Regulation of CK2 Activity by Phosphatidylinositol Phosphates

Received for publication, August 15, 2005 Published, JBC Papers in Press, September 12, 2005, DOI 10.1074/jbc.M508988200

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The process of clathrin-coated vesicle (CCV) formation/disassembly involves numerous proteins that act cooperatively. Phosphorylation is an important regulatory mechanism governing protein interactions in CCVs, and many of the core and accessory proteins of the CCV machinery are reversibly phosphorylated in vivo. CK2 is highly enriched in CCVs and is capable of phosphorylating a number of peripheral membrane proteins involved in the process of clathrin-mediated endocytosis. At least some of these phosphorylation events have been shown to be inhibitory for CCV assembly, and CK2 has been shown to be inactive when associated with intact CCVs. Here we show that CCV membranes inhibit CK2 activity even after incubation in trypsin, indicating that a component of the lipid bilayer may be the inhibitory factor. Consistent with this, we showed that liposomes containing phosphatidylinositol phosphates inhibit the activity of CK2 and that CK2 binds to those liposomes. Notably, liposomes containing phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), a component of CCVs, bind CK2 and inhibit its activity. Furthermore, we showed that the binding of CK2 to PtdIns(4,5)P2-containing liposomes is via the active site of CK2, thus providing a molecular explanation for the inhibition of CK2 activity when it is bound to PtdIns(4,5)P2-containing liposomes. Thus CK2 is inactive in CCVs because of the fact that it is bound to the CCV membrane via an interaction between PtdIns(4,5)P2 in the CCV membrane and the active site in CK2.

Several mechanisms are employed by eukaryotic cells for the internalization of material from their surroundings, one of these is clathrin-mediated endocytosis (CME). CME allows the internalization of nutrients, hormones, and other molecules from the plasma membrane into intracellular compartments. It occurs via a process of plasma membrane invagination and vesicle internalization that involves the formation of a clathrin coat on the cytosolic face of the plasma membrane. The coat consists of a lattice of clathrin triskelia, polymers of three clathrin heavy chains, each of which is bound to one of two clathrin light chains (LCa and LCb). Receptors and their ligands are recruited into CCVs forming at the plasma membrane, a process that involves a range of accessory proteins, including a specific adaptor complex (AP-2) that has the valency to interact with both clathrin and the cytosolic domains of receptors destined for internalization (reviewed in Ref. 1).

The assembly and disassembly of CCVs need to be well regulated and coherent processes if CME is to occur efficiently. Part of the mechanism by which these processes are regulated appears to involve protein phosphorylation; many of the core and accessory proteins of the CCV machinery are reversibly phosphorylated in vivo (e.g. see Refs. 2–6). However, although it is evident that phosphorylation is an important regulatory mechanism for CCV formation/disassembly, the exact role of the particular kinases involved remains unclear. Previously, it was shown that purified CCV fractions are highly enriched in the protein kinase CK2 and that CK2 is required for clathrin-dependent endocytosis (3, 7). CK2 is able to phosphorylate a number of endocytic proteins, including clathrin light chain, the α- and β2-subunits of the AP-2 adaptor complex, amphiphysin, dynamin, AP180, and synaptotagmin (2–6).

At least some of these phosphorylation events are inhibitory for CCV formation (4, 6, 8–10), so the presence of an active kinase on a CCV would be harmful for the integrity of that CCV. As CK2 is thought to be a constitutively active protein kinase, there must be one or more potent mechanisms to inhibit the enzyme when required. In agreement with this, we have shown that CK2 is inactive on intact CCVs but active after the uncoating of CCVs (2). The inactivity of CK2 when associated with CCVs is because of inhibition by an unknown component of lipid membranes.

Polyphosphoinositides play an important role in the process of CME. Different cellular membranes are characterized by specific sets of polyphosphoinositides, with PtdIns(4,5)P2 and PtdIns(3,4,5)P3 being of particular interest to those studying endocytosis. Their role is to recruit adaptor and accessory proteins to endocytic sites (reviewed in Refs. 11 and 12). Membrane recruitment of the AP-2 adaptor complex to polyphosphoinositides occurs via the lysine-rich N-terminal trunk domain of the α-subunit (13, 14), whereas there is also a PtdIns(4,5)P2 binding site in the μ2-subunit (15). Similarly, other proteins essential for CME (AP-180, epsin, and dynamin) are recruited to the plasma membrane via an interaction between a specific protein domain (ANTH, ENTH, and PH, respectively) and PtdIns(4,5)P2 (16–18). Furthermore, hydrolysis of PtdIns(4,5)P2 and PtdIns(3,4,5)P3 may be required for full uncoating of the CCV. The CCV-associated polyphosphoinositide 5’ phosphatase synaptojanin has been proposed to perform this role (19).

