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Protein kinase CK2 in development and differentiation (Review)

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Abstract. Among the human kinomes, protein kinase CK2 (formerly termed casein kinase II) is considered to be essential, as it is implicated in the regulation of various cellular processes. Experiments with pharmacological inhibitors of the kinase activity of CK2 provide evidence that CK2 is essential for development and differentiation. Therefore, the present review addresses the role of CK2 during embryogenesis, neuronal, adipogenic, osteogenic and myogenic differentiation in established model cell lines, and in embryonic, neural and mesenchymal stem cells. CK2 kinase activity appears to be essential in the early stages of differentiation, as CK2 inhibition at early time points generally prevents differentiation. In addition, the present review reports on target proteins of CK2 in embryogenesis and differentiation.

Contents

1. Introduction

Protein phosphorylation and dephosphorylation are important post-translational modifications in cellular regulation. According to Manning *et al* (1) the human kinome consists of 518 protein kinases, which catalyze the transfer of the terminal phosphate group of a nucleotide to a substrate protein. These various protein kinases are grouped into different families, which are further divided into subfamilies. Furthermore, these protein kinases are classified by sequence comparison of their respective catalytic domains, domain structure or by substrate specificities. Further distinctions can be drawn between tyrosine kinases and serine/threonine kinases (2). Protein kinase CK2, formerly termed casein kinase II, is a dual specific protein kinase, which phosphorylates either serine/threonine or tyrosine residues. Furthermore, in contrast to many other protein kinases, CK2 phosphorylates a plethora of different substrates and therefore this kinase participates in numerous different functions (3,4). With the steadily increasing number of physiological substrates of CK2, it becomes more evident that CK2 has the potential to participate in nearly every cellular process. There are excellent reviews regarding the role of CK2 in cell proliferation and survival (5), cancer (6,7), apoptosis (8), angiogenesis (9), DNA-damage and repair (10), the ER-stress response (11), the regulation of carbohydrate metabolism (12) and in the nervous system (13). With regard to these various functions, the mechanism by which a single enzyme regulates these different activities remains to be elucidated. One answer is the ubiquitous distribution in eukaryotic cells from the cell nucleus to the plasma membrane (14), where CK2 meets different substrates and may be specifically activated or inactivated in one particular compartment. Furthermore, CK2 most often appears as a tetrameric complex consisting of two catalytic α- or α'- and two non-catalytic β-subunits. The tetrameric holoenzyme may be composed of two CK2α, or two CK2α' or one CK2α and one CK2α' subunit, and two non-catalytic β-subunits. The non-catalytic β-subunit is not an on/off regulator of the catalytic activities of the CK2α or CK2α' subunits. It seems to regulate thermostability, substrate specificity and the ability to attach and penetrate the cell membrane (15-19). Furthermore, CK2 forms high-order oligomers depending on the ionic strength in the environment (20-24).

In addition, there is increasing evidence for functions of the CK2 subunits outside of the holoenzyme (25-29). The hypothesis that these observations point to an additional mode of regulation is supported by the identification of a number of cellular proteins, which regulate CK2 kinase activity (30). According to the large number of different substrates, as well as the various modes of regulation of the CK2 protein kinase activity, it follows that life without CK2α and CK2β is impossible (31-33). There is a notable review article regarding the role of CK2 in development of various organisms (34);

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however, the present review attempts to focus on the role of CK2 in development and differentiation in vertebrates.

