Accelerated Publications

A Nucleotide Binding Site in Caspase-9 Regulates Apoptosome Activation

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Received December 16, 2004; Revised Manuscript Received February 18, 2005

ABSTRACT: ATP or dATP is a required activator of Apaf-1 for formation of the Apoptosome and thereby activation of caspase-9 (Csp9) [Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997) *Cell 90*, 405−413]. Here we demonstrate that dATP or ATP may have an additional role in controlling Apaf-1-mediated Csp9 activation. In the presence of cytochrome *c* (CytC), dATP or ATP binds to Apaf-1 and triggers heptamerization of Apaf-1 leading to the activation of Csp9. At concentrations greater than 1 mM, dATP or ATP also functions as a negative regulator of apoptosis by binding to and inhibiting Csp9. The affinity labeling reagent, 3′-*O*-(5-fluoro-2,4-dinitrophenyl)-ATP (FDNP-ATP), was used to probe the binding of nucleotides to Csp9. Similar to ATP, but with a much more profound effect, FDNP-ATP binds to the full-length proCsp9 potently, with an IC_{50} of ≈5−11 nM. Neither ATP nor FDNP-ATP exhibits any effect on the prodomain-truncated enzyme ΔproCsp9 or p18/p10. FDNP-ATP covalently labels proCsp9 with a stoichiometry of 1:1, resulting in DNP-ATP-proCsp9 that is incapable of forming a productive Apoptosome with Apaf-1. Activity assays show that ATP and dATP, but not ADP or AMP, bind to the processed Csp9 p35/p10. This nucleotide binding site might play an important and previously unrecognized role in regulating proCsp9 activation.

Apoptosis is recognized as one of the most fundamental biological processes that controls events ranging from embryonic development to postnatal homeostasis in all multicellular organisms (for reviews, see refs *2* and *3*). Without exception, all studied apoptotic pathways employ caspases as the execution machinery. In all cells, caspases are synthesized as inactive zymogens, but are rapidly activated via a proteolytic chain reaction when cells receive proapoptotic signals to initiate apoptosis (*4*, *5*). Inasmuch as the activated caspases may lead to cell death, the activation of this protease family is tightly controlled in nonstimulated cells. Such regulation is carried out by at least two levels of regulatory mechanism, i.e., tight control at the activation of apical initiator caspases and well-orchestrated safeguard mechanisms that can terminate unintended activation of caspases. In cytochrome *c*-mediated apoptosis pathways, caspase 9 is the apical or initiator caspase. Initial activation of Csp9 requires a precise heptamerization of Apaf-1[1] to form the Apoptosome that is regulated by CytC and dATP or ATP (*6*, *7*). However, to ignite the full-blown chain reaction of caspase activation, not only are the initial and subsequent feed-forward (*8*) activation of Csp9 needed, but also a concomitant release of Smac is required to sequester IAPs from inhibiting caspases (*9*). In such a mechanism, CytC and ATP or dATP are the primary messengers that relay apoptotic signals from the mitochondria to the cytoplasm, whereas Smac can be viewed as a confirmatory signal that neutralizes the safeguard IAPs. Since CytC is normally sequestered in the mitochondria and is discharged into the cytoplasm only when mitochondria membrane potential is compromised, it seems sensible for CytC to serve as an apoptotic messenger for triggering the Apaf-1 action. However, the biological significance of ATP or dATP being an essential cotrigger in the Apaf-1-mediated pathway is not as apparent. Since the release of CytC from the mitochondria to the cytoplasm during the mitochondria-mediated apoptosis is an instant, all or nothing, and kinetically invariant event (*10*, *11*), mitochondrial ATP production is immediately disrupted when the discharge of CytC is initiated. Consequently, the cytosolic ATP level decreases because of the rapid cytosolic ATP turnover that typically takes a matter of seconds or minutes. Thus, if ATP as an effector of the Apoptosome−Csp9 complex is meant for coordinating the Csp9 activation in response to cytosolic ATP concentration decrease when a cell is to undergo apoptosis, it would have to be a negative regulator to be able to play such a role. This, however, is not supported by the observation that Apaf-1 requires ATP or dATP as a positive effector to function (*1*). Although it was suggested that ATP or dATP is not a messenger but an energy donor for formation of the Apoptosome (*17*, *19*), the observation that a nonhydrolyzable ATP analogue, AMP-PCP, could activate Apaf-1 (*12*) rules out the possibility. In our study, we demonstrate that ATP or dATP plays a dual role in regulating Apoptosome activity.