Currently protein phosphorylation and phospholipid metabolism are viewed as two independent mechanisms regulating endocytosis. Here we show that CK2 binds to polyphosphoinositides and that this binding inhibits CK2 activity while CK2 is present in intact CCVs. Further, we show that this interaction occurs directly via the active site of CK2, thus providing a mechanism for inhibiting CK2 activity in an intact CCV.

MATERIALS AND METHODS

Reagents were from Sigma, unless otherwise stated. Anti-α- and β2-subunits of CK2 antibodies were from Santa Cruz Biotechnology. hCK2 pT7–7 plasmid was kindly provided by Y.-S. Bae (Kyungpook National University, Taegu, Korea). Recombinant human CK2 was from Roche Applied Science. [γ32P]ATP was from PerkinElmer Life Sciences. Porcine brains were provided by Dr. M. Bailey (University of...
Preparation of Clathrin-coated Vesicles and Stripped Proteins—Clathrin-coated vesicles were purified to homogeneity from porcine brains as previously described (2). CCVs were uncoated using 1 M Tris, pH 7.0, with subsequent centrifugation at 100,000 × g. Clathrin was purified from the supernatant fraction according to Ref. 22. Stripped membranes were resuspended in Tris-buffered saline to a final protein concentration of 1–2 mg/ml and stored at −80 °C.

Proteolytic Digest of Stripped Membranes—50 μl of stripped membranes were incubated with 0, 100, or 500 μg/ml trypsin for 30 or 60 min. After incubation, trypsin was inhibited by the addition of 200 μg of soybean trypsin inhibitor, and membranes were separated from soluble protein by centrifugation for 30 min at 100,000 × g. Pellets were resuspended in 50 μl of 50 mM Tris-HCl, pH 7.5, and 5 mM MgCl2 buffer. In all other cases, liposomes were prepared by sonication as described above.

In Vitro Phosphorylation Assay—Liposomes containing single lipids (phosphatidylethanolamine (PE), phosphatidylycerine (PC), phosphatidylserine (PS), or phosphatidylinositol) were prepared from dried lipid films by sonication three times for 10 s in 50 mM Tris-HCl, pH 7.5, and 5 mM MgCl2 buffer. In all other cases, liposomes were prepared by sonication as described above from mixtures of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine in equimolar ratio as a backbone lipid supplemented with 20–90% phosphatidylinositol, 20% of cholesterol, phosphatidic acid, or diacylglycerol or 10% of the relevant diC16 phosphoinositide. 5 μg of purified bovine clathrin were phosphorylated by 2 microunits of purified CK2 for 10 min at room temperature in a 50-μl reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 100 μM ATP, 7.4 MBq ml−1 [γ-32P]ATP, and 25 μl of liposomes (final concentration of lipid was 1.1 mM) or stripped CCV membranes. The phosphorylation reaction was stopped by the addition of 12.5 μl of 5× SDS sample buffer. The samples were boiled for 5 min and loaded on SDS-PAGE gels (23). Dried gels were exposed to x-ray film.

Liposome Binding Assay—Liposome binding assays were performed as described elsewhere (24). In brief, lipid mixtures containing phosphatidylethanolamine, phosphatidylycerine, and phosphatidylserine were supplemented with 10% of the relevant diC16 phosphoinositide prior to being dried, resuspended in 20 mM Hepes, pH 7.4, 0.2 M sucrose, 20 mM KCl buffer, and sonicated 3 times for 10 s. The liposomes obtained were incubated with 250 ng of purified CK2 for 10 min at 30 °C and centrifuged for 30 min at 100,000 × g. Pellets were resuspended in 100 μl of SDS sample buffer. Following SDS-PAGE, samples were blotted to nitrocellulose, and the presence of CK2 was detected by immunoblotting as described previously (2).

