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The Raison D'Être of Constitutively Active Protein Kinases: The Lesson of CK2

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ABSTRACT

Protein kinases generally are tightly controlled signaling molecules that are switched on only in response to specific stimuli. Exceptionally few protein kinases are constitutively active, the most striking example being provided by CK2 (formerly "casein kinase 2"). Owing to unique structural features, the catalytic activity of CK2 is constantly on, although its targeting can be deeply influenced by the association of its two catalytic subunits (α and/or α′) with a dimeric non catalytic β subunit. Constitutive activity of CK2 reflects its extraordinary pleiotropy documented by its growing list of >300 protein substrates and is consistent with emerging evidence that CK2 plays an essential role in the cell by counteracting premature and/or unscheduled apoptosis, thus ensuring cell survival under stress conditions.

Introduction

Nearly all aspects of cell life are controlled by phosphorylation of Ser, Thr, and Tyr residues, which is the most common mechanism to reversibly regulate the biological activity of proteins. About one-third of mammalian proteins contain covalently bound phosphate, and there are almost 600 protein kinases which catalyze these reactions, encoded by the human genome.[1] In a sense the role of protein kinases can be likened to that of interpreters, who translate stimuli and signals into biochemical events. Not surprisingly, therefore, protein kinases are especially abundant along signal transduction pathways, at all levels, from the plasma membrane, where many receptors operate through their intracellular kinase domain, down to the nucleus where many effectors of gene expression, notably transcription factors, are under the control of protein kinases. This in turn implies that protein kinases are themselves subjected to tight control: there is plenty of evidence that this is indeed the case and that deregulation of protein kinases, giving rise to abnormally high catalytic activity, results in cell malfunction, eventually causing neoplastic growth and other diseases.[2] There are several mechanisms by which normally silent protein kinases are switched on, the most frequent being the binding of biological effectors, such as hormones, growth factors, and second messengers, and phosphorylation events which either displace autoinhibitory domains or confer an "open" conformation to a structural element named "activation loop" which can be likened to a gate at the entrance of the catalytic site. Quite often, an individual protein kinase is subjected to multiple controls. For example, cyclin dependent kinases (CDKs), which are controlling the whole cell division cycle, must pass at least four checkpoints before they can become active. Given these premises, it is quite understandable that biologists tend to think of kinases as signaling molecules that are switched on only in response to specific stimuli and phosphorylate a discrete set of targets appropriate to the response required. However, there are a few notable exceptions, provided by protein kinases that are constitutively active although their implication in signal transduction is well documented. Sometimes these constitutively active kinases result from genetic rearrangements responsible for tumors (e.g., Bcr-Abl and NPM-Alk, responsible for chronic myelogenous leukemia[3] and anaplastic lymphomas,[4] respectively), or they are the products of gene mutations which are exploited by some viruses as tools for their invasive strategy (e.g., tyrosine kinases of the Src family which have lost their C-terminal down-regulatory site, which is present in their cellular counterparts[5]).

More striking is the presence within normal cells of essential protein kinases which are constitutively active per se. The most telling example of these is provided by protein kinase CK2,[6,9] especially considering that its holoenzyme displays a quaternary structure, a feature which is generally held as the hallmark of tight regulation. Instead the catalytic subunits of CK2 (α and/or α′) are constitutively active with or without their non catalytic β-subunits. In hindsight it seems likely that this feature had a determinant role in making CK2, together with another constitutively active kinase, CK1, the first protein kinases to be discovered, in 1954,[10] by just incubating casein with liver extracts in the presence of radiolabeled ATP. As a matter of fact, neither CK2 nor CK1 needs any special condition for becoming active, and both readily phosphorylate casein as artificial substrate, though they are not physiologically related to it—hence the misnomers "casein kinase 1" and "casein kinase 2", used for decades before the acronyms CK1 and CK2 came into use in 1994.[11]

The feeling that the early discovery of CK2, owing to its constitutive activity, was in fact premature is grounded on the observation that for two decades after its identification as an individual kinase, CK2 remained an enzyme in search of its substrates. These started coming to light in the early 1980s and later underwent an impressive increase: to date, the repertoire of CK2 substrates includes 307 proteins, 243 of which have been shown to be phosphorylated also in vivo,[12] but these figures are

