Ecto-protein kinase CK2, the neglected form of CK2 (Review)

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Abstract. Ecto-protein kinases, including protein kinase CK2 (former name, casein kinase 2), have been the focus of research for more than 30 years. At the beginning of the ecto-kinase research their identification was performed with substrates and inhibitors whose specificity under the current knowledge was rather limited. Since all currently known ecto-kinases, including ecto-CK2, have intracellular counterparts, one has to exclude that an ecto-localization originates from intracellular counterparts after cell damage. Protein kinase CK2 is involved in cellular key processes such as cell cycle progression, inhibition of apoptosis, DNA damage repair, differentiation and many other processes. CK2 is composed of two catalytic CK2α or CK2α' subunits and two non-catalytic CK2β subunits. Progress in the ecto-kinase and in particular ecto-CK2 studies was made with the use of transfected tagged CK2 subunits, which allowed to follow their individual transport and localization on the cell surface after transfection. Furthermore, immunofluorescence studies with antibodies against CK2 subunits as well as affinity chromatography with a binding partner of CK2 subunits have improved ecto-kinase research. The use of new and more specific inhibitors as well as of substrates, which do not cross the plasma membrane, have further improved the specificity for ecto-CK2. From the various substrates of ecto-CK2, it can be concluded that ecto-CK2 plays a role in Alzheimer disease, cell adhesion, platelet aggregation, immune response and cellular signalling. New tools and techniques, to study ecto-CK2 activity, are required to identify new substrates and thereby new functional implications for ecto-CK2.

Contents

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1. Introduction

Cells need a versatile, fast mechanism to respond to changes in their environment. Such a fast response is possible by reversible phosphorylation of extracellular domains of cell surface proteins. Ecto (Greek word for outside)-phosphorylation is emerging as an important mechanism to regulate ligand interaction with their receptors, ion channels, signalling mechanisms, shedding from cell surfaces, cell-cell adhesion, immune response and proliferation and differentiation. Ecto-protein kinases are cell surface constituents of many cell types (1,2).

Ecto-kinase activities are involved in synaptogenesis, synaptic plasticity and long-term potentiation (3), activation of the complement system (4,5) and homeostasis (6,7). Ecto-kinases are powerful regulatory enzymes for protein phosphorylation at the cell surface. They are critical for intercellular communication and transduction of external stimuli. Potential substrates for ecto-kinases are cell adhesion molecules, growth factors and their receptors, coagulation factors and ion channels. In some cases, ecto-kinases are shed from the surface of cells in a substrate-induced manner. Ecto-kinases have been identified as extracellular versions of known intracellular kinases. The pathway involved in their export out of the cell is largely unknown. They utilize extracellular ATP, which is present in blood plasma at a concentration of approximately 2 μM (8). The cytosol of mammalian cells contains 5-10 mM ATP. This concentration gradient facilitates an increase in extracellular ATP concentration after cell activation or cell damage. It is well known that, the level of intracellular Ca^{2+} increases and ATP is released from endothelial cells into the medium when cells are activated by thrombin (9,10). Another important argument for ecto-kinases may be the addition of exogenous substrates, which do not enter the cells, or the use of inhibitors of kinases, which do not penetrate into cells into the labelling medium. Moreover, working on ecto-kinases, it is always an important point to exclude leakage from dead cells.

Originally, two ecto-kinases have been described, c-AMP dependent protein kinase (11) and a cyclic nucleotide independent kinase (12-14). Paas and Fishelson (4) reported on two types of ecto-kinases, a serine/threonine kinase and a tyrosine kinase. These ecto-kinases were assumed to bind to lipid-anchored molecules, some of which were additionally shed into the medium of cultured cells. In addition, there is also evidence for ecto-kinases, which are shed into the culture medium without being previously attached to the cell surface.

Notably, there is also evidence for the presence of ecto-phosphatase activity at least on endothelial cells, which was shown by using the membrane impermeable reagent, microcystin LR, which inhibits protein phosphatases PP-1 and PP-2a (15). Walter *et al* have identified protein kinases CK1 and CK2 as ecto-kinases on HeLa cells (14). Moreover, CK2-like kinases have been reported to be secreted from activated platelets, neutrophils and endothelial cells (7,16,17).