Molecular Modeling—All analysis and modeling of CK2 was performed using the program package Swiss-PdbViewer. The inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) molecule was introduced into the relevant region of the CK2 structure with the 1-phosphate position (the phosphate where the rest of the PtdIns(4,5)P2 molecule is attached) pointing away from the bulk of the CK2 molecule toward the most likely position where the membrane would be if PtdIns(4,5)P2 bound in that site. The Ins(1,4,5)P3 molecule was then moved and rotated to best fit into the space available while allowing potential interactions with nearby basic residues (Arg, Lys, and His). The aim of this modeling is only to show there is sufficient space for the Ins(1,4,5)P3 molecule and that there are potential basic residues nearby that could provide interactions with the Ins(1,4,5)P3. The structure of the CK2 molecule was not altered in any way to allow space for the Ins(1,4,5)P3 or to orientate any of the nearby basic residues into positions where they could make possible interactions with the Ins(1,4,5)P3. If the Ins(1,4,5)P3 did bind into either of these binding sites, it would be expected that the CK2 molecule would undergo at least some conformational changes to give the best shape of the binding site and to maximize any interactions with the Ins(1,4,5)P3. Residues chosen for site-directed mutagenesis were based on being localized and orientated to point in or nearby to the position of Ins(1,4,5)P3 in the model structure.

RESULTS

Which Component of CCV Membranes Inhibits CK2 Activity?—Previously we have shown that CK2 is not active when associated with CCVs and that it could be inhibited by CCV-derived membranes (2). We have already shown that heat treatment (70 °C for 15 min) of CCV membranes does not abolish their ability to inhibit CK2 activity (2), suggesting that the inhibitory factor was unlikely to be a protein component of the CCV membrane. Consistent with this, we found that incubation of CCV membranes in the presence of trypsin failed to abolish the ability of those membranes to inhibit CK2 activity (Fig. 1A). CCV membranes were stripped of peripheral membrane proteins by incubation in 1 M Tris-HCl and then treated with increasing concentrations of trypsin for different times. Membranes were then separated from soluble proteins by centrifugation, part of each fraction was subjected to SDS-PAGE, and the density of bands corresponding to Coomassie-stained proteins was measured. Another part of each membrane fraction was used for the inhibition of clathrin light chain phosphorylation by CK2 as described under “Materials and Methods.” The amount of phosphorylated clathrin light chain (determined densitometrically) was compared with the amount of protein present in the membrane fraction (as described above). We observed that untreated membranes inhibited phosphorylation of clathrin by up to 60%. This degree of inhibition was not significantly changed when membranes were trypsinated and the amount of protein in them was reduced by up to 80%.

These data argue against the possibility that the protein component of CCV membranes inhibits CK2 activity. A complex mixture of different lipids remains after trypsin cleavage of the proteins in CCV membranes. To determine which lipids might be able to inhibit CK2 activity, we initially screened simple liposomes (of single lipid composition) for their ability to inhibit the phosphorylation of clathrin light chain by CK2. Of those lipids initially tested, only phosphatidylinositol (PtdIns) inhibited the activity of CK2 (Fig. 1B). Liposomes composed of other phospholipids (i.e. PE, PC, or PS) did not have any effect on CK2 activity (Fig. 1B). Incorporation of cholesterol, phosphatidic acid, or diacylglycerol into “backbone liposomes” composed of equimolar amounts of PE, PC, and PS failed to affect the activity of CK2 (data not shown). However, the presence in backbone liposomes of PtdIns was able to inhibit CK2 activity but only when it was present at high concentrations (data not shown). This suggested that PtdIns is unlikely to be a physiologically relevant membrane-bound inhibitor of CK2 but did suggest that higher
phosphorylated forms of PtdIns might be worthy of investigation in this regard.

Polyphosphoinositides Inhibit and Bind CK2—To test whether polyphosphoinositides can bind and inhibit CK2, we performed kinase assays (using clathrin light chain as substrate for CK2) in the presence of liposomes of different composition. The liposomes consisted of the PE:PC:PS backbone mix alone or that mix including different polyphosphoinositides at 10% of the total lipid concentration. Although PE:PC:PS liposomes alone did not have any effect on CK2 activity (data not shown), all of those containing polyphosphoinositides inhibited the activity of CK2, although to different extents (Fig. 2). The most efficient inhibition was observed by PtdIns(3,4)P2 and PtdIns(4,5)P2 (\(\frac{1}{20}\) of initial activity).

PtdIns(4,5)P2 is known to be an important anchoring molecule in biological membranes, and it is of note that it mediates the attachment of numerous peripheral membrane proteins involved in CME to the cytosolic face of plasma membrane (reviewed in Ref. 25). Therefore, it was tempting to speculate that PtdIns(4,5)P2 could also anchor CK2 to the plasma membrane and at the same time render it inactive prior to inclusion in CCVs.