2. CK2 during embryogenesis

The first report on protein kinase CK2 in embryogenesis was published in 1986 when Schneider *et al* (35) demonstrated that CK2 activity increased on day 11 and reached a peak on day 12 of mouse embryonic development. These findings were confirmed for rat embryonic development (36) and similar results were reported for mRNA expression during chicken development (37). These early reports were followed by a study regarding the expression and distribution of CK2 in various tissues in mouse embryos at different stages (38). In the majority of cases the level of mRNA transcripts correlated well with the protein level of the two subunits, CK2α and CK2β . Only in skin was a marked difference between CK2α and CK2β transcripts and protein levels detected late in embryogenesis. In addition, CK2α' is the predominantly expressed catalytic CK2 subunit in testis. CK2α' knock-out testes demonstrated extensive germ cell degenerative processes at all stages of spermatogenesis (39,40) indicating a role for CK2α' in development and differentiation. CK2α' knock-out mice, however, are viable whereas a CK2α knock-out is lethal during embryonal development (31). These results indicate that the two catalytic subunits cannot substitute each other, indicating individual roles in development and differentiation. When the α-subunit of CK2 was knocked out in mice, embryos exhibited structural injuries in the heart and neural tube, and succumbed during embryogenesis (31,32). A combination of the two knock-outs, α and α', only resulted in offsprings when one of the catalytic subunits was sustained (25). However, all combinations demonstrate different and unique defects. Furthermore, these experiments have demonstrated that a combined loss of one CK2α allele or the two CK2α' alleles leads to abnormalities in growth and development of the offspring. In particular $CK2\alpha^{+/-}$ /$CK2\alpha'^{-/-}$ mice differ in weight, but not in size compared with their wild-type littermates. Furthermore, heterozygous knock-out mice were found to have ~50% of the body fat when compared with mice of other genotypes.

Knock-out of CK2β in mice leads to lethality (33). The lack of CK2β causes a diminished cell proliferation and the development of the murine embryos ends at the blastocyste stage with the resorption of the embryo. $CK2\beta^{-/-}$ blastocysts are not able to generate an inner cell mass (33). Notably, heterozygous CK2β ($CK2\beta^{+/-}$) knock-out mice do not exhibit an obvious phenotype (41) indicating a possible gene dosage effect. There was no correlation between CK2β levels in normal and $CK2\beta^{+/-}$ mice that were smaller in size or with gross abnormalities, but differently phosphorylated CK2β proteins were detected (41). The use of conditional CK2β knock-out mice revealed defects in proliferation and differentiation of embryonic neural stem cells (NSCs) (42). It was shown that CK2β was a positive regulator for the development of oligodendrocyte precursor cells. This regulation was achieved by binding of CK2β to, and phosphorylation of, the transcription factor, Olig-2. Thus, it has been concluded that CK2β may be essential in the development of the central nervous system. It was previously identified that a function of CK2β in the holoenzyme, and not a separate function of CK2β, was required for stem cell homeostasis (43).

3. CK2 and neuronal differentiation

The high abundance of all CK2 subunits in brain suggests that CK2 may be important in neural differentiation (44). The disruption of CK2β in embryonic NSCs leads to a restricted proliferation and a weaker differentiation of oligodendrocytes (42), potentially by regulating the activity of the transcription factor Olig-2. Previously, Blanquet (13) suggested that CK2 may be involved in the neuronal cell development program. This suggestion was based on the observations that numerous different substrates of CK2 were involved in neuritogenesis, such as the microtubule-associated protein (MAP)-1B, MAP-1A and tau (45,46). During neural cell differentiation, complexes between transcriptional corepressors of the Groucho/transducin-like enhancer of split (Gro/TLE) and various DNA binding proteins participate in the regulation of this process. One of the proteins within this complex, namely transcription factor, Hes-1, has been identified as a CK2 substrate (47). Inhibition of the enzyme activity of CK2 reduces the chromatin-association. Hes-1 inhibits neuronal differentiation, whereas the associated Hes-6 protein promotes neuronal differentiation. Hes-6 inhibits the interaction of Hes-1 with Gro/TLE and it promotes the proteolytic degradation of Hes-1. In addition, Hes-6 is a substrate for CK2 and its interaction with Hes-1 is regulated by CK2 phosphorylation (48). Finally, Gro/TLE has been identified as a substrate for CK2 (49). The CK2 phosphorylation of Gro/TLE occurs within a CCN motive, which is characterized by phosphorylation sites for cyclin-dependent kinase (cdk)1 (C) and CK2 (C) and a nuclear localization signal (N) (50). CK2 phosphorylation within this CCN motive regulates nuclear translocation for proteins, including Gro/TLE, and transcriptional repression (49). It was further demonstrated that phosphorylation of Gro/TLE by CK2 was required to inhibit the differentiation of cortical neural progenitor cells into neurons. Fibroblast growth factor-2, which enhances proliferation and neurogenesis of NSCs (51) interacts with CK2 via its regulatory β-subunit and stimulates the phosphorylation of nucleolin (52). cdk5 is another protein, which is implicated in the regulation of neuronal differentiation (53). Although there is a 60% sequence identity between cdk1, cdk2 and cdk5 (according to the Global Alignment Program of the National Center for Biotechnology Information; https://blast.ncbi.nlm.nih.gov/Blast.cgi), the latter is not primarily active as a cell cycle regulating enzyme. cdk5 exerts no enzymatic activity, and is activated by binding to the regulatory subunits p35 and p39, which are expressed in post-mitotic neurons. By pull-down experiments, size exclusion chromatography and co-immunoprecipitation the CK2α subunit was found to be associated with p35 and cdk5 (54). This association is independent of kinase activity. Furthermore, CK2α inhibits the kinase activity of cdk5 by preventing the binding of cdk5 to p35. This interaction appears to provide a protection mechanism, which supports cell viability in the nervous system.

