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The strange case of protein kinase CK2: how a constitutively active enzyme can mediate signal transduction

Lo strano caso della proteinchinasi CK2: come un enzima costitutivamente attivo può mediare la trasduzione del segnale

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Summary

Protein kinase CK2 is a Ser/Thr protein kinase composed of two catalytic (α and/or α') and two regulatory (β) subunits. It is ubiquitously expressed, constitutively active, and highly pleiotropic, with more than 300 protein substrates known so far (Meggio and Pinna, 2003); consequently, CK2 plays a key role in several physiological and pathological processes (Pinna, 2002; Ahmed *et al.,* 2002). Unlike the majority of the other protein kinases that are activated in response to specific stimuli by second messengers, phosphorylation events or association with regulatory molecules, CK2 activity is not regulated, thus understanding the mechanism by which it controls cellular events is challenging. Many of the efforts to understand its involvement in signaling pathways are directed to identify cellular substrates that are responsible for mediating its action in cells. This last consideration, together with the fact that the CK2 function in cells is well recognized to be pro-survival and anti-apoptotic, thus related to neoplastic transformation (Ruzzene and Pinna, 2010), has rendered CK2 a reasonable "druggable target" in many different pathologies in which it is involved (Guerra and Issinger, 2008), giving rise to the development of many specific inhibitors, useful not only for investigating its physiological role, but, possibly, also for therapy (Sarno and Pinna, 2008; Pierre *et al.,* 2011).

The main issue which gave rise to this work is therefore how can a constitutively active kinase mediate external stimuli, which need to be transient?

With this premise, we examined the CK2 involvement in different signaling pathways and in different contexts:

I. The role of protein kinase CK2 in the differentiation process of acute promyelocytic leukemia (APL) cells induced by retinoic acid (RA) treatment;

II. The role of CK2 in mediating plant response to salicylic acid (SA);

III. The CK2-dependent regulation of multimolecular chaperone complexes, under survival/apoptotic conditions, in normal and multidrug resistant (MDR) cells.

I. Role of protein kinase CK2 in the differentiation process of APL cells:

With this work we demonstrate that the CK2 activity is required for RA-induced APL cells differentiation since CK2 inhibitors block this response. Moreover, being the CK2 catalytic activity unchanged in response to RA, we focused on changes in the substrate phosphorylation pattern. A major change in phosphorylation revealed to concern β-actin, that was not known as a CK2 substrate before. By means of *in vitro*

kinase assays, we confirmed that actin is phosphorylated by CK2. Moreover, we found that RA induces an increase of β-actin expression level detectable both in the cytosol and in the nucleus; interestingly, the CK2 inhibition markedly reduced the nuclear β-actin increase in response to RA, suggesting a role of the CK2-dependent phosphorylation in actin nuclear translocation and its possible involvement in mediating the RA-induced APL cells differentiation.

II. Role of protein kinase CK2 in plant response to SA treatment:

SA treatment in plants is known to induce a complex cellular response, accompained by the production of nitric oxide (NO), which is prevented by inhibition of CK2 (Zottini *et al.,* 2007). We demonstrated that a major protein phosphorylated in *Arabidopsis* in response to SA treatment is the homologous of the human co-chaperone protein p23, known to be involved with Hsp90 in the cell chaperone machinery. Our experiments also showed that CK2 (from human and maize) phosphorylates *Arabidopsis* p23 *in vitro* with favourable kinetic parameters, and that endogenous *Arabidopsis* CK2 is the major kinase responsible for the phosphorylation of this protein. Moreover, we demonstrate that CK2 physically associates with p23 *in vitro* and that the two proteins co-localize *in vivo*. Although at present we do not know the exact function and effect of p23 phosphorylation. All together our results are consistent with a role of this protein in the requirement of CK2 for plant response to SA.

III. The CK2-dependent regulation of multimolecular chaperone complexes, under survival/apoptotic conditions, in normal and multidrug resistant (MDR) cells:

For this work we take advantage of a cell model, the CEM cells, which are available in two variants: the S-CEM, normally sensitive to drug-induced apoptosis, and the R-CEM, which are multidrug resistant (Dupuis *et al.,* 2003). This model is particularly interesting since we have previously demonstrated that R-CEM express a higher level of αCK2 respect to S-CEM. In these cells, we analysed a specific substrate of CK2, the co-chaperone protein Cdc37, a protein essential in the Hsp90 chaperone machinery committed to protein kinases folding and activation. We found that in the two cell variants, two different isoforms of Cdc37 are expressed: the protein in S-CEM is shorter at the C-terminus than that expressed in R-CEM. These two isoforms display different features: we found that Cdc37 of S-CEM is rapidly degraded in apoptosis, while the isoform of R-CEM is more stable, and, during apoptosis, undergoes the only

C-terminal cleavage. Surprisingly, despite the higher amount of CK2 exhibited by R-CEM , the CK2-dependent phosphorylation of Cdc37 Ser13 was not significantly different in the two cell lines. This suggested to us the possibility that, in dependence of the of the isoform expressed, Ser13 is differently accessible to the kinase and/or the phosphatase responsible for its modification. In agreement with this hypothesis, we found that during apoptosis, when the C-terminal part of Cdc37 in R-CEM is cleaved, a dephosphorylation of Ser13 also occurs. Moreover, the two Cdc37 isoforms seems to participate to different multimolecular complexes, as judge by their migration upon density gradient ultracentrifugation, and these complexes are differently susceptible to disruption by chaperone inhibitors.

In summary, in apoptosis resistant cells, a more fuctional chaperone machinery is observed, and CK2, expressed at particularly high levels in these cells, might exploit this substrate as a powerful tool to exert its anti-apoptotic function.

Riassunto

La proteinchinasi CK2 è un Ser/Thr chinasi composta da due subunità catalitiche (α e/o α') e due subunità regolatorie (β). E' ubiquitaria, costitutivamente attiva e altamente pleiotropica con più di 300 substrati noti sino ad oggi (Meggio e Pinna, 2003); proprio per queste sue caratteristiche, CK2 gioca un ruolo chiave in molti processi fisiologici e patologici (Pinna, 2002; Ahmed *et al.,* 2002). Diversamente dalla maggior parte delle altre chinasi, che in risposta a stimoli specifici sono attivate da secondi messaggeri, fosforilazioni o associazione con molecole regolatorie, l'attività di CK2 non è regolata, e questo rende particolarmente impegnativa la comprensione dei meccanismi tramite i quali essa controlla i diversi processi cellulari. Molti degli sforzi al riguardo sono diretti all'identificazione di substrati cellulari responsabili del suo intervento in tali processi. Queste considerazioni ed il fatto che è largamente riconosciuto il ruolo anti-apoptotico e pro-sopravvivenza che CK2 riveste nelle cellule, quindi legato alla trasformazione neoplastica (Ruzzene e Pinna, 2010), hanno reso CK2 un interessante bersaglio farmacologico nelle varie patologie nelle quali è stata riconosciuta avere un ruolo (Guerra e Issinger, 2008). Sono stati infatti sviluppati molti inibitori specifici per CK2, non solo allo scopo di studiare il suo ruolo fisiologico, ma anche, potenzialmente, per uso terapeutico (Sarno e Pinna, 2008; Pierre *et al.,* 2011).

La domanda principale che ha dato origine a questo lavoro di tesi è stata quindi: come può una chinasi costitutivamente attiva mediare stimoli esterni, che, per loro nature, devono essere transitori?

Con queste premesse, abbiamo quindi studiato il coinvolgimento di CK2 in differenti contesti e vie di segnalazione. I tre principali argomenti descritti in questa tesi sono:

I. Ruolo di CK2 nel differenziamento di cellule di leucemia promielocitica acuta (APL) in risposta ad acido retinoico (RA);

II. Ruolo di CK2 nella risposta delle piante al trattamento con acido salicilico (SA);

III. Regolazione da parte di CK2 di complessi *chaperone* multimolecolari, in condizioni di sopravvivenza ed apoptosi, in cellule normali e farmacoresistenti (MDR)

I. Ruolo di CK2 nel differenziamento di cellule di leucemia promielocitica acuta (APL) in risposta ad acido retinoico (RA):

Con questo lavoro, abbiamo dimostrato che l'attività di CK2 è necessaria per il differenziamento indotto da RA delle cellule di leucemia promielocitica acuta, infatti l'inibizione di CK2 blocca tale risposta cellulare. Inoltre, abbiamo dimostrato che l'attività catalitica di CK2 non è influenzata dal trattamento; abbiamo pertanto focalizzato la nostra attenzione su possibili variazioni di fosforilazione dei suoi substrati. Il principale cambiamento èstato osservato nel grado di fosforilazione della β-actina, una proteina mai annoverata sino ad ora tra i substrati di CK2. Attraverso saggi di fosforilazione *in vitro*, abbiamo confermato che l'actina è effettivamente un substrato di CK2. Abbiamo potuto inoltre appurare che il trattamento con RA induce un aumento dell'espressione della β-actina, individuabile sia a livello citosolico che nucleare; particolarmente interessante è risultato il fatto che l'inibizione di CK2 riduce fortemente tale incremento a livello nucleare, suggerendo un ruolo della fosforilazione di CK2 nella traslocazione della β-actina nel nucleo e, di conseguenza, la sua possibile importanza nel mediare l'effetto di RA sul differenziamento delle cellule APL.

II. Ruolo di CK2 nella risposta delle piante al trattamento con acido salicilico (SA);

Il trattamento con SA, nelle piante, è noto indurre una risposta di varia natura, accompagnata dalla produzione di ossido nitrico (NO), che viene però a mancare qualora CK2 sia inibita (Zottini *et al.,* 2007).

Con questo studio in *Arabidopsis*, abbiamo dimostrato che la principale proteina che viene fosforilata in risposta al trattamento con SA, è p23, una proteina omologa alla p23 umana che è un *co-chaperone* noto per essere coinvolto, con Hsp90, nel macchinario delle "chaperonine". I nostri risultati hanno dimostrato che CK2 (umana e di mais) fosforilano p23 di *Arabidopsis in vitro* con parametri cinetici favorevoli, e inoltre, che la CK2 endogena di *Arabidosis* è la principale chinasi responsabile della fosforilazione di questa proteina. Abbiamo anche dimostrato che CK2 e p23 possono interagire fisicamente *in vitro* e che hanno, *in vivo*, la stessa localizzazione subcellulare. Al momento non conosciamo la funzione di p23 e l'effetto della sua fosforilazione, ma, nell'insieme, i nostri dati sono coerenti con un suo ruolo nel mediare l'azione di CK2, in risposta all'SA nelle piante.

III. Regolazione da parte di CK2 di complessi *chaperone* multimolecolari, in condizioni di sopravvivenza ed apoptosi, in cellule normali e farmacoresistenti (MDR):

In questo lavoro ci siamo serviti del modello cellulare costituito dalle cellule CEM, che avevamo disponibili in due varianti: le S-CEM, che sono sensibili all'apoptosi indotta da farmaci, e le R-CEM, che sono invece farmacoresistenti (Dupuis *et al.,* 2003). Questo modello è particolarmente interessante, in quanto abbiamo dimostrato in precedenza che le cellule R-CEM esprimono un livello più elevato della subunità catalitica di CK2 rispetto alle S-CEM. In queste cellule, abbiamo analizzato un substrato specifico di CK2, la proteina *co-chaperone* Cdc37, che è essenziale nel macchinario *chaperone* di Hsp90 diretto al *folding* ed all'attivazione di proteinchinasi. Abbiamo visto che le due linee cellulari esprimono due isoforme diverse di Cdc37: le S-CEM esprimono una isoforma più corta al C-terminale rispetto all'isoforma presente nelle R-CEM. Le due isoforme dimostrano inoltre di avere caratteristiche diverse: quella delle S-CEM è rapidamente degradata in apoptosi, mentre l'isoforma delle R-CEM è più stabile e, in apoptosi, è sottoposta al taglio del solo C-terminale. Sorprendentemente però, nonostante l'elevata espressione di CK2α, nelle R-CEM, il livello della fosfo-Ser13, che è il residuo fosforilato in maniera specifica da CK2 in Cdc37, non è significativamente diverso nelle due linee cellulari. Questo ci ha suggerito l'ipotesi che, a seconda dell'isoforma espressa, la Ser13 sia diversamente accessibile alla chinasi e/o alla fosfatasi responsabili della sua modificazione. In accordo con ciò, abbiamo infatti osservato che in apoptosi, quando il C-terminale di Cdc37 delle R-CEM viene tagliato, si assiste anche alla defosforilazione della Ser13. Inoltre, le due isoforme sembrano partecipare a complessi multimolecolari diversi, come appurato tramite centrifugazione in gradiente di densità, che mostrano anche una diversa sensibilità alla disgregazione indotta da inibitori degli *chaperone*.
Nel complesso, i dati ottenuti suggeriscono che CK2, nelle cellule farmacoresistenti, dove è espressa a livelli particolarmente elevati, può servirsi di Cdc37, che in queste cellule sembra essere più funzionale, come un potente strumento per esplicare la sua funzione anti-apoptotica.

Aim of the study

In this thesis, three separate investigations are presented, but all of them concerned a special feature of protein kinase CK2, which, being a constitutively active enzyme, is not expected to respond to external stimuli but, is instead involved in mediating different signal transduction pathways.

Therefore we decided to focus our attention not on the CK2 catalytic activity, but mainly on its targets, trying to identifying proteins which change their CK2-dependent phosphorylation state in response to cell stimulation. The hypothesis considered was that, while the catalytic competence of the enzyme remains unchanged, the accessibility of some of its targets could be modified by a certain stimulus. Many explanations of this mechanism are possible, such as changes in protein structure (induced for example by phosphorylation operated by other signal-activated kinases), or changes in protein cellular localization (producing co-localization of CK2 and its substrates), or even changes in protein expression (making a certain substrate more readily available for phosphorylation). In this view, only one or few CK2 substrates are altered in their phosphorylation when cells are exposed to stimulation, but they are essential for the cell response, and they account for the requirement of CK2 in the transduction of the specific signal.

Within this general frame, a first aim was the investigation of the mechanism by which CK2 is required for the differentiation of acute promyelocytic leukemia (APL) cells treated with retinoic acid (RA); a second part of my work concerned the effect of salicylic acid (SA) on nitric oxide (NO) production in *Arabidopsis thaliana*, where CK2 is known to be required. A final part of the investigation was dedicated to a particularly important role of CK2, that is the regulation of the Hsp90/Cdc37 chaperone machinery; since this system is involved in the maintenance and activation of many protein kinases, it can represent a more general mechanism by which CK2, controlling the mediators of different stimuli, is a fundamental player in signal transduction.

Protein Kinases

Posttranslational modification by phosphorylation is a ubiquitous regulatory mechanism in both eukaryotes and prokaryotes. Phosphorylation, which affects an estimated one-third of all eukaryotic proteins (Cohen, 2001), is the most important and the best known reversible modification, consistent with its dominant role in protein-based signaling in eukaryotes. It is achieved by the largest family of enzymes, the protein kinases that in human are more than 500, and are often collectively referred to as the "kinome" (Manning *et al.*, 2002).

Addition of a phosphoryl group to serine, threonine, or tyrosine residues (in eukaryotes and occasionally in prokaryotes) confers properties that can have profound effects on protein conformation and function, thus changing the biological activity, stability, localization and interactions of the target protein (Johnson and Lewis, 2001). The simplicity, flexibility, and reversibility of this posttranslational modification, coupled with the ready availability of ATP as phosphate source, explains its selection as the most general regulatory device adopted by eukaryotic cells (Cohen, 2002). Because of the stability of the generated phosphate esters, other enzymes, protein phosphatases (Hunter, 1995), are required for their removal.

It is now recognized that many human diseases, including cancer, are frequently accompanied by abnormal phosphorylation, with perturbation of the activity of kinases and phosphatases which are mutated or altered in their expression. Nearly a half of the protein kinases encoded by the human genome are expressed from loci associated with specific diseases or regions amplified in human cancer (Blume-Jensen and Hunter, 2001; Manning, 2009; Brognard and Hunter, 2010). Moreover, naturally occurring toxins and pathogens often exert their pathogenic effects by altering the normal phosphorylation pattern of intracellular proteins (Cohen, 2001). Therefore, it is not surprising that the interest in elucidating protein phosphorylation machinery is growing up, and more and more protein kinases and phosphatases are becoming targets for drug development (Collins and Workman, 2006; Giamas *et al.,* 2007; Johnson, 2009; Eglen and Reisine, 2009).

Classification

Protein kinases are phosphotransferases belonging to one of the largest families of genes in eukaryotes. Based on the nature of the phosphoacceptor residue, two classes of protein kinases can be distinguished, the Ser/Thr- and Tyr-specific protein kinases. These enzymes use the γ-phosphate of ATP (or rarely GTP) to generate phosphate

monoesters using protein alcohol groups (on Ser and Thr) or protein phenolic groups (on Tyr) as phosphate acceptors. In addition to these two classes there are protein kinases belonging to the same family that phosphorylate histidine residues (Hanks and Hunter, 1995; Johnson and Lewis, 2001).

In eukaryotes, the protein kinase domain is the first, second, and third most common domain in the genome sequences of yeast, worm, and fly, respectively, indicating the importance of the phospho-signaling in these organisms (Johnson and Lewis, 2001). Phosphorylation on serine, threonine and tyrosine is approximately 86.4, 11.8 and 1.8%, respectively, in HeLa cells stimulated with epidermal growth factor (EGF) (Olsen *et al.,* 2006).

The completion of the human genome sequence project allowed the identification of the whole human kinome. Consequently, a new kinase classification was made by sequence comparison of their catalytic domains (Hanks and Hunter, 1995). The Hanks and Hunter human kinase classification was extended by Manning and co-workers (Manning *et al.,* 2002) and further implemented by more recent studies (Braconi-Quintaje and Orchard, 2008).

Classification takes into account the catalytic domain sequence similarity, but also other domain structures and the known biological functions, and refers to similar classification of the yeast, worm, and fly kinomes (Manning, 2002*). The total enzymes (518) predicted by Manning *et al.,* in 2002 were subdivided into 478 eukaryotic protein kinases (ePKs) and 40 atypical protein kinases (aPKs), but also 106 kinase pseudogenes were identified in the human genome. The aPKs lack sequence similarity to the ePKs catalytic domain but are known to have catalytic functional activity. Now it is believed that the number of human ePKs is 480 and, since the publication of the original list by Manning and co-workers, several more aPKs have been identified (Braconi-Quintaje and Orchard, 2008). The ePKs constitute one of the largest mammalian gene families comprising 1.7–2.5% of genes in eukaryotic genomes. The major ePK groups (Fig.1) are: tyrosine kinases (TK), cyclic nucleotide- and calcium-phospholipid-dependent kinases (AGC, which comprises PKA, PKG and PKC families), Ca^{2+}/calmodulin-regulated kinases (CAMK), the CMGC group [including cyclin-dependent kinase (CDKs), mitogen-activated protein kinase (MAPKs), glycogen synthase kinase (GSK), and casein kinase 2 (CK2)], homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases (STE), tyrosine kinase-like (TKL) and casein kinase 1 family (CK1); in addition an eighth group, RGC (receptor guanylate cyclase kinases),was added, and there is a further group labelled 'other'. The aPKs are

included since are proteins reported to have biochemical kinase activity, but which lack sequence similarity to the ePKs domain (Hanks, 2003; Park *et al.,* 2005; Braconi-Quintaje and Orchard, 2008).

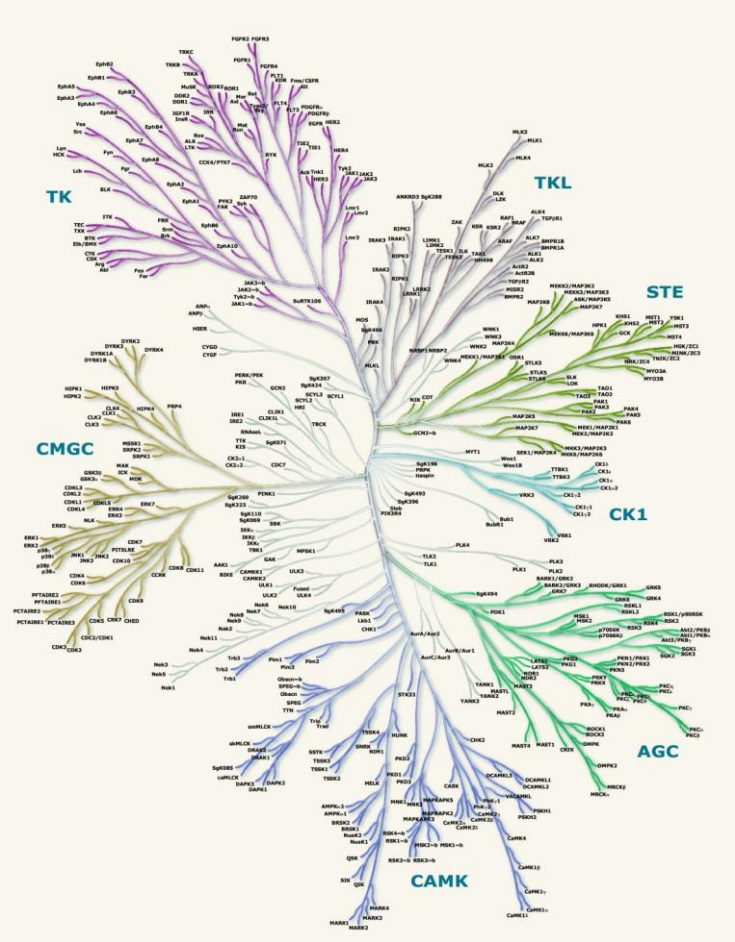

Figure 1. The Manning's kinome. The major groups of protein kinases are indicated. From Cell Signaling Technology.

Structure and function of protein kinases

Protein kinases transfer a phosphate group from nucleotides, ATP, or very rarely GTP, to a protein substrate molecule. Addition of a phosphoryl group affects the protein substrates conformation and function. At physiological pH, the double-negative charged phosphoryl group is likely to be dianionic, and its capacity to form extensive hydrogen-bond networks with the four phosphoryl oxygens confers special properties (Johnson and Lewis, 2001). Stoichiometry of phosphorylation is relatively low: in general, only a small fraction of the available intracellular pool of a certain

protein is phosphorylated at any given time as a result of a stimulus even in case of cells exposed to stimuli known to induce protein kinase activation (Mann *et al.*, 2002).
The highly conserved catalytic domain of about 300 amino acids of all eukaryotic protein kinases (Hanks e Quinn,1991) shows a very similar folding, as anticipated by sequence analysis, reflecting a common phosphotransferase mechanism. The structure comprises an N-terminal lobe mostly composed of β-sheets with one α-helix, termed C-helix, and a C-terminal domain that is mostly α-helical with a small amount of sheet. These two lobes are linked by a flexible hinge. The catalytic site for the ATP moiety is located into the deep cleft at the two lobes interface (Johnson and Lewis, 2001). The kinase domain of ePKs exerts three separate functions: binding and orientation of phosphate donor nucleotide as a complex with divalent cation (usually Mg^{2+} or Mn^{2+}); binding and orientation of the protein (or peptide) substrate; and transfer of the γ-phophate from ATP (or GTP) to the acceptor hydroxyl residue (Ser, Thr, or Tyr) of the protein substrate (Hanks and Hunter, 1995).
As protein kinases are implicated in a wide variety of cellular processes, but affect only one or few of the many potential phosphoacceptor residues in their targets, they must be endowed with very high selectivity (Pinna and Ruzzene, 1996). The pleiotropicity of protein kinases is extremely variable: some of them are dedicated enzymes, impinging on just one or few substrates (i.e. phosphorylase kinase), while others are very pleiotropic with hundreds protein targets subjected to their control (Salvi *et al.*, 2009). The sequence in the neighbourhood of a Ser/Thr or Tyr residue is an important determinant of specificity. Different protein kinases show different "consensus sequences" with respect to the neighbouring sequence of the residues to be phosphorylated (Fig. 2); however, this is not the only tool ensuring selectivity, in fact also co-localization of protein kinases and their substrates at distinct subcellular compartments and specific association mediated by targeting elements outside the catalytic domain, greatly enhance the specificity of the kinase reaction (Pinna and Ruzzene, 1996). Noteworthy, the recognition elements are not entirely located in the primary structure of the phosphoacceptor site but either constitute structural parts of the substrate protein distinct from the phosphorylation site or belong to it in the tertiary structure.

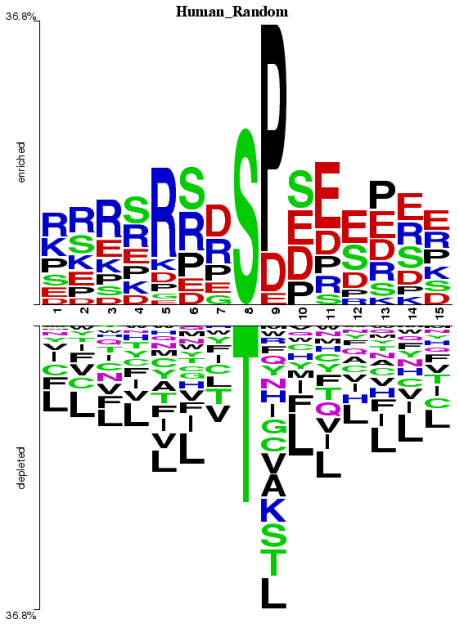

Figure 2. Two-sample logo based on 10,899 phospho-sites in humans (from Pinna, 2009, Screening, MedChem and ADMET Europe, Berlin, Germany). Upper, the most representative residues found in the neighbourhood of phosphorylated Ser/Thr in phosphopeptides; Bottom, the most depleted.

Protein kinases can exist in active and inactive conformations that are dependent on various different regulatory mechanisms (Johnson, 2009). The duration and extent of activation depends on the nature of the signal that induces the transition from the inhibited "off" state to the activated "on" state. The cell uses a multitude of mechanisms to control the activity of different protein kinases that include phosphorylation, regulation by additional domains that may target other molecules, binding of additional subunits, and protein–protein association. These controls operate mainly at two levels: via allosteric regulation of kinase activity and via co-localization of kinase and substrate protein. In addition to phosphorylating other proteins, many protein kinases are able to catalyse autophosphorylation reactions. The phosphate may be incorporated either in the enzyme catalytic domain or in the regulatory domain(s). One of the most dynamic regions of the protein kinase core, the activation loop, which retains the substrate in close proximity to the ATP binding site in such a manner that, in the presence of ATP, the kinase catalyzes the transfer of the phosphate moiety to the substrate, often contains one or more critical phosphorylation sites, and its phosphorylation represents a common tool regulating the activity of most protein kinases (Smith *et al.*, 1993).

Ser/Thr-specific protein kinases

Ser/Thr-specific protein kinases represent the majority of the kinome in eukaryotes, and they are divided in many different subfamilies. Among them, the following are included: protein kinases regulated by cyclic nucleotides (i.e. protein kinase A, PKA), diacylglycerol-regulated protein kinases (i.e. protein kinase C, PKC), Ca^{2+}/calmodulin-regulated protein kinases, G-protein-coupled receptor protein kinases, protein kinase CK1, protein kinase CK2, glycogen synthase kinase (GSK), cyclin-dependent kinases (CDK), and mitogen-activated kinases (MAP kinases). There are many other protein kinases that do not show any close relationship to these subfamilies.

It is worth to mention that among Ser/Thr protein kinases, enzymes with dual specificity are to be included, in that they can phosphorylate Ser/Thr and also Tyr residues. The only physiologically relevant example of dual specificity protein kinase is given by MAP kinase kinase (MEK) family (Pinna and Ruzzene, 1996).

Within the class of Ser/Thr-specific protein kinases, features underlying the preference for either seryl or threonyl residues remain unclear. As a general rule, Ser/Thr-specific protein kinases tend to prefer seryl over threonyl residues. Both from target sequences analysis and from studies with synthetic peptide substrates, it has emerged that Ser/Thr protein kinases are, with few exceptions, markedly sequence-specific. Indeed, they recognize specific consensus determinants in the substrate primary sequence that allow to divide Ser/Thr protein kinases class into three distinct groups: (1) basophilic protein kinases: they use basic and, often, also hydrophobic residues as specificity determinants, usually located in the N-terminal side of the target residue , this group includes the whole of the large ACG group, e.g. PKA, PKC, and all the members of CAMK group; (2) proline-directed protein kinases: they share the absolute requirement of the Ser/Thr-Pro motif and these comprise members of the CMGC group that belong to the families of both cyclin-dependent kinases and MAP kinases; (3) acidophilic and phosphate-directed Ser/Thr protein kinases: they use carboxylic and/or phosphorylated side chains as specificity determinants, among them there are CK2, GSK3, CK1 and the Golgi apparatus casein kinase G-CK (Pinna and Ruzzene, 1996). It should be noticed, on the basis of this classification, that a recent study (Salvi *et al.*, 2009) on the more updated phosphopeptides databases has shown that the majority of phosphopeptides appear to be not generated by basophilic protein kinase (the largest branch of Ser/Thr-specific protein kinases) but by proline-directed

and by acidophilic protein kinases. This latter represents a tiny minority of the kinome but, among them, CK2 might be responsible alone for the generation of a substantial proportion of the eukaryotic phosphoproteome.

Although some Ser/Thr-specific protein kinases are constitutively active in the cell, most of them are tightly regulated. The low basal activities of several regulatable protein kinases are often due to interactions of an "autoinhibitory" domain, located within the enzyme, with its catalytic site, thereby blocking binding of substrates. It has been proposed that the binding of allosteric activators induces conformational changes in the autoinhibitory domain that disrupt its interaction with the catalytic domain, hence activating the kinase to phosphorylate exogenous substrates (Soderling, 1990). An example of this regulation is provided by protein kinase CK1, which displays an autoinhibitory autophosphorylation site that *in vivo* is maintained in the dephosphorylated, active state by cellular protein phosphatases (Rivers, 1998).