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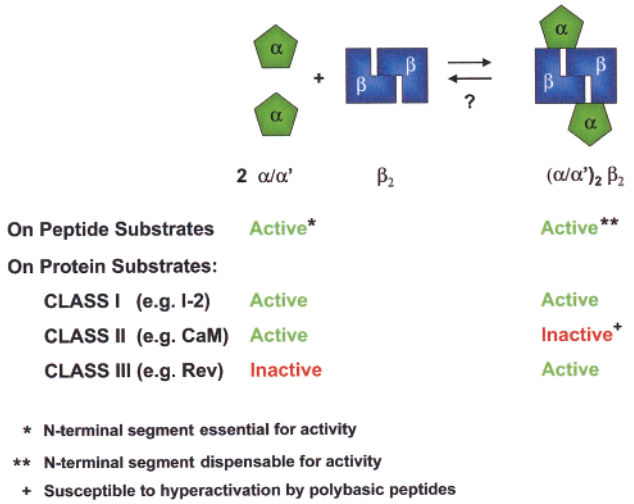

FIGURE 1. The catalytic subunits of CK2 are constitutively active with or without their "regulatory" β-subunits. The schematic representation of the heterotetrameric holoenzyme on the right takes into account the crystallographic information that each catalytic subunit makes contacts with both β-subunits and that its overall structure is not significantly altered by these interactions.[15] Drawn from Sarno et al.[14] and Marin et al.[17]Abbreviations: I-2, inhibitor 2 of protein phosphatase 1; CaM, calmodulin; Rev, human immunodeficiency virus type-1 Rev transactivator protein

increasing day after day. Such an extraordinary pleiotropy and constitutive activity are likely to be the two faces of the same coin: this kinase is in charge of so many tasks that its work is restless. Pleiptropy may also explain why CK2 is essential to cell viability although it does not appear to play any fundamental role in the hierarchy of cellular functions: even if, individually taken, none of its tasks might be indispensable, they cannot be collectively omitted without fatal consequences. However, how does this reconcile with a role in signaling? How can CK2 hit properly and timely its targets if it is not activated by specific stimuli? Could it happen that constitutive activity of CK2 becomes detrimental to the cell under special circumstances? These and other related questions will be addressed below.

A Free Lance in Signal Transduction. At variance with the great majority of protein kinases, which are normally silent enzymes turned on only in response to specific stimuli, CK2 is constitutively active despite its quaternary structure, which is generally held as a hallmark of tight regulation. As schematically depicted in Figure 1, both the free catalytic subunits of CK2 and the holoenzyme, composed with two catalytic subunits separately bound to a dimeric non catalytic β-subunit, display high catalytic activity as judged from the phosphorylation of specific peptide substrates. Neither specific ligands nor phosphorylation events such as those triggering the activation of most protein kinases are required for this basal activity of CK2, corroborating the view that the catalytic potential of CK2 within the cell is always "on" independently of specific stimuli and/or metabolic conditions. Also the level of CK2 subunits within the cell does not undergo remarkable changes depending on metabolic conditions, thus ruling out any acute regulation at translational level.

Structural insights[13] and mutational analyses[14] have shown that high constitutive activity of the isolated catalytic subunit is conferred by its unique N-terminal segment which makes extensive interactions with the active core, and by doing that, it keeps the activation loop in its "open" conformation without the need of external ligands and/or phosphorylation events. These interactions appear to be not required for the constitutive activity of the holoenzyme since nearly inactive mutants of human CK2 α with N-terminal truncations are reactivated to wild-type level upon association with the β-subunits.[14] Nevertheless the β-subunits neither interact with the activation loop nor induce any significant alteration in the catalytic core of the α subunits.[15] It has to be concluded therefore that CK2 is endowed with two mutually independent activation mechanisms aimed at keeping its catalytic potential on, irrespective of being or being not assembled with the β-subunits.