Indeed, liposome binding experiments demonstrated that CK2 binds to liposomes containing polyphosphoinositides. Moreover there is some correlation between binding and the inhibitory activity of those polyphosphoinositides (Fig. 3). However, it is probable that binding of CK2 to cellular membranes is not solely mediated by the interaction with PtdIns(4,5)P2, because others have shown that protein-protein interactions are also likely to play a role (26). This would explain why we were unable to see any effect on the amount of CK2 associated with cellular membranes when PtdIns(4,5)P2-protein interactions were blocked with neomycin or competed by overexpression of the PLCδ-PH domain.\(^3\)

Identification of a Polyphosphoinositide Binding Site on CK2—As CK2 does not contain any known specialized PtdIns(4,5)P2 binding domain, it was pertinent to determine the molecular mechanism by which CK2 interacts with polyphosphoinositides. The current view is that CK2 binds to lipid membranes via the acidic loop of its β-subunit, in particular through the region encompassing amino acid residues 51–110 (26, 27). Although it is unlikely that this acidic region could mediate interaction with the negatively charged head groups of polyphosphoinositides, we generated a construct encoding this sequence fused to glutathione S-transferase. As might be expected,
FIGURE 3. Binding of CK2 to liposomes containing polyphosphatidylinositides. Following liposome binding assays, the amount of CK2 in pelleted liposomes was detected by immunoblotting using an antibody to the α-subunit of CK2 followed by enhanced chemiluminescence detection and quantitation using Scion Image software (n = 4). A representative blot is shown beneath the graph. Data are presented as the mean, with error bars representing S.D.

FIGURE 4. Possible binding sites for PtdIns(4,5)P₂ on CK2. Schematic representation of the backbone of the model CK2 structure with Ins(1,4,5)P₃ (representing the head group of PtdIns(4,5)P₂; the 1-phosphate position, the phosphate where the rest of the PtdIns(4,5)P₂ molecule is attached, is indicated) bound in the heparin (1) and ATP (2) binding sites. A shows both binding sites in the context of the α-subunit of CK2; the structural features of CK2 are shown with α-helices in blue and β-strands in green. B shows the heparin binding site in more detail; the basic K₇₄K₃₃K₉₀ region has been highlighted in green, and Lys₇₆ and Arg₁₅₅ (also in green) are shown as larger sticks. C shows the ATP binding site in more detail. The bound Ins(1,4,5)P₃ molecules are shown as spacefill structures. The figure was generated using Swiss-PdbViewer and rendered using POV-Ray software.

FIGURE 5. A, heparin inhibits binding of CK2 to PtdIns(4,5)P₂-containing liposomes. Following liposome binding assays in the presence or absence of 10 μM heparin, CK2 was detected in liposome pellets by immunoblotting using an antibody to the α-subunit of CK2 followed by enhanced chemiluminescence detection and quantitation using Scion Image software. B, expression of recombinant mutant CK2 proteins. Recombinant mutant CK2 proteins were expressed and purified as described under “Materials and Methods,” separated by SDS-PAGE, and detected as in A, wt, wild type. C, all kinase-active CK2 mutants were inhibited by PtdIns(4,5)P₂-containing liposomes. The kinase activity of different CK2 mutants was assayed using clathrin light chain as substrate in the presence of backbone liposomes (PC:PE:PS) that either contained (+) or did not contain (−) 10% PtdIns(4,5)P₂. Reaction products were separated by SDS-PAGE and subjected to autoradiography. The band corresponding to phosphorylated clathrin light chain is shown. The autoradiograph is representative of three independent experiments. WT, wild type.

the glutathione S-transferase fusion protein neither bound to PtdIns(4,5)P₂-containing liposomes nor prevented binding of purified CK2 to PtdIns(4,5)P₂-containing liposomes (data not shown). Moreover, in contrast to others (27), we were unable to release CK2 from isolated membranes using this fusion protein.

The crystal structure of heterotetrameric CK2 was solved recently (28). This enabled us to screen for potential PtdIns(4,5)P₂ binding sites on the CK2 surface. In search for a site that would electrostatically interact with the negatively charged lipid head group, we first turned our attention to a basic cluster containing several lysines and arginines; i.e. K₇₄K₃₃K₉₀ and Arg₁₅₅ in the CK2 α-subunit (Fig. 4, A and B). This was an especially attractive candidate, as this region is thought to be a potential binding site for the strong CK2 inhibitor heparin (29). By analogy to heparin binding to this site and inhibiting CK2 activity, it is plausible to imagine that interaction of polyphosphoinositides with this region would also inhibit CK2 activity. This hypothesis was supported by data indicating that heparin inhibits binding of CK2 to PtdIns(4,5)P₂-containing liposomes (Fig. 5A).