2. Protein kinase CK2

Knowledge regarding protein kinases and in particular protein kinase CK2 has increased considerably. The human kinome consists of 518 protein kinases (18). The common feature of these protein kinases is the transfer of the terminal phosphate group of a nucleotide to a serine, threonine or tyrosine residue of substrate proteins. The majority of protein kinases employ ATP as a phosphate donor, and only a few other kinases, including CK2, can also use GTP as a phosphate donor. Most of the protein kinases are serine/threonine kinases, some are tyrosine kinases and only a few, such as CK2, are dual-specific kinases, phosphorylating serine, threonine and tyrosine (19).

The number of cell proteins, which are phosphorylated by CK2, is increasing rapidly (20) and therefore it is not surprising that CK2 is involved in almost every cell process regulating cell proliferation, cell survival (21), apoptosis (22), DNA damage and repair (23), development and differentiation (24) and the regulation of metabolism (25). CK2 is regarded as a constitutively active enzyme. With regard to the numerous substrates of CK2 and its implication in numerous basic cell processes it is, however, hard to believe that this kinase is not tightly controlled. In many organisms, CK2 is composed of two catalytic α-subunits or two α'-subunits and two non-catalytic β-subunits forming tetramers such as $(CK2\alpha CK2\beta)_2$, $(CK2\alpha' CK2\beta)_2$ or $(CK2\alpha CK2\alpha')/(CK2\beta)_2$, which can further aggregate into multimers of this basic tetramers (26,27). Since the tetramers and higher molecular weight complexes differ in their kinase activity, self-aggregation and dissociation seem to be involved in the regulation of CK2.

In addition to the tetramers and higher molecular complexes there is increasing evidence for the existence of CK2α-, α'- and β-subunits aside from the CK2 tetrameric holoenzyme. There are substrates, which are phosphorylated by the holoenzyme, in addition to substrates that are phosphorylated by CK2α or CK2α' alone and by the holoenzyme. Finally, there are substrates that are phosphorylated by CK2α, but not by the holoenzyme (28). These observations indicate a regulatory role for CK2β, which is supported by early findings, showing that CK2β confers stability to the holoenzyme (29), that it determines substrate specificity (30) and it increases the enzyme activity (31). It was shown by Cochet and Chambaz that CK2β can enhance the catalytic activity of CK2α 5- to 10-fold (32). By contrast, Meggio *et al* reported that CK2β downregulated the activity of CK2α against calmodulin as a substrate (33). Polybasic compounds such as polylysine or spermidine react with an acidic cluster between residues 55 and 64 of CK2β, thereby stimulating the kinase activity of the CK2 holoenzyme (34,35). These polybasic compounds do not directly affect the CK2α subunit. Recently, a new function of CK2β was identified by the cell-specific deletion of CK2β in T cells, which showed that CK2β plays an important role as a modulator of the immune response (36). There are some examples, where CK2β can also contribute this regulatory function to other protein kinases such as A-raf, c-mos and CHK-1 (37,38). Recently, a close interaction between CK2β, mTOR and IGFBP-1 in hepatocellular carcinoma cells was reported (39). On the other hand, there is an increasing number of proteins binding to the CK2 holoenzyme or to CK2α or CK2α' alone. These binding partners of CK2 are known to regulate CK2 kinase activity such as p53 (40-44) or PP2A and topoisomerase (45,46). Thus, there are obvious cell regulators of CK2 (47-49) and vice versa, as well as other proteins that are regulated by CK2 binding (49-52).

Phosphorylation and dephosphorylation is often the main mechanism for the regulation of proteins and their activities. Cdk1 is known to phosphorylate CK2α (53,54). The phosphorylation of CK2β by CK2α as well as by cdk1 seems to be, however, not essential for a fully active heterotetramer of CK2 (54). This led to the conclusion that phosphorylation and dephosphorylation do not play a major role in the regulation of CK2. The most important regulatory mechanism involved in CK2 seems to be its subcellular localisation. Since the beginning of research on CK2, it has become evident that CK2 is located in the nucleus and in the cytoplasm. However, an increasing number of studies reported that CK2 is located in almost every compartment of a eukaryotic cell. Moreover, the subcellular localisation of CK2 seems to be highly dynamic (55-58). This dynamic subcellular localisation enables the CK2 subunits to interact with proteins, which are specific for one particular cell compartment. Besides the nuclear and cytoplasmic localisation, CK2 was found at the plasma membrane (13,59-61). Plasma membrane preparations from A431 cells or from insect cells expressing CK2α and CK2β contain oligomeric forms of CK2 (62). The plasma membrane association of CK2 is mediated by a specific domain of the β-subunit (63).