Protein kinase CK2

Introduction

Protein kinase CK2 (acronym derived from the misnomer "casein kinase 2") is a Ser/Thr kinase member of the CMGC group of protein kinases (Manning *et al.,* 2002). This pleiotropic enzyme, ubiquitously expressed and constitutively active, is among the most highly conserved proteins thus indicating an important role in cell viability (Pinna, 1990). In fact by phosphorylating more than 300 substrates (Meggio and Pinna, 2003) distributed throughout the cell, CK2 demonstrates to be a regulator of fundamental cellular processes such as gene expression, cell cycle control, signal transduction, circadian rhythms, protein synthesis, apoptosis and tumorigenesis (Pinna, 2002; Ahmed *et al.,* 2002; Litchfield, 2003).

Structure and enzymatic features of CK2

In cells, CK2 exists primarily as an heterotetrameric (≈ 140 kDa) protein composed of two catalytic subunits, CK2α and/or CK2α' (42-38 kDa) and two regulatory CK2β (28 kDa) subunits. In mammals, the catalytic subunits exhibit greater than 80% sequence identity at the N-terminal (amino acids 1-329 of α and 1-330 of α'), despite being the product of distinct genes (Wirkner *et al.,* 1994; Yang-Feng *et al.,* 1994); on the contrary, the C-terminal domains are completely unrelated suggesting functional differences between the two isoenzymes (Lozeman *et al.,* 1990; Olsen *et al.,* 2008).
The human CK2 subunits were first cloned, bacterially expressed, purified and characterized by Grankowski and co-workers (1991). *In vitro* the recombinant catalytic subunits are active either alone or in combination with the regulatory subunits, whose presence however alters the protein substrate specificity (Pinna, 2002). The crystal structure of active tetrameric human recombinant CK2 (with a C-terminal deletion of about 60 amino acids) in the presence of Mg^{2+} and the non-hydrolysable ATP analogue AMPPNP, was determined by Niefind and co-workers (Niefind *et al.,* 2001), and revealed that the complex has the shape of a butterfly (Fig. 1), with a central building block composed of a CK2β dimer bridging the space between the two catalytic subunits; each CK2β monomer makes contacts with both CK2α subunits, which in contrast make no contact with each other; the shape of the complex resembles the ones from other kinases like c-AMP-depend protein kinases (Zhao *et al.,* 1998). In fact, both catalytic subunits show the typical bilobal structure of

the catalytic core of eukaryotic protein kinases composed of two major folding domains with the active site between them. The smaller N-terminal domain, whose constitutive element is an antiparallel β-sheet composed of five β-strand, is accompanied by a single long α-helix located next to the inter-domain cleft (helix αC) required for function, while the C-terminal domain is more constructed, having an α-helical fold, and at the inter-domain region, two small two-stranded β-sheets functionally important. Notably there is a strong attachment between N-terminal and activation segments (helix αC and activation loop) that is conserved between monomeric and tetrameric CK2, thus not influenced by CK2β. Each regulatory subunit can be roughly divided between an α-helical N-terminal domain, a Zn^{2+} containing domain (zinc finger) responsible for dimerization and a C-terminal tail that points away from the body of the CK2β, makes no contacts with its owner subunit and is stabilized by interactions with one CK2α and the other CK2β. In the N-terminal domain of CKβ, two autophosphorylation sites are present (Ser2 and Ser3) but their phosphorylation have negligible effects on CK2 kinetic parameters (Bodenbach *et al.,* 1994). In the tetramer structure, the CK2β-dimer arrangement is conserved despite complex formation, and the C-terminal tail of CK2β stabilizes the β/β and α/β contacts.

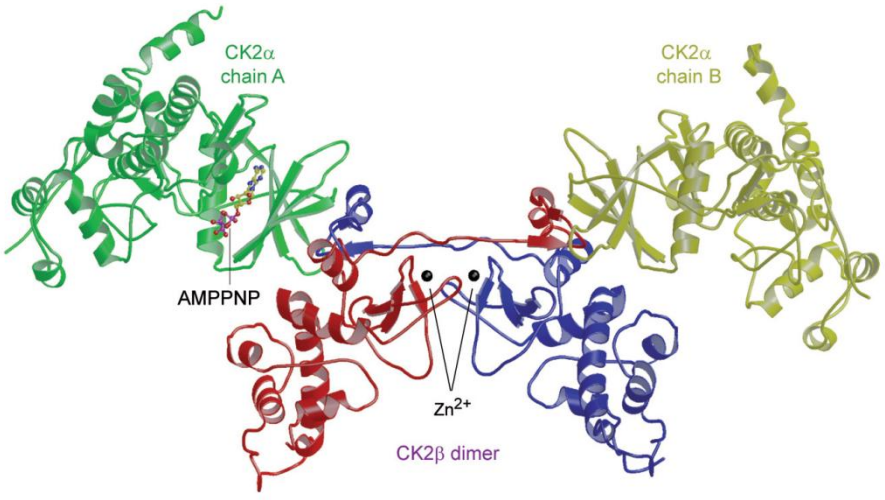

Figure 1. Overview of the CK2 holoenzyme complex. From Niefind and Issinger, 2010.

Either catalytic subunits make contacts with the CK2β dimer mainly through the N-terminal lobe, and precisely through part of the outer surface of the central β-sheet;

the main contact region, namely the glycine-rich ATP-binding loop and the β-strands on its sides, is the most important for the catalytic activity.

Essentially the CK2α crystal structure, either alone or in complex with CK2β, shows the stabilization of the activation segment in its active conformation, similarly to what happens in other regulated protein kinases in presence of their activating partners (e.g. cyclin-dependent protein kinases with cyclin), and this could be the explanation of its constitutive activity (Niefind *et al.,* 2001, 2009). The crystal structure determination therefore helped to clarify, even if not completely, the constitutively activity of this kinase, but also other peculiar enzymatic characteristics of CK2. In fact CK2 exhibits dual co-substrate specificity, being able to use both ATP and GTP as phosphate donors, and this is due to its particular binding cleft (Pinna, 2002; Niefind *et al.,* 1999). Moreover since the catalytic subunits of CK2 possess a large number of basic residues (including the basic cluster, $K^{74/75}KKKIKR^{80/81}$) at the beginning of the αC-helix, this works as substrate recognition site (Sarno *et al.,* 1997), and specificity is directed to proteins that possess multiple acidic residues near the phosphorylated site, in particular, downstream the phosphorylatable amino acid, that can be serine or threonine, acidic residues are preferred, with the minimum consensus sequence defined as X_{n-1}-S/T-X_{n+1}-X_{n+2}-E/D, where the presence of an acidic amino acid in position n+3 is crucial (Pinna and Ruzzene, 1996; Meggio and Pinna, 2003). Basic residues are extremely rare at any position between n-1 and n+4 in CK2 sites as they behave as negative determinants; the same applies to proline residues in position n+1, so in the consensus sequence described above X indicates any residue except basic ones or proline. Rarely, also tyrosine can be phosphorylated by CK2, as reported in the case of autophosphorylation (Chardot *et al.,* 1995; Donella-Deana *et al.,* 2001) and for other few cases (Wilson *et al.,* 1997; Marin *et al.,* 1999; Vilk *et al.,* 2008).

Regarding the CK2 activity regulation, as described above, CK2 is a constitutively active enzyme, due to its peculiar structure. So, at present, no regulatory phosphorylation events or physiological effectors are known. Therefore, the definition of CK2β as "regulatory" subunit can be considered not really proper. However, at least *in vitro*, it has been demonstrated that the β subunit has effects on the specificity, the stability and the activity of CK2; indeed, a classification of the CK2 substrates can be done with respect to the effect of CK2β on phosphorylation (Pinna, 2002; Fig. 2), since the CK2β subunit contains an acid region at the N-terminus (acidic loop residues 55-64) that down-regulates the activity of the enzyme towards some substrates; this inhibition could be counteracted by polylysine and increased ionic strength, resulting,

in vitro, in CK2 hyperactivation (Meggio *et al.,* 1994, Boldyreff *et al.,* 1994; Leroy *et al.,* 1997). In general, therefore, the catalytic activity of the holoenzyme is higher than that of isolated catalytic subunits; for some substrates the presence of CK2β is indispensable for substrate phosphorylation, however there are exceptions where the phosphorylation occurs only with the catalytic subunit and not with the holoenzyme; the phosphorylation of this latter group of substrates by the holoenzyme can be restored by polybasic peptides like polylysine (Pinna, 2002).

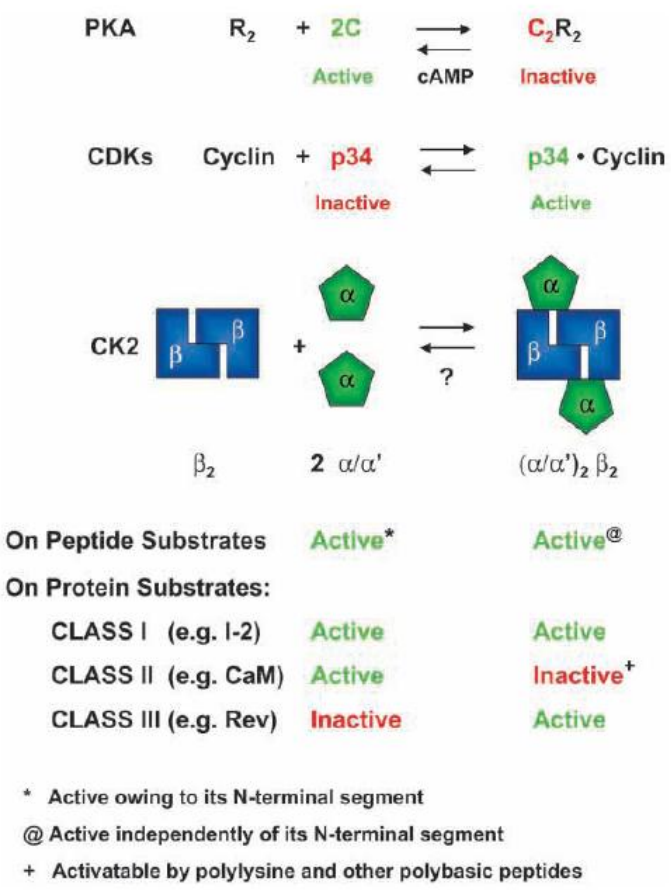

Figure 2. Mechanism of activation of PKA (Protein kinase A), CDKs (cyclin-dependent kinases) and CK2. For PKA, whose heterotetrameric structure is similar to that of CK2, the binding of cyclic AMP to the inactive tetrameric form leads to the release of the free active catalytic subunits. In the case of CDKs, the isolated catalytic subunits are inactive until their heterodimeric binding to the cognate cyclin and the phosphorylation of the activation loop. αCK2 is constitutively active and this is due to the unique interaction between the activation loop and its N-terminal segment and the association with βCK2 whose presence indeed influences the substrate specificity, summarized in the three classes: class I substrates phosphorylated with the same efficiency by both CK2 forms, class II substrates phosphorylated only by monomeric CK2 and by the holoenzyme only in the presence of polylysine or polybasic peptides and class III substrates phosphorylated only in the presence of βCK2.
From Pinna, 2002.

These observations corroborate the view of a regulatory subunit of CK2 that operates more as a targeting molecule and/or a docking platform for the binding of substrates and effectors and for the assembly of multimolecular complexes, rather than being a strictly regulatory element (Bibby and Litchfield 2005; Arrigoni *et al.,* 2008). This hypothesis has also to be taken in mind when the *in vivo* CK2 regulation is considered, since, due to constitutive activity of this enzyme, other mechanism of regulation should take place, like cell compartment shuttling or different subunit composition (Filhol and Cochet, 2009) and others recently reviewed by Montenarh (2010).

CK2 inhibitors

Since CK2 is involved in different pathological processes like tumorigenesis but also neurodegenerative, inflammatory, vascular and bone tissue diseases (Guerra and Issinger, 2008; Sarno and Pinna, 2008), the development of selective small molecule inhibitors, besides being useful for the dissection of the signaling pathways, represents also a potential source of drugs for human diseases. The distinctive CK2α binding cleft contributes to the binding of many inhibitors described to date: by its hydrophobicity surface, it establishes hydrophobic interactions and van der Waals contacts with the compounds; this, in addition to the shape and the reduced dimension of the CK2 active site in comparison to other kinases, accounts for the high specificity of many inhibitors, and, on the contrary for the low potency of staurosporine, which is a good inhibitor of most protein kinases but not of CK2 (Meggio *et al.,* 1995; Battistutta *et al.,* 2001; Mazzorana *et al.,* 2008). The importance of selectivity is also underlined by the number of studies in which CK2 inhibitors were tested on wide panels of different protein kinases (Prudent *et al.,* 2008; Pagano *et al.,* 2008; Sandholt *et al.,* 2009): they demonstrated that usually selectivity is very high, and only few other enzymes can be affected by some compounds, mainly PIM 1, 2 and 3, DYRK 1a, 2 and 3, HIPK2, 3 and in some cases GSK3β and CDK2.
The CK2 inhibitors can be divided in different families according to scaffold similarities (Cozza *et al.,* 2010):

- Benzoimidazole derivatives;
- Carboxyl acid derivatives;
- Anthraquinone, xanthenone, and fluorenone;
- Natural derivatives: ellagic acid, flavonoids, and coumarins;
- Pyrazolotriazine scaffold;
- Other ATP-competitive inhibitors;
- Non ATP-competitive CK2 inhibitors.

The protein kinase CK2 is one of the kinases with the highest number of known crystal structures in complex with inhibitors.
TBB (4,5,6,7-tetrabromobenzotriazole), one of the most used and studied inhibitor, belongs to the first group; it has a K_i=0.4 μM *in vitro* and it has been demonstrated to persistently inhibit CK2 *in vivo* inducing time- and dose-dependent cell death (Ruzzene *et al.,* 2002); moreover even if it inhibits also PIMs, DYRKs and HIPK2, the

IC_{50} for this kinases is 3-5 fold higher than for CK2 (IC_{50} =0.15 μM) (Pagano *et al.,* 2008).

Notable compounds in the second group are TBCA (tetrabromo cinnamic acid, K_i=0.077 μM) (Pagano *et al.,* 2007); IQA ([5-oxo-5,6-dihydro-indolo(1,2-a)quinazolin-7-yl]acetic acid, K_i=0.17 μM) (Sarno *et al.,* 2003) which does not affect DYRK1a and other compound from Cylene Pharmaceutical. This company from San Diego (CA, USA) among other CK2 inhibitors produces CX-4945, the first CK2 inhibitor in clinical trial, that is very effective (IC_{50} =2 nM) and selective as well (Pierre *et al.,* 2011).

To the third family belongs emodin (K_i=1.5 μM), a natural compound that has also antiviral and antimicrobial activity, but low specificity for CK2 since it inhibits also SGK, GSK3β and DYRK1a (Sarno *et al.,* 2003). Interesting examples in the same group are the anthraquinone inhibitors MNA and MNX: MNA has a good efficacy towards the CK2 holoenzyme (IC_{50} = 0.30 μM) but is much less effective towards the isolated catalytic subunit (IC_{50} = 2.5 μM); on the contrary, the closely related MNX is equally effective on holoenzyme and monomeric CK2 (IC_{50} =0.4 μM) (De Moliner *et al.,* 2003; Meggio *et al.,* 2004); they are therefore a useful tool to discriminate the activity of momomeric or tetrameric CK2 (Salvi *et al.,* 2006), and in particular, in CEM cell, these compounds were useful since multidrug resistant R-CEM cells express free catalytic αCK2 (Di Maira *et al.,* 2007).

Between natural compounds ellagic acid is a very potent (K_i= 20 nM) and specific inhibitor for CK2, with noteworthy ability to simultaneously interact with the hinge region and the phosphate-binding part of the CK2 ATP-binding pocket; actually, this represents a unique binding motif among all the known CK2 inhibitors (Cozza *et al.,* 2006; Sekiguchi *et al.,* 2009). Recently from a natural compounds screening, resorufin has emerged as a good inhibitor for CK2, being selective over a panel of 52 protein kinases (Sandholt *et al.,* 2009). The development of non ATP-competitive inhibitors has raised in recent years leading to the identification of compounds such podophyllotoxine indolo-analogues for the inhibition of CK2 assembly, polioxometalates and others (Laudet *et al.,* 2007,2008; Prudent *et al.,* 2008,2008*).

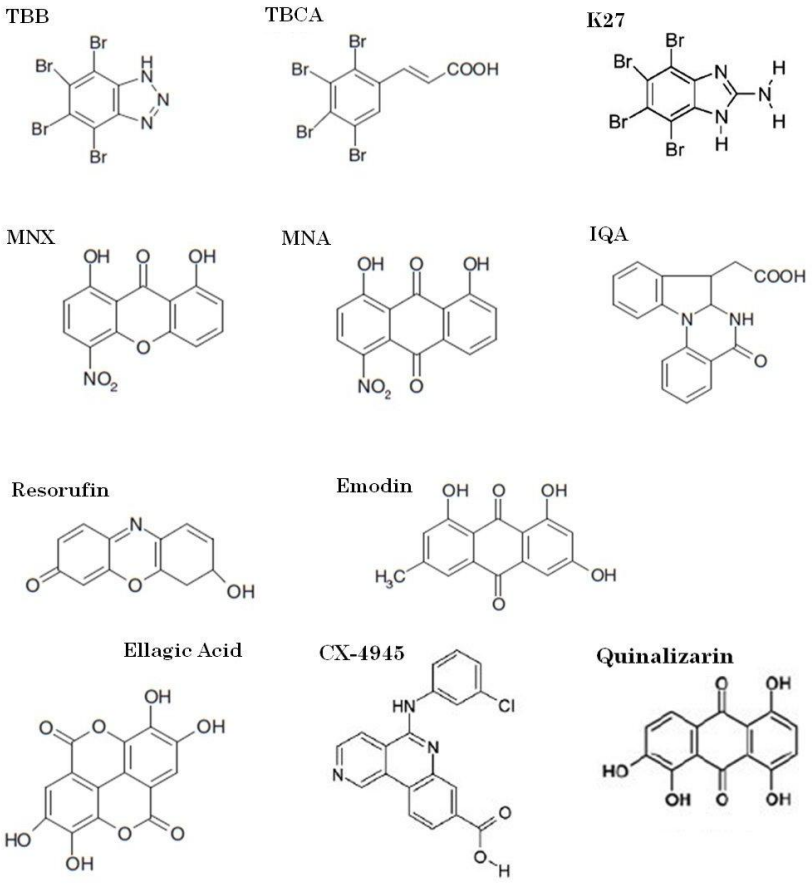

Figure 3. Structure of some CK2 inhibitors mentioned above and/or used in this thesis work.

Physiopathological role of CK2

CK2 is one of the most conserved protein kinases in evolution and the deletion of both its catalytic subunits is lethal in *S. cerevisiae* (Padmanabha *et al.,* 1990), *S. pombe* (Roussou and Draetta, 1994), *D. discoideum* (Kikkawa *et al.,* 1992) and in mouse (Lou *et al.,* 2008). Knockout mice lacking the CK2α' gene are viable but infertile with spermatogenesis defects (Xu *et al.,* 1999), while the depletion of both CK2β alleles leads to early embryonic lethality (Buchou *et al.,* 2003).
As said previously more than 300 substrates have been identified si far, and for most of them the phosphorylation has been demonstrated also *in vivo* (Meggio and Pinna 2003); but a recent WebLogo analysis, performed on a database of near 11000 naturally occurring phosphorylated sites, revealed that more than 20% of them display the unique acidic pattern that identifies CK2 consensus sites (Salvi *et al.,* 2009), underlying once more again the massive CK2 involvement in cell physiology.

CK2 and cell cycle

CK2 regulates many aspects of the cell life, first of all the cell cycle where it has been implicated in every progression stage. In yeast CK2 is required for G1/S and G2/M transitions (Glover, 1998) while in mammals it is required also for G0/G1 transition (Lorenz *et al.,* 1993, 1994; Pepperkok *et al.,* 1994). For example, CK2α forms a complex with and phosphorylates Cyclin H (Faust *et al.,* 2002; Schneider *et al.*, 2002), one of the components, with Cdk7 and MAT1, of the Cdk activating kinase (CAK) that activates in part, at the appropriate time, cyclin-dependent kinases (Cdks), important mediators of the cell cycle (Nasmyth, 1996; Lolli and Johnson, 2005). The CK2-dependent phosphorylation of cyclin H does not affect the CAK complex formation, but is critical for its full activity (Schneider *et al.*, 2002). In G1/S transition, CK2 has additional roles in regulating the initiation of DNA replication and preparation for division; essentially acting in the DNA damage checkpoint in addition to several proteins involved in checkpoint signaling, CK2 phoshorylates the tumor suppressor p53 and p53 regulatory proteins (Meek *et al.,* 1990; Cox and Meek 2010). Moreover other proteins involved in p53 function are CK2 substrates: SSRP1 whose phosphorylation decreases its DNA binding, halting transcription (Li *et al.,* 2005), and MDM2, which, when phosphorylated, is less efficient in p53 degradation and binding to retinoblastoma (pRb) protein (Götz *et al.,* 1999, 2005; Hjerrild *et al.,* 2001; Allende-

Vega *et al.,* 2005). Other CK2 substrates involved in the regulation of the cell cycle are Cdk inhibitor proteins such as $p21^{WAF1/CIP1}$ (Götz *et al.,* 2000; Romero-Oliva and Allende, 2001) and $p27^{KIP1}$ (Tapia *et al.,* 2004).

Interestingly, CK2 itself undergoes modifications during the cell cycle: during mitosis, it is phosphorylated in its regulatory and α catalytic subunit by Cdk1 (Litchfield *et al.,* 1991,1992; Bosc *et al.,* 1995), and it has been demonstrated that the disruption of the phosphorylation sites on the catalytic subunit impairs mitosis, suggesting that these sites are crucial for mitotic progression (St-Denis *et al.,* 2009). Moreover the phosphorylation of the catalytic subunit at the C-terminus by Cdk1 mediates its interaction with Pin1, a peptidyl-prolyl isomerase, and results in a decrease of the CK2 dependent phosphorylation of Topoisomerase IIα (Messenger *et al.,* 2002). CK2 is also involved in the G2/M transition and mitosis: it interacts with and phosphorylates many proteins involved in this process such as β-tubulin (Faust *et al.,* 1999), CDC25B (Theis-Febvre *et al.,* 2003), Tau and Microtubule-associated proteins 1A and 1B (Avila *et al.,* 1994), Condensin (Takemoto *et al.,* 2006), PP2A (Hériché *et al.,* 1997), Wee1, Plk1 and Cdk1 (Watanabe *et al.,* 2005), while its interaction via the regulatory subunit with checkpoint kinase Chk1 regulates the degradation of CDC25A, a phosphatase involved in the removal of inhibitory phosphorylations on Cdk1 and Cdk2 (Kreutzer and Guerra, 2007). Other protein interactors/substrates in the DNA damage checkpoint are the already mentioned Chk1 (Guerra *et al.,* 2003), Chk2 (Bjørling-Poulsen *et al.*, 2005), Topoisomerase II (Daum and Gorbsky, 1998; Escargueil *et al.,* 2000), BRCA1 (O'Brien *et al.,* 1999) and other cell cycle regulators substrates such Cdc34 (Block *et al.,* 2001), Cdk1 (Russo *et al.,* 1992), and Six1 (Ford *et al.,* 2000).

CK2 is also implicated in transcription regulation, and a consistent part of the CK2 substrates known to date are transcription factors (Meggio and Pinna, 2003). CK2 directly regulates the activity of human RNA polymerases I, II and III (Panova *et al.,* 2006; Lin *et al.,* 2006; Cabrejos *et al.,* 2004; Johnston *et al.,* 2002) and phosphorylates many transcription-associated factors: in the case of RNA polymerase I, we can mention upstream binding factor-UBF, selectivity factor 1 (SF1) subunit TAF_I100 and RNAP Iβ-associated Topoisomerase IIα (Voit *et al.,* 1992; Panova *et al.,* 2006), transcription initiator factor TIF-IA (Bierhoff *et al.,* 2008). The control of RNA Polymarase III (RNAP III) is mediated by phosphorylating the general transcription factor TFIIIB with both positive and negative effects (Ghavidel and Schultz, 2001; Hu *et al.,* 2003). In the case of RNA polymerase II, CK2 phosphorylates the polymerase

itself at the C-terminal domain (CTD) of the largest subunit, the phosphatase FCP1 responsible for CTD dephosphorylation, and other transcription factors associated to RNA polimerase II (Cabrejos *et al.,* 2004; Dahmus, 1981; Abbott *et al.,* 2005). Many other transcription factors are regulated by CK2 such NFκB (Wang *et al.,* 2000), CREB (Yamaguchi *et al.,* 1998), c-Myc (Bousset *et al.,*1994) and others, as reviewed by St-Denis and Litchfield (2009).

CK2 is also involved in the translation machinery; as an example we can mention that it phosphorylates eIF2β (Llorens *et al.,* 2006), eIF5 (Majumdar *et al.,* 2002, Homma *et al.,* 2005) and also others in plants (Dennis and Browning, 2009).

CK2 and apoptosis

The role of protein kinase CK2 in protecting cells from apoptosis has been deeply investigated, since it was evident that cell treatment with CK2 inhibitors produces cell death (Ruzzene *et al.,* 2002). Although a precise and comprehensive mechanism can not be easily described, at present numerous pro- or anti-apoptotic proteins are included among the CK2 targets, and many different roles of CK2 in counteracting apoptosis has been reported (reviewed in Litchfield, 2003; Ahmad *et al.,* 2008). A well-described and elegant mechanism is represented by the phosphorylation of proteins which consequently become refractory to the degradation by caspases, the key proteases which allow the execution of the apoptotic program. Proteins whose phosphorylation by CK2 leads to a protection from the caspase cleavage are Bid (Desagher *et al.,* 2001), Max (Krippner-Heidenreich *et al.,* 2001), hematopoietic lineage cell-specific protein 1 (HS1) (Ruzzene *et al.,* 2002), Presenilin-2 (Walter *et al.,* 1999), Connexin 45.6 (Yin et at. 2001), PTEN (Torres *et al.,* 2003) and caspase 9 (McDonnell *et al.,* 2008). Especially noteworthy is the observation that these proteins show an overlap between the CK2 consensus site and the consensus sequence for caspase cleavage suggesting a convergence of protein kinase and caspase signaling pathway (Duncan *et al.,* 2010) (Fig.3). Besides this regulation of caspase functions, acting at the level of the substrates, CK2 controls caspases also directly, as in the case of caspase 2: its phosphorylation by CK2 inhibits its dimerization and activation (Shin *et al.,* 2005).

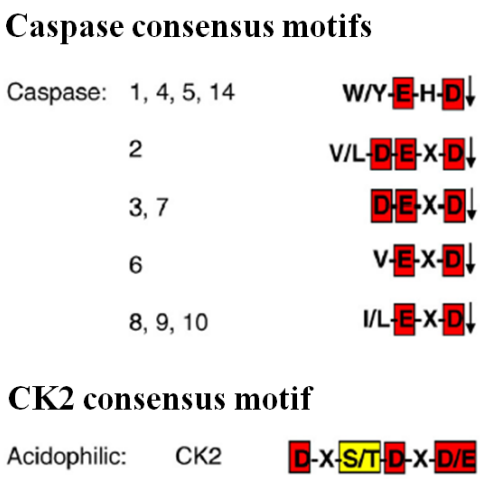

Figure 4. Convergence of caspases and CK2. Consensus motifs for caspases and for CK2 with acidic residues highlighted in red and phospho-acceptors in yellow. The arrows indicate caspase cleavage site. Modified from Duncan et al. 2010.

Another level of intervention of CK2 is the control of caspase effectors, such as ARC (apoptosis repressor with caspase recruitment domain), which requires CK2 phosphorylation to inhibit caspase 8 activation (Li *et al.,* 2002). Moreover survivin (member of the inhibitors of apoptosis proteins, IAP) is up-regulated whenever CK2 expression is increased (Tapia *et al.,* 2006). It has been also demonstrated that CK2 co-operates with other anti-apoptotic pathways to promote cell survival. This is the case of the CK2/Akt(PKB) connections: not only the already mentioned PTEN (Torres *et al.,* 2003) but also Akt itself (Di Maira *et al.,* 2005, 2009) are phosphorylated by CK2, with the final effect of potentiating its activity and amplifying its pro-survival potential.

A more general mechanism is represented by the phosphorylation/activation of the chaperone machinery which assists many different protein kinases; this mechanism, which will be described in details below, ensures the maintenance and the activaty of several pro-survival enzymes, whose function is therefore indirectly dependent or at least modulated by CK2.

Other survival signaling pathways potentiated by CK2 are those dependent on NF-κB and β-catenin transcription factors (as reviewed in Dominguez *et al.,* 2009). In the case of NF-κB, its inhibitor IκB is sent to proteolytic degradation when phosphorylated by CK2 (Chu *et al.,* 1996; McElhinny *et al.,* 1996), allowing the NF-κB transcription factor subunits to migrate to the nucleus and to function in transcription of pro-survival and anti-apoptotic genes. CK2 acts also at different levels in this pathway: it is involved in the regulation of the expression of IKK kinases (Eddy *et al.,* 2005) that are responsible for the canonical pathway of IκB degradation. Moreover, the p65 subunit of NF-κB itself is phosphorylated and regulated by CK2

(Wang *et al.,* 2000; Chantôme *et al.,* 2004). For β-catenin, the same result is obtained with an opposite mechanism, since its phosphorylation by CK2 preserves the protein from ubiquitination and degradation, and potentiates its effects in mediating the Wnt proliferation and survival signal (Song *et al.,* 2003). CK2 can affect this pathway at multiple levels (Song *et al.,* 2000), in fact in addition to the already mentioned β-catenin, CK2 phosphorylates several components of the "destruction complex" which targets β-catenin to degradation such as dishevelled (Dvl), promoting its stabilization (Willert *et al.,* 1997; Song *et al.,* 2000); moreover CK2 phosphorylates transcription factors of the T-cell-specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF) family, for which β-catenin acts as co-factor, facilitating their association to partner molecules (Wang and Jones, 2006).
Collectively, it is clear that CK2 has multiple, overlapping roles in promoting survival and preventing apoptosis, which almost certainly contributes to its oncogenic activity, as it will be described in a further paragraph.