Because heparin inhibits binding of CK2 to PtdIns(4,5)P₂-containing liposomes, we used an in silico approach to generate a model of how PtdIns(4,5)P₂ might bind to this region of CK2 and identified several residues in CK2 that would be critical for direct interaction with PtdIns(4,5)P₂ (Fig. 4B). Two of these residues (Lys₇₆ and Arg₁₅₅) were individually and collectively mutated to alanine or glutamic acid. All single and double mutants were expressed in E. coli and purified the same as for wild type CK2 (Fig. 5B). All mutants still bound to heparin-Sepharose as efficiently as the wild type CK2. Those mutants containing R1₅₅E were inactive, whereas R1₅₅A mutants retained ~50% of their activity. In phosphorylation assays (using clathrin light chain as substrate) all active mutants were inhibited by PtdIns(4,5)P₂-containing liposomes as efficiently as the wild type CK2 (Fig. 5C). This argues...
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against a role for the basic cluster of the CK2 α-subunit in binding to and being inhibited by polyphosphoinositides.

Finally, we turned our attention to the ATP binding site of the CK2 α-subunit. As the published structure (28) and our modeling data (Fig. 4A and C) show, this region also contains a number of basic residues that can potentially interact with the head group of PtdIns(4,5)P2. In the case of this model, inhibition of CK2 activity would be a direct consequence of the lipid head group blocking the active site and not allowing substrate access. Because in the crystal structure of CK2 (28) the active site cleft is blocked with a non-hydrolyzable analogue of ATP (AMPPNP), we have included AMPPNP in liposome binding assays and shown that it prevents binding of CK2 to liposomes containing polyphosphoinositides, the most complete inhibition of binding being that to PtdIns(4,5)P2-containing liposomes (Fig. 6).

DISCUSSION

We have previously shown that the protein kinase CK2 is present but catalytically inactive in CCVs and that CK2 is capable of phosphorylating the majority of peripheral membrane proteins present within the CCV that are substrates for phosphorylation (2). CK2 is activated as the CCV starts to uncoat, leading to the phosphorylation of peripheral membrane proteins (2). Several of these membrane proteins have been shown to be assembly-incompetent when phosphorylated (4, 6, 8–10), thus their phosphorylation as the CCV uncoats would ensure that the CCV coat does not reform on the vesicle from which it has just been released but would allow the coat components to be recycled for another round of CME following dephosphorylation.

We have now shown that CK2 binds to liposomes containing the polyphosphoinositide PtdIns(4,5)P2 (a known component of CCV membranes) and that binding of CK2 to PtdIns(4,5)P2-containing liposomes inhibits the activity of CK2. A molecular explanation for CK2 being inhibited by binding to PtdIns(4,5)P2-containing liposomes is provided by our observation that CK2 binds to PtdIns(4,5)P2-containing liposomes via the active site of the enzyme.

It is of note that another component of CCVs is the polyphosphoinositide 5′-phosphatase synaptojanin which has been implicated as playing a role in the uncoating of CCVs (19). Thus, we propose that the PtdIns(4,5)P2 present in the membrane of forming and nascent CCVs would provide both an anchor for CK2 and a means of ensuring that CK2 is inactive as the CCV is generated. Following CCV formation and the activation of synaptojanin, PtdIns(4,5)P2 would be hydrolyzed to PtdIns (4)P, releasing the inhibition on CK2 and allowing it to phosphorylate those CCV peripheral membrane proteins it recognizes as substrates. The regulation of CK2 activity by its binding to PtdIns(4,5)P2 would therefore impose some directionality on the process of CCV coat assembly and disassembly by allowing coat assembly when CK2 is in the PtdIns(4,5)P2-bound inactive state and inhibiting coat reassembly when PtdIns(4,5)P2 has been hydrolyzed to PtdIns (4)P and CK2 is no longer inactive.

Acknowledgments—We thank Dr. Y.-S. Bae for the CK2 plasmid, Dr. M. Bailey for pig brains, and Prof. P. Cullen for reagents and advice.

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