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[1] Abbreviations: Apaf-1, apoptotic protease-activating factor 1; Smac, second mitochondrial activator of caspases; IAP, inhibitor of apoptosis protein.

10.1021/bi047360+ CCC: $30.25
Published on Web 03/09/2005

While it is an activator of Apaf-1, it is also an inhibitor of caspase-9. Such dual roles of ATP or dATP may allow the Apoptosome to respond to ATP concentration fluctuation prior to apoptosis in a highly sensitive manner.

EXPERIMENTAL PROCEDURES

Recombinant Proteins. Recombinant Apaf-1, procaspase-9, and procaspase-3 proteins were produced in a baculovirus expression system as described previously (*8*). Expression plasmids for Apaf-1 and procaspase-9 were generous gifts from X. Wang (University of Texas, Dallas, TX). Pro domain-truncated caspase-9 (ΔproCsp9) was prepared according to the procedure described in ref *18*. All proteins were purified to homogeneity based on SDS−PAGE Coomassie Blue staining.

Preparation of S100 Cytosolic Fractions. The HeLa cell S100 cytosolic extracts were prepared according to procedures published previously (*1*). The activation of the Apoptosome activity in the S100 extract required exogenous additions of 200 nM CytC and 200−500 μM ATP or dATP. Without the added ATP or dATP and CytC, no activation was detected in the Apoptosome activity assay (see Caspases and Apoptosome Assays).

The endogenous ATP concentration in one representative S100 sample was determined. One hundred λ samples of the S100 prep containing 34 mg/mL proteins were centrifugally gel-filtered through Sephadex G-50-80 that had been pre-equilibrated with buffer A [20 mM Hepes-NaOH, 10 mM KCl, and 7.5 mM $MgCl_2$ (pH 7.0)]. Small molecules retained in the Sephadex G-50-80 were then eluted off the column using 0.5 mL of buffer A. A_{259} of the eluate was measured, and the adenosine concentration was determined using 15.4×10^3 as the molar extinction coefficient of adenosine. With a 5-fold dilution factor between the original 100 λ of the S100 sample and the 500 λ of the eluate, the total adenosine concentration was calculated to be 116.2 μM for all nucleotides and nucleosides in the original S100 sample. Thus, in a typical Apoptosome activity assay mixture that contains diluted S100 (total protein concentration = 1 mg/mL), the endogenous ATP concentration is no more than 116.2/34 = 3.4 μM.

Caspases and Apoptosome Assays. Caspase-9 and Caspase-3 activity assays were conducted according to the method described in ref *18*. The Apoptosome activity of the HeLa S100 cytosolic extract was assayed in a 30 μL reaction mixture containing 7 mM Hepes-NaOH, 3.3 mM KCl, and 2.5 mM $MgCl_2$ (pH 7.0) (buffer R). The S100 extract prepared from HeLa cells (1 mg/mL total proteins) was incubated with CytC (200 nM) in the presence of dATP or ATP at the indicated concentrations for 30 min at 30 °C. At the end of the incubation, 175 μL of buffer C (25 mM Hepes-NaOH, 1 mM EDTA, 0.1% CHAPS, 0.5 mg/mL BSA, and 10% sucrose) containing 23 μM Ac-DEVDamc and 6 mM DTT (pH 7.5) was added to quench the Apoptosome reaction and to assay the activated Csp3. The mixture was incubated for an additional 1 h at 30 °C. The Ac-DEVDamc cleavage activity of Csp3 was determined using a Cytofluor fluorescence photometer (λ_{ex} = 360 nm and λ_{em} = 460 nm).