CK2 and the chaperone machinery

As already mentioned, CK2 regulates the activity of the Hsp90/Cdc37 chaperone system, and thus the function of many signaling molecules with special reference to oncogenic protein kinases. CK2 was first linked to the Hsp90 chaperone-machinery when it was found to co-purify with a 90-kDa substrate that was later identified as Hsp90 (Meggio *et al.,* 1985; Dougherty *et al.,* 1987); in addition, CK2 was co-immunoprecipitated with Hsp90 from cell extracts (Miyata and Yahara, 1995). CK2 phosphorylates both α and β isoforms of Hsp90 *in vitro* and *in vivo*, and this phosphorylation may influence Hsp90 and clients activity (Szyszka *et al.,* 1989). Hsp90 forms two major chaperone complexes, one specific for the steroid hormone receptors, and one related to the protein kinase folding. In both of them, besides Hsp90, CK2 phosphorylates other proteins which exert co-chaperone functions. In the case of the steroid hormone receptors, CK2 phosphorylates FKBP52, a co-chaperone member of the immunophilin family (Miyata *et al.,* 1997; Cox *et al.,* 2007). In the case of the kinase-specific complex, CK2 phosphorylates Cdc37, a co-chaperone protein particularly relevant for the subject of this thesis, and whose action will be described in details in a specific chapter. Here, I anticipate that the CK2-dependent phosphorylation of Cdc37 at Ser13 (Ser14 in yeast) is essential for its co-chaperone function, which results in the stabilization and activation of many tumor-related

signaling protein kinases (Miyata, 2009-Fig.5). It has also been proposed that CK2 is itself a Cdc37 client kinase (Kimura *et al.,* 1997; Bandhakavi *et al.,* 2003) and that a positive feedback loop can occur between the two proteins in promoting multiple client kinases (Miyata, 2009).

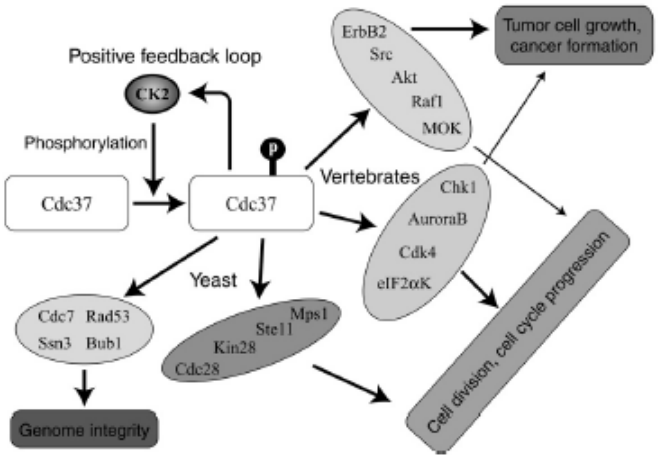

Figure 5. CK2 and Cdc37 constitute a positive feedback loop that results in promoting multiple client protein kinases in yeast and in vertebrates. CK2-dependent phosphorylation is essential for the role of Cdc37 in maintaining Hsp90-Cdc37-kinase heterocomplexes and thus the physiological function of multiple client protein kinases.
In the picture many other Cdc37-dependent signaling client kinases are omitted for simplicity.
From Miyata, 2009.

CK2 and cancer

Although oncogenic mutations of CK2 have never been reported, arguments supporting the implication of CK2 in cancer are numerous, as described previously, but the more convincing are that it is abnormally high, both in terms of protein and activity, in a wide variety of tumors respect to normal cells (Tawfic *et al.,* 2001; Unger *et al.,* 2004; Trembley *et al.,* 2009). High CK2 activity favours neoplastic growth also by enhancing the transforming potential of oncogenes (Seldin and Leder, 1995; Channavajhala and Seldin, 2002), by counteracting the efficacy of anti-tumor drugs like imatinib and melphalan (Mishra *et al.,* 2007; Piazza *et al.,* 2006), by contributing to the multi-drug resistant phenotype (Di Maira *et al.,* 2007) and by supporting neo-vascularization (Ljubimov *et al.,* 2004). The idea is that elevated CK2 is neither the causes nor the consequence of neoplastic transformation, but an element that benefits cancer cells; in this view the correlation between tumor malignancy and CK2 level seems to be consistent and useful as a prognostic marker (Unger *et al.,* 2004; Kim *et al.,* 2007). CK2 acts as an anti-apoptotic and pro-survival agent not only regulating general functions required for cell growth and proliferation as described above but also intervening in different, well characterized signaling pathways promoting survival and proliferation, which are often up-regulated in cancer. Among these, as

described above, we can mention the NF-κB, The Wnt/β-catenin, and the PI3K/Akt pathways (Dominguez *et al.*, 2009). All these examples highlight an atypical participation of CK2, which appears as a "lateral player" in "longitudinal" pathways (Ruzzene and Pinna, 2010). This means that the CK2 constitutive activity is not switch on by an external stimulus, but acts in response to it, on elements of a pathway that indeed respond to the signal. This view implicates that high levels of CK2 could ensure a favourable environment for the development of malignancy and has raised the concept that CK2 acts through a mechanism denoted as "non oncogene addiction" (Ruzzene and Pinna, 2010). According to this concept, once oncogenic mutation(s) take place in the genome, those cells where CK2 level reaches a critical threshold will be "selected" by the tumor and will be more prone to malignant transformation than cells where CK2 is normally represented. Some examples of this condition are T-cell acute lymphoblastic leukemia (T-ALL) that, even if not defective in PTEN, display lower PTEN activity due to its hyper-phosphorylation by over-expressed CK2 (Silva *et al.,* 2008); the loss of the tumor suppressor PML (promyelocytic leukemia gene) induced by a CK2-dependent degradation mechanism (Scaglioni *et al.,* 2006); the cooperation of CK2 and oncogenes, such as c-myc, whose malignant potential is increased by co-expression of CK2 (Seldin and Leder, 1995); the effect of NF-κB in inducing highly invasive mammary tumors, which is potentiated by CK2 (Belguise *et al.,* 2007). In conclusion, addiction to high CK2 levels represents a common denominator of several different kinds of tumors; this suggests that malignancy could be effectively counteracted by lowering abnormally high CK2 levels below a critical threshold, and that CK2 is a valuable druggable target, as demonstrated by the fact that despite the concern to target a kinase essential for cell viability, a CK2 inhibitor, CX-4945, has entered in Phase I clinical trials for different cancers with promising results (Pierre *et al.*, 2011).

CK2 and viruses

Being constitutively active and having a general pro-survival activity in the cells, CK2 is exploited by many viruses as a source of phosphorylation for viral proteins important for their life cycle (Table 1). The virus list include Epstein-Barr Virus (EBV) (Chi *et al.,* 2002; El-Guindy and Miller, 2004; Medina-Palazon *et al.,* 2007), Herpes Simplex Viruses (HSV) (Conner, 1999; Wadd *et al.,* 1999), Hepatitis B and C Viruses (HBV, HCV) (Enomoto *et al.,* 2006; Dal Pero *et al.,* 2007; Franck *et al.,* 2005),

Human Immunodeficiency Virus (HIV) (Meggio *et al.,* 1996), Human Cytomegalovirus (CMV) (Alvisi *et al.,* 2005), Human Papilloma Virus (HPV) (Firzlaff *et al.,* 1989), and Human T-cell Leukemia Virus-1 (HTLV-1) (Bidoia *et al.,* 2010).

The phosphorylation of many proteins of these viruses by CK2 may have different effects, ranging from enhanced nuclear localization (as for CMV) to regulation of viral enzyme activity (as for EBV and HIV).

Table 1. Viruses and their CK2-phosphorylated proteins

Virus	Viral protein	Function
Borna disease	P protein	Transcriptional cofactor of viral RNA polymerase
Canine distemper	P protein	Transcriptional cofactor of viral RNA polymerase
Measles	P protein	Transcriptional cofactor of viral RNA polymerase
Vesicular stomatitis	P protein	Transcriptional cofactor of viral RNA polymerase
Respiratory syncytial	P protein	Transcriptional cofactor of viral RNA polymerase
Bovine papilloma (BPV-1)	*E1*	ATP-dependent helicase activity
Hepatitis delta	Small HDAg	Nuclear phosphoprotein involved in genome replication
Epstein-Barr	ZEBRA protein	DNA binding and activation of transription from promoters of early viral lytic cycle genes
Epstein-Barr	EBNA-2	Indispensable for immortalization of B-lymphocytes *in vitro*
Influenza	PA polymerase subunit	Involved in polymerase action
Herpes simplex	VP22	Tegument protein involved in several aspects of viral replication
Herpes simplex	VP16	Induces expression of the viral immediate-early genes
Herpes simplex	Protein α22	Regulatory functions, *e.g.* to transactivate the transcription of viral genes
Herpes simplex	R1 subunit	Ribonucleotide reductase
Herpes simplex	Glycoprotein E	Blocking some host responses to infection
Varicella zoster human herpes v	Glycoprotein I (gpI)	Promotion of AP-1 recruitment onto Golgi membranes
Varicella zoster v	Gene 63 protein	Nuclear phosphoprotein
HIV	Vpu	Integral membrane phosphoprotein
Polyoma	VP1	Major capsid protein: in virus assembly and virus-cell recognition
Human papilloma	E7	Oncoprotein
Human papilloma	E7	Oncoprotein
SV40	Large T	Oncoprotein
SV40	Large T	Oncoprotein

Table 1. Examples of viral proteins phosphorylated by CK2. From Guerra and Issinger, 1999.

The involvement of CK2 in the life cycle of these viruses suggests that it may be a useful target for antiviral drugs: however, in order to successfully achieve this, much more needs to be known about the specific regulatory events in which CK2 is involved.

Importance and methods of CK2 activity assessment in cells

On the basis of what described so far, a detailed investigation of CK2 activity in cells can be particularly interesting, especially in cancer cells, where its quantitative measure may not only represent a criterion for "grading" malignancy, but also a prognostic marker to evaluate the propensity of cells to undergo neoplastic transformation. However, at difference with the majority of the other kinases, which are activated by specific phosphorylation on their molecules and whose *in vivo* activation can be detected with the aid of phospho-specific antibodies, CK2, as repeatedly said, lacks of any phosphorylation-dependent activation mechanism, and this prevents the analysis of its activity by simple western blot techniques. On the contrary, and fortunately, some of the CK2 features make its enzymatic quantification quite handy and reliable: in particular, its high catalytic activity towards peptide substrates, its peculiar site specificity, its ability to use both ATP and GTP as phosphate donors, and the availability of many inhibitors extremely specific for it. Exploiting these features, many tools are available to evaluate CK2 activity in crude extracts, and part of them where specifically developed during the work performed for this thesis (Ruzzene *et al.*, 2010).

The first developed and probably more important method to measure CK2 activity in cell lysates exploits the extreme specificity and acidophility of this kinase, and is based on specific peptides which are good targets of CK2, but unaffected by basophilic or proline-directed kinases, and also able to discriminate CK2 from the other rare acidophilic Ser/Thr protein kinases (Marin *et al.,* 1994). Determining the phosphorylation rates of these peptides in biological preparations (crude extracts, different kinds of cells and tissues) provides a first choice criterion to make a reliable comparative estimate of CK2 catalytic activity, and to establish if the level of this kinase is "regular" or abnormally high. Two different peptides have been successfully developed and employed for this aim, the CK2-tide $R_3AD_2SD_5$, and a peptide reproducing the N-terminal segment of the eukaryotic translation initiation factor 2β (eIF2β); while the CK2-tide is readily phosphorylated by either monomeric or tetrameric CK2, the eIF2β peptide displays the properties of class III substrates (Fig.2), since it is phosphorylated only by the holoenzyme; thus, the combined use of both peptides can be useful to discriminate between activity of the holoenzyme or the

isolated catalytic subunit (Salvi *et al.,* 2006; Poletto *et al.,* 2008). A common advantage of these peptides is the fact that they are extremely specific, and their phosphorylation in crude extracts containing many protein kinases can be ascribed definitely only to CK2.

The selectivity of the peptide assays can be further improved by replacing ATP with GTP. This strategy can be also advantageous when the CK2 activity has to be assayed with a protein substrate, notably casein that, unlike the peptides, can be phosphorylated by other kinases.

Another valuable method to assess CK2 activity in crude extracts is the so-called "in gel kinase assay" (Ruzzene *et al.*, 2010): in this kind of experiments, a protein substrate is included in the gel matrix, and cellular proteins are separated by SDS-PAGE, according to their apparent molecular weight in denaturing conditions; then, after proteins renaturation, the gel is incubated with a radioactive phosphorylation mixture, which allows the detection of the kinase activity as a radioactive band in the position where the catalytic subunit migrates. The applicability and sensitivity of this assay depends on the ability of the kinase to recover its active conformation once SDS is removed; CK2 displays a very good recovery of activity and, importantly, a full correlation between the radioactive signal detected and the amount of active kinase; therefore this method provides an accurate assay whenever a measure of the activity of the catalytic subunit alone (separated from its regulatory subunit) is required. It also allows to appreciate the contribution of the two different CK2 catalytic subunits possibly present in a biological sample (α and α', whose Mw is slightly different), or the generation of truncated form of CK2 catalytic subunits which are still active (Sarno *et al.*, 2002; Tapia *et al.*, 2002).

A commonly used tool to evaluate the in-cell protein phosphorylation is the ^{32}P-radiolabeling of intact cells; in the case of CK2, the availability of specific inhibitors (that are administered to the cells simultaneously to the [^{32}P]-orthophosphate) gives the great advantage of allowing the identification of the CK2-dependent phosphorylations (Ruzzene *et al.*, 2010).

As far as phospho-specific antibodies is concerned, they can be used to evaluate the phosphorylation state of known CK2 target sites; several of them have been identify and produced, such as the antibodies directed to pT117 of Bad (Klumpp *et al.*, 2004), pS129 of Akt1 (Di Maira *et al.*, 2005), pS17 of S6K1 (Panasyuk *et al.*, 2006), pS13 of Cdc37 (Miyata and Nishida, 2007), pS282 and pS559 of Estrogen Receptor α (Williams *et al.*, 2009), and pS201 of yeast Sic1 (Coccetti *et al.*, 2006). However, this

method of CK2 activity measurement based on phospho-directed antibodies critically depends on the cellular environment, and in particular on the expression of the protein whose phosphorylation has to be checked. To overcome this limit, another tool is represented by the possibility to overexpress a convenient CK2 target, whose phospho-specific antibody is available.

One of the most powerful tools, when CK2 activity is studied, is represented by a large panel of CK2-specific inhibitors (see previous paragraph). They are useful in many experiments, especially when it is necessary to establish if CK2 is actually responsible for the phosphorylation of a certain substrate, or when the dissection of signaling pathways suspected to be altered by abnormally high CK2 activity is pursued. It is important to note that many different and structurally unrelated CK2 inhibitors are available, and, in general, the use of more than one compound is recommended to be completely sure that CK2 is indeed the kinase involved. The concentrations required *in vivo* are higher than those effective *in vitro*; this is a general rule due to many factors, such as cell permeability, trapping and dilution in the medium and in cell compartments, high intracellular ATP concentration (being most inhibitors competitive with respect to ATP). Concentrations 50 to 100-fold higher than *in vitro* are frequently required to effectively inhibit the kinase in cells.

Another useful approach to evaluate CK2 activity in cell lysates is the so-called "autophosphorylation" of endogenous proteins: this is achieved by incubating cell lysates with a radioactive phosphorylation mixture without adding any kinase or other artificial phosphoacceptor substrate. Also in these experiments, the addition of CK2 inhibitors is essential to ascribe phosphorylation of a certain band to CK2, and, again, the use of GTP instead of ATP increases specificity. In this case the CK2 inhibitors should be added at concentration two- to five-fold higher than their *in vitro* IC_{50} and should induce effects similar to those observed if they are used for cell treatment. It is worth to note that these experiments do not necessarily correspond to real *in vivo* phosphorylation of substrates, since the accessibility of a target can be completely different in intact cells respect to cell lysates; however, they provide an idea of the global CK2 kinase activity in a certain cell type or condition, and give indications of possible specific substrates to be further investigated.

Of course, and especially for *in vitro* determination, studies on CK2 are often based on its expression in bacteria or in mammals cells followed by its purification. On the other hands, its activity can be modulated in cells essentially using transient overexpression or inducible expression vectors (Turowec *et al.,* 2010). In general, these

experimental procedures have the main purpose to identify the signaling pathways in which CK2 is involved, and to find out specific substrates, whose phosphorylation constitutes a fundamental aspect in signal transduction.

Role of protein kinase CK2 in the differentiation process of acute promyelocytic leukemia cells

Introduction

Acute myeloid leukemia (AML) represents a group of clonal hematopoietic stem cell disorders in which both failure in differentiation and overproliferation in the stem cell compartment result in accumulation of non-functional cells termed myeloblasts (Stone *et al.,* 2004). Acute promyelocytic leukemia (APL) is a distinct subtype of AML, morphologically identified as AML-M3 by the French-American-British (FAB) classification, characterized by a clear differentiation block of the granulocytic lineage at the promyelocytic stage. Cytogenetically, APL is characterized by reciprocal and balanced translocations always involving the retinoic acid receptor α (RARα) gene on chromosome 17 (Melnick and Licht, 1999). The majority of APL patients harbours the t(15;17)(q22;q21) translocation, in which the RARα gene translocates on chromosome 15 to the promyelocytic leukemia gene (PML), thus encoding the PML/RARα fusion oncoprotein (de Thé *et al.,* 1991; Goddard *et al.,* 1991; Kakizuka *et al.,* 1991; Pandolfi *et al.,* 1991). Rarely the RARα gene fuses to the promyelocytic leukemia zinc finger (PLZF) or the nuclear mitotic transducer (NuMA) gene both on chromosome 11 (Chen *et al.*, 1993; Wells *et al.,* 1997), or to the nucleophosmin (NPM) gene on chromosome 5 (Redner *et al.,* 1996), or to the signal transducer and activator of transcription 5b (STAT5b) on chromosome 17 (Licht *et al.,* 1995; Arnould *et al.,* 1999). Also in these cases the translocation (not reciprocal only in the case of NuMA and STAT5b) lead to the production of fusion proteins (Table 1). Interestingly, all of them are associated with leukemias morphologically recognized as APL, but the different fusion proteins are associated with different response to treatment with retinoic acid (RA) (Redner, 2002).

Table 1. Variant translocations

Translocation	Translocation partner	Product
t(11;17)(q23;q21)	PLZF (promyelocytic leukemia zinc-finger protein)	PLZF-RAR and RAR-PLZF
t(5;17)(q35;q21)	NPM (nucleophosmin)	NPM-RAR and RAR-NPM
t(11;17)(q13;q21)	NUMA (nuclear mitotic appararatus)	NUMA-RAR (no reciprocal product)
der(17)	STAT5b (signal transducer and activator of transcription)	STAT5b-RAR (no reciprocal product)

From Redner, 2002.

The majority of APL, respond in fact to pharmacological doses of all-trans retinoic acid (ATRA), an active metabolite derived from vitamin A, that was introduced in APL therapy in 1980s and changed the history of this disease, leading to a dramatic clinical improve of patients and to the concept of differentiation therapy for cancer (Huang *et al.,* 1988; Castaigne *et al.,* 1990; Sell, 2005; Petrie *et al.,* 2009; review on history of APL differentiation therapy by Wang and Chen, 2008).

Vitamin A and its active derivatives, referred to as retinoids, are non-steroid hormones which exert their action by regulating the expression of specific genes within target tissues (Collins *et al.*, 2008), thus playing a critical role in development and homeostasis, regulating cell differentiation, proliferation and apoptosis (Ross *et al.*, 2000; Altucci and Gronemeyer, 2001). The retinoid signal is transduced by two families of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs); each family consists of three isotypes (α, β and γ) encoded by separate genes (Leid *et al.*, 1992; Mangelsdorf and Evans, 1995; Chambon *et al.*, 1996) and work as RXR/RAR heterodimers (Kastner *et al.*, 1997; Mark *et al.*, 1999). RARs are activated by all-trans retinoic acid (ATRA) and its 9-cis isomer, while RXRs are only activated by 9-cis retinoic acid (RA). In the absence of ligand, retinoid receptors are found primarily in the nucleus, where they act as RAR/RXR heterodimers and binds to specific DNA sequences termed RA response elements (RAREs); this results in transcriptional repression through the recruitment of corepressors such as NCoR and SMRT and others (Dilworth and Chambon, 2001). The binding of ATRA to the receptors causes a conformational change that results in dissociation of co-repressors and recruitment and association with co-activators thus leading to transcription activation (Bastien and Rochette-Egly, 2004).

In the pathogenesis of APL, the PML/RARα acts in a dominant-negative manner, being able to form homodimers sequestering RXR and/or PML proteins leading to a repression of transcription of RARα genes through the recruitment on RAREs of co-repressors (CoR) (de Thé *et al.,* 1991; Lin and Evans, 2000; Di Croce *et al.,* 2002); since RARα signaling regulates myeloid differentiation (Kastner *et al.,* 2001; Kambhampati *et al.,* 2004) its inhibition explains the block in differentiation in APL cells.

The addition of pharmacological concentrations of ATRA convert PML–RARα into a transcriptional activator, thus enhancing expression of crucial RARα targets and restoring the normal differentiation. However, the only deregulated RARα transcriptional activity is not sufficient to explain APL pathogenesis, and several other PML-RARα additional properties have been reported to contribute to the APL

phenotype (de Thé and Chen, 2010). The dominant-negative action of PML-RARα is exerted also by sequestering and impairing the function of PML (Scaglioni and Pandolfi, 2007). PML is a tumor suppressor that plays a critical role in growth control, transformation suppression, induction of apoptosis, and replicative senescence, it localizes to nuclear matrix-associated macromolecular structures known as PML nuclear bodies, which are dependent on PML for assembly (Shen *et al.,* 2006). PML-RARα disrupts PML nuclear bodies through its ability to bind to PML (Bernardi and Pandolfi, 2007), abrogating its functions and contributing to APL phatogenesis (Lallemand-Breitenbach and de Thé, 2010).

The role of CK2 in AML has not been extensively studied, but its indirect involvement in APL disease was suggested since it was found that CK2 regulates PML protein levels by phosphorylating it and promoting its ubiquitin-mediated degradation (Scaglioni *et al.,* 2006). Actually, direct evidences of the CK2 involvement in AML were provided by very old studies, which showed that CK2α is almost undetectable in normal granulocytes, but markedly increased in highly proliferating myeloblastic cells from patients with acute myelogenous leukemia (AML) or with chronic myelogenous leukemia in blastic crisis (BC-CML) (Phan-Dinh-Tuy *et al.,* 1985). A more recent study (Kim *et al.,* 2007) confirmed that the CK2α level is low in bone marrow specimens and elevated in a substantial proportion of AML cells from the patients examined, with a correlation between CK2α levels and activity; furthermore in AML patients with normal karyotype, the disease-free survival and overall survival rates were lower for the patients with high CK2α expression levels respect to the ones with low CK2α expression levels, indicating that CK2 could be an unfavorable prognostic marker in AML. Moreover CK2 inhibition through cell permeable inhibitors was able to induce apoptosis especially in those cells expressing high levels of CK2α, in particular among them the NB4 and HL60 cell lines that are acute promyelocytic leukemia cell lines. Another paper reports the synergistic effect of CK2 and PI3K/Akt inhibition in inducing apoptosis in human myeloid leukemia cells, while sparing healthy hematopoietic stem cells (Cheong *et al.,* 2010).

Unpublished results from Dr. F. Piazza (Padua, Italy) show that, while almost undetectable in normal granulocytes, CK2 is markedly increased in highly proliferating myeloblasts from patients with AML or with chronic myelogenous leukemia in blast crisis, as well as in APL cell lines, and this is in agreement to what found also by others. Moreover CK2 inhibition by specific inhibitor, or knockdown by RNA interference, affects the G1 arrest of APL cells, and prevent the phenotypical,

morphological and functional differentiation normally triggered by retinoic acid. It has also been found that RARα-dependent transcription was greatly impaired by CK2 inhibition. With these premises we investigate the role of CK2 in the differentiation process of NB4 cells, an APL cell line: we wondered if its requirement for the cell response to retinoic acid was related to a change in its catalytic activity (not expected for a constitutively active enzyme), or rather to the phosphorylation of some specific proteins with a role in cell differentiation.

Results

RA-induced differentiation of NB4 (APL) cells is inhibited by CK2 specific inhibitors

In order to evaluate the role of CK2 in the RA-induced differentiation in APL cells, firstly we tested the effect of CK2 inhibitors on the differentiation of NB4 cells (an APL cell line).

As shown in Fig. 1, the RA effect is easily detectable with the NBT assay for cell differentiation, but the combined treatment of cells with RA and the CK2 inhibitors TBB and K27 (Pagano *et al.,* 2004) inhibited cell differentiation. On the contrary, cell viability was unaffected by CK2 inhibition (Fig. 1), as expected considering the low doses used of the inhibitors, which were able to induce to induce cell death only at concentrations higher than 25 μM (not shown).

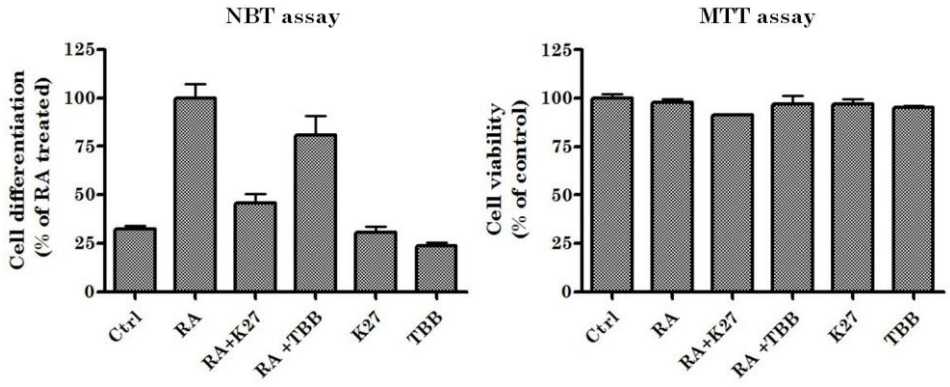

Figure 1: Cell differentiation and viability assays. Cell were plated 5 x 10^5/ml and treated for 48h with vehicle (Ctrl), 9-cis retinoic acid (RA, 1 μM) and the CK2 specific inhibitors K27 (5 μM) or TBB (5 μM) with RA or alone. Cells were then incubated with NBT (0.5 mg/ml) and activated with PMA (0.1 μg/ml) 1 h prior to the end of treatment. For MTT assay, treated cells were incubated with MTT (0.5 mg/ml) 1 h prior to the end of treatment. In both cases, the absorbance of the formazan produced was measured at λ=570nm. Vertical bars indicate the standard deviation to the mean obtained from three separated experiments.

CK2 expression and activity do not change upon RA treatment

Since we and others (Piazza *et al.,* unpublished) showed that CK2 is involved in the differentiation process induced by RA, we investigated if this treatment caused any change in CK2 expression and/or activity. We treated NB4 cells for different times (1-6-24-48 hours) with RA alone or in combination with K27 or TBB, and we found that the CK2 subunits α, α' and β (not shown) levels did not change upon RA treatment at any time tested (Fig. 2).

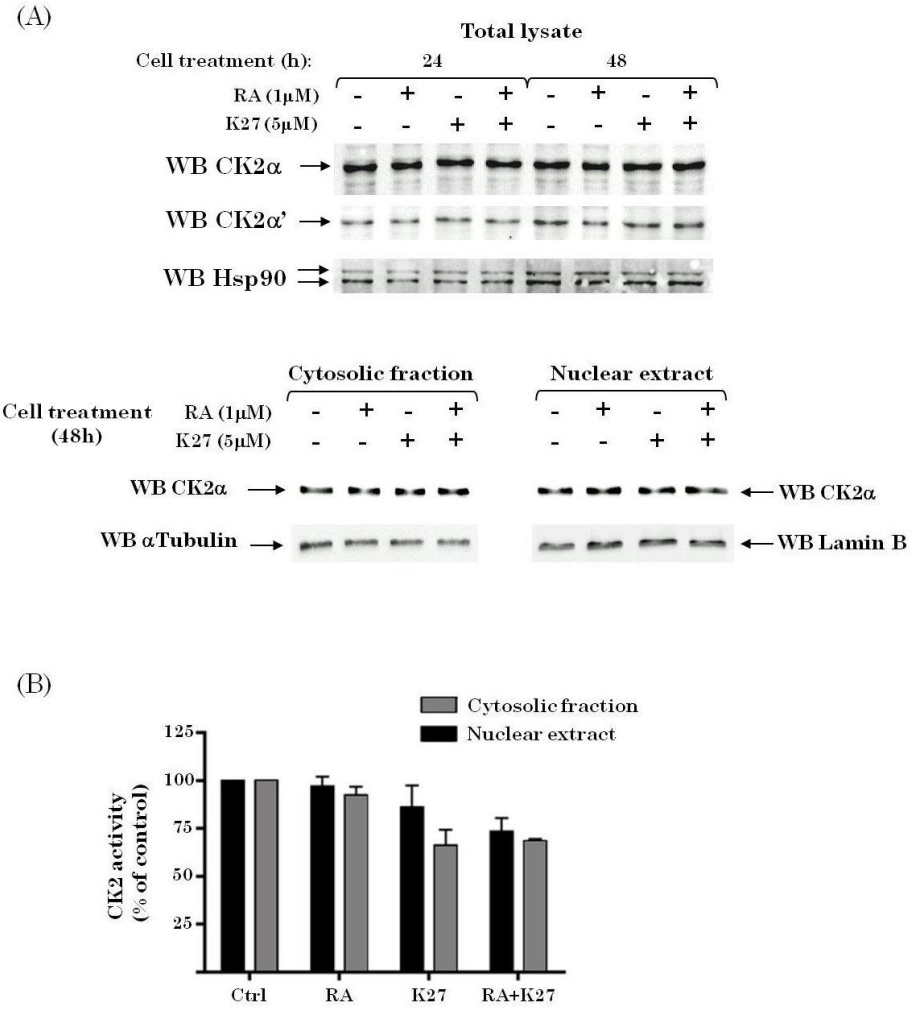

Figure 2: CK2 expression and activity in treated cells. Cells were treated as indicated and lysed differently to obtain a total cell lysate or cytosolic fraction and nuclear extract. (A) 10 μg of proteins from the different lysates were analysed by SDS-PAGE and WB with the indicated antibodies Hsp90 was used as a loading control for total lysates, while α-tubulin and Lamin B for cytosol and nuclear extracts, respectively. (B) **E**ndogenous CK2 activity in cell lysates was measured on the specific peptide $R_3AD_2SD_5$. Vertical bars indicate the standard deviation to the mean obtained from three separated experiments. See "Materials and Methods" for details.