Bone morphogenetic proteins (BMPs) restrict neural differentiation. Activation of the BMP-2 signalling pathway leads to increased levels of inhibitor of DNA binding 1, HLH protein, which negatively regulates mammalian achaete scute

homolog-1 (Mash1)-dependent transcription in neurogenic precursor cells by sequestering E proteins away from Mash1 and by enhancing its degradation (55). Conversely, Mash1 is phosphorylated by protein kinase CK2 at serine 152. This phosphorylation is triggered by its complex partner E47 and increases further heterodimer interaction, thus positively regulating neurogenesis.

N-methyl-D-aspartate receptors (NMDARs) belong to the class of ionotropic glutamate receptors, which are ubiquitously expressed in the nervous system (56). NMDARs are composed of NR1, NR2 A-D and NR3 A-B which form tetramers composed of two NR1 and two NR2 or NR3 subunits (57). CK2 phosphorylates NR2B (58), which drives the endocytosis of NR2B resulting in an increased expression of synaptic NR2A. This switch from NR2B to NR2A occurs at cortical synapsis during development (59).

4. CK2 and adipogenic differentiation

For more than 40 years the cellular and molecular mechanisms of adipocyte differentiation have been extensively investigated. The majority of these studies were performed with pre-adipocyte models, for example the mouse cell lines 3T3-L1, 3T3-F442A or C3H10T1/2. As adipocytes are derived from multipotent mesenchymal stem cells, there have also been attempts to analyse mechanisms of adipogenesis in this experimental system. A first indication for the role of CK2 during adipogenesis stems from a study by Sommercorn and Krebs (60) in 1987, which described an increase in CK2 kinase activity early after induction of differentiation. As differentiation progressed, the quantity of CK2 and kinase activity decreased to a level that was almost undetectable. This early observation was recently confirmed and extended for all three CK2 subunits and for CK2 kinase activity. Pharmacological inhibition of CK2 revealed that differentiation was abrogated when CK2 was inhibited at early time points after differentiation commenced (61). After day six of differentiation, inhibition of CK2 activity demonstrated no effect on the differentiation process. Very similar results were reported for the adipogenic differentiation of human mesenchymal stem cells (62). In this case it was, however, shown that mesenchymal stem cells respond differently to the type of inhibitor for the CK2 enzyme activity. For example, quinalizarin efficiently inhibited the differentiation process, whereas CX-4945 failed to inhibit differentiation. Furthermore, there was an asymmetric loss of CK2α and CK2β subunits during the course of differentiation. CK2 inhibition by quinalizarin was accompanied by a loss of the nuclear localization of peroxisome proliferator-activated receptor (PPAR)γ2 to a more cytoplasmic localization. As PPARγ2 is one of the key transcription factors for successful differentiation these data may indicate the method by which CK2 is implicated in the regulation of adipogenesis. In a recent study one of the members of the CCAAT-enhancer-binding proteins (C/EBP) family of transcription factors, namely C/EBPδ was identified as a substrate for CK2 (63). Co-expression of CK2 together with C/EBPδ enhanced the transcription factor activity of C/EBPδ, and pharmacological inhibition of CK2 reduced the transcriptional activity of C/EBPδ. Given that C/EBPδ is an upstream transcription factor of PPARγ2, reversible phosphorylation of this transcription factor appears to be an additional regulatory mode for CK2 during adipogenesis. The zinc finger protein (ZNF), ZNF638 is another transcription factor that has been identified as an early regulator of adipocyte differentiation. ZNF638 physically interacts and transcriptionally co-operates with C/EBPβ and C/EBPδ in the regulation of PPARγ (64). According to an *in silico* analysis (http://www.expasy.org/), ZNF638 should be a CK2 substrate.