In the reconstituted Apoptosome system that consists of highly purified recombinant Apaf-1 (2 nM), proCsp9 (2 nM), and proCsp3 (50 nM) prepared as described previously (*17*), 0.5 mg/mL BSA and CytC (200 nM) in 30 μL of Buffer R, dATP, or ATP at desired concentrations were introduced to activate the Apoptosome that ultimately led to Csp3 activation. Reactions were allowed to run for 20 min at 30 °C, followed by assaying the activated Csp3.

In assays containing inhibitors, IC_{50}'s were determined according to the method described in ref *18*.

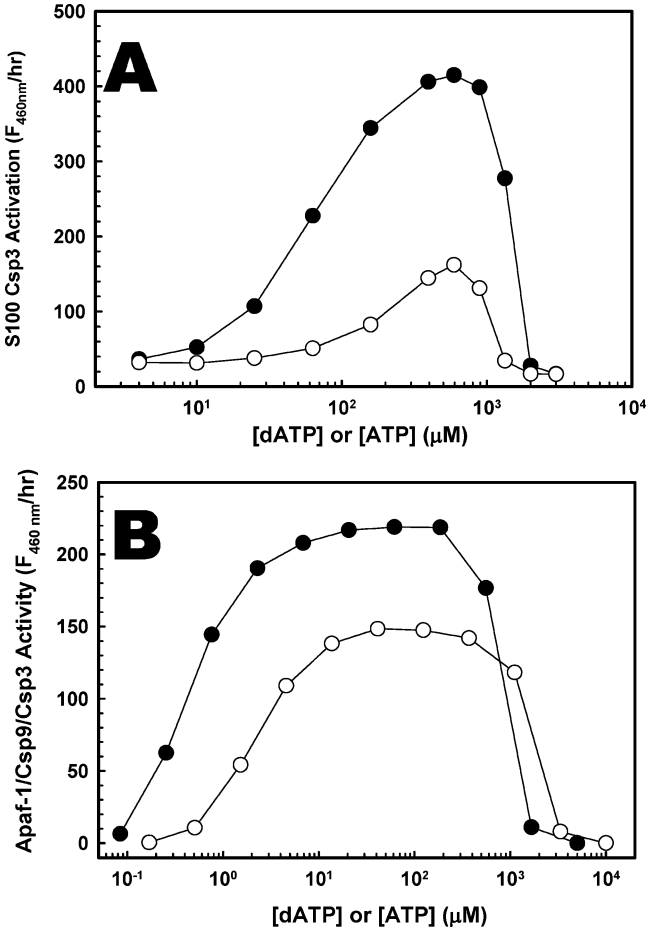

FIGURE 1: Effects of dATP or ATP on Apoptosome-catalyzed Csp-3 activation in cytosolic extract (A) and in a reconstituted Apoptosome system (B). (A) In a 30 μL reaction mixture containing 7 mM Hepes-NaOH, 3.3 mM KCl, and 2.5 mM $MgCl_2$ (pH 7.0) (buffer R), cytosolic extracts prepared from HeLa cells (1 mg/mL total proteins) were incubated with CytC (200 nM) in the presence of dATP (●) or ATP (○) at indicated concentrations for 30 min at 30 °C. At the end of the incubation, the reaction product, i.e., activated Csp3, was assayed using DEVDamc as described in Experimental Procedures. (B) A reconstituted Apoptosome system that consisted of highly purified recombinant Apaf-1 (2 nM), proCsp9 (2 nM), and proCsp3 (50 nM) was prepared as described previously (*17*) except that 0.5 mg/mL BSA was included. CytC (200 nM) and dATP (●) or ATP (○) at indicated concentrations were introduced to activate the Apoptosome that led to Csp3 activation. Reactions were carried out for 20 min at 30 °C. Reaction product Csp3 was monitored using the Csp3 activity assay.