Consistent with the above observations, many attempts to insert CK2 at well-defined positions within individual signal transduction pathways ultimately proved unsuccessful.[16] On the other hand, it is hard to deny that CK2 must play a global role in signal transduction and cell regulation if we give an even cursory glance to the list of its multitude of substrates. In fact, 87 out of 307 proteins included in the latest repertoire of CK2 targets[12] are signaling proteins; if these are implemented with the 60 transcription factors, which are the ultimate executors of signals impinging on gene expression, and with 50 proteins committed to the synthesis and regulation of DNA/RNA functions, it has to be concluded that a substantial majority of CK2 substrates are dealing with the network of cellular signaling. Given these premises, it is not conceivable that CK2 might be not implicated, eventually in a subtle way, in signaling. How could this be reconciled with the lack in CK2 of the stigmata of signal transducing molecules, i.e., to become active upon request?

An at least partial answer could come from the observation that although the intrinsic catalytic activity of CK2 is not turned on nor off by the β−subunit, this latter can deeply influence the phosphorylation of two subsets of protein substrates, one of which is entirely dependent on β for phosphorylation while the other is phosphorylated by the free catalytic subunits but not by the holoenzyme, unless polylysine or other very basic polypeptides are also added (see Figure 1). This, in conjunction with the observation that the β subunit has little influence, if any, on the site specificity dictated by local determinants, strongly supports the view that the β-subunit, rather than switching on/off activity, as the regulatory subunits of other protein kinases, notably cAMP-dependent protein kinase (PKA) and CDKs, do, operates as a docking platform for binding substrates and eventually substrate directed effectors, thus mediating signals which impinge on the targeting rather than on the activity of CK2. This would be in agreement with the detection of many cellular partners of β by the two hybrid system approach.[18] It is also conceivable that the β-subunit plays a role by recruiting CK2 to given subcellular compartments, similar

to what protein kinase-A anchoring proteins (AKAPs) do with PKA. The crystal structure of CK2 holoenzyme[15] would be also consistent with this scenario: while the overall structure of the two individual catalytic subunits is not modified by interaction with the β dimer, this latter provides a large surface potentially exploitable by binding molecules. The nature and the area of the contacts between the β and the α subunits (832 A^2) moreover make plausible the reversibility under physiological conditions of the reaction of holoenzyme formation. Although the dissociation of the holenzyme within the cell is still a matter of debate, there are a number of arguments supporting such a possibility. Especially telling is in this respect a recent study on the mechanism of polymerase III (polIII) regulation by CK2. Being committed to the synthesis of tRNA and 5SrRNA, polIII is expected to be constantly active in living cells except under special circumstances in which, due to DNA damage, transcription has to be interrupted. Phosphorylation of a component of the polIII complex, the TATA-binding protein (TBP), by CK2 promotes a remarkable increase in polIII activity. Ghavidel and Schultz[19] have shown that CK2 normally associates through its β-subunit with TBP, and by doing that, it phosphorylates TBP and sustains polIII transcription. Transcriptional repression promoted by DNA damage is mediated by down-regulation of TBP associated CK2, which occurs through the release of the catalytic subunits from the complex. Therefore, the molecular mechanism leading to CK2 down-regulation would be the mere dissociation of the catalytic subunits from the $\beta-\beta$ dimer anchored to the complex, an event plausible in the context of the crystallographic data just mentioned above. Perhaps this kind of negative regulation could become the key for deciphering many paradoxical behaviors of CK2 in the living cell.