5. CK2 and myogenic differentiation

Mesenchymal stem cells are also capable of myogenic differentiation. As with adipogenic differentiation of mesenchymal stem cells, myogenic differentiation is also controlled by various cytokines, growth factors, extracellular matrix molecules and transcription factors (65). One of these transcription factors is the paired box 3 (Pax3) protein, which was found to be phosphorylated (66). Finally the kinase, which is responsible for this phosphorylation at serine 205, has been identified as CK2 (67). This CK2 phosphorylation has been found in proliferating, but not differentiated, primary myoblasts (66). The phosphorylation is part of a priming event where phosphorylation at serine 205 is a first event required for a subsequent phosphorylation at serine 201 by glycogen synthase kinase 3β. Serine 205 phosphorylation is a further requirement for the phosphorylation at serine 209, which is also by CK2. Serine 205 phosphorylation is associated with proliferation whereas serine 209 phosphorylation is more associated with myogenic differentiation (68). Notably, in mutated Pax3, which is typically associated with rhabdomyosarcoma, this phosphorylation is maintained and may be responsible for the proliferating properties of these types of tumour cell (68).

The myogenic regulatory factors (MRFs) are a subclass of transcription factors, which are characterized by a common helix-loop-helix (bHLH) motif. They interact with another bHLH protein from the E protein family. Two members of the MRF family, MyoD and MRF4, are phosphoproteins and have been identified as CK2 substrates (69). Using MRF4 and MyoD mutants, which are no longer phosphorylated by CK2, and CK2 overexpression resulted in elevated transcriptional activity and enhanced myogenic activity demonstrating that phosphorylation of MRF4 and MyoD by CK2 was not responsible for this activity (69). Rather, one of the E proteins, E47 was phosphorylated by CK2 and this phosphorylation inhibited its DNA binding activity. Furthermore, Johnson *et al* (69) suggested a model where the phosphorylation of E47 by CK2 may increase the pool of E proteins that bind to MRF proteins. These MRF/E heterodimers may then transactivate muscle gene expression.

Myogenic factor (Myf)-5 is a member of a family of muscle-specific transcription factors, which are important for myogenic cell determination and differentiation. Winter *et al* (70) reported that Myf-5 was phosphorylated at two serine residues, one in the N-terminal and another one in the C-terminal domain of the protein. The two CK2 phosphorylation sites are required for the transcriptional activity of Myf-5 (70). Thus, it has been suggested that the N-terminal CK2 phosphorylation site regulates nuclear translocation of Myf-5.