RESULTS

Activation of the Apoptosome in cytosolic extracts requires addition of exogenous CytC and ATP or dATP (*1*, *19*). Figure 1A shows that in an in vitro system using cytosolic fractions from HeLa cells that were essentially free of CytC, adding exogenous CytC does not initiate the Apoptosome-catalyzed Csp9 activation at typical cellular ATP concentrations that are normally greater than 1 mM (*19*−*23*). However,

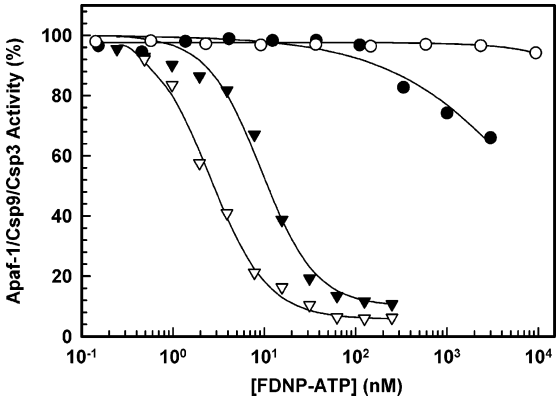

FIGURE 2: Pretreatment of proCsp-9 with FDNP-ATP impedes Apoptosome activity. In a reconstituted Apoptosome system described in Figure 1B, Apaf-1 (●), proCsp9 (▼ and ▽), or proCsp3 (○) was pretreated with FDNP-ATP at indicated concentrations for 1 h prior to the assay. The pretreatment of Apaf-1 (100 nM) was conducted in buffer R containing 0.5 mg/mL BSA, 200 nM CytC, and 30 μM dATP at 22 °C. Aliquots were diluted 50-fold to dissociate noncovalently bound FDNP-ATP, and subsequently mixed with proCsp-9 (2 nM), proCsp-3 (50 nM), CytC (200 nM), and dATP (30 μM) to initiate the Apoptosome reaction. The reaction was carried out for 20 min at 30 °C, and monitored for active Csp3 production using the Csp3 activity assay. Pretreatments of unprocessed proCsp9 [p50 (▼)] and partially processed proCsp9 p35/p10 (▽) were conducted in buffer C (pH 6.5). The pretreatment of proCsp3 was carried out in buffer C (pH 7.5). Apoptosome assays were run in a manner identical to that for FDNP-ATP-pretreated Apaf-1, except that untreated Apaf-1 and FDNP-ATP-pretreated Csp9 or proCsp3 were used.

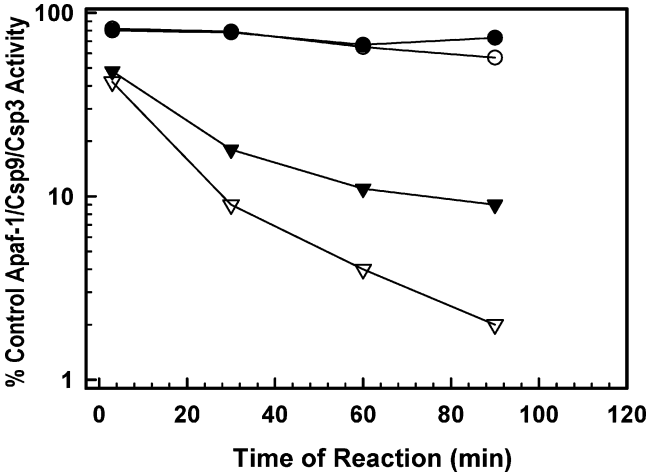

FIGURE 3: Affinity labeling of proCsp9 with FDNP-ATP. In a solution containing 1 mg/mL BSA in buffer C (pH 6.5), 250 nM proCsp9 p50 (▼ and ●) or processed Csp9 p35/p10 (▽ and ○) was incubated at 22 °C with (▽ and ▼) or without (○ and ●) 0.75 μM FDNP-ATP. At intervals, 50 μL aliquots of each reaction mixture were removed and centrifugally filtered through Sephadex G-50-80 that had been pre-equilibrated with buffer C. The resultant filtrates were immediately assayed for Apoptosome activity in a 30 μL buffer A solution containing 2 nM Apaf-1, 50 nM proCsp3, 200 nM CytC, and 30 μM dATP. Amounts of the filtrates used for the assay were adjusted on the basis of the protein concentration that gave 2 nM Csp9 in the final 30 μL reaction mixtures.

