 mg, 1.58 mmol), in 61% yield. Compound **6a** (288 mg, 0.57 mmol) was suspended in methanol (25 mL) and hydrogenated with a catalytic amount of palladium hydroxide. After 1.5 h, the solution was filtered through celite and concentrated to give **6b** (212 mg, 0.57 mmol) in quantitative yield. Compound **6b** (205 mg, 0.55 mmol) was then dissolved in acetonitrile (1 mL) and potassium carbonate (191 mg, 1.38 mmol) was added followed by iodomethane (392 mg, 2.76 mmol). The solution was stirred overnight, concentrated, suspended in dichloromethane, and filtered. The filtrate was concentrated and the crude compound was dissolved in dichloromethane (2 mL) and trifluoroacetic acid (2 mL) was added. The solution was stirred for 1.5 h and then concentrated, suspended in fresh dichloromethane, and extracted with water. The aqueous layer was concentrated and dried overnight over phosphorus pentoxide to give **6** as an orange, sticky solid in 85% yield. ^{1}H NMR (300 MHz, D_2O) δ: 7.88 (s, 1H), 7.66–7.78 (m, 3H), 7.28–7.40 (m, 3H), 3.99 (t, J = 6.60 Hz, 1H), 3.06–3.15 (m, 2H), 2.84 (s, 9H), 1.83–1.94 (m, 2H), 1.66 (m, 2H), 1.34 (m, 2H). ^{13}C NMR (125 MHz, D_2O) δ: 168.48, 162.78, 133.88, 133.41, 131.29, 129.43, 127.94, 127.84, 127.33, 126.38, 121.10, 119.01, 66.12, 53.81, 53.05, 30.65, 22.31, 21.32. IR (neat) ν (cm^{-1}): 3049, 1671, 1198, 1128, 832, 799, 720. HRMS (ESI) [M]$^+$: calcd for [$C_{19}H_{28}N_3O$]$^+$ m/z = 314.2232, found m/z = 314.2223.

4.2.6. 5-Guanidino-2-morpholin-4-yl-pentanoic acid naphthalen-2-ylamide (7)

Boc-Orn(Z)-OH (1 g, 2.73 mmol) was dissolved in dichloromethane (50 mL). 2-Naphthylamine[44] (470 mg, 3.28 mmol) was added followed by HOAt (929 mg, 6.83 mmol) and EDC (785 mg, 4.09 mmol). Reaction progress was monitored by TLC. The resulting material was purified by flash chromatography, eluting with dichloromethane/ethyl acetate (9:1) to give **7a** (570 mg, 1.56 mmol, 49% yield). Compound **7a** (320 mg, 0.65 mmol) was dissolved in dichloromethane (2 mL) with trifluoroacetic acid (2 mL) and stirred at room temperature overnight. The solution was concentrated and used for the next step without further purification. The crude compound was dissolved in acetonitrile (6 mL) and refluxed with potassium carbonate (360 mg, 2.6 mmol) and bis(2-bromoethyl)ether (181 mg, 0.78 mmol) overnight. The next day, the solution was filtered and purified by flash chromatography (first 1:1 ethyl acetate/dichloromethane then 100% ethyl acetate) to give **7b** (100 mg, 0.22 mmol) as a yellowish solid in 33% yield. Compound **7b** was hydrogenated in methanol (12 mL) with catalytic amount of palladium hydroxide. After 2 h, the solution was