In recent years, more substrates for CK2 have been identified at the plasma membrane. Since plasma membrane phosphorylation of proteins by CK2 were neglected thus far, the present review aims to address the role of CK2 as an ecto-kinase. An early observation identified that bone phosphoproteins were phosphorylated by CK2 isolated from detergent extracts of membranous fractions of a 12-day embryonic chick tibia (64). Although there are many early reports on ecto-CK2, little is known regarding the mechanism of how CK2 is exported to the cell surface. Tagged CK2 subunits were transfected in HEK293T cells to study their export to the cell surface. It took approximately 5-7 h after transfection before tagged subunits were detectable on the cell surface (65). When the subunits were expressed individually, they were not detectable externally. However, after transfection of both CK2α and CK2β, approximately 3-4% of the CK2 holoenzyme was detectable on the cell surface. Transport to the surface of the plasma membrane is independent of the kinase activity because a kinase-negative CK2α mutant is also transported to the cell surface when CK2β is co-transfected. *De novo* protein synthesis is not required for the presence of ecto-CK2 on the cell surface (65). By using deletion and point mutants of CK2β, the same group found that a region between amino acid 20 and 33 in the N-terminus of CK2β was

necessary for the localization of the holoenzyme on the cell surface (63). In the course of these experiments, Rodriguez *et al* also reported that CK2β can be exported out of the cells but it is not retained at the plasma membrane. A construct where the region from amino acid 20 to 33 of CK2β was linked to the C-terminus of CK2β was able to bind the CK2α to form a holoenzyme, but it was unable to transport the holoenzyme to the ecto-kinase location. These results suggest that the region between amino acid 20 and 33 of CK2β is necessary but not sufficient for the extracellular localization of CK2 (63). Free CK2β interacts with several other protein kinases such as A-raf, c-mos and CHK-1 (66). One might speculate that these kinases are also transported to the plasma membrane by their interaction with CK2β. In contrast to an early observation by Kübler *et al* (12), the kinase activity on the cell surface is rapidly restored (13). Ecto-CK2 is not released from the cell surface by incubation with phospholipase C, suggesting that ecto-CK2 is not anchored in the plasma membrane via glycosyl-phosphatidyl-inositol-linkage.

Since CK2 is known to be elevated in many cancer cells and the observation that an inhibition of CK2 kinase activity leads to apoptosis of at least cancer cells (67,68), there has been a search to identify new efficient and specific inhibitors of CK2 worldwide. Most of the currently known CK2 inhibitors are ATP competitors. Recently, bifunctional inhibitors have been designed, which, on the one hand bind ATP competitively, and on the other hand, mimic phospho-acceptor substrates (69-71). On the basis of 4,5,6,7-tetrabromo-1H-benzimidazol (TBI), new derivatives were generated, which compete with the phospho-acceptor sites of substrates. These bifunctional inhibitors of CK2 have impaired cell permeability, which qualifies them for the inhibition of ecto-CK2 (70).

3. Substrates of ecto-CK2

Table I lists the substrates, which are shown to be phosphorylated by ecto-CK2. The β-amyloid precursor protein (βAPP) is phosphorylated within its ecto-domain and CK2 was identified as one of the ecto-kinases that phosphorylates βAPP at the outer face of the plasma membrane (72). Cell surface labelling of βAPP was analysed by adding radiolabelled γATP or γGTP to the supernatant of HEK293T cells overexpressing βAPP1-695. The ecto-kinase activity was inhibited by heparin and by DRB, two compounds that are known to inhibit CK2 kinase activity (73,74). Further experiments revealed that, not only membrane-bound βAPP, but also secreted βAPP from cell membranes, was phosphorylated by ecto-CK2.

Already in 1993, a CK2-like activity was described in thrombin-activated platelets and in the supernatant of these activated platelets. It is known that thrombin triggers at least in endothelial cells, the release of intracellular ATP (10). The presence of elevated extracellular ATP concentration and the presence of ecto-CK2 are an ideal combination for the phosphorylation of cell surface proteins.