lowering the ATP concentration to 0.2−0.3 mM effectively activates the system, though further decreasing the ATP concentration eventually stops the activation. When the ATP or dATP concentration < 10 μM, the system cannot be activated. Similar observations were obtained in a reconstituted Apoptosome system that contained highly purified Apaf-1, proCsp9, and proCsp3 (Figure 1B).

While these observations are consistent with previous data which show that ATP or dATP is a required positive effector for activating the Apoptosome−proCsp9 complex (*1*), these data also suggest an additional role of ATP or dATP. At normal physiological levels, ATP actually prevents activation of the Apoptosome−proCsp9 complex. The Apoptosome−Csp9 complex appears to be very sensitive to dATP and ATP concentration in the narrow range of 0.5−2 mM. Within this range, ATP or dATP turns on or turns off, respectively, the enzymatic activity in a very responsive manner. These dual roles of ATP and dATP may suggest that there are two distinct types of dATP and ATP binding sites in the Apoptosome−Csp9 complex or among the pre-Apoptosome components. One is required to promote Apoptosome formation in the presence of CytC and thereby activate Csp9, whereas the other is to inhibit such activation. The inhibitory nucleotide binding site appears to have a lower affinity for dATP or ATP than the first site such that it only functions at a high dATP or ATP concentration. In an attempt to locate this negative modulator site, we used the covalent affinity ATP analogue, 3′-*O*-(5-fluoro-2,4-dinitrophenyl)-ATP (FDNP-ATP), as a probe. Effects of pretreatment of Apaf-1, proCsp9, or proCsp3 with this probe on the initiation of the Apoptosome activation are shown in Figure 2. FDNP-ATP was originally synthesized to probe ATP binding sites of F_1-ATPase (*13*, *14*), and was subsequently shown to have high affinity and specificity for the ATP binding sites of various ATP-binding proteins (*13*−*16*). As expected, FDNP-ATP antagonizes the dATP- or ATP-binding site of Apaf-1, as the pretreatment of Apaf-1 with FDNP-ATP in the presence of 30 μM dATP and 200 nM CytC for 60 min prior to the mixing with proCsp9 and proCsp3 abolishes the ability of Apaf-1 to activate proCsp9 [Figure 5 (●); $IC_{50} \sim 2$ μM]. On the other hand, pretreating proCsp9 with FDNP-ATP before mixing with the Apaf-1−CytC−dATP complex and proCsp3 results in a much more potent inhibitory effect [Figure 2 (▼); $IC_{50} \sim 11$ nM]. A similar pretreatment of proCsp3 with FDNP-ATP exhibits no effect (○). While the loss of function in the FDNP-ATP-pretreated Apaf-1 sample may be explained by the covalent antagonistic binding that prevents dATP from triggering Apaf-1, the observation that the functional Apaf-1−CytC−dATP complex fails to activate FDNP-ATP-pretreated proCsp9 may indicate a nucleotide binging site in proCsp9 that binds FDNP-ATP with high affinity and keeps proCsp9 from being activated by the Apoptosome.

The weakly reactive 3′-*O*-5-fluoro-2,4-dinitrophenyl group of FDNP-ATP could in principle react in situ with a nearby Lys, Tyr, or Cys in the binding pocket. It has been reported from different labs that FDNP-ATP specifically labeled the Lys in the ATP binding sites (*14*, *16*) and a Cys in UCP (*15*). This affinity reagent has also been shown to be reactive only when it is bound to the ATP binding pocket with close-by nucleophiles available, or otherwise inert (*13*, *14*). Figure 3 shows that this affinity probe inhibits proCsp9 from being activated by Apaf-1 in a time-dependent manner, indicative of an irreversible inhibition. The rate of the covalent inactivation is slow because of the weak reactivity of the FDNP group, but the covalent labeling is highly specific as demonstrated in Figure 4. In the activity titration experiment shown in Figure 4, Csp9 was incubated with FDNP-ATP