We analysed the CK2 expression in total lysate and also in the cytosolic and nuclear fractions (Fig. 2A) to confirm that the unchanged level of CK2 corresponds also to an

unchanged distribution in the cell compartments. The CK2 activity in the cytosol and in the nuclear extract was also not influenced by RA, but as expected, it was reduced by CK2 inhibitors. Since there was no change in the CK2 global catalytic activity, we decided to analysed the protein phosphorylation pattern, performing the phosphorylation of the endogenous proteins of cells treated as described above.

When we looked at the phosphorylation pattern of endogenous proteins, we found that K27 alone, at these doses, only induced a slight decrease of the radioactive bands (not shown); on the contrary, there were many proteins whose phosphorylation level was affected by RA treatment, in particular in the cytosolic fraction. We focused on those bands whose phosphorylation was increased by RA and decreased by simultaneus addition of K27, suggesting that they were putative CK2 substrates (Fig. 3A). We tried to enhance the differences, performing the same experiment avoiding the addition of protein phosphatase inhibitors to the lysis buffer, to raise the number of

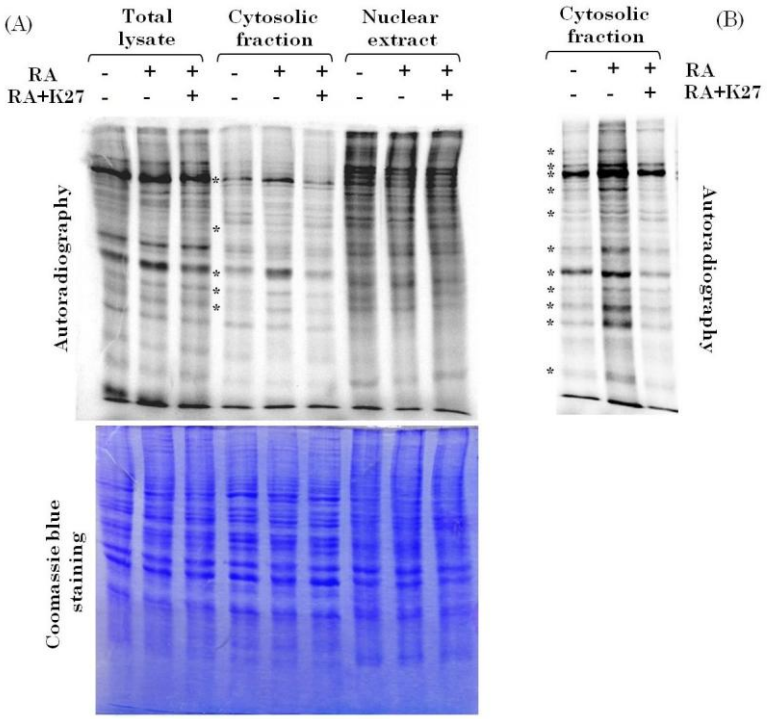

Figure 3. RA-induced CK2-depend protein phosphorylation pattern. (A) 10 μg of proteins of cell total lysate, cytosolic fraction or nuclear extracts from NB4 cells treated 48h as indicated (control (–) with vehicle, 1 μM RA, 1 μM RA + 5 μM K27) were incubated with a radioactive phosphorylation mixture (see "Materials and methods" for details. Proteins were separated by SDS-PAGE and analysed by Coomassie blue staining (shown in the lower part of the figure) as loading control and by autoradiography. (B) The same experiment was performed using lysates without protein phosphatase inhibitors; the autoradiography of the gel is shown. Asterisks denotes those bands whose radioactivity was increased by RA and decreased by K27.

dephosphorylated sites that were accessible to the kinases present in the lysates (Fig. 3B): indeed we obtained an increased number of the CK2-depend RA-induced phosphorylated bands.

β-actin phosphorylation is increased in response to RA treatment

With the aim to identify the CK2 substrates involved in RA-induced cell differentiation, we chose the cytosolic fraction, where the phosphorylation changes were more evident (see Fig. 3), and we performed two-dimensional gel electrophoresis experiments. We focus on those radioactive spots that having comparable staining intensity, but a high variation in their phosphorylation degree; in particular, we selected spots more phosphorylated in response to RA, but not when also a CK2 inhibitor (K27 or TBB), was present and we sent them to mass spectrometry analysis.

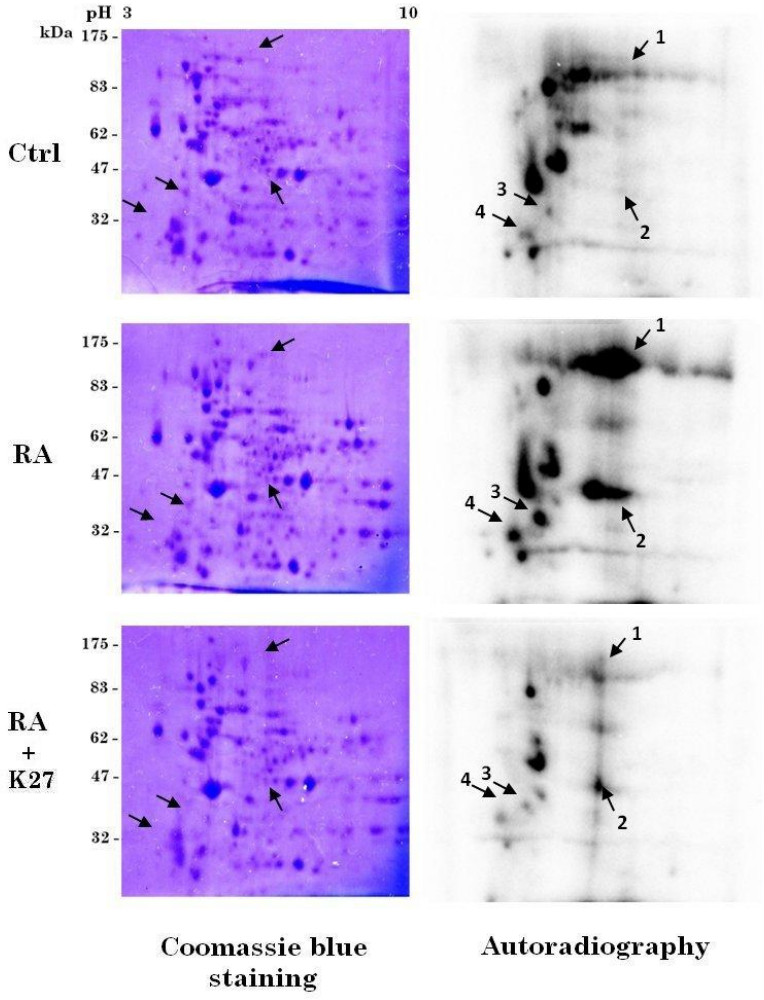

Figure 4. 2D-PAGE analysis of radioactive phosphorylation of cytosolic proteins from NB4 cells, treated for 48 h with vehicle (Ctrl) or with 1 μM RA, alone or in combination with 5 μM K27. 1st dimension: IEF, pH 3-10; 2nd dimension: 11% SDS-PAGE. Arrows indicate spots whose radioactivity was induced by RA and reduced by CK2 inhibitors, and that were excised and analysed by mass spectrometry for identification. Three separate experiments were performed with similar results.

The Fig. 4 shows a 2D-PAGE representative experiment, in which four main spots (indicated by the arrows) were assigned as RA-induced and CK2-dependent.

From the mass spectrometry analysis, we were able to identify three proteins (in at least two separated experiments) out of the four spots cut: the spot indicated as 1 was not unambiguously identified, spots 3 and 4 were identified as nucleophosmin (B23) and nascent polypeptide associated complex α (αNAC), respectively, both are already known as CK2 substrates (Lawson *et al.,* 2005; Quélo *et al.,* 2005); spot 2 was identified as β-actin, which, interestingly, has never been reported to be phosphorylated by CK2 so far. β-actin is a highly conserved protein that forms cytoskeletal microfilaments but plays many other functions; it is also present in the nucleus, where it is supposed to be involved in several process, such as transcription and chromatin remodelling (Vartiainen, 2008; Skarp and Vartiainen, 2010).

Therefore, we focused our attention on β-actin to confirm that its phosphorylation by CK2 changed in NB4 cells treated with RA, in combination or not with CK2 inhibitors. We performed again the phosphorylation of endogenous proteins of lysate from NB4 cells treated as mentioned above, and we detected β-actin by Western blot with the specific antibody. As expected, we found that β-actin co-migrates with one of the major CK2-dependent phosphorylated bands evoked by RA treatment (Fig. 5A); to confirm that CK2 is actually responsible for the phosphorylation of this band we performed the same experiment in the presence of different kinase inhibitors, and we observed that only CK2 specific inhibitors (TBB, K27, both *in vitro* and *in vivo*, and IQA; Cozza *et al.,* 2010) were able to reduce the phosphorylation of this band, while staurosporine, an aspecific kinase inhibitor not affecting CK2 (Meggio *et al.,* 1995), was ineffective (Fig. 5B). As clearly evident from the Western blot presented in Fig. 5A, it turned out that the amount of β-actin significantly increases in response to RA, both in the cytosol and in the nucleus; it is therefore conceivable that the increased phosphorylation is only apparent, being actually due to a greater amount of the protein. However, it is interesting to note that CK2 inhibition, while having no effects on the β-actin amount in the cytosol, prevents its increase in the nucleus (Fig. 5C), suggesting that CK2 activity is required for β-actin nuclear localization. This is already detectable after 6h of RA treatment, but it becomes much more evident at longer times.

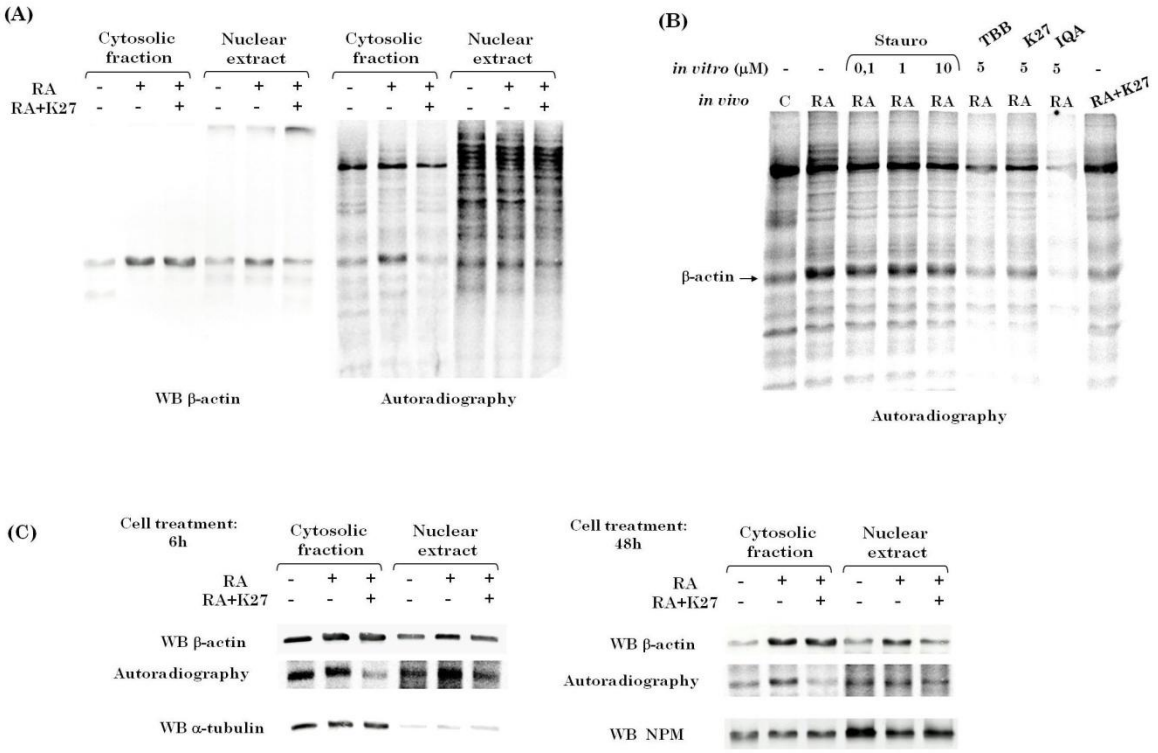

Figure 5. β-actin phosphorylation and expression in response to RA. (A) NB4 cells were treated for 48h as indicated (where present RA was 1 μM, K27 was 5 μM); 10 μg of cytosolic or nuclear proteins were incubated with a radioactive phosphorylation mixture and loaded on 11% SDS-PAGE and analysed by WB for β-actin antibody (left panel) followed by autoradiography (right panel). (B) NB4 cells treated for 48h as indicated "*in vivo*" (1 μM RA, 5 μM K27); 10 μg of cytosolic proteins were incubated with a radioactive phosphorylation mixture, with the addition of the indicated concentrations of different protein kinase inhibitors ("*in vitro*", Stauro indicates staurosporine); samples were then separated by 11% SDS-PAGE and blotting, and the autoradiography of the blot is shown. The migration of β-actin is also indicated. (C)NB4 cells were treated as indicated (RA was 1 μM, K27 was 5 μM) for 6h (left panel) , or 48h (right panel).); 10 μg of cytosolic or nuclear proteins were incubated with a radioactive phosphorylation mixture and separated 11% SDS-PAGE followed by blotting. The WB for β-actin and the radioactivity of the corresponding area of the blots are shown. WB for α-tubulin or nucleophosmin (NPM) were used for ormalization.

β-actin is phosphorylated by CK2

Analysing the β-actin sequence, we found that it displays some consensus sites for CK2 (S/T-X-X-Acidic residues, Meggio and Pinna, 2003) (Fig.6), so we decided to investigate if CK2 directly phosphorylates β-actin *in vitro*.

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MDDDIAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQS 60
KRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKMT 120
QIMFETFNTPAMYVAIQAVLSLYASGRTTGIVMDSGDGVTHTVPIYEGYALPHAILRLDL 180
AGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKSY 240
ELPDGQVITIGNERFRCPEALFQPSFLGMESCGIHETTFNSIMKCDVDIRKDLYANTVLS 300
GGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQ 360
EYDESGPSIVHRKCF                                               375
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Figure 6. CK2 consensus site in β-actin sequence. Generic serine and threonine are indicated in red while residues insert in a CK2 consensus sequence are highlighted in yellow.

First we demonstrate that β-actin immunoprecipitated from NB4 cell lysates is actually phosphorylated by CK2 (Fig. 7A). Then, with the aim to obtain a higher amount of purified β-actin with the final intent of identifying the phosphorylated sites, we performed a DEAE Sepharose purification of β-actin from NB4 cells. We obtained a good yield of protein (not shown) but, the phosphorylation produced by CK2 was very low (Fig. 7B). However, since the purification protocol required the presence of high ATP concentration not only in all the purification steps but also in the lysis buffer, in order to avoid polymerization (Mommaerts, 1951; Korn *et al.,* 1987), we reasoned that the purified actin was already phosphorylated by the endogenous kinases. As a confirm of this hypothesis, a significant improve in β-actin phosphorylation by CK2 was obtained by pretreating with the generic λ-PPase before the incubation with CK2 (Fig. 7B).

On this bases, we decided to use a commercial recombinant human β-actin to better characterize its phosphorylation by CK2: to this purpose, we looked for a commercial β-actin whose preparation was devoid of ATP (see Methods) and we found that it is phosphorylated by recombinant CK2, with a stoichiometry of about 16% (not shown). Interestingly, monomeric αCK2 seemed to be more active on β-actin than the tetrameric holoenzyme (not shown), as observed only for few CK2 substrates (Meggio and Pinna, 2003) (Fig. 8). The kinetics values are quite favourable, as shown in Fig. 8.

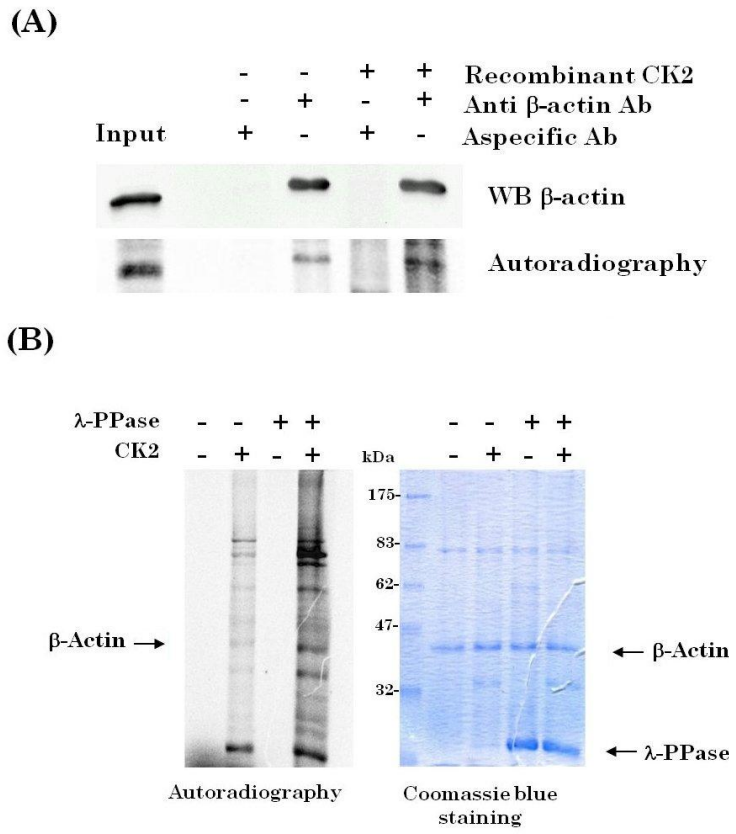

Figure 7. Phosphorylation of purified β-actin. (A) Immunoprecipitation from NB4 cytosolic extract was performed with a β-actin antibody or an aspecific one, as a negative control. Recombinant CK2 was added to the immunoprecipitates, where indicated, in the presence of a radioactive phosphorylation mixture. Samples were analysed by WB and autoradiography. (B) DEAE-Sepharose purified β-actin was pretreated or not with λ-PPase (500 U), then incubated, where indicated, with recombinant CK2 $\alpha_2\beta_2$ (200 ng), in presence of a radioactive phosphorylation mixture. Samples were analysed by SDS-PAGE, Coomassie blue staining and autoradiography.

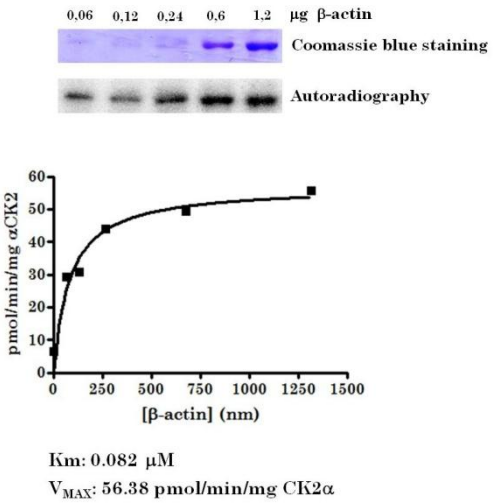

Figure 8. Phosphorylation of recombinant human β-actin. Increasing amounts of recombinant β-actin were incubated with 200 ng of human recombinant CK2α in the presence of a radioactive phosphorylation mixture. Proteins were analysed by SDS-PAGE, followed by Coomassie blue staining and autoradiography. For ^{32}P incorporation, the phosphorylated bands were excised and counted in a scintillation counter. Initial rate data were fitted to the Michaelis–Menten equation with the program Prism (GraphPad Software) to obtain Km and Vmax values.

Discussion

Whit this study we confirm that the CK2 activity inhibition by using subletal doses of specific CK2 inhibitors is able to block the RA-induced differentiation of NB4 cells. We also demonstrate that the CK2 expression level and activity are not affected by the treatment. However, an important change is seen in the endogenous protein phosphorylation pattern; in particular, many proteins are affected in their phosphorylation level by RA and many of them are dependent on CK2 activity, since the application of CK2 inhibitors results in a decreased phosphorylation. Hence, we focused on those putative CK2 substrates (affected by the inhibitors), whose phosphorylation increases in response to RA, and we tried to identify them by a proteomic approach. Therefore, we performed two-dimensional gel electrophoresis of lysates from cells treated with RA, K27 or a combination of both, and sent four selected spots to mass spectrometry analysis. This analysis enable the non-ambiguous identification of three proteins: two of them, nucleophosmin and nascent polypeptide associated complex α, are already known as CK2 substrates, while the third one, β-actin, has never been reported to be phosphorylated by CK2 so far. Actins are globular (≈42-kDa), highly-conserved proteins, which take part as monomeric units to microfilaments, one of the three major components of the cytoskeleton, and also in muscle cells to thin filaments, part of the contractile apparatus. Thus actins participate in many important cellular processes including muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signaling, establishment and maintenance of cell junctions and cell shape, and, noteworthy, also transcription and chromatin remodelling (Vartiainen, 2008). Mammals have at least six actin isoforms coded by separate genes (Vandekerckhove and Weber, 1978) which are divided into three classes (α, β and γ) according to their isoelectric point. In general, α actins are found in muscle, whereas β and γ isoforms are prominent in non-muscle cells. In the dynamic of filaments formations, monomeric globular ATP-binding (G)-actin can undergo cycles of self-assembly into filamentous (F)-actin, ATP hydrolysis, and depolymerization.

Analyzing β-actin phosphorylation in lysates from NB4 cells, we found that the β-actin, detected with the specific antibody, co-migrates with one of the major CK2-dependent phosphorylated bands evoked by RA treatment. With experiments of *in vitro* phosphorylation of cell lysates in the presence of different protein kinase

inhibitors, we confirmed that CK2 is actually involved in the phosphorylation of this band since its phosphorylation level is affected by CK2 specific inhibitors and not by staurosporine.

Another important observation has been that RA treatment induces an increase of β-actin expression level, detectable both in the cytosol and in the nucleus, and that the CK2 inhibition markedly affects the nuclear β-actin increase in response to RA without preventing its increase in the cytosol. We can therefore speculate that CK2 phosphorylates β-actin independently of RA stimulation, but an increased phosphorylation is apparently detected in response to RA due to the higher protein amount. Moreover, the effect of CK2 inhibition in preventing the RA-induced increase of β-actin in the nucleus can suggest that the CK2-dependent phosphorylation of β-actin is required for its nuclear translocation.

β-actin sequence presents some CK2-consensus sites and indeed we demonstrated its direct phosphorylation *in vitro* by CK2, when actin is provided as immunoprecipitated protein from NB4 cell lysates or as recombinant purified protein. These experiments demonstrated that CK2 can phosphorylate β-actin with a stoichiometry of about 16% and with good kinetic parameters; also interestingly, the monomeric CK2α seems to be more active than the tetrameric holoenzyme toward actin as occurs only for few CK2 substrates (Meggio e Pinna, 2003). Our results are the first reported evidence for actin phosphorylation by CK2. Together with numerous reports of CK2 involvement in cytoskeleton dynamic and cell morphology (Canton and Litchfild, 2006; Kramerov *et al.,* 2010), they suggest that CK2 could play a role in NB4 differentiation by phosphorylating β-actin and thus regulating it. At present, we do not know the exact effect of the CK2-dependent phosphorylation of β-actin, which can possibly affect its polymerization/depolymerization cycle; it is worth to mention that a role in this context has already been proposed for the phosphorylation of actin by PKA (Ohta *et al.,* 1987). Our results rather suggest a possible role of CK2 in the actin shuttling in to the nucleus, where it is known to exert some important functions, especially regard gene expression. However further studies must be done to validate this model; moreover, it will be necessary to identify the CK2-dependent phosphorylation site, for the final aim to investigate its importance in cell response to RA.

It is also worth to say that several other proteins seems to be more phosphorylated by CK2 in response to RA; the two proteins already identified (NPM and αNAC) are known to be involved in cell differentiation, but their role in mediating the CK2 involvement in this process should be considered. Further investigation will be

necessary, also taking into account that more other proteins, not identified in this study, can be involved.

For the moment, the important message resulting from this study is that CK2 mediates the differentiation process induced by RA in APL cells: although the CK2 constitutive activity does not change in response to the stimulus, the phosphorylation of some of its substrates is altered. Therefore its inhibition can be detrimental for the therapeutic effect of RA, and this should be kept in mind when CK2 is considered a therapeutic target.

Materials and methods

Materials

RA (9-*cis*-retinoic acid), PMA (phorbol 12-myristate 13-acetate), NBT, MTT and λ–PPase were from Sigma-Aldrich. [γ-^{33}P] ATP was from PerkinElmer. The CK2 inhibitor TBB (4,5,6,7 tetrabrome benzotriazole) was provided by Prof. Meggio (Padua, Italy), IQA ([5-oxo-5,6-dihydroindole (1,2-a) quinazolin-7-yl]-acetic acid) was synthesized as in Sarno *et al.,* 2005, the TBB-derivative K27 (2-amino-4,5,6,7-tetrabromo–1*H*-benzimidazole) was synthesized and kindly provided by Dr. Z. Kazimierczuk (Warsaw, Poland). All this compounds were diluted in 100% dimethylsulphoxide (DMSO) at the indicated concentrations, and the same amount of the solvent was used for control experiments.

Recombinant human CK2 was α and $\alpha_2\beta_2$ were kindly provided by Dr. Stefania Sarno (Padua, Italy). Recombinant human β-actin was from GenWay Biotech. Prestained blue Protein Marker broad range, was from Cell Signaling.

Antibodies

Antibodies for Hsp90, Lamin B and CK2α' were purchased from Santa Cruz Biotechnology. Anti β-actin, α-tubulin and c-myc antibodies were from Sigma. CK2α antisera were raised in rabbit against the sequence of the C-terminus (376-391) of the human protein. Nucleophosmin (B23) antibody was from Invitrogen. Secondary antibodies were from Amersham (anti-rabbit IgG biotinylated , streptavidin-horseradish peroxidase conjugate), Calbiochem (anti-mouse IgG biotinylated), Pelkin Elmer (anti-rabbit and anti-mouse HRP-labeled) and Sigma-Aldrich (anti-goat IgG biotinylated).

Cell culturing and treatment

NB4 promyelocytic leukemia cells, were maintained in RPMI 1640 (Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich), in an atmosphere containing 5% CO2. For the treatments, cells were at 0.5-1×10^6 cells/ml and treated for 6, 24 and 48 h with 1 μM RA and/or 5 μM K27 or TBB; for the 48h treatments, an addition of 50% fresh media with the stimulus was performed. At the end of the incubations, cells

were centrifuged (at 800 rpm without brake), washed with PBS and lysed as described below.

Cell viability and differentiation

Cell viability was detected by means of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent, a tetrazolium salt that is metabolized by mitochondrial dehydrogenases and produces a purple precipitate (formazan) in viable cells. Cell suspension (100 µl; 10^5 cells) was incubated in each well of a 96-well plate under different conditions. Then, 1 h before the end of the incubations, 10 µl of MTT solution (5 mg/ml in PBS) where added to each well. Incubations were stopped by addition of 20 µl of lysis solution at pH 4.7, consisting of 20% (w/v) SDS, 50% (v/v) N,N-dimethylformamide, 2%(v/v) acetic acid and 25 mM HCl. Formazan absorbance was read at λ590 nm, in a Titertek Multiskan Plus plate reader (Flow Laboratories). Cell differentiation was assayed by means of NBT (2,2′-bis(4-Nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene) ditetrazolium chloride) reagent, this soluble salt is metabolized by superoxide production in differentiated granulocytes activated with phorbol myristate acetate (PMA) (Levinsky *et al.,* 1983) to an insoluble intracellular blue formazan. Cell suspension (100 µl; 10^5 cells) was incubated in a 96-well plate under the different conditions indicated; 1h prior to the stop of the treatment, 5 µl of NBT (10 mg/ml in sterile H_20) and PMA 0.1 µg/ml were added. Incubations were stopped by the addition of 20 µl of the stop solution described above and plates were read also at λ590 nm.

Cell lysis

Cells were centrifuged, washed, and lysed to obtain a total lysate, by adding an ice-cold buffer consisting of 20mM Tris-HCl, pH 7.5, 150mM NaCl, 2mM EDTA, 2mM EGTA, 0.5% (v/v) Triton X-100, 2mM dithiothreitol, protease inhibitor cocktail Complete (Roche), 10 mM NaF, 1 µM okadaic acid, 1mM Na vanadate. After 20 min incubation on ice, the lysates were centrifuged at 14000 *g* for 10 min, at 4°C. The supernatants contained the cell-soluble fraction, whose protein concentration was determined by the Bradford method.

For cytosolic fraction and the nuclear extract preparation, cells were lysed by the addition of ice-cold hypotonic buffer consisting of 10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, protease inhibitor cocktail, 10 mM NaF and 1 µM okadaic acid. After 20 min of incubation on ice, 0.625% (v/v) Nonidet P-40 was added, and the lysates were centrifuged immediately at 14000×*g* for 30 s. The

supernatants contained the cell soluble fraction, whereas the pellets corresponded to the nuclear fraction and were incubated for 20 min on ice with a hypertonic buffer consisting of 20 mM Hepes pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, protease inhibitor cocktail, 10 mM NaF and 1 μM okadaic acid. The nuclear extracts was obtained by centrifugation for 10 min at 14000×*g* and 4 °C. Protein concentration was determined by the Bradford method.