The Acidophilic Character of CK2. The great majority of Ser/Thr protein kinases recognize sites which are specified by basic and/or prolyl residues, often in conjunction with hydrophobic side chains.[20] By sharp contrast, basic residues and proline are nearly absent in the CK2 sites,[20] and they have been shown to act as negative determinants in peptide substrates phosphorylated by this kinase.[21,22] CK2 is in fact very acidophilic in nature: its phosphoacceptor sites are specified by multiple acidic residues (on the average >5 in the 307 sites analyzed so far),[12] the ones at positions $n + 3$, and, to a lesser extent, at position $n + 1$ relative to the target residue, being of crucial importance. This point is highlighted by the observations that the sporadic sites with just one acidic residue (9 out of 307) invariably have this residue at position $n + 3$ and that every time the acidic determinant at position $n + 1$ is lacking, it is found at position $n + 3$ and vice versa. Interestingly, phosphorylated residues (especially serine and tyrosine) can effectively replace carboxylic residues as specificity determinants. This includes CK2 in a small group of "phosphate-directed" Ser/Thr kinases implicated in "hierarchical" phosphorylations[23] where a "priming" kinase creates the site for subsequent phosphorylation by either the same enzyme or another kinase. Note that by this device a constitutively active kinase can be controlled at substrate level by other kinases which could be responsive to specific stimuli. This may also apply to CK2 in sporadic cases, since some CK2 sites are entirely or partially phosphate directed for having a phosphoresidue at the crucial $n + 3$ position or nearby. In a yeast phosphoproteome analysis the motif pS-x−x-pS−P is found in 5 phosphopeptides,[24] suggesting that in these cases the phosphorylation of the N terminal serine by CK2 might be primed by a proline directed kinase (either a CDK or a mitogen activated protein kinase (MAPK)) which, once activated, phosphorylates the residue adjacent to proline. Interestingly, in at least one case, CK2 phosphorylates a tyrosyl residue, in yeast immunophilin Fpr3.[25] This phosphorylation is also specified by acidic residues although the consensus appears to be significantly different from the canonical one recognized in Ser/Thr sites.[26]

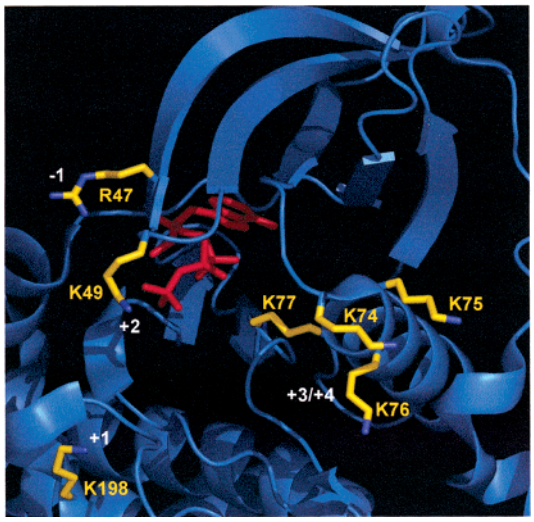

FIGURE 2. Mapping basic residues responsible for the recognition of acidic determinants at CK2 phospho-acceptor sites. Basic residues (yellow) making contacts with the specificity determinants at individual positions (white) relative to serine in the phospho-acceptor peptide RRR-ADDSDDDD are shown. The AMP scaffold found in the ATP binding site is shown in red. Based on mutational analyses[27−29] and crystallographic data.[13]

Why is CK2 so markedly acidophilic? A systematic mutational analysis[27−29] has led to the identification of a number of basic residues which are responsible for interactions with different acidic determinants within the optimal peptide substrate RRR-A-DDSDDDDD: these residues are conserved across CK2 catalytic subunits from different species, while they are replaced by non basic residues in the majority of other kinases. They belong to distinct structural elements, namely, a KKKK quartet (residues 74−77) at the beginning of helix-C (responsible for the interaction with the crucial determinant at position $n + 3$ and probably also with others down stream from it), to the $n + 1$ loop (K-198, interacting with the determinant at position $n + 1$), and to the Gly-rich loop where R47 and K49 interact with the acidic determinants at positions $n - 1$ and $n + 2$, whenever present. As shown in Figure 2, these basic residues are clustered around the

catalytic site in the 3D structure of CK2α. Although the crystal structure of the peptide bound to the catalytic subunit of CK2 is not available, it can be inferred from Figure 2 that the peptide will bridge across the catalytic cleft by making its main interactions with both the upper lobe (lysines 74−77) and the lower lobe (Lys-198) of the kinase.