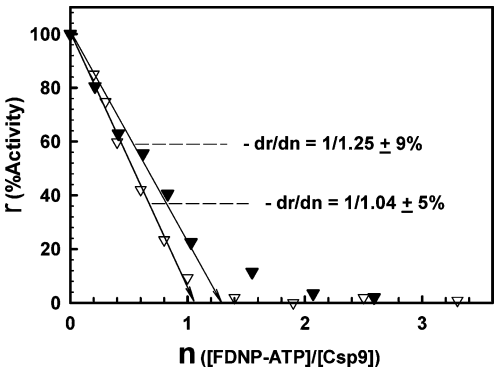

FIGURE 4: Stoichiometric inhibition of proCsp9 by FDNP-ATP. A solution containing 91 nM p50 proCsp-9 (▼) or p35/p10 (▽) was incubated with FDNP-ATP at a concentration ranging from 15 to 300 nM in buffer C (pH 6.5) for 75 min at 22 °C. At the end of the incubation, 0.75 μL aliquots of each reaction mixture were removed and assayed for Apoptosome activity in a 30 μL assay mixture containing 2 nM Apaf-1, 50 nM proCsp3, 200 nM CytC, and 30 μM dATP in buffer R. The assay was run in duplicate for 15 min at 30 °C, and the product Csp3 was monitored using the Csp3 activity assay described in the legend of Figure 1. The control Csp9 was treated similarly but without being exposed to FDNP-ATP. The activity of the control Csp9 remained constant throughout the 75 min incubation at 22 °C in buffer C. r denotes the ratio of the activity of the partially labeled Csp9 in the Apoptosome assay to that of the unlabeled Csp9. n represents the molar ratio of FDNP-ATP to Csp9 in the reaction mixtures. Each data point represents the averaged result of duplicate measurements. Solid lines are linear regressions of data points for which $n \leq 0.8$.

ranging in concentration from 0 to 3.3 times the Csp9 concentration. The affinity labeling was carried out at 22 °C for 75 min to allow the reaction to reach completion. Aliquots were then diluted into the Apoptosome assay mixture to determine the activity of FDNP-ATP-labeled Csp9. Thus, the degree of Csp9 inhibition can be correlated with the number of FDNP-ATP labels per enzyme (n). The linear correlation with the intercepts very close to 1 shown in Figure 4 suggests that the covalent labeling of Csp9 with FDNP-ATP is highly specific. Even at nanomolar concentrations of FDNP-ATP, Csp9 can be completely inhibited with only 1.04 ± 0.05 or 1.25 ± 0.11 equiv of the inhibitor. Thus, nearly every FDNP-ATP in the samples for which $n < 1$ covalently labels Csp9 and inhibits the enzyme. These results also suggest that there is one FDNP-ATP binding site per proCsp9 molecule.

Figure 5 shows that unmodified proCsp9 can be fully processed by the Apoptosome within 10 min under the indicated experimental conditions. However, the processing of FDNP-ATP-labeled proCsp9 (proCsp9−DNP−ATP) was significantly impeded as ~60% of p50 remained unprocessed even after reaction for 30 min. The processed 40% of the Csp9−DNP−ATP complex did not yield an active Apoptosome−Csp9 complex even though the modified proCsp9 had been converted to p35/p10. A similar impediment in Csp9 processing was also observed in the mixture containing Apaf-1, unmodified Csp9, CytC, and 10 mM dATP or ATP (data not shown). Since the preprocessed Csp9, i.e., p35/p10, is fully accessible to FDNP-ATP inactivation (Figure 2), it is not expected that the p35/p10 derived from the proCsp9−DNP−ATP complex is enzymatically active for cleaving proCsp3. Taken together, these data suggest that although the DNP−ATP−proCsp9 complex retains the accessibility for forming the Apoptosome complex with