filtered through celite and concentrated. The resulting compound was dissolved in acetonitrile (1 mL) and *N*,*N*′-bis-tert-butoxycarbonylpyrazole-1-carboxamidine[45] (27 mg, 0.87 mmol) was added followed by DIPEA (15 mg, 0.113 mmol). The reaction was stirred overnight, concentrated and purified by flash chromatography, eluting with ethyl acetate/hexanes (4:1) to give **7c** (20 mg, 0.035 mmol) as a sticky, white solid in 41% yield. Compound **7c** was dissolved in dichloromethane (1 mL) and trifluoroacetic acid (1 mL) was added. The solution was stirred overnight and then concentrated, suspended in dichloromethane, and extracted with water. The aqueous layer was concentrated and dried overnight over phosphorus pentoxide to give **7** in 85% yield. ^{1}H NMR (300 MHz, D_2O) δ: 7.96 (s, 1H), 7.70–7.85 (m, 3H), 7.34–7.48 (m, 3H), 3.18–4.05 (m, 9H), 3.11 (t, J = 6.46 Hz, 2H), 1.86–2.15 (m, 2H), 1.45–1.72 (m, 2H). ^{13}C NMR (125 MHz, D_2O) δ: 166.00, 162.95, 156.95, 133.31, 131.47, 129.47, 127.91, 127.36, 126.56, 120.97, 119.33, 117.67, 115.35, 69.27, 63.74, 40.48, 24.62, 23.86. IR (neat) ν (cm^{-1}): 3360; 2877, 2718, 1668, 1563, 1508, 1435, 1364, 1200, 800, 723, 707. HRMS (ESI) [M+H]$^+$: calcd for $C_{20}H_{27}N_5O_2$ m/z = 370.2243, found m/z = 370.2224.

4.2.7. Phosphoric acid mono-(2-methylamino-ethyl) ester (9)

2-Methylaminoethanol (500 mg, 6.66 mmol) was stirred with sodium bicarbonate (1.12 g, 13.31 mmol) in diethyl ether (6.6 mL). Benzyl chloroformate, 30–35% in toluene, (3.6 g, 6.66 mmol) was slowly added at 0 °C. The solution was warmed to room temperature after 15 min. After 1 h, the solution was concentrated and purified by flash chromatography, eluting with dichloromethane/ethyl acetate (1:1) to give **9a** in 63% yield. Compound **9a** (300 mg, 1.43 mmol) was dissolved in diethyl ether (5 mL) and diphenyl chlorophosphate (578 mg, 2.15 mmol) was added followed by triethylamine (290 mg, 2.87 mmol). After 2 h, the solution was filtered and purified by flash chromatography, eluting with dichloromethane/ethyl acetate (9:1) to give **9b** (633 mg) in 81% yield. Compound **9b** (500 mg, 1.13 mmol) was added to a solution containing sodium hydride, 57–63% in oil, (86 mg, 2.15 mmol) and benzyl alcohol (368 mg, 3.42 mmol) in diethyl ether (5 mL). After 1 h, the solution was purified by flash chromatography, eluting with dichloromethane/ethyl acetate (9:1) to give **9c** (324 mg) in 60% yield. Compound **9c** (324 mg, 0.69 mmol) was hydrogenated in methanol (25 mL) with catalytic amount of Pd/C (10% Pd, 50% wet with water) for 1 h. The solution was filtered through celite, and the celite pad was washed with water. The solution was concentrated and dried overnight over phosphorus pentoxide to give **9** (90 mg, 0.58 mmol) as a white solid (mp 204–206 °C) in 84% yield. ^{1}H NMR (300 MHz, D_2O) δ: 3.76–3.94 (m, 2H), 2.97–3.13 (m, 2H), 2.49 (s, 3H). ^{13}C NMR (75 MHz, D_2O) δ: 60.25, 49.11, 48.99, 32.77, 32.41. IR (neat) ν (cm^{-1}): 3017, 2729, 2520, 1146, 1075, 1026, 923, 815. HRMS (ESI) [M+H]$^+$: calcd for $C_3H_{10}NO_4P$ m/z = 156.0426, found m/z = 156.0424.