Western blot analysis

Equal amounts of protein from the different cell lysates were loaded on 11% SDS-PAGE (Laemmli,1970) and blotted on Immobilon-P membranes (Millipore) in a TE 22 Mini Tank Transfer Unit (GE Healthcare), at 60V for time varying from 60 to 90 min, using a buffer containing 10 mM CAPS-NaOH (3-(Cyclohexylamino)-1-propanesulfonic acid pH 10, DTT 3 mM and 1% (v/v) methanol. Dried PVDF membranes were then washed with TBS buffer (Tris-HCl 50 mM pH 7.5, NaCl 50 mM) with 1% (w/v) BSA (Sigma-Aldrich) and then processed with the indicated antibodies diluted in the same buffer. For membrane development, biotinylated or HRP-conjugated antibodies were incubated for at least 30 min (15 min for streptavidin-HRP) in the same buffer, washes between steps were made in TBS buffer. Antibodies signals were detected by ECL (enhanced chemiluminescence, Amersham Biosciences). Quantitation of the signals was obtained by chemiluminescence detection on a Kodak Image Station 4000MM Pro and with Carestream Health Molecular Imaging software.

Endogenous CK2 activity in cell lysate

For the CK2 activity assay, 1-2 μg of proteins from the different cell lysates were incubated in the presence of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 μM [γ-^{33}P] ATP (1000-2000 c.p.m/pmol) and 0.1 M NaCl, with the specific peptide $R_3AD_2SD_5$ (0.1 mM) in a total volume of 20 μl. Incubation was carried out at 30°C for 10 min and stopped by sample absorption on phospho-cellulose P81 paper. Papers were washed three times with 75 mM phosphoric acid, dried, and counted in a scintillation counter. Phosphorylation of endogenous proteins was performed with 5-10 μg of lysate proteins under the same conditions for 20 min, but without the addition of the peptide substrate. The reactions were stopped by the addition of Laemmli buffer and the proteins were analysed by SDS-PAGE, Coomassie blue staining or WB, and autoradiography (PerkinElmer's Cyclone Plus Storage Phosphor System).

Two-dimensional gel electrophoresis

Phosphorylation of endogenous proteins was performed with 100-125 μg of lysate proteins under the same conditions described above with 10 μM [γ-^{32}P] ATP (2000 c.p.m/pmol), in a total volume of 150 μl. For SDS-PAGE analysis, Laemmli buffer was added to 5 μl of the reaction mixture while, for IEF, the rest of the mixture was blocked with 80% (v/v) ice-cold acetone and incubated for 1h at -80°C. Precipitated proteins were centrifuged at 14000×*g*, for 20 min at 4°C. Acetone was discarded and pellets were dried at room temperature, resuspended in 125 μl of IEF buffer (8M urea, 2 M thiourea, 4% CHAPS (w/v), 0.5% ASB-14, 1% DTT, 0.8% Bio-lyte Ampholyte (3-10 pI range, Bio-Rad) and 0.002% bromophenol blue) and applied onto 7 cm immobilized pH gradient (IPG) strip (linear, pH 3-10, Bio-Rad). The first dimension was carried out on a Protean IEF Cell system (Bio-Rad) at 20°C following preset method for 7 cm strips. After IEF, the IPG strips were equilibrated in a buffer (6 M urea, 30% glycerol, and 2% SDS in 5mM Tris-HCl buffer, pH 8.8) containing 125 mM DTT first, and then 250 mM iodoacetamide (IAA), each incubation lasted 20 min. The second dimension was carried out on 11% SDS-PAGE followed by Coomassie blue staining.

Immunoprecipitation experiments

Immunoprecipitation was performed incubating 100-200 μg of proteins from NB4 cytosolic fraction lysates over night at 4°C, with anti β-actin antibody (2.5 μl) or with the aspecific antibody anti c-myc (2.5 μl), as a negative control, followed by addition of protein A-Sepharose (Sigma-Aldrich) for 40 min at 4°C. Immunoprecipitates were washed once with NET buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0,05% (v/v) Nonidet P-40, 0,2% BSA) and twice with 50 mM Tris-HCl pH 7.5 and used for phosphorylation assays.

β-actin purification

For β-actin purification we followed the method by Liebes *et al.,* (1983) with slight modifications: 200×10^6 NB4 cells were homogenized at 4°C with 2 to 3 volumes of Buffer A (Tris-HCl 3 mM , 0.1 mM $CaCl_2$, 0.5 mM ATP, and 0.75 mM 3-mercaptoethanol, pH 7.5 and protease inhibitor cocktail). The homogenate was centrifuged at 14000×*g* for 20 min and then for 1 h at 100000×*g* at 4°C and applied to an 1×10 cm DEAE-Sepharose column (Whatman) equilibrated with Buffer A and saturated with ATP (saturation monitored by OD measuring at λ260 nm). Proteins

were eluted by linear gradient of 2×25 ml 0-0.5 M KCl in Buffer B (Tris-HCl 10 mM, 0.1 mM $CaCl_2$, 0.5 mM ATP, and 0.75 mM 3-mercaptoethanol, pH 7.5), 1.2 ml fractions were collected. 20 µl of odd fractions were analysed by 11% SDS-PAGE and WB or Coomassie blue staining for β-actin presence.

Phosphorylation of purified and recombinant β-actin

For immunoprecipitate phosphorylation, the pellets were incubated in the presence of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 µM [γ-^{33}P] ATP (1000-2000 c.p.m/pmol) and 0.1 M NaCl with 0.1 µg recombinant CK2, as indicated in the figure legend. Reactions were stop by the addition of Laemmli sample buffer, samples were then boiled for 5 min at 100°C and analysed by SDS-PAGE/WB and autoradiography.

For purified β-actin dephosphorylation, 5 µl of DEAE-Sepharose fractions were incubated with 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 µM [γ-^{33}P] ATP (1000-2000 c.p.m/pmol) and 0.1 M NaCl, in the presence of 0.1 µg of recombinant tetrameric CK2 ($\alpha_2\beta_2$) where indicated. After 20 min incubation at 30°C, the reaction was stopped with Laemmli sample buffer and proteins were analysed by SDS-PAGE, Coomassie blue staining and autoradiography. When previous dephosphorylation was required, the actin fractions were incubated with 1.25 µl (500 U) of λ–PPase (Sigma-Aldrich), supplemented with the provided PPase buffer and 10 mM $MnCl_2$. After 1h incubation at 30°C, samples were incubated 20 min at 70°C to inactivate the phosphatese.

For recombinant β-actin phosphorylation, variable amounts of actin were incubated with 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 20 µM [γ-^{32}P] ATP (2000 c.p.m/pmol) in the presence of 0.2 µg of recombinant CK2α for 20 min at 37 °C, reaction was stopped by the addition of Laemmli sample buffer and sample were analysed by SDS-PAGE, Coomassie blue staining and autoradiography. For calculation of ^{32}P incorporation, the phosphorylated bands were excised and counted in a scintillation counter. Initial rate data were fitted to the Michaelis–Menten equation with the program Prism (GraphPad Software) to obtain Km and Vmax values. In experiments for the determination of phosphorylation stoichiometry, 0.24 µg of β-actin were incubated under the conditions described above with 0.2 µg of recombinant αCK2 for 20 min with further addition of 0.2 µg of αCK2 for 20 min.

The co-chaperone protein p23 is a novel substrate of CK2 in *Arabidopsis thaliana*

Introduction

Protein kinase CK2 is well studied especially in animals and yeast, but much less is known about CK2 in plants. In human, three genes has been identified to encode for the α, α', and β subunits of CK2 (Pyerin and Ackermann, 2003; Ackermann *et al.,* 2005) while in plants many isoforms of the catalytic or regulatory subunits have been identified, for example maize express three different α and three different β subunits of CK2 (Riera *et al.,* 2001,*) and a new, fourth αCK2 isoform has been recently identified (Lebska *et al.,* 2009), while in *Arabidopsis* there are four genes encoding for each CK2 subunit (Salinas *et al.,* 2006). Both monomeric and oligomeric forms with CK2-like activity were isolated from different plant sources such as maize (Dobrowolska *et al.,* 1989), broccoli (Klimczak *et al.,* 1992) or pea (Li *et al.,* 1992), and the characterization of the different forms of CK2 has been made from recombinant proteins from different species including maize (Riera *et al.,* 2001, 2003), tobacco (Salinas *et al.,* 2001), wheat (Yan and Tao, 1982) and *Arabidopsis* (Sugano *et al.,* 1998; Dennis and Browning, 2009). Studies concerning the CK2 function in plants have revealed its involvement in many vital physiological processes such as light signaling and growth control (Lee *et al.,* 1999), cell division and cell cycle regulation (Espunya *et al.,* 1999, 2005; Moreno-Romero *et al.,* 2008), transcription (Klimczak *et al.,* 1995; Sugano *et al.,* 1998, 1999; Lee *et al.,* 1999; Hardtke *et al.,* 2000), translation (Dennis and Browning, 2009), abscisic acid (ABA) signaling pathways (Riera *et al.,* 2004), circadian rhythm (Portolés and Más, 2010) and salicylic acid (SA)-mediated defence (Hidalgo *et al.,* 2001; Salinas *et al.,* 2001; Kang and Klessig, 2005; Zottini *et al.,* 2007). SA influences many functions in plants like seed germination, cell growth, respiration and thermogenesis but plays a predominant role in plant defence signaling (Vlot *et al.,* 2009). SA is necessary for stress defence responses induced by pathogens not only at local level (Alvarez, 2000) and in hypersensitive response (Mur *et al.,* 2008) but also in systemic acquired resistance (SAR) (Durrant and Dong, 2004; Loake and Grant, 2007). These responses allow plants to survive pathogen infection and acquire a long-lasting systemic resistance responsible for the protection from further infections (Grant and Lamb, 2006; Shah, 2009). It has been demonstrated that SA accumulates upon infections of various pathogens, treatment with elicitors from pathogens, and stress conditions, and the blockage of its level increment severely compromises defence response (Gaffney *et al.,* 1993; Delaney *et al.,* 1994; Kubota and Nishi, 2006).

Moreover, exogenous application of SA or its analogs to plants, is sufficient to evoke disease resistance (Vlot *et al.,* 2009). The resistance to infections triggered by SA seems to be mainly due to activation of specific defence genes, some of which encoding for proteins involved in cellular redox balance control (Klessig *et al.,* 2000; Blanco *et al.,* 2009, Maier *et al.,* 2011). In particular a feedback loop between SA and nitric oxide (NO) has been proposed due to the evidence that NO donors induce SA accumulation and NO signaling in defence requires SA (Durner *et al.,* 1998; Wendehenne *et al.,* 2004, Moreau *et al.,* 2010). In a paper published in 2007 by Zottini and co-workers, with the collaboration of my laboratory group, the relationship between NO and SA in *Arabidopsis thaliana* was studied; measuring NO levels in *Arabidopsis* seedlings and in cultured cells, they show that SA triggers NO synthesis in a dose dependent manner. In order to identify the metabolic pathways involved, they adopted both genetic and pharmacological approaches. By means of the double mutant for nitrate reductase (NR) *nia1,nia2* they demonstrated that the SA-induced NO production occurs independently from NR activity. For the investigation of the components involved in NO synthesis upon SA treatment, they used also protein kinase inhibitors to disclose the importance of the phosphorylation events in the SA-induced signaling cascade leading to NO production. A general Ser/Thr protein kinase inhibitor (K252a) and a protein kinase CK2 specific inhibitor (TBB) were used, and a significant reduction of NO production was determined only in the presence of TBB, thus demonstrating that the CK2 activity is required for SA-induced NO production. This data are in agreement with others in which the CK2 involvement in SA signaling is also demonstrated by its role in phosphorylating specific proteins implicated in this pathway (Hidalgo *et al.,* 2001; Kang and Klessig, 2005). However, in contrast to the results published by others (Kang and Klessig, 2005), Zottini *et al.,* did not find any changes in the CK2 catalytic activity in response to SA. This is expected for a constitutively active enzyme, and suggested to us the possibility that CK2 intervenes in SA signaling by specifically modulating the phosphorylation level of only one or few proteins among its substrates, which become available as targets only upon an SA-mediated event, such as changing in protein expression, cellular translocations, post-translational modifications catalyzed by other enzymes. On these bases, we performed this study, looking for putative CK2 substrates, which become phosphorylated only in response to SA. Using a proteomic approach we could identify a new CK2 substrate in *Arabidopsis*, homologous to the co-chaperone p23 protein, whose phosphorylation was investigated in detail.

Results

Identification of the p23-like protein of *Arabidopsis* as a substrate of CK2

Looking for putative SA-dependent CK2 substrates, we treated 8-day-old *Arabidopsis* seedlings with SA, with or without the specific CK2 inhibitor TBB (Sarno *et al.*, 2001). We performed radioactive phosphorylation of endogenous cytosolic proteins, incubating plant lysates with a radioactive phosphorylation mixture, without the addition of any kinase, and we analysed proteins by SDS-PAGE and autoradiography, searching for those bands whose phosphorylation was increased upon SA treatment and decreased in the presence of TBB.
The SDS-PAGE and autoradiography of one representative experiment is shown in Fig. 1.

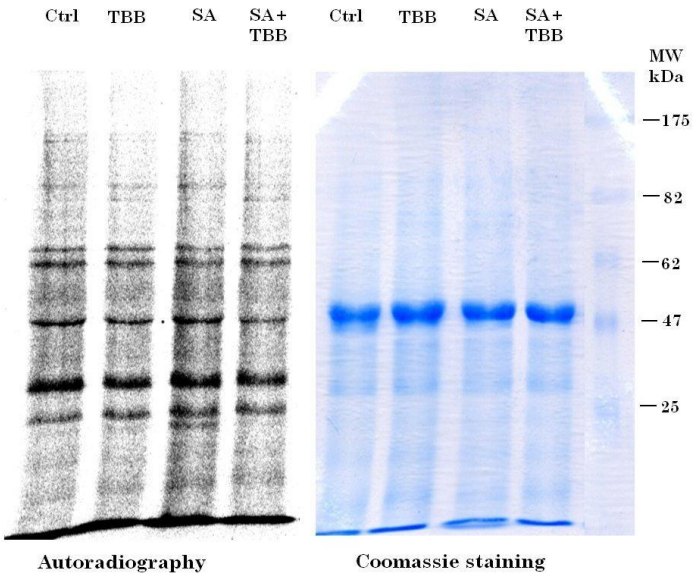

Figure 1: Cytosolic extracts (10 μg proteins) from 8-day-old *Arabidopsis* seedling treated or not with SA (1mM) and/or TBB (30μM), were incubated with a phosphorylation mixture containing [γ^{33}P] ATP as phosphate donor. Protein phosphorylation was evaluated by SDS-PAGE and autoradiography. The Coomassie blue staining of the gel is shown on the right side, where also the migration of protein molecular weight markers (MW) is indicated.

The major change in the phosphorylation pattern in SA-treated samples was represented by a band of about 23 kDa, that appeared in response with SA but disappeared if CK2 was inhibited by TBB.

To obtain a more complete analysis of this material, and in order to identify this and/or other proteins, we performed two-dimensional gel electrophoresis (Fig. 2), in particular comparing, the samples that displayed the highest (SA-treated plants) and the lowest (TBB treated plants) phosphorylation degree of this band.

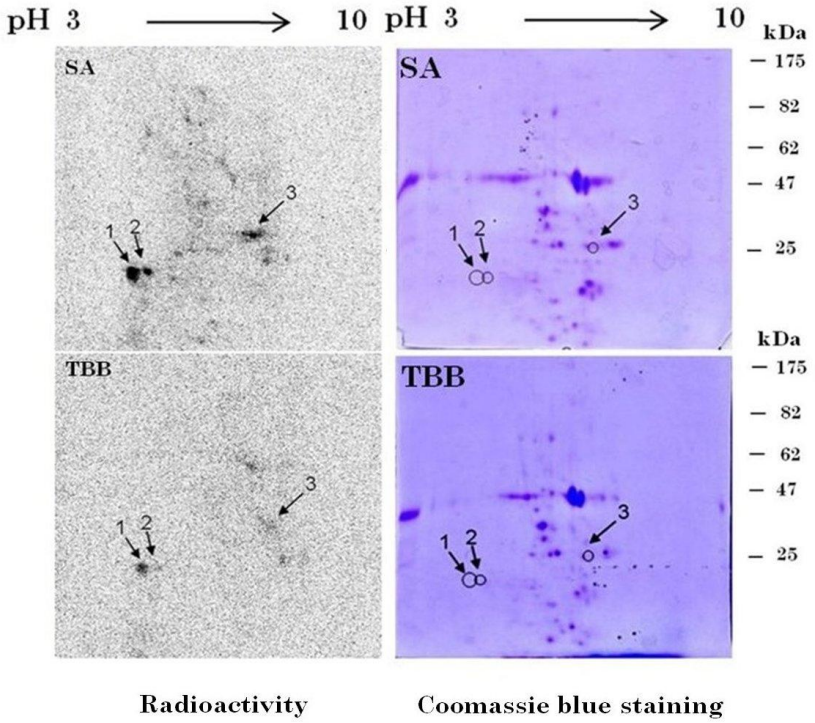

Figure 2: Two-dimensional gel electrophoresis of radioactive phosphorylation of endogenous cytosolic proteins. Cytosolic extracts (100 μg of proteins) from 8-day-old *Arabidopsis* seedling treated or not with SA (1mM) and/or TBB (30μM), were incubated with a phosphorylation mixture containing [γ^{32}P] ATP as phosphate donor. Protein were then subjected to two-dimensional gel electrophoresis and analysed by Coomassie blue staining and autoradiography. The Coomassie blue staining of the gels is shown on the right side, where also the migration of molecular weight (MW) markers is indicated. Arrows indicate spots whose phosphorylation is increased upon RA treatment and decreased by TBB. For details see "Materials and methods".

The protein staining of the two samples were similar enough to allow a comparative analysis of the radioactivity, which, as expected, was instead significantly different; spots indicated in the figure were excised and sent to mass spectrometry for identification (Fig. 2). While spot 1 and 3 did not give an unambiguous identification, spot 2 was identified as the *Arabidopsis* co-chaperone p23, a protein which associates to Hsp90 (Kadota *et al.,* 2008).

In *Arabidopsis*, two isoforms of the p23 protein are expressed, Atp23-1 and Atp23-2 (Zhang *et al.,* 2010). It has been demonstrate that human p23 is phosphorylated by CK2 (Kobayashi *et al.,* 2004), and despite the *Arabidopsis* p23-like isoforms, share low amino acid identities (less than 30% for both) with the human p23, their sequence reveal the presence of putative CK2-consensus sites (Pinna and Ruzzene, 1996).

Interestingly, one of the reported CK2-phosphorylation sites on human p23 is similar also in *Arabidopsis* isoforms (Fig. 3).

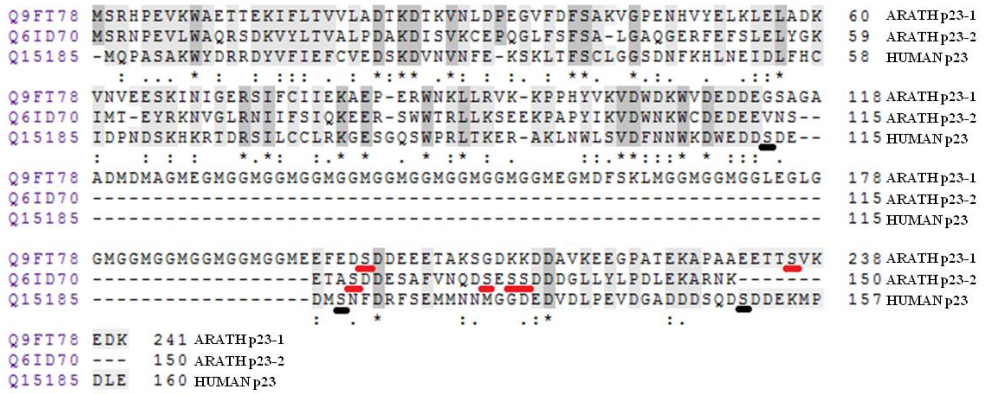

Figure 3: Alignment of human and *Arabidopsis* p23 proteins. ClustalW alignment performed on UniProt. On the left UniProtKB entries of the p23 proteins. In dark grey conserved amino acids indicated also with (*), amino acid differences that do not influence structure are indicated with (:) while amino acid differences that influence structure are indicate with (.). The CK2 phosphorylation sites in human p23 are underlined in black while serine residues in CK2 consensus sequences in *Arabidopsis* p23 are underlined in red.

Since we had the clone for the Atp23-2 isoform available and, as shown previously, it has consensus sites for CK2, we decided to express an purify it.

We expressed the recombinant p23 in *E. coli*, and we used the purified protein to characterize its phosphorylation by CK2 *in vitro*.

From this point onwards, the *Arabidopsis* p23-like protein isoform 2, Atp23-2, is referred to as p23 for simplicity.

p23 is efficiently phosphorylated by recombinant human CK2

In the first set of experiments, we used the recombinant human CK2 (hrCK2) for the phosphorylation of p23, using the model substrate β-caseins for comparison. We found that p23 is phosphorylated by hrCK2 *in vitro*, with an efficiency similar to that obtained with more than five-fold higher concentration of β-casein (Fig. 4).

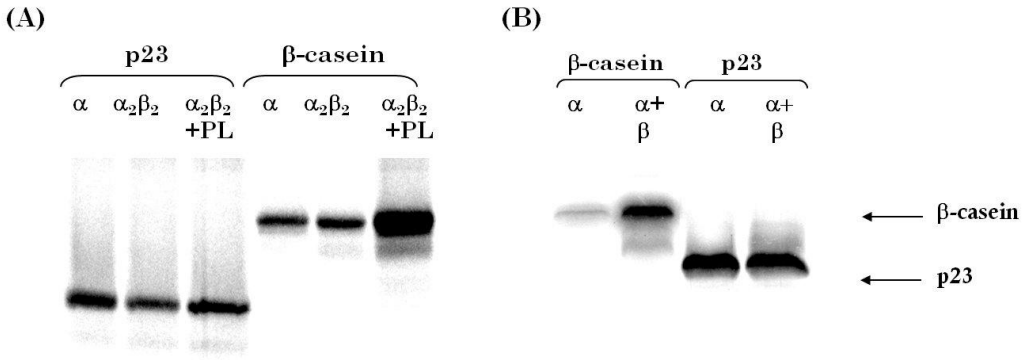

Figure 4: Phosphorylation of p23 by human recombinant CK2. (A) Equi-active amounts on β-casein of monomeric αCK2 (15 ng) and tetrameric ($\alpha_2\beta_2$) CK2 (4.4 ng) were incubated with recombinant p23 (0.1 μg-0.29 μM) or β-casein (1 μg-1.72 μM) β-casein in the presence of a radioactive phosphorylation mixture, polylysine (400 μg), a CK2 holoenzyme activator, was also added where indicated. After 10 min incubation at 30° C. Proteins were the analysed by SDS-PAGE, Coomassie blue staining and autoradiography. (B) Recombinant p23 (0.1 μg) and β-casein (0.5 μg) were incubated with recombinant human αCK2 (100 ng) with or without recombinant human βCK2 (100 ng) in the presence of a radioactive phosphorylation mixture and incubated 10 min at 30°C. The proteins were analysed as described above.

The stoichiometry of phosphorylation reached 1.5 mol Pi / mol protein, with the monomeric CK2α (not shown); an amount of the tetrameric CK2 ($\alpha_2\beta_2$) sufficient to produce a phosphorylation degree similar to CK2α on β-casein was slightly less efficient on p23 (Fig. 4A). In agreement to that, when recombinant βCK2 was added to αCK2 during the phosphorylation assay, while a significant increase in phosphorylation was obtained on β-casein, no appreciable effect was induced towards p23 (Fig. 4B). Since p23 seems to be a substrate better recognized by α than $\alpha_2\beta_2$, we tested the effect of polylysine, an *in vitro* CK2 activator absolutely required for the phosphorylation of those substrates better recognized by α than $\alpha_2\beta_2$ (Pinna, 2002), but only a minimal increase was induced on p23 phosphorylation if compared with the strong activation toward β-casein (Fig. 4A). We performed kinetics studies on p23 phosphorylation by CK2 and the data are shown in Fig. 5, where Km and Vmax values are indicated.

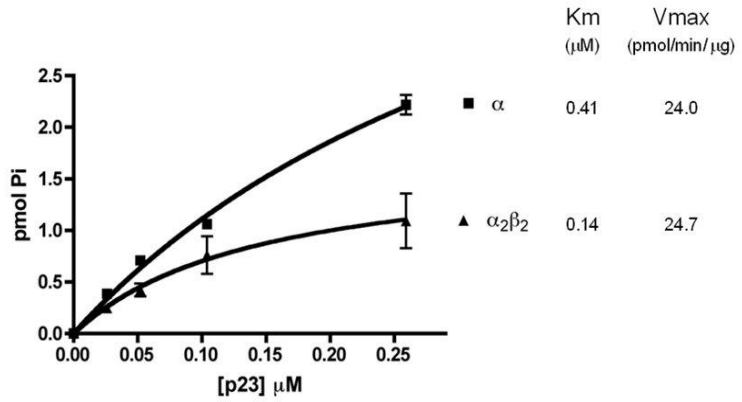

Figure 5: Kinetics of the p23 phosphorylation by human CK2. Increasing amounts of recombinant p23 were incubated with equi-active (on the model substrate β-casein) amounts of human recombinant CK2 in the monomeric or tetrameric form or recombinant maize αCK2, in the presence of a radioactive phosphorylation mixture. Proteins were analysed by SDS-PAGE, Coomassie blue staining and autoradiography. For ^{32}P incorporation, the phosphorylated bands were excised and counted in a scintillation counter. Initial rate data were fitted to the Michaelis–Menten equation with the program Prism (GraphPad Software) to obtain Km and Vmax values. For details see "Materials and methods".

Then we decide to confirm some of these results with a plant CK2, evolutionary more similar to *Arabidopsis* CK2 than the human enzyme, the available recombinant and purified enzyme was αCK2 from *Z. mays*. The results were similar to those obtained with human αCK2 (Fig. 6).

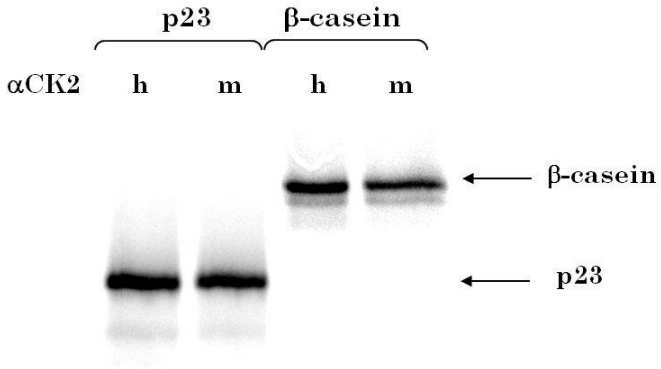

Figure 6: Comparison between Atp23 phosphorylation by human (h) and maize (m) recombinant αCK2. Equiactive amounts of enzymes on β-casein were used. Recombinant p23 (0.1 μg) was incubated with human (h) recombinant αCK2 (40 ng) or maize (m) recombinant αCK2 (20 ng) in the presence of a radioactive phosphorylation mixture. After 10 min incubation at 30°C, the reaction was stopped by Laemmli sample buffer addition. Proteins were the analysed by SDS-PAGE, staining and autoradiography.

p23 is phosphorylated by a CK2-like activity in *Arabidopsis*

We decided to verify if a CK2-like activity able to phosphorylate p23 was present also in *Arabidopsis* extracts: to this purpose, we performed *in vitro* phosphorylation of recombinant p23 using *Arabidopsis* seedlings cytosolic lysates as source of kinases.

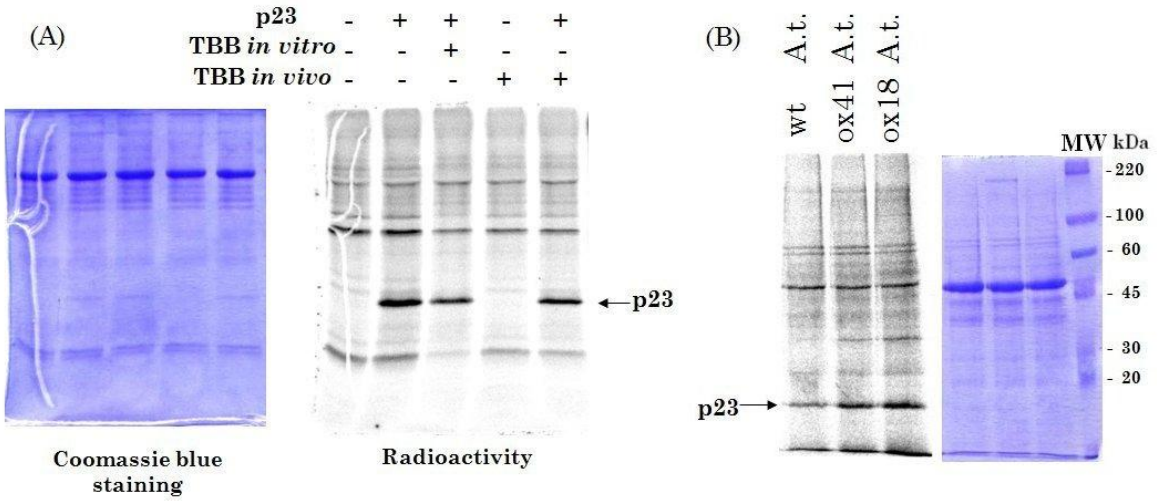

Figure 7: p23 phosphorylation by kinases in *Arabidopsis* cytosolic extracts. Recombinant p23 (0.1 μg) was incubated with 10 μg of proteins from cytosolic extracts of *Arabidopsis* seedlings treated or not with TBB (30 μM) in the presence of a radioactive phosphorylation mixture. TBB (2 μM) was also added *in vitro* were indicated. Incubation was carried out for 10 minutes at 30°C and stopped by Laemmli sample buffer addition. Proteins were then analysed by SDS-PAGE, Coomassie blue staining and autoradiography. (B) Recombinant p23 was incubated, in the conditions described above, with cytololic extracts (10 μg of proteins) from transgenic *Arabidopsis* plants overexpressing CK2β3 indicated as ox41 and ox 18 as in Sugano et al. 1999.