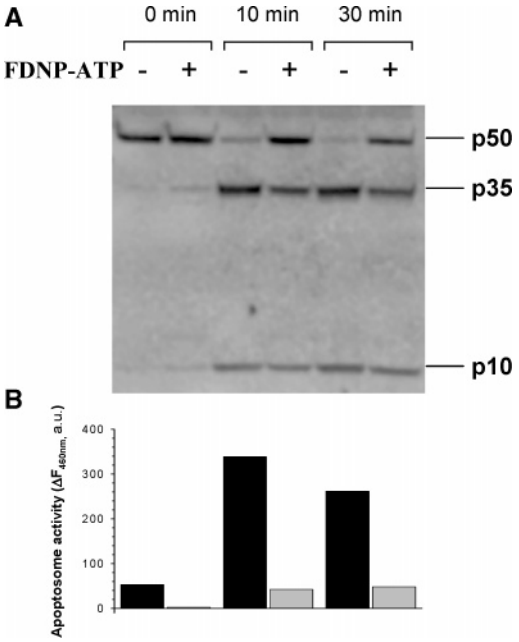

FIGURE 5: Processing of proCsp9 and the proCsp9−DNP−ATP complex by the Apoptosome. p50 proCsp9 (120 nM) was incubated with or without 480 nM FDNP-ATP in buffer C (pH 6.5) for 30 min at 22 °C. Both reaction mixtures were subsequently mixed with 2× volume of buffer R containing 60 nM Apaf-1, 300 nM CytC, and 45 μM dATP and incubated at 30 °C to allow Csp9 autoprocessing. At intervals, aliquots of reaction mixtures were removed for Western blot analysis (A) and an activity assay (B). The Apoptosome activity assay was carried out as described in the legend of Figure 1. Western blot analysis was conducted according to the procedure published previously (*8*).

Apaf-1 and is slowly processed to the p35/p10−DNP−ATP complex, the resultant Apoptosome−Csp9 holoenzyme is enzymatically incapable of processing proCsp3.

We have previously demonstrated that in the absence of the Apaf-1−CytC−dATP complex, the processed Csp9 p35/p10 exhibits weak proteolytic activity using LEHDamc as the substrate (*8*). This form of Csp9 may be used to demonstrate the binding of natural nucleotides. Typically, the LEHDamc cleavage activity of p35/p10 is ~2% of Apoptosome−Csp9 activity. The 50-fold reduction in the activity in the absence of the Apoptosome is attributed to the fact that only 2% of the Csp9 population forms the active conformation as determined by the active site titration, while nearly 100% remains active in the presence of the Apoptosome. As such, each Csp9 molecule of this 2% population is a fully competent enzyme and is legitimate for testing the binding of nucleotides. Table 1 summarizes results of p35/p10 Csp9 intrinsic activity inhibited by ATP, dATP, ADP, and AMP in the absence of Apaf-1. Notably, ATP inhibits this form of Csp9 with an IC_{50} of 121 μM, though ADP and AMP are also weakly inhibitory with IC_{50} values of 1.1 and 4.3 mM, respectively. The inhibition by ATP is consistent with that observed in the native cytosolic extracts (Figure 1A). Weak inhibitory effects of ADP and AMP were also obtained in cytosolic extracts where the Apoptosome was activated by the addition of 100 μM dATP and 200 nM CytC (data not shown). As a control experiment, the prodomain-truncated Csp9 p18/10 was examined. None of the nucleotides exhibited an inhibitory effect (Table 1).

Table 1: Effects of Nucleotides on p35/p10 Csp9 and Pro Domain-Truncated p18/p10 ΔproCsp9[a]

	IC_{50} (μM)	
nucleotide	processed Csp9 p35/p10	ΔproCsp9 p18/p10
ATP	121	ND[b]
dATP	149	ND[b]
ADP	1080	ND[b]
AMP	4310	ND[b]

[a] p35/p10 was assayed in buffer C (pH 6.5) for 30 min at 30 °C using proCsp3 (25 nM) as the substrate. Formation of product Csp3 was followed using the Csp3 activity assay. Δpro-Csp9 p18/p10 was assayed using LEHDamc (50 μM) as the substrate described previously (*8*). IC_{50} values were determined using a nonlinear regression two-parameter fit program. [b] Not detectable at 10 mM nucleotide.