CK2 as a Pharmacological Target. In general the transforming potential of oncogenic protein kinases is conferred by mutations that subtract the kinase from its normal mode of control rendering it constitutively active.[2] No natural mutations of CK2 are known, either related or not to diseases. Intriguingly, however, constitutive activity, which is a misfortunate outcome for oncogenic kinases, is a physiological trait of CK2, suggesting that under special circumstances increased expression of CK2 could lead to cell malfunction. This concept is corroborated by a number of observations, the most striking, albeit coincidental in nature, being the elevated level of CK2 invariably observed in a wide variety of tumors.[8,30] A more direct cause-effect relationship between CK2 and transformation has been provided by animal[31−33] and cell[34,35] experimental models where transfection of the catalytic subunits of CK2, besides being causative of transformation per se, becomes dramatically oncogenic in concommitance with altered expression of oncogenes or anti-oncogenes. Also, the inclusion of many viral proteins among CK2 targets strongly suggests a role for CK2 in virally mediated pathologies, including cancer. Considering on the other hand that CK2 has been recently shown to counteract apoptosis,[36] a task which could be part of its emerging role as a promoter of cell survival,[37] it becomes conceivable that under special conditions, e.g., in stressed or injured cells, its zealous determination to keep the cell alive could have detrimental effects. All the more so if it is born in mind that there is no fast and general way to turn its catalytic activity off. Consistent with this scenario, modest alterations in the levels of CK2α are sufficient to induce dramatic effects on cell fate by cooperating with other factors.[31,35] This has led Twafic et al.[30] to hypothesize that CK2α "might impart an oncogenic potential to the cell such that in cooperation with certain oncogenes it produces a profound enhancement of the tumor phenotype".

Given these premises, it is not surprising that CK2 starts becoming an attractive target for anti-neoplastic and anti-infectious drugs. By doing that it joins a growing list of protein kinases that nowadays already represent the second largest group of drug targets, accounting for 20−30% of the drug discovery programs of many companies, with many inhibitors of protein kinases that are already either in clinical practice or undergoing human clinical trials.[38−40] On the other hand, the objection that, being essential to viability, CK2 is not amenable as drug target has been overcome by the empirical observation that other kinase activities (e.g., the MAPK cascade) are indispensable but nevertheless their inhibitors have successfully entered clinical trials. The essential role of these kinases, CK2 included, may be restricted to early phases of embryogenesis and differentiation, becoming less crucial for normal functions in adults. Also, the unexpected finding that brief inactivation of another proto-oncogene, MYC, leads to a permanent loss of the neoplastic phenotype [41] corroborates the view that CK2 might be a target worthy of attention. Another dogma which was disproved in the course of the search for selective and potent protein kinase inhibitors was that being the ATP binding site highly conserved among kinases and being the intracellular ATP concentration much higher than the Km values of ATP for kinases, effective and selective in vivo inhibitors of kinases cannot be competitive with respect to ATP. Instead, almost all the most promising inhibitors of protein kinases, including compounds already in clinical practice/trials actually, are ATP site-directed ligands. This is due to the fact that the sensu stricto conserved ATP binding sites are surrounded by a region of structural elements, the so-called "pharmacophore"[42] which are quite variable from one enzyme to another and where a variety of compounds can bind with remarkable affinity and selectivity, preventing the binding of ATP.

This also applies to CK2, in which a hydrophobic pocket partially overlapping the adenine binding subsite is smaller than in the majority of other protein kinases owing to the presence of three unique bulky side chains, Val-66, Met-163, and Ile-174. It has been demonstrated by crystallographic[43] and mutational studies[44] that the unique smallness of this cavity is indeed the basis for selective inhibition of CK2 by tetrabromo-benzotriazole (TBB). This compound affects CK2 activity much more drastically than that of a panel of >30 other kinases[45] and is proving useful for unraveling the role of CK2 in intact cells with special reference to its ability to counteract apoptosis by rendering caspase sites refractory to cleavage.[46] As shown in Figure 3A, the sizes of TBB and of the hydrophobic pocket of CK2 are perfectly complementary, like those of a hand and its glove, so that TBB is retained into the cavity by van der Waals interactions which would be much weaker if the cavity were larger, like in most other other kinases.