The results (Fig. 7) show that p23 is phosphorylated by a kinase present in *Arabidopsis* cytosol, that is sensitive to the CK2 inhibitor TBB (Sarno *et al.,* 2001) added both *in vitro* (during the phosphorylation assay) and *in vivo* (during the seedling culture), as demonstrated by a decrease in p23 phosphorylation level (Fig. 7A). Another supporting evidence of the p23 phosphorylation by *Arabidopsis* CK2 came from an experiment performed using cytosolic extracts from two transgenic *Arabidopsis* lines overexpressing CK2β3 and reported to have an increased CK2 activity respect to wild type (Sugano *et al.,* 1999): a higher p23 phosphorylation level was present with protein extracts from transgenic lines respect to the wild type *Arabidopsis* (Fig. 7B). Taken together, these data strongly suggest that p23 is phosphorylated by endogenous *Arabidopsis* CK2 in seedlings extracts.

To further confirm the data shown above, we decided to test the efficacy of different protein kinase inhibitors and effectors on Atp23 phosphorylation by *Arabidopsis* cytosolic extracts; essentially, we performed the same experiments described previously, but in the presence of these molecules. As shown in Fig. 8, the phosphorylation level of p23 was significantly decreased only in the presence of the CK2 specific inhibitors TBB and quinalizarin (Cozza *et al.,* 2009), while with staurosporine and K252a, which are two general Ser/Thr kinase inhibitors not effective on CK2 (Meggio *et al.,* 1995; Zottini *et al.,* 2007) or with EGTA (for calcium deprivation), the p23 phosphorylation was only slightly decreased.

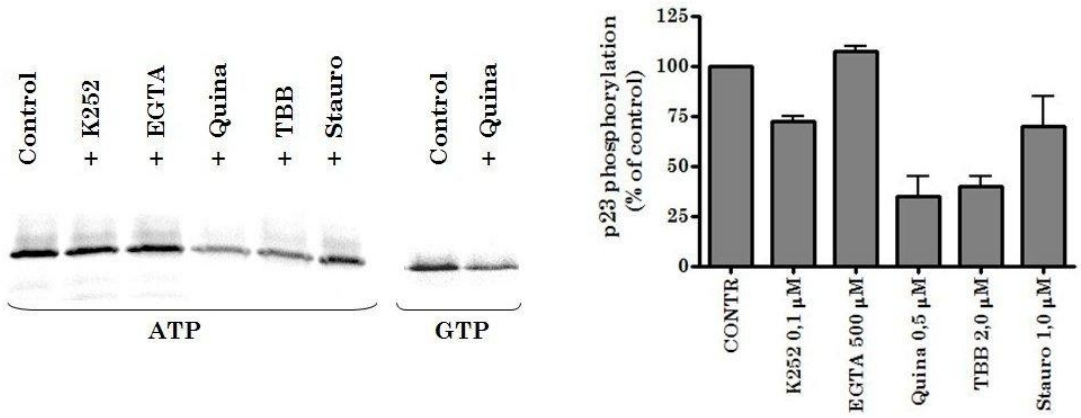

Figure 8: Effect of different protein kinase effectors on p23 phosphorylation. Recombinant p23 (0.1 µg) was incubated with 10 µg of cytosolic proteins from *Arabidopsis* seedlings extracts in the presence of a radioactive phosphorylation mixture containing either ATP or GTP (20 µM). Different protein kinase effectors were added during the phosphorylation reaction as indicated, at the following concentrations: 2 µM TBB and quinalizarin (Quina), 1 µM staurosporine (Stauro), 0.1 µM K252a, (the same amount of the solvent (DMSO) was added for control samples) and 500 µM EDTA. Reaction was carried out for 10 min at 30°C and stopped by Laemmli sample buffer addition. Proteins were then analysed by 11% SDS-PAGE, Coomassie blue staining and autoradiography. In the left part of the figure it is shown the autoradiography of the phosphorylated p23 protein, while on the right it is shown the quantification of p23 phosphorylation expressed as percentage respect to control; vertical bars indicate the standard deviation to the mean obtained from three separated experiments.

Moreover, p23 was still phosphorylated if ATP was replaced by GTP, as phosphate donor that can be used by CK2 but not the majority of the other protein kinases (Allende and Allende, 1995; Cheek *et al.*, 2005) (Fig. 8). Next, to assess if the main endogenous enzyme responsible for p23 phosphorylation was a casein kinase and its size consistent with that of CK2 catalytic subunit, we performed a set of in-gel kinase assays, including either casein or p23 in the gel, and performing the radioactive phosphorylation of these substrates by enzymes present at certain migration positions, after SDS-PAGE and protein renaturation.

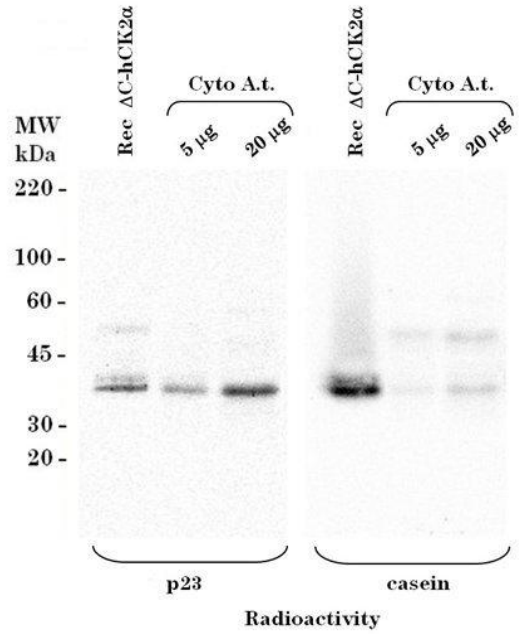

Figure 9: In-gel kinase assay of *Arabidopsis* cytosolic extracts, on p23 and β-casein substrates. 5 or 20 µg of proteins from *Arabidopsis* lysate were loaded on a gel containing 10 µg/ml p23 (left) or 500 µg/ml β-casein (right). After protein separation, proteins on the gel were renaturated, then the gel were incubated with a radioactive phosphorylation mixture and analysed by autoradiography. 10 ng of recombinant truncated human αCK2 (ΔC-hCK2α) were loaded as a positive control since it as the same size expected for Arabidopsis CK2α.

These experiments (Fig. 9) demonstrate that the only kinase that phosphorylates p23 in *Arabidopsis* migrates at about 39 kDa, which is consistent with the molecular weight of the most *Arabidopsis* αCK2 isoforms (Salinas *et al.,* 2006) and identical to the migration of a band able to phosphorylate casein. This strongly suggests that the major p23 kinase in Arabidopsis is a casein kinase with the size expected for CK2α.

CK2 and p23 physically associate

Considering that p23 is phosphorylated by CK2 *in vitro*, we wondered if also a stable association can occur between the two proteins. To verify this hypothesis, we performed BIAcore experiments, injecting p23 over a surface where αCK2 (the human recombinant protein) was immobilized. As shown in Fig. 10, a concentration dependent signal, that corresponds to p23 binding to αCK2, was observed.

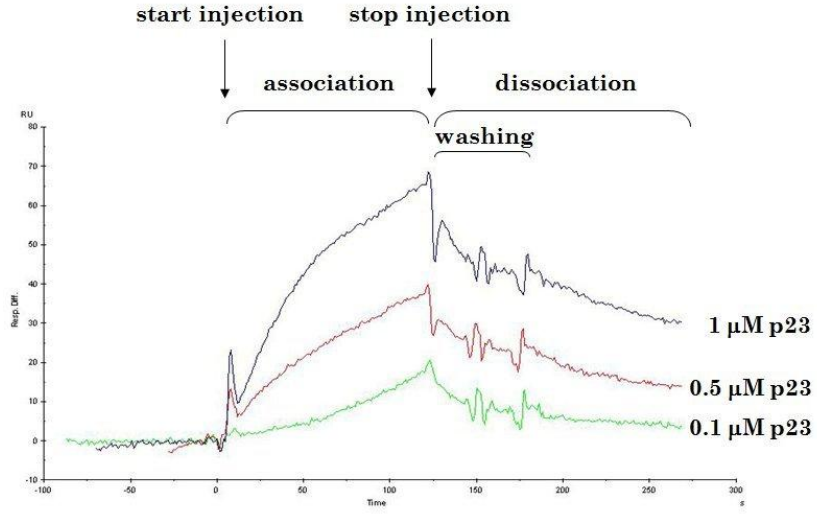

Figure 10: Detection of αCK2-p23 interaction by means of surface plasmon resonance. For the Surface Plasmon Resonance (SPR) analysis, a BIAcore X system was used, as described in (Ruzzene 1999). Human αCK2 was covalently coupled to a CM5 sensor chip (carboxymethylated dextran surface), by the amine-coupling chemistry, to a final density of 1600 RU (resonance units); a flow cell with no immobilized protein was used as a control. p23 solutions were injected at the indicated concentrations in HBS buffer (10 mM Hepes, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% P20) at a flow rate of 10 µl/min; the same buffer flowed before injections and during the dissociation phase; each sensorgram (time course of the SPR signal) was corrected for the response obtained in the control flow cell, and normalized to baseline. Resp. diff., response difference. Each curve corresponds to the indicated p23 concentration.

p23 co-localize with CK2

In order to test if CK2 and p23 association could also occur *in vivo*, we assessed the subcellular localization of these two proteins. In *Arabidopsis* three non plastidial CK2α subunits have been identified (A, B, C) (Salinas *et al.,* 2006) and among them the CK2αC is the most similar to the human and maize isoforms. We then fused the green fluorescent protein (GFP) at the C-terminal end of the CK2 αC and expressed it in *Arabidopsis* (Fig. 11) and tobacco mesophyll protoplasts (data not shown).

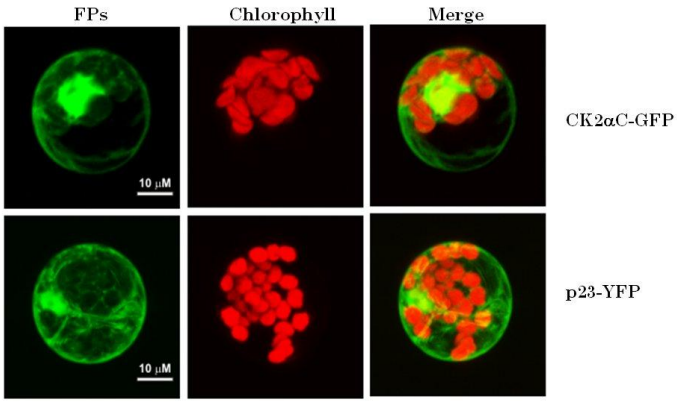

Figure 11: CK2αC and p23 *in vivo* localization. Confocal images of an Arabidopsis mesophyll protoplast transformed with CK2αC fused with GFP (green) and with p23 fused with YFP (yellow).

The confocal microscopy analyses showed that the GFP signal was clearly distinguished in the cytoplasm and nucleus (Fig. 11), confirming published data (Salinas *et al.,* 2006). We then tested the subcellular localization of p23 by fusing it to the yellow fluorescent protein (YFP) and expressed it in *Arabidopsis* mesophyll protoplasts. The confocal microscopy analyses show the presence of the signal in cytoplasm and nucleus (Fig. 11). In order to confirm the p23 nuclear localization, we also expressed the p23::YFP in tobacco epidermal cells where the nuclei are easy to detect compared with mesophyll protoplasts, and, indeed, we confirmed the p23 nuclear localization (data not shown).

At present, we have no clear-cut evidence of a physical *in vivo* interaction between p23 and CK2, and future experiments will be necessary to unequivocally demonstrate this point. However, our present results indicate that the two proteins localize in the same cellular compartments, suggesting that their association is conceivably possible.

Discussion

We have demonstrated that CK2 phosphorylates a major protein in *Arabidopsis* in response to SA treatment, and, by two-dimensional electrophoresis and mass spectrometry we identified this protein as the p23-1 protein, homologous to the human p23 co-chaperone, which associates with Hsp90, a protein required for the folding, assembly and maintenance of a subset of proteins (Taipale *et al.,* 2010). p23 was first identified as a component of the progesterone receptor (PR)–Hsp90 complex (Johnson *et al.,* 1996) and since then, it is widely recognized as a co-chaperone of Hsp90 (Felts and Toft, 2003; Pratt and Toft, 2003). In particular, p23 binds to the ATP-bound form of Hsp90, blocking its ATPase activity, thereby stabilizing that state and thus client protein binding and activation time (McLaughlin *et al.,* 2006; Ali *et al.,* 2006); moreover, p23 possesses passive chaperone activity itself in that it can suppress the aggregation of denatured proteins (Bose *et al.,* 1996; Freeman *et al.,* 1996). Recently the *Arabidopsis* p23-like proteins has been characterized: two genes in *A. thaliana* encode for p23-like proteins, p23-1 and p23-2 which shares respectively 27% and 25% of amino acid identities with the human p23 and both are expressed under normal conditions. p23-1 and p23-2 binding to Hsp90 requires ATP and only p23-1 seem to have a small inhibitory effect on Hsp90 ATPase activity (Zhang *et al.,* 2010). Both isoforms have CK2 consensus sites, but we had available the only clone for p23-2 thus we expressed and purified the p23-2 isoform for *in vitro* phosphorylation studies. We demonstrate that p23-2 is phosphorylated by recombinant CK2 from human and maize with favourable kinetic parameters. We also find a CK2-like activity in *Arabidopsis* extracts, that is able to phosphorylate recombinant p23-2: in fact, its activity on p23-2 is reduced in the presence of specific CK2 inhibitors, both *in vitro* and *in vivo*, while other protein kinase effectors, not active on CK2, are unable to change the p23-2 phosphorylation level. Moreover, p23 phosphorylation is increased in lysates from *Arabidopsis* mutants overexpressing CK2β3 and reported to have higher CK2 activity respect to the wild type plants (Sugano *et al.,* 1999). This data, together with the results obtained by means of in-gel kinase assay experiments, demonstrate that CK2 is the major kinase that phosphorylates p23 in *Arabidopsis*. From BIAcore expriments we also demonstrate that a physical association occurs between human αCK2 and p-23. Preliminary results obtained with the newly cloned p23-1 (not shown) show that both the p23

isoforms of *Arabidopsis thaliana* are substrate of CK2, as aspected considering the presence of CK2 consensus sites in their sequences (Fig. 3).

This work originated from the observation that CK2 is required for the NO synthesis upon SA treatment in *Arabidopsis*. Since the catalytic activity of CK2 does not change in response to SA, our hypothesis was that some specific substrates are phosphorylated upon stimulation, due to changes in their site accessibility. With this work we identified p23 as a possible mediator of CK2 involvement in SA signaling. At present we do not have any explanation on the reason why only in SA-treated plants p23 is phosphorylated, we can only say that the two proteins, CK2 and p23, can physically associate and co-localize. Further work will be necessary to assign a specific physiological role to the CK2-dependent p23 phosphorylation. Our results are now in the hands of our collaborators working on *Arabidopsis* physiology, (group of Prof. Lo Schiavo, Padua, Italy) which have produced *Arabidopsis* mutants not expressing p23, and are presently investigating on SA signaling of these mutants. Further plans concern the identification of the exact phosphorylation sites on p23 (1 and 2), with the final goal to mutate them to not-phosphorylatable residues and to determine the effects of such mutations on SA signaling.

Materials and methods

Materials

The CK2 inhibitor TBB (4,5,6,7 tetrabrome benzotriazole) was provided by Prof. Meggio (Padua, Italy), Quinalizarin (>99%analytically pure) was provided by Produits Chimiques ACP Chemicals. Staurosporine and K252a were from Sigma-Aldrich. All these compounds were diluted in 100% dimethyl sulphoxide (DMSO).

Recombinant human and maize CK2 (α and $\alpha_2\beta_2$) were kindly provided by Dr. Stefania Sarno (Padua, Italy), β-casein was from Sigma. [γ-^{33}P/^{32}P] ATP/GTP was from PerkinElmer. Prestained blue Protein Marker broad range, was from Cell Signaling, while ColorBurst™ colored electrophoresis protein markers were from Sigma.

Plant material, growth and treatment conditions

All plant material was provided by Dr. Alex Costa (group of Prof. Lo Schiavo, Padua). Plants and protein extracts were obtained as in Zottini *et al.,* 2007.

Expression and purification of recombinant *Arabidopsis* p23 proteins

The recombinant *Arabidosis* p23 proteins were also provided by Dr. Alex Costa and the protein purification was performed as in Sparla 1999.

Kinase assays with *Arabidopsis* seedlings extracts

Phosphorylation of endogenous proteins was performed with 5-10 μg of cytosolic proteins from seedlings treated as described above, incubated in the presence of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 20 μM [γ-^{33}P/^{32}P] ATP/GTP (1000-2000 cpm/pmol) in a total volume of 20 μl. Incubation was carried out at 30°C for 10 min and stopped by the addition of Laemmli buffer. The proteins were analysed by SDS-PAGE, Coomassie blue staining, and autoradiography (PerkinElmer's Cyclone Plus Storage Phosphor System).

Two-dimensional gel electrophoresis

Phosphorylation of endogenous proteins was performed with 100-125 μg of cytoplasmic proteins under the same conditions described above with 20 μM [γ-^{32}P] ATP (2000 c.p.m/pmol), in a total volume of 150 μl. The first dimension separation was isoelectric focusing (IEF): the incubation was blocked with 80% (v/v) ice-cold

acetone followed by freezing at -80°C for 1h. Precipitated proteins were centrifuged at 14000×*g*, for 20 min at 4°C. Acetone was discarded and pellets were dried at room temperature, resuspended in 125 µl of IEF buffer (8M urea, 2 M thiourea, 4% CHAPS (w/v), 0.5% ASB-14, 1% DTT, 0.8% Bio-lyte Ampholyte (3-10 pI range, Bio-Rad) and 0.002% bromophenol blue) and applied onto 7 cm immobilized pH gradient (IPG) strip (linear, pH 3-10, Bio-Rad). The first dimension was carried out on a Protean IEF Cell system (Bio-Rad) at 20°C following preset method for 7 cm strips. After IEF, the IPG strips were equilibrated in a buffer (6 M urea, 30% glycerol, and 2% SDS in 5mM Tris-HCl buffer, pH 8.8) containing 125 mM DTT first, and then 250 mM iodoacetamide (IAA), each incubation lasted 20 min. The second dimension was carried out on 11% SDS-PAGE followed by Coomassie blue staining and autoradiography (PerkinElmer's Cyclone Plus Storage Phosphor System).

Phosphorylation of recombinant p23 by recombinant CK2

For the phosphorylation of recombinant p23, 100 ng of protein were incubated with equivalent active (on the model substrate β-casein – 1-500 µg) amounts of human recombinant CK2 in the monomeric (α) or tetrameric form ($\alpha_2\beta_2$) or recombinant maize αCK2, in the conditions described above for kinase assays with *Arabidopsis* extracts, with the addition of 100 mM NaCl in the case of tetrameric CK2. For kinetic studies, different amounts of p23 (0.025-0.05-0.1-0.25 and 0.5 µg) were incubated with human recombinant αCK2 (15 ng), the human CK2 holoenzyme $\alpha_2\beta_2$ (4.5 ng) or the maize recombinant αCK2 (15 ng) in the presence of a radioactive phosphorylation mixture described above with with 20 µM [γ-^{32}P] ATP (2000 c.p.m/pmol) for 10 min at 30°C. Reactions were stopped by Laemmli sample buffer addition and the proteins were separated on 15% SDS-PAGE and analysed by autoradiography. For ^{32}P incorporation and kinetic parameters determination, the phosphorylated bands were excised and counted in a scintillation counter. Initial rate data were fitted to the Michaelis–Menten equation with the program Prism (GraphPad Software) to obtain Km and Vmax values. In experiments for the determination of phosphorylation stoichiometry, 0.1 µg of recombinant p23 was incubated under the conditions described above with 0.05 µg of recombinant αCK2 for 10 min with two further additions of 0.05 µg of αCK2 for 10 min.

In-gel kinase assay

For this assay, a protein substrate (10 μg/ml of p23 or 500 μg/ml of β-casein) was included in the solutions (running and stacking) of a SDS-PAGE where cytosolic proteins (5-20 μg) from *Arabidopsis* seedling extracts and 10 ng of recombinant human ΔC-αCK2 (as positive control) were separated according to Laemmli *et al.,* 1970 on 11% SDS-PAGE. After the electrophoresis, SDS was removed by two 30-min washings of the gel, in a buffer composed of 50 mM Tris-HCl, pH 8.0, 20 % (v/v) 2-propanol, at room temperature (RT). After a quick rinse in 50 mM Tris-HCl, pH 8.0, the gel was incubated for 1 h at RT in a buffer consisting of 50 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol (Buffer A) and subsequently in Buffer A plus 6 M guanidine, also at RT for 1h. For protein renaturation, the gel was incubated overnight at 4°C in Buffer A plus 0.04 % (v/v) Tween-20. For the protein phosphorylation, the gel was incubated with a phosphorylation mixture containing 50 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 20 μM ATP, [γ-^{32}P/^{33}P]ATP (specific radioactivity ~1000-5000 cpm/pmol). To remove the excess of ATP, the gel was exhaustively washed with trichloroacetic acid (TCA) 5% and sodium pyrophosphate 1%. All incubations were performed with gentle shaking in a volume ensuring total covering of the gel. The protein phosphorylation was then evaluated by autoradiography (PerkinElmer's Cyclone Plus Storage Phosphor System) while proteins were stained with Coomassie blue staining.

BIAcore experiments

For the Surface Plasmon Resonance (SPR) analysis, a BIAcore X system was used, as described in (Ruzzene 1999). Human αCK2 was covalently coupled to a CM5 sensor chip (carboxymethylated dextran surface), by the amine-coupling chemistry, to a final density of 1600 RU (resonance units); a flow cell with no immobilized protein was used as a control. The p23 solutions were injected under the conditions specified in the figure legend by using HBS-EP buffer (10 mM Hepes, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% P20 (GE Healthcare)); each sensorgram (time course of the surface plasmon resonance signal) was corrected for the response obtained in the control flow cell, and normalized to baseline. After each injection the surface was regenerated by injecting 1 M NaCl for 1 min; this treatment restored the baseline to the initial resonance unit value.

Co-localization experiments

These experiments were performed by Dr. Alex Costa using the specific p23 and CK2 constructs according to procedures as in Bregante 2008, and Sheen, J. 2002, A transient expression assay using *Arabidopsis* mesophyll protoplasts.
http://genetics.mgh.harvard.edu/sheenweb/

Protein kinase CK2 and molecular chaperones: implications in the control of cell survival

Introduction

Molecular chaperones are a special groups of conserved proteins which assist the proper folding of newly synthesized proteins, prevent unnecessary and harmful protein-protein interactions during synthesis and mediate refolding of misfolded proteins also breaking up protein aggregates and mediating multimolecular complexes dynamics. Many of these proteins are upregulated in response to heat shock and are therefore termed *heat shock proteins* (HSPs). Hsp90 is one of the most conserved HSPs, present from bacteria to mammals; it is highly abundant even in unstressed cells (Borkovich *et al.,* 1989) and associates with a wide variety of proteins, termed clients, that depend on its chaperoning function to acquire their active conformations (Taipale *et al.,* 2010). The two major isoforms of Hsp90 in humans, Hsp90α and Hsp90β, encoded by two distinct genes, share approximately 81% sequence homology (Passarino *et al.,* 2003; Chen *et al.,* 2005) and they seems to play different roles. While Hsp90β is constitutively expressed at high abundance in most tissues, Hsp90α is typically inducible in response to various cellular stress conditions (Csermely *et al.,* 1998, Sreedhar *et al.,* 2004; Pearl *et al.,* 2008). The Hsp90 structure consists of three domains: an amino terminal region (N-domain) that contains an ATP-binding site, a middle (M) domain for the interaction with client proteins, and a carboxy-terminal (C) domain for dimerization that is necessary for chaperone function. This function depends also on its weak intrinsic ATPase activity (Grenert *et al.,* 1999; Panaretou *et al.,* 1998; Pearl and Prodromou, 2006) that drives the

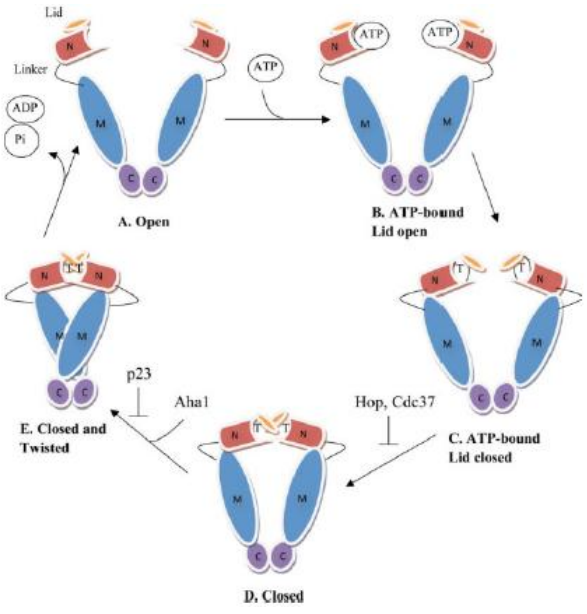

Figure 1. Hsp90 ATPase cycle. In the absence of ATP (A), Hsp90 dimerizes via its C-terminus and acquires the "open" conformation. Upon ATP binding (B), the N-terminal domains change their conformation, this result in closing of a "lid" over the bound nucleotide (C) with the formation of a second dimerization interface (D, the "closed" conformation). Rearrangements of the closed conformation still continue allowing interaction between the N-terminal and the middle domains, and this results in the "closed and twisted" conformation which is able to hydrolyze ATP (E). After ATP hydrolysis the lid opens, the N-terminal domains release from one another and Hsp90 returns to the open conformation (A). Co-chaperones binding to Hsp90 modulates its ATPase cycle. Hop/Sti1 and Cdc37 have an inhibitory effect, keeping Hsp90 in the open conformation. Aha1 stimulates ATP hydrolysis by promoting formation of the closed conformation, while Sba1 stabilizes the closed and twisted conformation.
From Zuehlke and Johnson, 2010.

chaperone cycle, mediating the conformational changes that makes Hsp90 switch from the open ADP-bound to the closed ATP-bound conformation, being ATP hydrolysis coupled to additional structural rearrangements (Prodromou *et al.,* 2000, Vaughan *et al.,* 2008) essential for client maturation (Fig. 1). The chaperone activity of Hsp90 is influenced by the association with many co-chaperones (Zuehlke and Johnson, 2010; http://www.picard.ch), which guide client protein recognition and modulate also its ATPase activity. There are many diverse Hsp90 client proteins involved in signal transduction, cellular trafficking, chromatin remodelling, cell growth and differentiation, among them two main groups have been well studied: the nuclear steroid receptors and the protein kinases. Regarding this last group, the protein kinase folding depends on the presence of the co-chaperone protein Cdc37 (Cell division cycle protein 37) that contributes to protein kinase activation by targeting them to the Hsp90-based chaperone machine; for this reason Cdc37 has been described as a substrate-specificity factor that directs Hsp90 to kinases (Hunter and Poon, 1997). Cdc37 has a general role in kinase biogenesis and maturation, and acts on nascent chains of protein kinases, either during or immediately after translation, protecting them from misfolding, aggregation and degradation (MacLean and Picard, 2003; Gray *et al.,* 2008); moreover it has been demonstrated to be essential for cell viability in different species (MacLean and Picard, 2003). Cdc37 interacts with the catalytic domain of kinases (Terasawa *et al.,* 2006; Caplan *et al.,* 2007) through its N-terminal domain (Shao *et al.,* 2003), which is the most highly conserved region among Cdc37 homologs; the middle domain binds the N-terminus of Hsp90 and has also a putative dimerization region, while the C-terminal part is involved in Hsp90 binding and is probably required for the dimer formation (Fig.2).

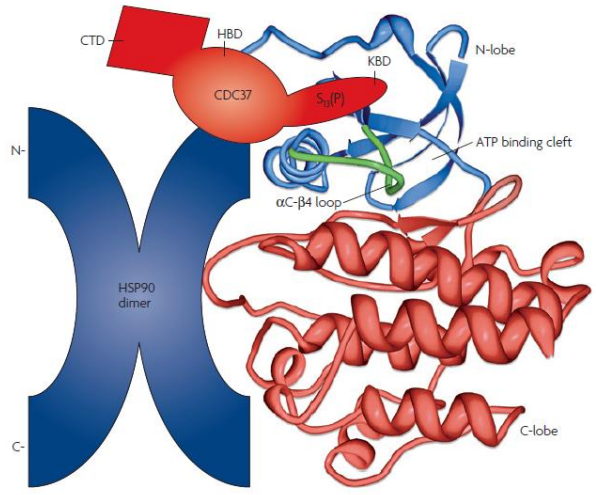

Figure 2. Hsp90-Ccd37-protein kinase interaction. Cdc37 can be divided in three domains: the amino-terminal domain for the client protein kinase binding (KBD) with the phospho-Ser13 residue, the central domain for Hsp90 binding (HBD) and dimerization, and a carboxyl-terminal domain (CTD) involved probably in Hsp90 binding and dimerization. The catalytic domains of protein kinases is depicted in the ribbon diagram with the N-lobe in blue and the C-lobe in red. Cdc37 (red) interacts with the N-lobe with its KBD whereas Hsp90 (blue) binds to both lobes and to the HBD of Cdc37. From Gray *et al*. 2008.