Ecto-CK2 phosphorylated bovine coagulation factor Va and human factor VIII (6). Phosphorylated factor Va was more sensitive for active protein C than the non-phosphorylated form, triggering its degradation. This result indicates that ecto-CK2 may play a role in the downregulation of coagulation. Recently, an interesting result was reported for megakaryocytes and platelets from CK2β$^{-/-}$ mice. Münzer *et al* showed an abnormal microtubule structure and a significantly increased fragmentation within the bone marrow (75). Aggregation of CK2β$^{-/-}$ platelets was abrogated and thrombus formation was reduced. Whether this effect is due to an impaired localization of CK2 on the cell surface remains to be elucidated. Another protein that is phosphorylated by CK2 is C3 (5). Phosphorylated fragments derived from C3 cleavage show an increased binding to IgG in serum over that of non-phosphorylated C3. Since CK2 phosphorylation of C3 increases its susceptibility to elastase cleavage, these results suggest an effect of platelet-derived CK2 phosphorylation of C3 to enhance the opsonisation of immune complexes (5).

Rat liver endothelial cells, which were activated with thrombin release up to 10% of the total protein kinase activity into the culture medium. This protein kinase phosphorylates casein and fibrinogen and the enzyme is inhibited by heparin. These features suggest ecto-CK2 (17). There was a very similar observation, i.e., cultivated hamster and chicken cells have ecto-CK2, which phosphorylates human and bovine fibrinogen as well as fibrin (76).

The C9 protein, which is a component of the lytic activity of the complement system was found to be phosphorylated by an ecto-kinase (4), which was subsequently identified as CK2 (77). In this case, the ecto-kinase activity was determined with intact cells and purified C9 protein, ATP and Mg^{2+}. The ecto-kinase is neither influenced by Ca^{2+} nor by cAMP. The ecto-kinase is shed from the plasma membrane and this shed protein kinase also phosphorylates C9 protein (77). C9 phosphorylation was achieved on intact Raji cells and also by shed proteins from the surface of Raji cells. Ecto-CK2 was identified by immunofluorescence flow cytometry. Kinase activity was inhibited by emodin, TBB and DRB, which are known inhibitors of CK2 (74,78,79). The three inhibitors did not reduce cell viability. In addition, CK2α and CK2β were detected in the supernatant of K562 and Raji cells using CK2 subunit-specific antibodies. C9 is a

Table I. Substrates for ecto-CK2.

Substrates	Refs.
β-amyloid precursor protein	(72)
Human factor VIII	(6)
Coagulation factor Va	(6)
Complement factor C3	(5)
Complement factor C9	(4)
Casein	(17)
Fibrinogen	(17,76)
Fibrin	(76)
Phosvitin	(59)
Vitronectin	(82)
Laminin-1	(85)
Collagen XVII	(87)
Osteopontin	(88)
Stanniocalcin-2	(89)
Nucleolin	(90)

blood plasma protein that binds to the C5b-8 complex of the complement system (80). C9 phosphorylation by ecto-CK2 is a protective mechanism against complement-mediated lysis (81). Phosphorylated C9 has a reduced haemolytic activity whereas the inhibition of ecto-CK2 kinase activity enhanced cell killing. In addition, C9 ecto-CK2 phosphorylated calmodulin and a highly CK2-specific peptide substrate. The level of ecto-CK2 on peripheral blood mononuclear cells (PBMC) and B cells was lower than that on Raji and K562 cells (81).

Other examples of substrates for ecto-CK2 are phosvitin and vitronectin. Vitronectin is a glycoprotein, which is present in blood and in the extracelluar matrix. At least for vitronectin, it is known that its phosphorylation is inhibited by DRB. Phosphorylation of vitronectin by ecto-CK2 regulates the adhesion of cells to the extracellular matrix (82-84). Both substrates induced the release of ecto-CK2 when vascular smooth muscle cells (VSMC) were incubated with phosvitin or vitronectin (82). When the cells were adhered to vitronectin, ecto-CK2 was enriched in clusters on the cell surface and then underwent a displacement from the VSMC surface (82).

Ecto-CK2 regulates monocyte migration through laminin-1 phosphorylation (85). Ecto-kinase activity was inhibited by heparin and CK2 was identified with CK2-specific antibodies. An interesting feature of phosphorylated laminin-1 is its interaction with heparin, where phosphorylated laminin-1 binds better to heparin than non-phosphorylated laminin-1. In cell adhesion experiments, a significantly higher amount of cells adhere to phosphorylated laminin-1 (85). Furthermore, phosphorylated laminin-1 promotes cell proliferation as well as monocyte migration.