4.2.8. Phosphoric acid mono-(2-dimethylamino-ethyl) ester (10)

2-Dimethylaminoethanol, **10a**, (500 mg, 5.61 mmol) was dissolved in ether (5.6 mL) and diphenyl chlorophosphate (1.8 g, 6.73 mmol) was added followed by triethylamine (1.135 g, 11.22 mmol). After 1 h, the solution was concentrated and purified by flash chromatography, eluting with dichloromethane/triethylamine (9:1) to give **10b** (1.37 g) in 76% yield. Compound **10b** (540 mg, 1.68 mmol) was added to a solution containing sodium hydride, 57–63% in oil, (121 mg, 3.03 mmol) and benzyl alcohol (545 mg, 5.04 mmol) in ether (5.4 mL). After 1 h, the solution was concentrated and purified by flash chromatography, eluting with ethyl acetate/methanol (19:1) to give **10c** (340 mg) in 58% yield. Compound **10c** (166 mg, 0.48 mmol) was hydrogenated in methanol (25 mL) with catalytic amount of palladium hydroxide for 1 h. The solution was filtered through celite, concentrated, and dried overnight over phosphorus pentoxide to give **10** (70 mg) as a white solid (mp 155–157 °C) in 87% yield. ^{1}H NMR (300 MHz, D_2O) δ: 3.92 (m, 2H), 3.19 (m, 2H), 2.71 (s, 6H). ^{13}C NMR (75 MHz, D_2O) δ: 57.87, 42.95, 42.43. IR (neat) ν (cm^{-1}): 3415, 2629, 2404, 1163, 1149, 1093, 1026, 993, 951, 931, 768. HRMS (ESI) [M+H]$^+$: calcd for $C_4H_{12}NO_4P$ m/z = 170.0582, found m/z = 170.0609.

4.2.9. Trimethyl-(3-phosphonooxypropyl)-ammonium iodide (11)

3,3-Dimethylamino-1-propanol (500 mg, 4.846 mmol) was dissolved in ether (4.8 mL) and diphenyl chlorophosphate (1.56 g, 5.816 mmol) was added, followed immediately by triethylamine (1 g, 9.692 mmol). After 1 h, the solution was concentrated and purified by flash chromatography, eluting with ethyl acetate/triethylamine (19:1) to give **11a** (1.45 g) in 92% yield. Compound **11a** (1.45 g, 4.32 mmol) was added to a solution of sodium hydride, 57–63% in oil, (519 mg, 12.97 mmol) and excess benzyl alcohol in diethyl ether (14.5 mL). After 1 h, the reaction was concentrated and the crude was purified by flash chromatography, eluting with ethyl acetate/triethylamine (19:1) to give **11b** (246 mg) in 15% yield. Compound **11b** (246 mg, 0.68 mmol) was dissolved in acetonitrile (1 mL) and iodomethane (192 mg, 1.35 mmol) was added. After 1 h, the solution was concentrated to give **11c** (337 mg), which was used in the next step without further purification. Compound **11c** (337 mg, 0.67 mmol) was first hydrogenated in 50 mL methanol/water (1:1) with a catalytic amount of palladium hydroxide. These conditions removed only one of the two benzyl groups. The second benzyl group was removed by latex balloon hydrogenation in 150 mL 30% (v/v) aqueous formic acid with a catalytic amount of Pd/C. The reaction contents were filtered through a celite pad, concentrated under reduced pressure, and washed with dichloromethane. The aqueous layer was further evaporated under reduced pressure, and the residue was exhaustively dried overnight over phosphorus pentoxide to give **11** (80 mg) as a white solid (mp 230 °C dec) in 35% yield. ^{1}H NMR (300 MHz, D_2O) δ: 3.75 (m, 2H), 3.14–3.34 (m, 2H), 2.93 (s, 9H), 1.92 (m, 2H). ^{13}C NMR (75 MHz, D_2O) δ: 64.41, 60.60, 52.96, 24.29, 24.20. IR (neat) ν (cm^{-1}): 3379, 3020, 2957, 2939, 2887, 2432, 1190, 1054, 932. HRMS (ESI) [M]$^+$: calcd for $C_6H_{17}NO_4P^+$ m/z = 198.0890, found m/z = 198.0879.