DISCUSSION

Data presented in this report indicate the existence of a nucleotide binding site in Csp9 that binds ATP as a negative modulator to prevent the activation of Csp9 by the Apoptosome. In nonapoptotic cells, the cytosolic ATP concentration is normally between 1 and 5 mM (*19*−*23*). Assuming a K_d of ≈120 μM for ATP binding to proCsp9 as approximated by its IC_{50} (Table 1), 1−5 mM cytosolic ATP would be sufficient to constrain 90−98% of the Csp9 zymogen in the cytosol. When mitochondria-mediated apoptosis takes place, CytC is released and the cytosolic ATP level is diminished due to mitochondria dysfunction. While the released CytC binds to Apaf-1/dATP and promotes assembly of the Apoptosome, the decrease in ATP concentration in the cytosol results in dissociation of ATP from proCsp9. Consequently, proCsp9 is available for the Apoptosome activation. Thus, a successful activation of the Apoptosome−Csp9 complex requires concurrent actions of triggering (Apoptosome formation) and deregulation (dissociation of ATP from Csp9), much analogous to setting off the safety before firing a rifle. However, it is not immediately obvious if there is added value in a bi-messenger mechanism that uses both CytC and ATP compared to a mono-messenger mechanism that uses CytC as the sole signal.

Since discharging CytC from the mitochondria to the cytoplasm during mitochondria-mediated apoptosis is a rapid and synchronized event among all mitochondria (*10*, *11*), it would inevitably result in halting the cellular ATP production. Consider a situation where a small population of the mitochondria in a nonapoptotic cell is damaged, causing leakage of CytC and SMAC into the cytoplasm. Such release of CytC and SMAC could easily trigger Apaf-1-mediated Csp9/Csp3 activation if proCsp9 is not safeguarded by the bound ATP at its ATP binding site. However, since the cell is not undergoing apoptosis and the ATP production machinery in most of the mitochondria remains intact, the overall ATP production is not significantly impeded; hence, the cytosolic ATP level remains relatively steady. Consequently, the undesirable proCsp9 activation due to accidental leakage of CytC and Smac is avoided.

For the nucleotide binding site of Csp9 to function effectively as a molecular switch, it needs to respond to ATP concentration variations in an efficient manner that ideally turns all Csp9 molecules on or off at critical thresholds of ATP signal. Figure 1 shows that the Apoptosome−Cps9 complex appears to be regulated by ATP or dATP in just such a manner. It switches from the all-off state at 2 mM ATP to maximal activity at 300 μM ATP, requiring an only 7-fold change in ATP concentration. Notably, since there is only one nucleotide binding site in Csp9 (Figure 4), the binding of ATP to Csp9 most likely would follow single-site binding kinetics that would require a 342-fold-decrease in ATP concentration to shift the equilibrium from 95 to 5% saturation of proCsp9·ATP. Data in Figure 2 suggest that in the absence of the Apoptosome during the preincubation, FDNP-ATP indeed follows a single-site noncooperative kinetic mode in binding to both unprocessed proCsp9 p50 and the processed enzyme p35/p10, as indicated by the fact that the Hill coefficient for both curves is approximately equal to 1. Dose−response curves for determining the inhibition of p35/p10 by ATP or dATP in Table 1 also suggest a single-site binding (data not shown). While the Apoptosome−Csp9 complex being extremely sensitive to the change of ATP concentration seems to reflect the biological adaptation for effective functioning, how such sensitivity is achieved at the molecular level is not clear. It is likely that the interaction with Apaf-1 within the Apoptosome allows Csp9 to respond to ATP concentration variation in a highly cooperative, and hence very sensitive, manner.

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BI047360+