The regulatory function of Cdc37 is exerted by arresting the Hsp90 ATPase cycle, and with a mechanisms which is based on the interaction with residues crucial for ATPase activity in the N-terminal domain of Hsp90, which precludes the access of the middle domain catalytic loop to the ATP-binding pocket; block of the ATP-binding pocket of Hsp90 maintaining its molecular lid (Fig. 1) in an open, inactive conformation, prevention of Hsp90 N-terminal domain association, thus dimerization, in the closed state of the ATPase cycle (Siligardi *et al.,* 2002; Roe *et al.,* 2004; Zhang *et al.,* 2004). Cdc37 associates *in vitro* with Hsp90 in a dimer-dimer complex in the absence of a bound client kinase (Zhang *et al.,* 2004; Roiniotis *et al.,* 2005), while *in vivo* studies indicate that Cdc37 interacts with the kinase as a dimer, but with the kinase and Hsp90 as a monomer (Vaughan *et al.,* 2006). Interestingly Cdc37 has been demonstrated to possess chaperone activity on its own, independently from Hsp90 (Kimura *et al.,* 1997; Lee *et al.,* 2002; Turnbull *et al.,* 2005).

In mammals and yeast Cdc37 is post-translationally regulated in particular through phosphorylation at Ser13 (Ser14 in yeast) by protein kinase CK2 and the CK2-dependent phosphorylation is required for multiple Hsp90-client signaling protein kinases (Miyata and Nishida, 2004). The general importance of this phosphorylation, the only reported for Cdc37 *in vivo*, has been revealed by many studies: the mutation of the phosphorylation site in yeast severely affects the phenotype (Dey *et al.,* 1996; Bandhakavi *et al.,* 2003) and the mutants are defective in supporting many kinases (Abbas-Terki *et al.,* 2000; Mandal *et al.,* 2007). The phosphorylation of Cdc37 is essential for the binding to numerous kinases in mammalian cells (Miyata and Nishida, 2004), and is important for the recruitment of Hsp90 to protein kinase-Cdc37 complexes, while is not required for the Hsp90 binding to Cdc37 (Shao *et al.,* 2003*, Miyata and Nishida, 2004). Moreover the inhibition of CK2 activity, exerted by treating mammalian cells *in vivo* with CK2-specific inhibitors, decreases the phospho-Cdc37 level and the amount of Cdc37-dependent kinases (Miyata and Nishida, 2004), whose diversity and variety also suggest that their interaction with Cdc37 takes place when it is in the phosphorylated state (Miyata and Nishida 2007; Vaughan *et al.,* 2008). Taken together, all these data suggest that the CK2-dependent phosphorylation of Cdc37 is indispensable for the maintenance of the Hsp90-Cdc37-protein kinase heterocomplexes, and for the proper activation (Arlander *et al.,* 2006) and thus the physiological role of these clients (Miyata and Nishida 2004; Gray *et al.,* 2008). CK2 itself has been included among the Cdc37 client kinases (Kimura *et al.,* 1997; Bandhakavi *et al.,* 2003), and a positive feedback loop promoting multiple client

kinases, has been suggested between the two proteins (Bandhakavi *et al.,* 2003; Miyata, 2009).

The concept of cycle could also be applied to the function of Cdc37 if we consider its phosphorylation state: in fact, it has been proposed that not only the Ser13 phosphorylation is required, but also its dephosphorylation, in order to obtain the full activation of the machinery; indeed, this could explain why, when Ser13 is mutated to a phosphomimetic (but not dephosphorylatable) residue such as Glu, the protein is inactive (Shao *et al.,* 2003*). Concerning the phosphatase implicated in Ser13 dephosphorylation it has been demonstrated that the phosho-Ser13 is differently accessible to specific or not dephosphorylation if Cdc37 is in the monomeric form or in the Hsp90-Cdc37-Cdk4 complex (Vaughan *et al.,* 2006); in particular isolated phospho-Cdc37 is rapidly dephosphorylated by the non-specific λ-phosphatase, while when present in complex with Hsp90 and Cdk4, only the association of the phosphatase PP5 with the same Hsp90 dimer to which Cdc37 is bound, results in its dephosphorylation (Vaughan *et al.,* 2008). Anyway, the precise mechanism of the phosphorylation-dependent regulation is not completely elucidated, also because Cdc37 crystal structures lack the N-terminal domain where Ser13 in located.

Protein kinases are key players in signal transduction networks (Manning 2002) and their mutations and/or overexpression are critical for neoplastic cell growth; since Cdc37 has a specialized role in kinome maintenance, it may participate in maintaining the malignant phenotype (Pearl, 2005; Smith and Workman, 2009). Actually Cdc37 is upregulated in cancer cells and, importantly, in pre-cancerous states (Stepanova *et al.,* 2000,*). For all this reasons, Cdc37 can be considered a therapeutic target (Smith and Workman, 2009, Smith *et al.,* 2009), and compared to Hsp90 (Whitesell and Lindquist, 2005) has the advantage to be specific for kinases. The Cdc37 activity disruption can be achieved not only by blocking its functional interactions, but also by preventing its phosphorylation: the feedback-loop mechanism between Cdc37 and CK2 suggests that a modest CK2 inhibition may possibly disrupt the kinome chaperone system (Miyata, 2009).

In this study, we analysed the importance of Cdc37 and its correlation to the phenomenon of apoptosis resistance, taking advantage of the CEM cells model, acute T-lymphoblastoid leukemia cells, which are available in our laboratory in two variants: the S-CEM, normally sensitive to drug-induced apoptosis, and the R-CEM, derived from the parental one by selection with vinblastine treatment, but displaying

a cross-resistance to many other chemotherapeutic drugs (Dupuis *et al.,* 2003) and expressing the P-glycoprotein (Juliano e Ling, 1976; Molinari *et al.,* 2002).

We have previously demonstrated that these two cell lines differ also in the CK2 content; in particular, the R-CEM cells express a higher level of the catalytic (α) subunit of CK2, and thus they have higher CK2-dependent phosphorylation of endogenous proteins, respect to the normal S-CEM cells. In this model, the study of the role of Cdc37 phosphorylation in regulating its function could be facilitated by the comparison of two conditions with different CK2 activity; on the other hand the model also gives the possibility to assess if Cdc37 has a role in the regulation of αCK2 level.

Results

S-CEM and R-CEM cells express different isoforms of Cdc37

First of all we examined the expression level of Cdc37 in both apoptosis-sensitive (S-CEM) or resistant (R-CEM) cells by western blot, using different antibodies from Santa Cruz Biotechnology; surprisingly we obtained conflicting results: with the antibodies defined as Ab E and Ab G, the Cdc37 protein was detectable only in R-CEM, while with the antibody called Ab F the Cdc37 expression level was similar in the two cell lines (Fig.1).

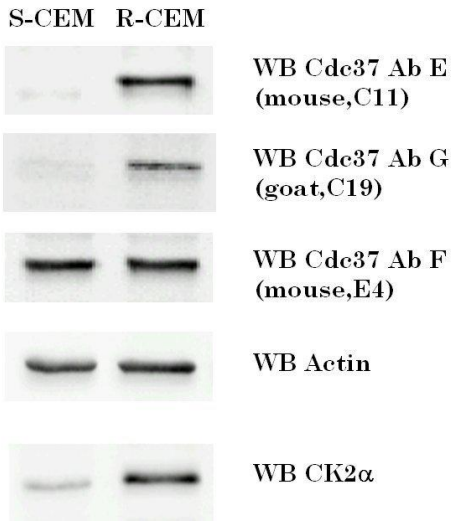

Figure 1: Ccd37 protein level in S and R-CEM cells. 10 μg (20 μg for Ab G) of proteins from total cell lysates were analysed by 11% SDS-PAGE and WB with the indicated antibodies. Actin was used for normalization. CK2α is also shown.