In response to injury or a disease, skeletal muscle cells are dependent on a stem cell population termed satellite cells

in order to guarantee compensatory growth regeneration. Establishment of the satellite cell lineage is controlled by the paired-box transcription factor, Pax7. Loss of Pax7 results in a severe reduction of muscle regenerative capacity. It has been shown that Pax7 is subjected to caspase 3-directed proteolytic degradation (71). Caspase 3 is a member of the caspases, which are implicated in the proteolytic cascade of apoptosis regulation. By using a combination of bioinformatics, peptide arrays and peptide cleavage assays, and by comparing overlapping protein kinase phosphorylation sites with caspase recognition motifs, CK2 was identified as a global regulator of apoptosis (72). Pax7 was identified as a CK2 substrate and the CK2 phosphorylation site was identified to be overlapping with a caspase 3 consensus site (71). Furthermore, phosphorylation of Pax7 by CK2 prevents caspase 3 cleavage and promotes satellite cell self-renewal (71). Thus, these results clearly indicate that CK2 is involved in the differentiation process, as well as the life of muscle stem cells by limiting the self-renewal process.

6. CK2 and osteogenic differentiation

Throughout human life, bone is maintained in balance by the complex coordination of multiple types of bone marrow cell. Bone formation by osteoblasts and resorption of osteoclasts are tightly regulated processes. The imbalance between bone formation and resorption is responsible for various diseases. Osteoblasts regulate osteoclast activity through cell-to-cell contacts whereby the osteoblast surface receptor activator of nuclear factor (NF)-κB ligand, receptor activator of nuclear factor κ-B ligand (RANKL) binds to its receptor of NF-κB RANK. Ikaros was originally described as a hematopoietic cell-specific zinc finger DNA binding protein, which is responsible for lymphocyte development (73). Ikaros was identified as a CK2 substrate (74). Subsequently, CK2 phosphorylation of Ikaros was demonstrated to be essential in the regulation of alkaline phosphatase, which is a major osteoplastic differentiation marker (75). Son *et al* (76) showed that pharmacological inhibition of CK2 by CX-4945 significantly inhibited the RANKL-induced osteoclast differentiation. Conversely, the BMP-2-induced osteoblast differentiation was enhanced (76). This CK2 inhibitor was originally described as an anti-cancer agent either alone or in combination with other therapeutic agents (77). CX-4945 inhibited the formation of RANKL-induced tartrate-resistant acid phosphatase (TRAP) in bone marrow-derived macrophages, which are induced to differentiate. Furthermore, CX-4945 inhibited the RANKL-mediated induction of nuclear factor of activated T-cells, cytoplasmic 1 and Akt phosphorylation (76).

BMPs are essential during embryonal development and regulate such diverse processes as neurogenesis, hematopoiesis and bone formation. In murine C2C12 mesenchymal precursor cells BMPs bind to a BMP receptor (BR) and induce a SMAD- and p38-dependent signalling cascade. CK2β was identified as a binding factor for the BMP receptor type 1a (BRIa). BMP-2, which is a crucial factor for osteoblast differentiation, binds to BRIa and displaces CK2β from BRIa (78,79). Inhibition of this CK2β-BRIa interaction led to an activation of the Smad signalling pathway. However, overexpression of CK2β or a dominant negative form of CK2α increased BMP signalling and BRIa was phosphorylated by CK2 at three different sites. Peptides (length, ~30 amino acids) were designed, which specifically blocked each site. These peptides included various amino acids flanking each phosphorylation site, which are important for the binding of CK2 to BRIa (80). Mutants of the CK2 phosphorylation sites abrogated the binding of CK2 to BRIa and induced osteogenesis or adipogenesis. Recently, it was shown that one of these peptides, namely CK2.1, induced chondrogenesis, but not osteogenesis in the mesenchymal stem cell line C3H/T101/2 (81). In addition, blocking the interaction of CK2 with BRIa induces differentiation of C2C12 cells into osteoblasts and adipocytes. Notably, the peptides CK2.1 and CK2.3 appeared to signal through more specific signalling pathways than binding of BMP-2 to its receptor (81,82). By using one of these blocking peptides it was shown that an injection of this peptide into the tail vein of mice resulted in increased bone mineral density (82). The differentiation into osteoblasts is regulated via SMAD/mechanistic target of rapamycin or mitogen-activated protein kinase kinase (MEK) signalling pathways, whereas differentiation into adipocytes is dependent on SMAD4, p38 and MEK signalling. Furthermore, administration of this peptide led to an induced phosphorylation of extracellular signal-regulated kinases (ERK), but not Smad in osteocytes and osteoblasts. Mice treated with this peptide exhibited a decreased osteoclast differentiation and osteoclast activity (82). This was consistent with a previous observation regarding the crucial role of BMP-2, the downstream targeting BRIa and CK2 in regulating osteogenesis (80). The mechanism of action of these peptides remains unknown. Whether these peptides prevent binding of CK2 to BRIa or binding and phosphorylation of BRIa requires further investigation. Previous studies demonstrated that blocking peptides could be used in vivo to increase bone growth, which indicated that peptides may be a novel therapeutic strategy for bone fracture (78-82). Furthermore, CK2 may serve as a target for treatment of bone fractures or for osteoporosis.