Another scaffold useful to develop CK2 inhibitors could be that of emodin (6-methyl-1,3,8-trihydroxy-antraquinone, see Figure 4), the active principle of an herbal medicine extracted from the rhizomes of *Rheum palmatum* reported to inhibit CK2,[47] as well as some receptor protein tyrosine kinases.[48] Emodin, whose structure in complex with CK2α has been solved,[49] is somewhat less potent and less selective than TBB (see Figure 4). By screening a library of about 300 compounds provided by Dr. G. Zagotto (university of Padova), however, several derivatives somehow related to emodin have been identified which are more effective and selective than emodin itself.[44,50] In contrast, no significant improvement of inhibitory properties could be attained till now by altering the TBB scaffold. A common denominator of some of the most effective emodin-related inhibitors is the presence of nitro-group(s) alone causing a magnitude drop up to 2 orders in K_i value, as exemplified by comparing the K_i

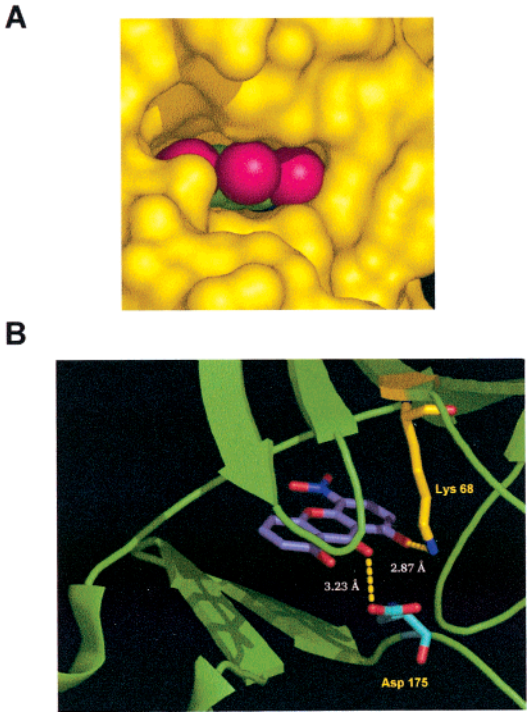

FIGURE 3. Mode of binding of tetrabromo-benzotriazole (TBB) (A) and 1,8-dihydroxy-4-nitro-xantenone (MNX) (B) into the active site of CK2α. Based on the coordinates of the complexes of maize CK2α with TBB[43] and MNX 50, respectively.

values of 1,8-dihydroxy-xanthenone and 1,8-dihydroxy-*4-nitro*-xanthenone (Figure 4). Recent structural studies support the view that the low K_i value of the latter compound is not accounted for by direct interaction(s) of its nitro group with structural elements in the active site of the kinase but is a consequence of an inductive effect on the dissociation constant of the phenolic groups.[50] Thus, at physiological pH, the nitro derivative is present in its anionic form, prone to strong electrostatic interaction with the positively charged side chain of Lys-68, as illustrated in Figure 3B. This interpretation is further validated by the finding that by increasing the pH from 7.5 to 9.5, the K_i value of 1,8-dihydroxy-xanthenone is significantly decreased, as expected due to the increased ionization of the phenolic group, while the K_i value of its 4-nitro-derivative is not affected.

Conclusions and Open Questions. All data available are consistent with the view that constitutive activity of CK2 is instrumental in its extraordinary pleiotropy and emerging role as a promoter of cell survival. In fact, CK2 is committed to the phosphorylation of so many targets, implicated in such a variety of cellular functions that a mechanism keeping its catalytic activity off unless in the presence of specific stimuli would not make sense. In this respect CK2 cannot be compared with "classical" protein kinases which operate in a hierarchical and "vertical" manner along one or few individual signaling pathways and whose activation is a sporadic requirement. In contrast, CK2 intervenes "transversally" on a multitude of pathways, behaving like an independent executor whose interplay with the signaling network is genetically