4.3. Low molecular weight activator stock solutions

Cyclic peptide 38 (cp38), kindly provided by Appella,[22] was dissolved in water (1 M) and then serially diluted with same. Dephosphorylation of cp38 with calf intestinal alkaline phosphatase (CIP; Promega) and YOP Protein Tyrosine Phosphatase (NEB) were carried out as described for other proteins.[11] The possibility of exposure of the cleavage factors to residual CIP activity was eliminated by using an established and validated procedure.[11] (*S*)-(+)-Abscisic acid (ABA) was dissolved in DMSO (500 mM) and then serially diluted with water. Compounds **1**–**7** were placed in water and treated with 1–3 equiv of 1.00 M HCl to dissolve the compounds. Compound **1** was purchased as the HCl salt and dissolved in water directly. Compounds **8**–**11** were dissolved in water and treated with NaOH until the pH reached 6–7.9. (1.1 equiv of NaOH was added to compound **8** and **9**; 1.0 equiv of NaOH was added to compound **10**; 1.5 equiv of NaOH was added to compound **11**; 0.25 equiv of NaOH was added to compound **12**.)

4.4. Cleavage factor fractionation from HeLa cell nuclear extract

HeLa cell pellets were purchased from the National Cell Culture Center (Biovest International). Nuclear extract and 3′ cleavage

factor DEAE-sepharose fractions (CPSF, CstF and CFm) were prepared as described in detail elsewhere.[11,25] Active fractions from each cleavage factor were pooled, concentrated by 70% ammonium sulfate precipitation and dialyzed 2 × 3 h at 4 °C against Buffer D50AS (20% glycerol, 20 mM Na-HEPES, pH 7.9, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT and 50 mM ammonium sulfate). Total protein concentration of cleavage factor fractions was estimated by Bio-Rad Bradford protein assay with bovine serum albumin as the standard and determined to be: CPSF (3 mg/mL): CstF (3.5 mg/mL): CFm (3 mg/mL). The amount of 3′ cleavage factor fractions used in a 12.5 μL cleavage reaction were: CPSF (0.8 μL); CstF (0.6 μL); CFm (3 μL).

4.5. RNA substrate

SV40 late pre-mRNA (233 nt) was transcribed in vitro by SP6 RNA polymerase (Promega) from the pG3SVL-A plasmid linearized at the DraI site.[24] Substrate RNA was uniformly labeled by including [α-^{32}P]-UTP (Perkin–Elmer Life Sciences) and 5′ capped during transcription using the 5′Me^{7}G(5′)ppp(5′)G-Cap analog (NEB). The transcript was purified on a 0.4 mm denaturing polyacrylamide gel, located by shadowing on film, and extracted as previously described.[11]

4.6. In vitro 3′ cleavage reactions

Cleavage reactions were carried out as described in detail elsewhere[11,14] in 12.5 μL and contained, in addition to the cleavage activators indicated in the figures, tRNA (0.1 mg/mL), pH 8 EDTA (2 mM), 2′-dATP (2 mM), DTT (0.41 mM), BSA (Roche; 40 ng/mL), placental RNase inhibitor (Promega, 0.32 u/mL), polyvinyl alcohol (2.5%), 10% glycerol, 10 mM HEPES-NaOH (pH 7.9), 25 mM ammonium sulfate, the cleavage factor fractions described in Section 4.4, and the RNA substrate (1–5 nM). The reactions were incubated at 30 °C for 2 h and then digested with Proteinase K, phenol–$CHCl_3$ extracted, ethanol precipitated, and resolved on a 6% denaturing polyacrylamide gel. After drying, the gel was exposed to a Molecular Dynamics Storm PhosphorImager screen and the bands were quantitated using ImageQuant software. Relative cleavage (R.C.) was calculated as [5′ fragment/(5′ fragment + uncleaved RNA)] × 100. The total recovered RNA varied from lane to lane due to losses during phenol–$CHCl_3$ extraction and ethanol precipitation. Use of the R.C. ratio standardizes the amount cleaved and allows comparison of gel lanes with different amounts of total recovered RNA.

Acknowledgments

This work was supported by Grant 5SC1GM083754 from the National Institutes of Health (NIH). Additional infrastructural support at the City College of New York (CCNY) was provided by the NIH National Center for Research Resources (2G12RR03060-26A1) and the National Institute on Minority Health and Health Disparities (8G12MD007603-27). We thank E. Appella and R. Hayashi for providing cp38, and L. Yang (CCNY) for mass spectrometry.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.12.006.

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