BMP-2 induced the expression of alkaline phosphatase, which is a marker for osteoblast differentiation (83) and this induction was enhanced following CK2 inhibition. In addition, the mRNA level for osteogenic BMP-4 was enhanced subsequent to CK2 inhibition with CX-4945. Analyzing the signalling pathway following CK2 inhibition indicated that CX-4945 enhanced BMP-2 induced phosphorylation of ERK1/2.

7. CK2 in hematopoietic differentiation

Ikaros is essential in thymocyte differentiation where it undergoes dephosphorylation (84). At least two amino acids have been shown to be dephosphorylated during thymocyte differentiation, which were previously phosphorylated by CK2. These data indicated the role of CK2 and the phosphorylation of Ikaros during T cell differentiation (85,86). Certain Ikaros phosphorylation sites, which are mediated by CK2, were identified within a typical PEST sequence, which function as a recognition motif for protein degradation. Whereas alanine mutants at the CK2 phosphorylation sites within the PEST sequence stabilized Ikaros (85). These data are consistent with earlier observations where an overexpression of CK2α contributed to the development of T cell leukaemia and

lymphoma (87-90). Notably, these results correlate well with impaired Ikaros functions (73,91).

$CD4^+$ regulatory T cells (T_{reg} cells) are essential in the maintenance of peripheral tolerance. Recently, it was shown that the suppression of an allergic immune response by T helper type 2 (T_H2) cells was dependent on CK2 (92). In a mouse model in which only T_{reg} cells lacked CK2β, a marked increase in infiltration of immune cells into the lung, but not in other organs was observed. This result indicates that CK2β in particular may be implicated in a cell-type and tissue-specific uncontrolled T_H2 response.

Recently it was demonstrated that CK2 is involved in the molecualr decision between T_H17 and T_{reg} cells (93). T_H17 cells represent a subset of T cells, which are important for the immune response against bacteria and fungi, and T_{reg} cells are important for the maintenance of immune tolerance. The balance between these two subsets of T cells contributes to the severity of a disease. Ulges *et al* (93) reported that the inhibition of CK2 kinase activity, as well as downregulation of the expression of CK2β in $CD4^+$ T cells prevents the development of T_H17 cells and promotes the generation of T_{reg} cells.

8. Conclusion

There is no differentiation or development without CK2. Organisms and organs cannot develop without CK2, and in recent years there have been increasing observations regarding the role of CK2 in embryogenesis and differentiation. Although, CK2 is indispensable for the development of organisms, in the majority of cases CK2 has a negative impact on the ongoing differentiation process of adult stem cells. However, its presence appears to be necessary for the early phase or the initiation of differentiation. It is tempting to speculate that CK2 is dispensable in the adult organism and in terminally differentiated cells, as inhibition of CK2 activity is not detrimental for the survival of normal cells (94). However, with high probability, cells of the stem cell niche, which are also present in different tissues and organs of the adult organism, have to be excluded from this statement. Thus, it is hypothesized that a lack of CK2 may inhibit the self-renewal and repair capacity of an organism.

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