All the antibodies were supposed to recognize the C-terminal half of the protein, but the exact epitope was unknown. Since the signal with Ab G was very weak, we decided to focus on Ab E and Ab F: they are both raised against amino acids 108-378 of human Cdc37 but Ab E recognizes only human Cdc37, while Ab F reacts also with mouse and rat isoforms. The main differences in the Cdc37 sequence from human, mouse and rat origin, are at the C-terminus and in particular in the last ten amino acids (Fig. 2); so we reasoned that, conceivably, Ab E recognizes the extreme C-terminus of the protein, while the epitope for Ab F is more internal in the Cdc37 sequence.

```
Q63692  MVDYSVWDHIEVSDDEDETHPNIDTASLFRWRHQARVERMEQFQKEKEELDRGCRECKRK  60    CDC37_RAT
Q61081  MVDYSVWDHIEVSDDEDETHPNIDTASLFRWRHQARVERMEQFQKEKEELDRGCRECKRK  60    CDC37_MOUSE
Q16543  MVDYSVWDHIEVSDDEDETHPNIDTASLFRWRHQARVERMEQFQKEKEELDRGCRECKRK  60    CDC37_HUMAN
        ************************************************************
Q63692  VAECQRKLKELEVAEGGGQVELERLRAEAQQLRKEERSWEQKLEDMRKKEKNMPWNVDTL  120   CDC37_RAT
Q61081  VAECQRKLKELEVAESDGQVELERLRAEAQQLRKEERSWEQKLEDMRKKEKNMPWNVDTL  120   CDC37_MOUSE
Q16543  VAECQRKLKELEVAEG-GKAELERLQAEAQQLRKEERSWEQKLEEMRKKEKSMPWNVDTL  119   CDC37_HUMAN
        ***************. *:.*****:******************:******.********
Q63692  SKDGFSKSMVNTKPEKAEEDSEEAREQKHKTFVEKYEKQIKHFGMLHRWDDSQKYLSDNV  180   CDC37_RAT
Q61081  SKDGFSKSMVNTKPEKAEEDSEEAREQKHKTFVEKYEKQIKHFGMLHRWDDSQKYLSDNV  180   CDC37_MOUSE
Q16543  SKDGFSKSMVNTKPEKTEEDSEEVREQKHKTFVEKYEKQIKHFGMLRRWDDSQKYLSDNV  179   CDC37_HUMAN
        ****************:******.**********************:*************
Q63692  HLVCEETANYLVIWCIDLEVEEKCALMEQVAHQTMVMQFILELAKSLKVDPRACFRQFFT  240   CDC37_RAT
Q61081  HLVCEETANYLVIWCIDLEVEEKCALMEQVAHQTMVMQFILELAKSLKVDPRACFRQFFT  240   CDC37_MOUSE
Q16543  HLVCEETANYLVIWCIDLEVEEKCALMEQVAHQTIVMQFILELAKSLKVDPRACFRQFFT  239   CDC37_HUMAN
        **********************************:************************
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Q61081  KIKTADHQYMEGFKYELEAFKERVRGRAKLRIEKAMKEYEEEERKKRLGPGGLDPVEVYE  300   CDC37_MOUSE
Q16543  KIKTADRQYMEGFNDELEAFKERVRGRAKLRIEKAMKEYEEEERKKRLGPGGLDPVEVYE  299   CDC37_HUMAN
        ******:******: ********************************************
Q63692  SLPEELQKCFDVKDVQMLQDAISKMDPTDAKYHMQRCIDSGLWVPNSKSGEAKEGEEAGP  360   CDC37_RAT
Q61081  SLPEELQKCFDVKDVQMLQDAISKMDPTDAKYHMQRCIDSGLWVPNSKSGEAKEGEEAGP  360   CDC37_MOUSE
Q16543  SLPEELQKCFDVKDVQMLQDAISKMDPTDAKYHMQRCIDSGLWVPNSKASEAKEGEEAGP  359   CDC37_HUMAN
        ************************************************:.**********
Q63692  GDPLLEAVPKAGNEKDISA  379   CDC37_RAT
Q61081  GDPLLEAVPKAGNEKDVSA  379   CDC37_MOUSE
Q16543  GDPLLEAVPKTGDEKDVSV  378   CDC37_HUMAN
        **********:*:***:*.
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Figure 2: Alignment of rat, mouse and human Cdc37 isoforms. ClustalW alignment performed on UniProt. On the left UniProtKB entries of Cdc37 isoforms. In dark grey conserved amino acids indicated also with (*), amino acids whose difference do not influence structure are indicated with (:) while amino acid differences that influence structure are indicate with (.).

Based on this speculation, we decided to synthesize a peptide whose sequence (PKTGDEKDVSV) reproduces the last eleven amino acids of human Cdc37, and we used it as a competitor for the reactivity of antibodies E and F towards Cdc37 protein; we therefore performed the western blot experiments in the presence of 300 μM peptide, and the results are shown in Fig.3.

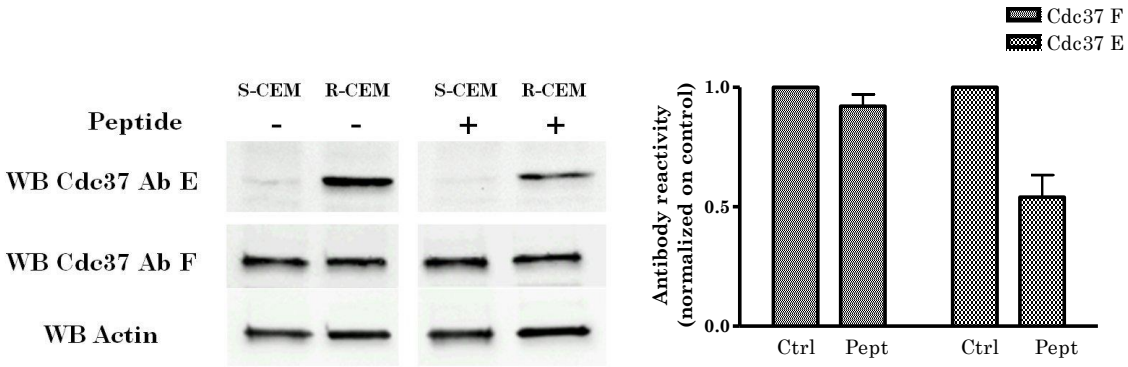

Figure 3: WB analysis of Cdc37 in the presence of the blocking peptide. 10 μg of proteins from S- and R-CEM total cell lysates were analysed by 11% SDS-PAGE and WB with the antibodies Ab E and F, in the presence, where indicated (+) of 300 μM peptide PKTGDEKDVSV. On the right it is shown the graph of the quantification of western blots with Ab E and F in R-CEM cells, by Kodak Image Station 4000MM Pro. The signals obtained with the indicated antibody in the presence of the peptide (Pept) were normalized on controls (Ctrl) without it; vertical bars indicate the standard deviation to the mean obtained from three separated experiments.

The reactivity of the Ab E antibody was markedly reduced, while the signal obtained with Ab F was not significantly influenced by the presence of the peptide, hence we confirmed that these antibodies recognize different regions and in particular that the Ab E epitope is at the C-terminus of the protein, while the Ab F one is in a more internal region. All together these western blot experiments demonstrate that the Cdc37 protein level is similar in S- and R-CEM, but in S-CEM the protein is slightly shorter, since it lacks the region recognized by Ab E, which instead is present in R-CEM Cdc37.

To determine if the difference of Cdc37 in the two cell lines was due to a proteolytic cleavage or to the expression of a different protein, we performed an RT-PCR with two different combinations of primers in order to amplify the full length or a shorter form of the *CDC37* coding sequence.

ATGGTGGACTACAGCGTGTGGGACCACATTGAGGTGTCTGATGATGAAGACGAGACGCACCCCAACATCGACACGGCCAGTCT
CTTCCGCTGGCGGCATCAGGCCCGGGTGGAACGCATGGAGCAGTTCCAGAAGGAGAAGGAGGAACTGGACAGGGGCTGCCGCG
AGTGCAAGCGCAAGGTGGCCGAGTGCCAGAGGAAACTGAAGGAGCTGGAGGTGGCCGAGGGCGGCAAGGCAGAGCTGGAGCGC
CTGCAGGCCGAGGCACAGCAGCTGCGCAAGGAGGAGCGGAGCTGGGAGCAGAAGCTGGAGGAGATGCGCAAGAAGGAGAAGAG
CATGCCCTGGAACGTGGACACGCTCAGCAAAGACGGCTTCAGCAAGAGCATGGTAAATACCAAGCCCGAGAAGACGGAGGAGG
ACTCAGAGGAGGTGAGGGAGCAGAAACACAAGACCTTCGTGGAAAAATACGAGAAACAGATCAAGCACTTTGGCATGCTTCGC
CGCTGGGATGACAGCCAAAAGTACCTGTCAGACAACGTCCACCTGGTGTGCGAGGAGACAGCCAATTACCTGGTCATTTGGTG
CATTGACCTAGAGGTGGAGGAGAAATGTGCACTCATGGAGCAGGTGGCCCACCAGACAATCGTCATGCAATTTATCCTGGAGC
TGGCCAAGAGCCTAAAGGTGGACCCCCGGGCCTGCTTCCGGCAGTTCTTCACTAAGATTAAGACAGCCGATCGCCAGTACATG
GAGGGCTTCAACGACGAGCTGGAAGCCTTCAAGGAGCGTGTGCGGGGCCGTGCCAAGCTGCGCATCGAGAAGGCCATGAAGGA
GTACGAGGAGGAGGAGCGCAAGAAGCGGCTCGGCCCCGGCGGCCTGGACCCCGTCGAGGTCTACGAGTCCCTCCCTGAGGAAC
TCCAGAAGTGCTTCGATGTGAAGGACGTGCTGATGCTGCAGGACGCCATCAGCAAGATGGACCCCACCGACGCAAAGTACCAC
ATGCAGCGCTGCATTGACTCTGGCCTCTGGGTCCCCAACTCTAAGGCCAGCGAGGCCAAGGAGGGAGAGGAGGCAGGTCCTGG
GGACCCATTACTGGAAGCTGTTCCCAAGACGGGCGATGAGAAGGATGTCAGTGTGTGA

Figure 4: Full length coding sequence of human *CDC37* mRNA. The primers used for PCR amplification are highlighted in yellow and red (for full length amplification) or yellow and green (for shorter form amplification).

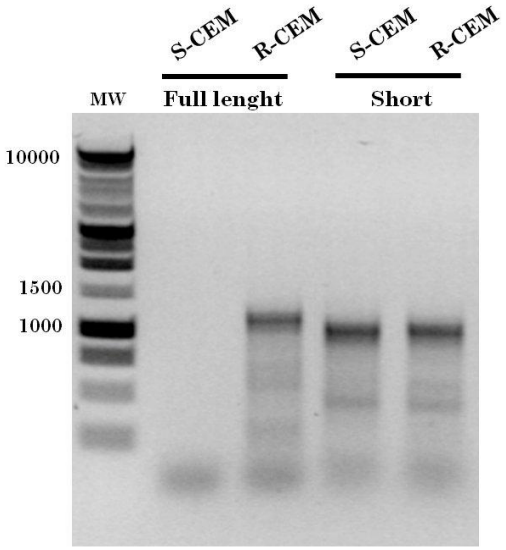

Figure 5: Amplification products obtained from RT-PCR with the primes described in Fig. 4. Amplicons were separated on 1% agarose gel. MW markers migration is shown on the left.

These experiments, performed by Dr. Alex Costa (Padua, Italy), revealed that the full length coding sequence of *CDC37* was amplified only in R-CEM, where, using the primers that hybridize at the beginning and at the end of the full coding sequence

(indicated in yellow and in red, respectively in Fig.4), we obtained a strong signal; when the amplification was performed with a primer indicated in green in Fig. 4 that hybridize in a more internal region, a shorter form of the *CDC37* coding sequence was also found both in R-CEM and in S-CEM (Fig. 5). This demonstrates that the CEM cells express two different isoforms of Cdc37, and in particular that the protein in S-CEM lacks a fragment at the C-terminus.

Cdc37 is degraded during apoptosis

Since the major difference between S- and R-CEM is the sensitivity to drug-induced apoptosis, as said previously, we decided to study the function of Cdc37 during apoptosis: we looked for a possible different behaviour of the two Cdc37 isoforms described above, particularly interesting considering that in cancer the imbalance between survival and apoptosis is often due to an overexpression of kinases that are known as Cdc37 clients (Smith and Workman, 2009).

We treated the cells with different apoptotic stimuli (not shown), and we found that staurosporine, an alkaloid protein kinase inhibitor, characterized as a strong inducer of apoptosis in many different cell types (Gani and Engh, 2010), was effective also in R-CEM and was able to give a similar cell death response in both cell lines (Fig.6).

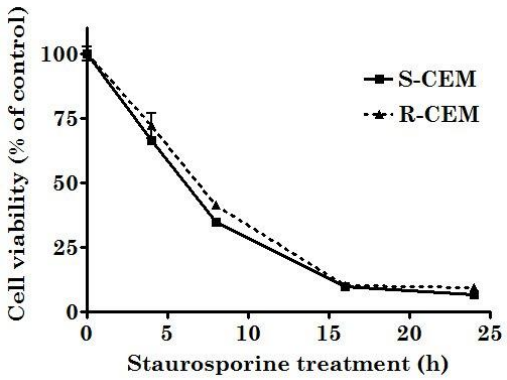

Figure 6: Cell viability upon staurosporine treatment. CEM cells were treated for 4-8-16 and 24 hours with 1μM staurosporine. Cell viability was measured with the MTT assay. The percentage of viable cells is expressed as percentage respect to the controls, treated with vehicle (DMSO).
Vertical bars indicate the standard deviation to the mean obtained from three separated experiments.

Using this stimulus, we first analysed the effect of apoptosis on Hsp90 amount and we found that it is degraded in apoptosis, and that the degradation is faster in S- than in R-CEM (Fig. 7).

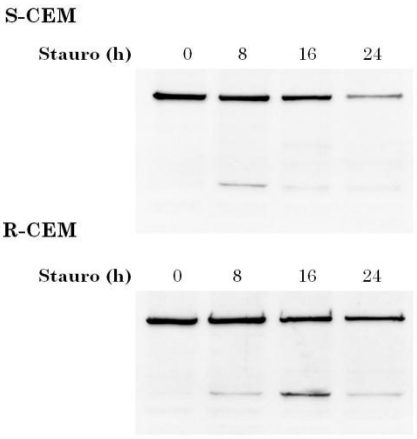

Figure 7: Expression level of Hsp90 in apoptosis. Cells were treated for the indicated times with 1 μM staurosporine. 10 μg of proteins from total cell lysates were analysed by 11% SDS-PAGE and WB with Hsp90 antibody.

We then analysed the levels of Cdc37 in total lysates obtained from cell treated for different times with staurosporine.

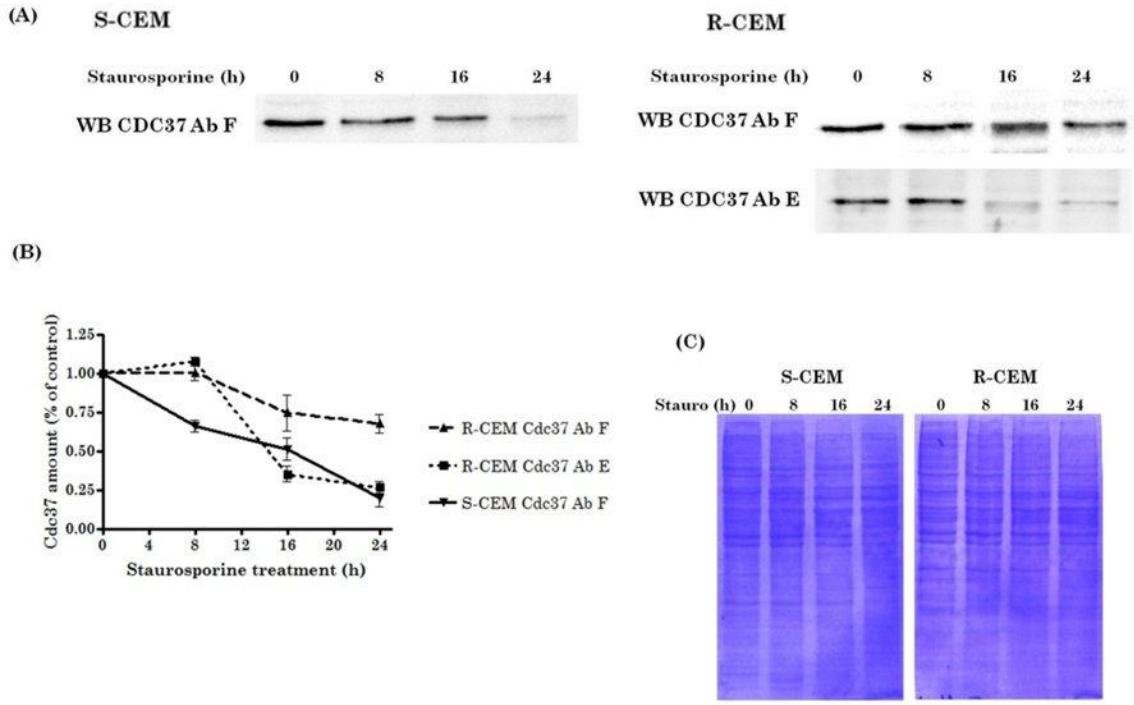

Figure 8: Level of Cdc37 in apoptosis. Cells were treated for the indicated times with 1 μM staurosporine. (A) 10 μg of proteins from total cell lysates were analysed by 11% SDS-PAGE and WB with the indicated antibodies.
(B) Quantification of Cdc37 degradation. Quantification of signals obtained with the antibodies indicated, with Kodak Image Station 4000MM Pro. The amount of Cdc37 is expressed as percentage of the control represented by cells treated with vehicle (DMSO); vertical bars indicate the standard deviation to the mean obtained from three separated experiments. (C) Coomassie staining of WB membranes performed as loading control.

Interestingly, we found that also the level of Cdc37 decreases in apoptosis; however, also in this case its disappearance is particularly evident in S-CEM where the protein, detected with Ab F, is already decreased after 8 h of treatment and completely absent after 24 h (Fig 8A). On the contrary, in R-CEM Cdc37 appears to be more stable, since the signal does not decrease significantly after 24h, when detected by Ab F. However, its C-terminus is apparently more rapidly cleaved, as judged by the decreased signal during apoptosis obtained with Ab E, which recognized the extreme C-terminus of Cdc37, as described above (Fig. 8A). These results suggests that in apoptosis Cdc37 in S-CEM is faster degraded while in R-CEM the main body of the protein is quite stable and only the C-terminus is rapidly degraded. Fig. 8B shows the quantification of the Cdc37 bands obtained with different antibodies in the two cell lines. It has to be noticed that other proteins are degraded besides Cdc37 mainly in S-CEM at long times of treatment (not shown) while in R-CEM they are much more stable, but this degradation does not involve all the proteins, as shown by the membrane staining performed as loading control (Fig. 8C).

To confirm that the C-terminus degradation in R-CEM Cdc37 occurs before the cleavage of the main body of the protein, we used a different apoptotic stimulus, the anti-human Fas activating antibody, exploiting the fact that R-CEM are well responsive to receptor-apoptosis signals.

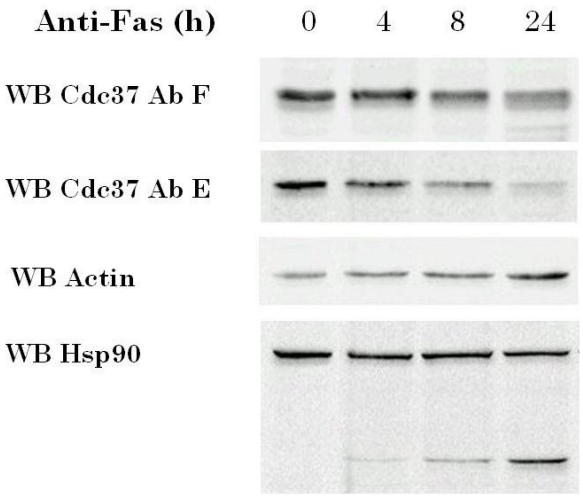

Figure 9: Effects with anti-human Fas activating antibody. Cells were treated with anti-Fas antibody (50 ng/ml) for the indicated time. 10 μg of proteins from total lysates were analysed by SDS-PAGE and WB with the indicated Ab.

Also in this case, the changes in Cdc37 signal are different with the two antibodies, confirming that only the C-terminus of the protein is rapidly cleaved. The Hsp90 degradation is also confirmed (Fig. 9); so, taken together, these results overlap with those obtained with staurosporine.

The CK2-dependent phosphorylation level of Cdc37 in CEM cells is similar

It has been already demonstrated that Cdc37 is a CK2 substrate and that the CK2-dependent phosphorylation at Ser13, the only phosphorylation reported for this protein until now, is essential for the recruitment of Hsp90 to the Cdc37-client kinase complexes (Miyata, 2009). We analysed the phosphorylation level of Cdc37 in CEM cell with a phospho-specific antibody directed against phospho-Ser13.

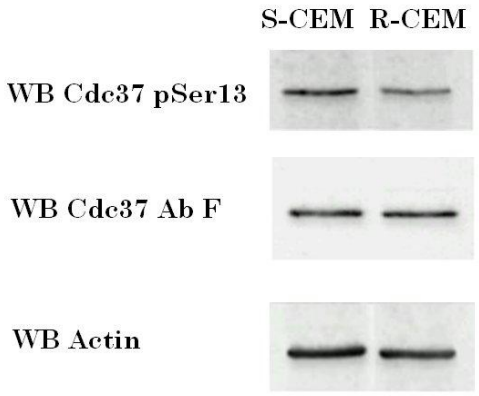

Figure 10: CK2-dependent phosphorylation level of Cdc37. 20 μg of proteins from total lysates were analysed by SDS-PAGE and WB with the indicated Ab.

Since R-CEM cells express a higher level of the catalytic subunit of CK2 respect to S-CEM, we expected a higher CK2-depend phosphorylation of Cdc37 in these cells, nevertheless we found that it is similar or even lower in R-CEM respect to S-CEM (Fig. 10). It has been reported that a specific protein phosphatase, PP5 (Vaughan *et al.*, 2008); is responsible for the dephosphorylation of phospho-Ser13, and that the cycle between the phosphorylated and the dephosphorylated state of Cdc37 is important for its function. On these bases, we hypothesized that our observations on Cdc37 phosphorylation level in S- and R-CEM was due to a different arrangement of the proteins in complex with Cdc37 in case of different isoform expressed; the possibility exists that in the two cell lines different partners are associated in the complexes, or that they are differently accessible to the kinase and/or the phosphatase.

On this regard, by analysing the phospho-Ser13 level during apoptosis, we obtained some evidences that the presence/absence of the C-terminus of Cdc37 is related to the Ser13 phosphorylation. As shown in Fig. 11, we found that the cleavage of the C-

terminus observed in R-CEM treated with staurosporine, is accompanied by phospho-Ser13 dephosphorylation, suggesting that the absence of the C-terminus renders the site more accessible.

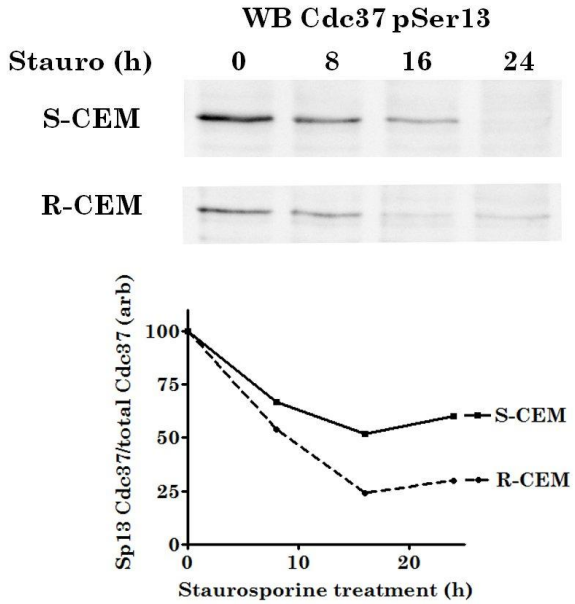

Figure 11: CK2-dependent phosphorylation level of Cdc37. Cells were treated with 1 μM staurosporine for increasing length of time and 20 μg of proteins from total lysates were analysed by SDS-PAGE and WB with the indicated Ab (shown in the upper panel of the figure). On the lower panel, it is shown the graph of bands quantification by Kodak Image Station 4000MM Pro. The phospho-Ser13 signal was normalized to the total amount of Cdc37 protein.

Cdc37 is present in different complexes in S- and R-CEM

We decided to analyse the multimolecular complexes formed by Cdc37 in the two cell lines; to this purpose, we separated protein complexes of the cell lysates by ultracentrifugation on glycerol gradients, and we analysed the gradient fractions by WB.

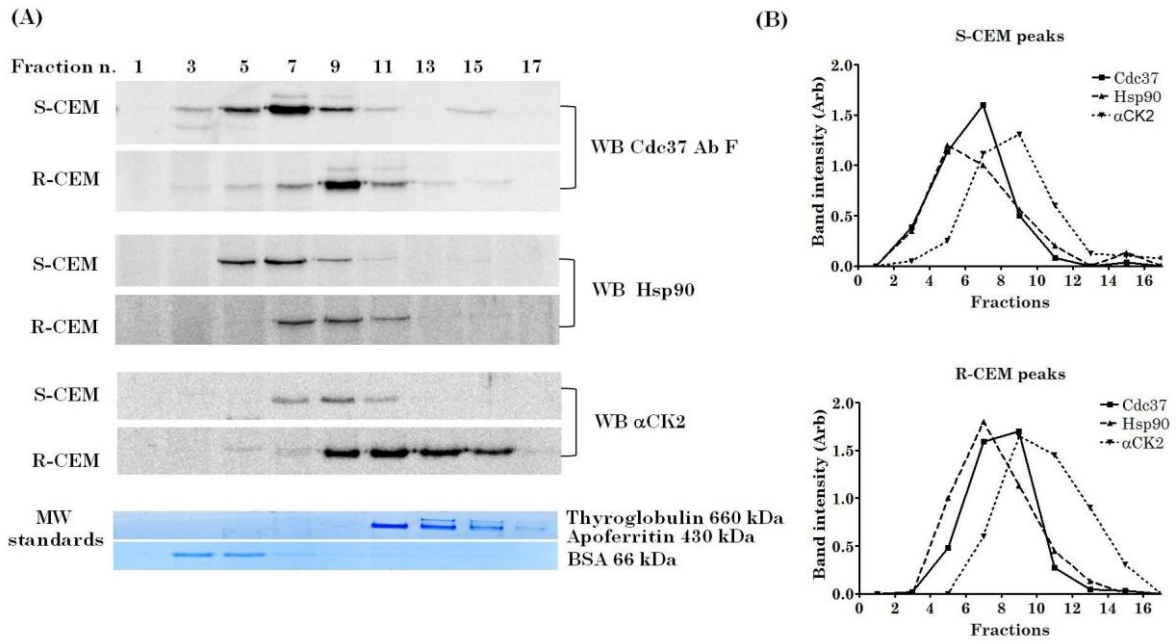

Figure 12: Cdc37, Hsp90 and αCK2 distribution in the fractions of the glycerol gradient. Cytosolic fraction lysate (250 μg of proteins) from CEM cells was loaded on top of a linear glycerol gradient (10%-40%) and centrifuged as detailed in "Material and methods". Nineteen fractions were collected and 15 μl of odd fractions were analysed by WB with the indicated antibodies. For molecular weight estimation, molecular weight standards have been run in parallel and 25μl of odd fractions were analysed by SDS-PAGE and coomassie blue staining (A). Trends in Cdc37, Hsp90 and αCK2 distribution on glycerol gradients. A graph, obtained by quantifying the WB signal of the indicated proteins, with Kodak 4000MM Pro Image Station, is representative of experiments performed in triplicate.

We found that the migration of Cdc37 and Hsp90 on the gradients is different in the two cell lines and, in particular, in R-CEM the position of the proteins is shifted to higher molecular weights respect to S-CEM (Fig. 12). This suggests that the composition of Cdc37-Hsp90 multimolecular complexes in the two cell lines is different and that in R-CEM, where Cdc37 has a complete C-terminus, there could be some additional partners.

We also analysed the migration of the CK2 α catalytic subunit, and interestingly, we noticed that in R-CEM there is a higher amount of αCK2 that migrates independently from the chaperone complexes (Fig.12). This could be related to the lower

phosphorylation level of Ser13 observed in R-CEM, despite the higher CK2α expression.

S-CEM are more sensitive to Hsp90 inhibition

To obtain further evidence for the occurrence of different chaperone complexes in S- and R-CEM, we treated the cells with the Hsp90 inhibitor BIIB021 (formerly CNF2024). This inhibitor binds to the ATP-binding pocket of Hsp90, interferes with its chaperone function, and results in client protein degradation and tumor growth inhibition. It has been demonstrated from *in vitro* studies, that treatment of tumor cells with BIIB021 leads to degradation of the Hsp90 client oncogenic signaling proteins (Lundgren *et al.,* 2009; Yin *et al.,* 2010, Zhang *et al.,* 2010). Furthermore, oral administration of BIIB021 to athymic mice bearing a variety of human tumors resulted in client protein degradation and dose-dependent inhibition of tumor growth (Lundgren *et al.,* 2009). In cell proliferation assays, BIIB021 potently inhibited the growth of cell lines from a variety of tumor types; particularly interesting for our purpose, it is also effective in multidrug resistant cell (Zhang *et al.,* 2010). We therefore decided to use this Hsp90 inhibitor to treat S- and R-CEM cells, and to evaluate if it is differently effective.

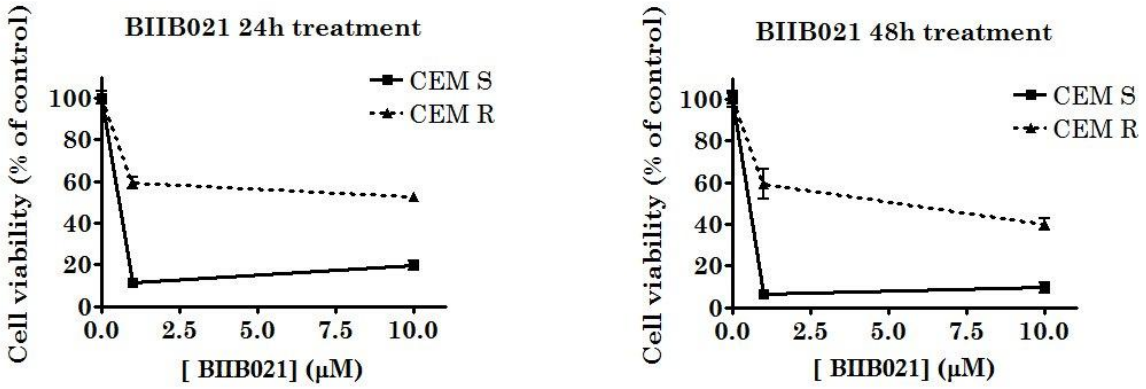

Figure 13. CEM cells viability upon BIIB021 treatment. Cells seeded at $1x10^6$/ml were treated with 1 or 10 μM BIIB021 for 24 and 48 hours. Cell viability was measured with MTT assay. These graphs are representative of experiments done in triplicate.

We found that it is more effective in inducing cell death in S- respect to R-CEM, whose viability is decreased near the 50% only at 48 hours with 10 μM inhibitor concentration, whereas viable S-CEM at 24 hours are already less than 20 % with 1 μM inhibitor (Fig. 13). This result support the hypothesis that the chaperone complex in R-CEM is different, thus more resistant to inhibition than in S-CEM.
However, we were still afraid that R-CEM were refractory to the treatment because they express the P-glycoprotein (P-gP), as occurs in the case of other Hsp90 inhibitors, such as geldanamycin (Zhang *et al.,* 2010) (not shown). For this reason, we performed the same treatment using another cellular model, the U2OS cells, that are a human osteosarcoma cell line, available in the two variants S-U2OS, apoptosis sensitive and R-U2OS resistant to drug-induced apoptosis and expressing P-gP.

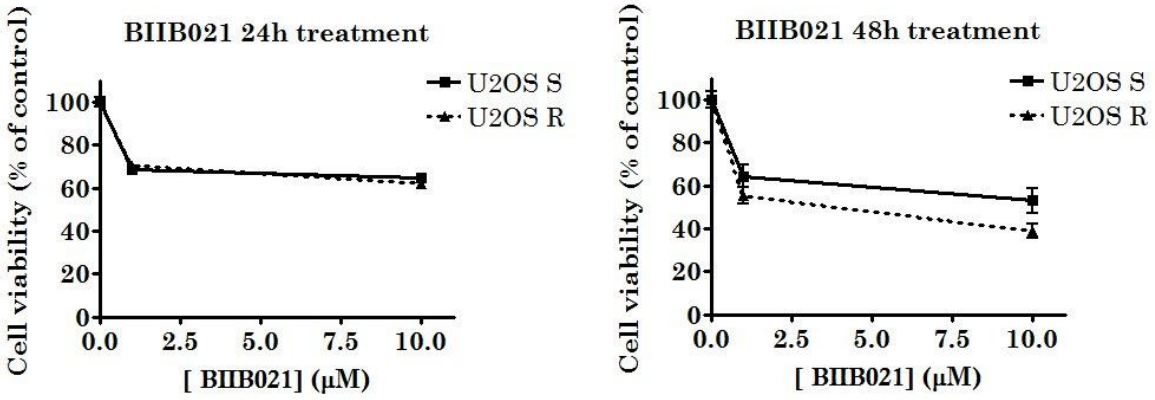

Figure 14. U2OS cells viability upon BIIB021 treatment. 15×10^6 cells were seeded in each 96-well plate and treated with 1 or 10μM BIIB021 for 24 and 48 hours. Cell viability was measured with MTT assay. These graphs are representative of experiments done in triplicate.

The effect of BIIB021 was similar in S- and R-U2OS cells, that are both quite resistant to inhibitor-induced cell death, that reaches 50% only after 48 h of treatment at 10 μM inhibitor concentration (Fig. 14). This result confirms that the effect of BIIB021, as reported in literature, is not influenced by the expression of P-gP. It is important to note that U2OS cells express both the full length isoform of Cdc37 (Fig. 15), so their similar response to BIIB021 is expected. Moreover if we compare the effect of Hsp90 inhibition in CEM and U2OS cell lines, the full length isoform expression seems to be protective (Fig. 16).

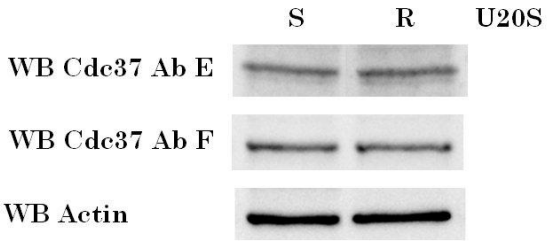

Figure 15. Cdc37 expression in U2OS cells. 10 µg of proteins from total lysates were analysed by SDS-PAGE and WB with the indicated Ab.

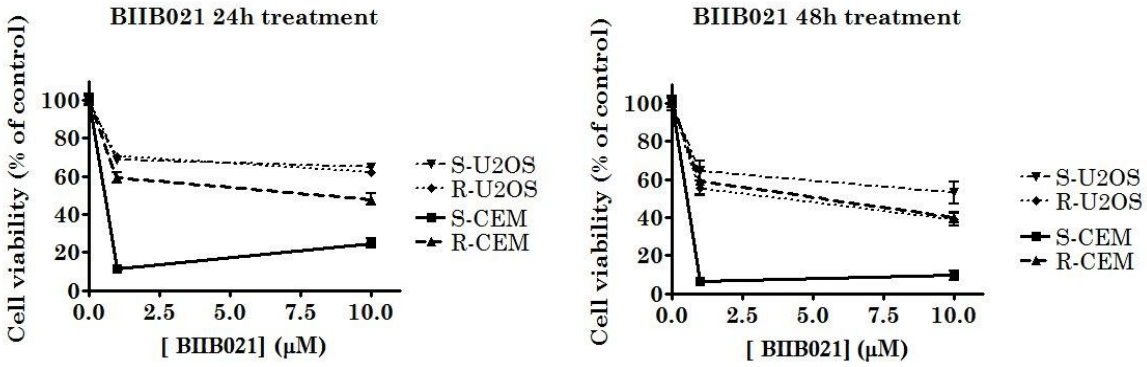

Figure 16. Comparison between CEM and U2OS viability upon BIIB021 treatment. Cell viability measured by MTT assay expressed by percentage of the control treated with vehicle (DMSO). These graphs are representative of experiments done in triplicate.

Discussion

With this work, we analysed the expression of Cdc37 in two different cell line variants, either sensitive or resistant to drug-induced apoptosis; we found that the protein expression is similar, but the sensitive S-CEM cells express an isoform shorter at the C-terminus respect to the isoform of the resistant R-CEM. We also provided data demonstrating that the shorter form is not the product of a proteolytic cleavage, since also a different mRNA isoform is detectable. At present we do not know the reason of this difference and further investigation has to be done however a possible hypothesis could be alternative splicing. Also the exact length of the C-terminal fragment absent in the S-CEM Cdc37 isoform is unknown; from the RT-PCR experiments, we can only say that it is less than 36 amino acids long, consistently with the very similar migration in SDS-PAGE of the two isoforms expressed in S- and R-CEM (see Fig. 1).

It is worth to note that the two Cdc37 isoforms are not necessary associated to the sensitive or resistant phenotype, since two other cell line variants that we have analysed, S-U2OS and R-U2OS, both express the full length Cdc37 protein. So the aim of this work was not to correlate one Cdc37 isoform to the multidrug resistance, but to exploit the S-CEM and R-CEM model for more detailed investigation of the Cdc37 function, and in particular of the role of the C-terminal part of the protein, which is still unclear (Shao *et al.,* 2003).

Our results report for the first time, to our knowledge, of an apoptotic-dependent cleavage of Cdc37. This provide a strong support to the frequently proposed pro-survival role of Cdc37. Interestingly the C-terminus of the protein seems to be particularly important in this role: not only S-CEM, more susceptible to apoptosis, express an isoform devoid of this domain, but also the C-terminal fragment, when present (in R-CEM) is the first target of the proteases in apoptosis, suggesting that its removal could be instrumental for the cell death. Of course, further investigation will be necessary to confirm this hypothesis and to identify the enzyme(s) responsible and the exact mechanism for the Cdc37 degradation. Also Hsp90 is cleaved during apoptosis, and, as found for Cdc37, its degradation is faster and more pronounced in S-CEM than in R-CEM.

Surprisingly when we analyse the CK2-dependent phosphorylation level of Cdc37 with a phospho-specific antibody directed to phospho-Ser13 (Miyata and Nishida,

2008), we found that the phosphorylation level is similar or even lower in R-CEM respect to S-CEM, and this is unexpected since R-CEM express a higher level of αCK2; although the exact reason of this result is still under investigation, we can hypothesize that it is due to a different accessibility of the two isoform both to the kinase and the to phosphatase (Vaughan *et al.,* 2008).

All together, our results suggest that the two Cdc37 isoforms are present in different multimolecular complexes; to assess this hypothesis, we performed the separation of the protein complexes by ultracentrifugation on glycerol gradient of the cell lysates. Indeed we found that Cdc37 in R-CEM forms higher molecular weight complexes respect to the shorter Cdc37 isoform in S-CEM. The occurrence of different Cdc37/Hsp90 complexes was also supported by the effects of Hsp90 inhibition: in particular, we treated the cells with the BIIB021 inhibitor, chosen because it is known to be effective also in multidrug resistant cells, nevertheless we observed that it is more effective in inducing cell death in S-CEM respect to R-CEM. This suggests that the chaperone system present in S-CEM is more susceptible to dissociation and inhibition and this could be due to the shorter Cdc37 isoform expressed in this cells. To further confirm this data, we used BIIB021 to treat another cell line, available in the two sensitive (S) and resistant (R) variants, the U2OS osteosarcoma cell lines. Both cell lines express the full length isoform of Cdc37, and we found that they respond in the same manner to the BIIB021 treatment, confirming that the results in CEM cells are most likely due to the different isoform expressed and not to the resistance of R-CEM to the treatment. Interestingly, from the comparison of the effect on cell viability in all cell lines upon BIIB021 treatment, it is evident that only the expression of the shorter isoform of Cdc37 results in a marked cell death (see Fig. 16).

Taken together these data suggest that Cdc37 is involved in the apoptotic process and its C-terminus, whose function and structure are not yet elucidated, could have a role in the control of cell survival possibly by regulating the association with partners in multimolecular complexes.

Materials and methods

Materials

Antibodies for Cdc37 C11, C19 and E4 and for Hsp90 were all purchased from Santa Cruz Biotechnology. β-actin was from Sigma. CK2α antiserum was raised in rabbit against the sequence of the C-terminus (376-391) of the human protein (Sarno *et al.*, 1996). Anti Cdc37 Sp13 was from Abcam. Anti-human Fas-activating antibody was from Millipore. Secondary antibodies were from Amersham (anti-rabbit IgG biotinylated , streptavidin-horseradish peroxidase conjugate), Calbiochem (anti-mouse IgG biotinylated), Pelkin Elmer (anti-rabbit and anti-mouse HRP-labeled) and Sigma-Aldrich (anti-goat IgG biotinylated).

Vinblastine and staurosporine were from Sigma-Aldrich, BIIB021/CFN2024 was from Axon Medchem, okadaic acid was from Alexis Biochemicals.

Cell culturing and treatment

Human T lymphoblastoid CEM cell line (normal sensitive, S-CEM and its MDR variant, R-CEM, selected with 0.1 μg/ml vinblastine) were maintained in RPMI 1640 (Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, in an atmosphere containing 5% CO2. To the R-CEM medium, 0.1 μg/ml vinblastine was routinely added. Human osteosarcoma U2OS cell line (normal sensitive, S-U2OS and its MDR variant, R-U2OS (Cenni 2004)) were cultured in D-MEM (Sigma-Aldrich) supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, in an atmosphere containing 5% CO2. For the treatments, CEM cells were seeded at 10^6 cells/ml and treated for the indicated times in culture media as indicated in the figure legend. Staurosporine and BIIB021/CFN2024 were dissolved in 100% DMSO, which was added to cells suspension at concentration not higher than of 0.5% (v/v). Controls were always treated with the same amounts of vehicle. For R-CEM treatment, 50 ng/ml of Anti-human Fas-activating antibody was diluted in culture media. At the end of the incubations, cells were centrifuged, washed with PBS and lysed as described below.

Cell viability

Cell viability was detected by means of 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) reagent, a tetrazolium salt that is metabolized by mitochondrial dehydrogenases and produces a purple precipitate (formazan) in viable cells. Cell suspension (100 µl; 10^5 cells for CEM, 10^4 for U2OS cells) was incubated in each well of a 96-well plate in culture media under different conditions. Then, 1 h before the end of the incubations, 10 µl of MTT solution (5 mg/ml in PBS) was added to each well. Incubations were stopped by addition of 20 µl of lysis solution at pH 4.7, consisting of 20% (w/v) SDS, 50% (v/v) N,N-dimethylformamide, 2%(v/v) acetic acid and 25 mM HCl. Plates were read at λ590 nm, in a Titertek Multiskan Plus plate reader (Flow Laboratories).

Cell lysis

Cells were centrifuged, washed, and lysed by the addition of ice-cold buffer consisting of 20mM Tris-HCl, pH 7.5, 150mM NaCl, 2mM EDTA, 2mM EGTA, 0.5% (v/v) Triton X-100, 2mM dithiothreitol, protease inhibitor cocktail Complete (Roche), 10 mM NaF, 1 µM okadaic acid, 1mM Na vanadate. After 20 min incubation on ice, the lysates were centrifuged at 14000×*g* for 10 min, at 4°C. The supernatants contained the total soluble cell fraction, whose protein concentration was determined by the Bradford method.

In case of the gradient, cell were lysed by the addition 2-3 volumes of ice-cold hypotonic buffer consisting of 10 mM Hepes, pH 7,9, 10 mM KCl, 0,1 mM EDTA, 0,1 mM EGTA, protease inhibitor cocktail (Roche), 10mM NaF, 1 µM okadaic acid, 1mM Na vanadate. After 20 min of incubation on ice, 0,025% (v/v) Nonidet P-40 was added and the lysates were centrifuged immediately at 14000× *g* for 30 s. The supernatant contained the cell soluble fraction, whereas the pellet correspond to the nuclear fraction.

Western blot analysis

Equal amounts of protein were loaded on 11% SDS-PAGE (Laemmli,1970) and blotted on Immobilon-P membranes (Millipore) in a TE 22 Mini Tank Transfer Unit (GE Healthcare), at 60V for time varying from 60 to 90 min, using a buffer containing 10 mM CAPS-NaOH (3-(Cyclohexylamino)-1-propanesulfonic acid pH 10, DTT 3 mM and 1% (v/v) methanol. Dried membranes were then washed with TBS buffer (Tris-HCl 50 mM pH 7.5, NaCl 50 mM) with 1% (w/v) BSA (Sigma-Aldrich) and then processed

with the indicated antibodies diluted in the same buffer. For membrane development, biotinylated or HRP-conjugated antibodies were incubated for at least 30 min (15 min for streptavidin-HRP) in the same buffer, washes between steps were made in TBS buffer. Antibodies signals were detected by ECL (enhanced chemiluminescence, Amersham Biosciences). The antibody blocking peptide PKTGDEKDVSV was synthesized by Prof. Oriano Marin, and added to the antibodies Ab E and Ab F dilutions at the concentration of 300 μM; the membranes were the incubated with these antibodies dilution for the same time as the controls that consisted in antibodies dilution without peptide. Membranes were then processed normally as described above. Quantitation of the signals was obtained by chemiluminescence detection on a Kodak Image Station 4000MM Pro and with Carestream Health Molecular Imaging software.

RT-PCR analysis of HsCdc37 in S and R CEM culture lines

These experiments were performed by Dr. Alex Costa (Prof. Lo Schiavo group, Padua, Italy).

Total RNA extraction from CEM cells was performed by using TRIZOL (Invitrogen) according to manufacturer's instructions. 10 μg of RNA for each sample were then DNAse treated with RQ1 RNase-Free DNase (Promega) following the manufacturers specifications. For the cDNA synthesis 1 μg of RNA was used and the cDNA retrotranscription was performed with a commercial kit (ImProm-II™ Reverse Transcription System, Promega), using oligo (dT) 18 primers. 1 μl of cDNA reaction was then used as template in the PCR reactions.

For the amplification of full length (For and Rev1) and truncated form (For and Rev2) of *HsCdc37* the following primers were used:

Cdc37 For CATGaagcttATGGTGGACTACAGCGTGTGGGA

Cdc37 Rev1 CATGgaattcTCACACACTGACATCCTTCTCAT

Cdc37 Rev2 ACCCAGAGGCCAGAGTCAATGCA

The PCR was performed by using the GoTaq® Hot Start Polymerase (Promega), with the following parameters: 95°C for 2 min, 35 cycles of: 98°C for 45 sec - 58°C for 30 sec - 72°C for 2 min and 30 sec, and then 72°C for 5 min. The amplicons were separated on 1% (w/v) agarose gel.

Fractionation by centrifugation on glycerol gradient

CEM cells (20-40×10^6) were lysed with hypotonic buffer as described above. A total of 225-300 µg of cytosolic proteins (in 300µl) were loaded on a 3,9-ml glycerol (10%-40%) linear gradient in 25 mM Hepes, pH 7.4, 0.5 mM EDTA, protease inhibitor cocktail Complete (Roche), phosphatase inhibitor cocktail II and III (Sigma-Aldrich), 10mM NaF, 1mM Na vanadate. The tubes were centrifuged 18 hours at 100000×*g* in a SW60Ti rotor (Beckman Coulter) at 4°C and fractionated from the top into 19 fractions. As molecular weight standards, BSA (serum bovine albumin, 66kDa), apoferritin (443kDa) and thyroglobulin (669 kDa, Sigma-Aldrich) were run on separated tubes for protein complexes molecular weight estimation.

General conclusions

CK2 is a constitutively active and highly pleiotropic kinase, with more than 300 substrates known to date (Meggio and Pinna, 2003) and, its involvement in many crucial cellular processes depicts a complex scenario where apparently the capability to mediate the transduction of transient signals hardly reconciles to its features. In fact if we consider the view of signaling pathways as vertical cascades, a kinase with a constant activity and so many targets seems to be inadequate; on this regard, it has been proposed that CK2 acts as a "lateral" player, phosphorylating different substrates involved in the response to the stimuli, thus modulating signaling cascades primarily dependent on other molecules (Ruzzene and Pinna, 2010).

All the research projects investigated in this thesis have provided results that fit in this model. In fact, in the study on the differentiation process of APL cells, we found that CK2 activity is required for RA-induced differentiation, where, without any change in its catalytic activity, a major change occurs in the CK2-dependent phosphorylation of β-actin in response to the treatment. The β-actin level increases in response to RA, and this can account for the higher phosphorylation detected; interestingly, the CK2 inhibition markedly reduces the amount of β-actin in the nucleus, suggesting a role of the CK2-dependent phosphorylation on actin nuclear translocation.

In the study on the role of CK2 in *Arabidopsis* response to SA, we found that in response to this treatment, CK2 phosphorylates a major protein, that we identified as the p23 protein, homologous of the human p23 co-chaperone protein, known to be involved in the Hsp90 chaperone machinery.

Although we can not assume that β-actin and p23 are the only CK2 substrates important for the RA and SA signaling, respectively, both these studies highlight that, in a crowded environment full of possible substrates, CK2 acts modifying the phosphorylation level of only few of them. In this view CK2, can provide a useful task to mediate the external signals in the cell, quickly and precisely: its constitutive activity is not a disadvantage, since its action is controlled at the level of the substrate. In a similar view, an inducible kinase, whose activity is switched on by a signal would be inappropriate inasmuch as all its substrates, especially if many, would be affected.

The last topic of this thesis concerns the Hsp90/Cdc37 chaperone machinery which is under the control of CK2. Even if apparently unrelated, this study pertains to another mechanism by which CK2 can mediated the cell responses to different stimuli: if we consider that Cdc37 regulates a wide repertoire of protein kinases committed to signal transduction, CK2, by regulating the Cdc37 function (Miyata, 2009), can provide a multifaceted modulation of the cellular responces.

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Abbreviations

Amino Acids

Ala	Alanine	A
Arg	Arginine	R
Asn	Asparagine	N
Asp	Aspartic acid	D
Cys	Cysteine	C
Gln	Glutamine	Q
Glu	Glutamic acid	E
Gly	Glycine	G
His	Histidine	H
Ile	Isoleucine	I
Leu	Leucine	L
Lys	Lysine	K
Met	Methionine	M
Phe	Phenylalanine	F
Pro	Proline	P
Ser	Serine	S
Thr	Threonine	T
Trp	Tryptophan	W
Tyr	Tyrosine	Y
Val	Valine	V

Acronyms

2D-PAGE	Two dimensional PAGE
ADP	Adenosine diphosphate
AMPPNP	5′-adenylyl-β,γ-imidodiphosphate)
ATP	Adenosine 5'-triphosphate
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
CHAPS	3- ((3-Cholamidopropyl)dimethylammonio)-1-Propanesulfonic Acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FBS	Fetal bovine serum
GTP	Guanosine 5'-triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IC_{50}	Half maximal inhibitory concentration
IEF	Isoelectric focusing
IgG	immunoglobulins
kD	KiloDalton
Ki	Inhibition constant
Km	Michaelis constant
MTT	Tetrazolium
MW	Molecular weight
NBT	Nitro blue tetrazolium chloride
OD	Optical Density
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate
PVDF	Polyvinylidene difluoride
RA	Retinoic acid
RNA	Ribonucleic acid
RT-PCR	Retrotranscription-polymerase chain reaction
SA	Salicylic acid

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TFA	Trifluoroacetic acid
TRIS	Tris (hydroxymethyl) aminomethane
WB	Western blot