Ecto-CK2 was also detected on mast cells RBL-2113 by its capacity to phosphorylate a CK2-specific peptide, the use of [^{32}P]γATP as well as [^{32}P]γGTP, by western blot analysis and immunofluorescence with CK2-specific antibodies (86). CK2 was co-immunoprecipitated with an anti Fc-R antibody, indicating that ecto-CK2 is tightly bound to a receptor molecule.

Collagen XVII is an example of an integral membrane protein with an extracellular domain, which was phosphorylated by ecto-CK2 at serine 542 and 544. Collagen XVII seems to be an interesting example for hierarchical phosphorylation, because serine 544 is the first phosphorylation event, which generates an acidic environment, which then allows the serine 542 phosphorylation by CK2.

The pharmacological inhibition of CK2 with 4,5,6,7-tetrabromobenzimidazol (TBB) as well as the use of a non-phosphorylatable alanine mutant revealed that CK2 phosphorylation of collagen XVII regulates ecto-domain shedding. By contrast, the overexpression of CK2α inhibited the cleavage of collagen XVII (87). An antibody against a phospho-peptide derived from a collagen XVII sequence from amino acid 535 to 551 detected CK2-phosphorylated collagen XVII on the cell surface. As a control for the specificity, a dominant negative mutant of CK2 was expressed and shown to be present on the cell surface. This mutant ecto-CK2 failed to phosphorylate collagen XVII (87). CK2 phosphorylation of collagen XVII inhibits its degradation by metalloproteases (87). The authors of that study suggested that ecto-CK2 phosphorylation is a novel mechanism involved in the regulation of adhesion and motility of epithelial cells (87).

Ecto-CK2 seems to be involved in ossification by direct influencing mineral formation through the phosphorylation of osteopontin (88). This observation is in agreement with the detection of stanniocalcin-2 (STC-2), a substrate of ecto-CK2 (89). STC-2 is a proteohormone, which is involved in the regulation of calcium and phosphate homeostasis (89).

Using affinity chromatography on urokinase-conjugated Sepharose4B and nano-electrospray mass spectrometry and by immunoblotting, ecto-CK2 was found in a complex with urokinase and with nucleolin (90), and this complex seems to be highly dynamic on the cell surface. Ecto-CK2 phosphorylated the cell membrane-associated protein nucleolin. Nucleolin is a phosphoprotein, which shuttles between the nucleus and cytoplasm and which is located on the cell surface (91). Urokinase activates ecto-CK2, leading to the phosphorylation of nucleolin and this phosphorylation is responsible for the translocation of nucleolin into the cell nucleus (90). By contrast, intracellular CK2 is insensitive in the activation by urokinase. Another example of a complex of ecto-CK2 with cell surface proteins was mentioned earlier, that of the association with Fc-R on monocytes (86).

4. Conclusion and future perspectives

There is a long history of a subclass of CK2 as an ecto-kinase. CK2 was identified by using casein as a substrate and with heparin as an inhibitor. It is now clear that casein is not a natural substrate of CK2. The kinase committed to the phosphorylation of casein in the Golgi apparatus of the lactating mammary gland is conventionally termed genuine or Golgi casein kinase (92).

Heparin, which was used as an inhibitor of CK2, interacts non-specifically with proteins such as cytokines, growth factors, adhesion molecules and proteases (93). DRB, not only inhibits CK2, but also RNA polymerase II (94). Thus, in both cases off-target effects cannot be excluded. The direct identification of CK2 subunits on the cell surface by immunofluorescence or by immunoprecipitation were steps forward in the identification and characterisation of ecto-CK2. Furthermore, the development of new, highly specific, cell impermeable inhibitors of CK2 and phosphorylation experiments with substrates that cannot penetrate into cells have improved the specificity for ecto-CK2. Transfection of the tagged subunits of CK2 has shown that only the holoenzyme and/or the CK2β subunit are present on the cell surface. It remains an open question whether there are also high molecular aggregates of the CK2 holoenzyme on the cell surface. Since CK2β binds also to other protein kinases and since it is responsible for the cell surface localization of CK2, it remains to be determined whether these other protein kinases are also present as ecto-kinases.

The proteins already identified earlier as substrates for ecto-CK2 show that ecto-CK2 plays a role in blood homeostasis, in thrombosis, in cell adhesion, in Alzheimer disease, calcium homeostasis and in the regulation of the immune system. Other plasma membrane-associated proteins such as ion channels and receptors for hormones and growth factors, are excellent candidates for ecto-CK2.

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Competing interests

The authors declare that they have no competing interests.

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