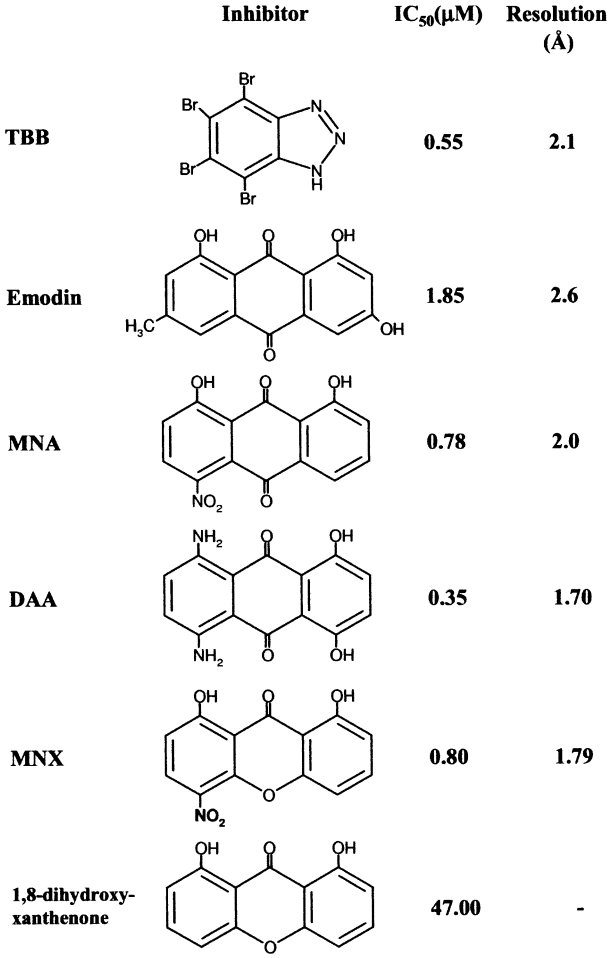

FIGURE 4. CK2 inhibitors whose structure in complex with maize CK2α has been solved. Based on data published elsewhere.[43,44,49,50]

predisposed but may be not readily adjustable. A circumstance this latter which could account for oncogenic potential, becoming evident whenever a deviation from the predetermined level of CK2 activity is accompanied by the deregulation of other protooncogenes. Its "global" role, however, does not exempt CK2 from the need of devices that regulate its targeting. In this respect there are emerging clues that despite constitutive catalytic activity, there are specific means of regulating CK2 activity at least within a given process. This is the case, e.g., of polymerase III regulation, where the turning off of CK2 is attained through the release of the catalytic subunits from the complex.[20] In a similar vein, CK2 activity has been reported to undergo stimulation and inhibition upon association with the activated p38 MAP kinase[51] and adenomatous polyposis coli (APC) protein,[52] respectively. A critical role in determining precise targeting by CK2 could be also played by the β-subunit, which, as outlined in Figure 2, cannot turn off nor on catalytic activity but can switch CK2 activity from one subset of substrates to another. In other words the possibility should be considered that within the cell virtually "active" CK2 molecules are de facto devoid of activity toward a large number of their potential targets depending on a number of as yet poorly understood regulatory devices.

The emerging role of CK2 as an antagonist of apoptosis and a promoter of cell survival "at all costs" raises the question of whether there are biological devices to silence its catalytic activity when the "battle is lost" and any further delay of programmed cell death would be detrimental to the whole organism. An enticing, albeit merely speculative, possibility would be that the interactions between the N-terminal tail and the activation loop, which are essential to keep the catalytic subunits constitutively active, could be disrupted, either reversibly by unknown effectors or irreversibly by proteolytic cleavage of the N-terminal segment. This latter event would be intriguingly reminiscent of the inactivation of MAP kinase kinase by lethal factor (LF) a component of antrax toxin. This has been shown to display proteolytic activity which cleaves a short N-terminal stretch of the MAP kinase kinase that is consequently inactivated.[53] It may be worthy to explore the possibility that CK2 undergoes a similar mechanism of inactivation by bacterial toxins or eventually by endogenous proteases expressed by the cell under conditions demanding apoptosis.

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