Characterization of Human Lactoferricin as a Potent Protein Kinase CK2 Activator Regulated by A-Kinase in Vitro

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Lactoferrin (LF), an iron-transport protein transferrin,\(^1\) (i) is found in high concentrations in mammalian milk and in the specific granules of neutrophils,\(^2\) (ii) is an inflammatory response protein synthesized exclusively by mucosal epithelium and polymorphonuclear leukocytes,\(^2\)\(^3\) and (iii) is released from the granules of neutrophils in response to microbial infections.\(^4\) Lactoferrins (LFcins), at the N-terminal positions 1—47 of human LF (hLF) and positions 17—41 of bovine LF (bLF) (Fig. 1), are obtained from lactoferrins (LFs) hydrolyzed by pepsin in vitro.\(^5\) hLFcin contains a basic amino acid-rich region near the N-terminus of LFs with a higher antimicrobial activity than intact hLF.\(^6\) Recently, we reported that (i) hLFcin contains a binding site of glycyrhizin (GL, an anti-inflammatory substance isolated from the root of licorice)\(^7\); and (ii) both bLF and hLF function as phosphate acceptors of protein kinase CK2 (CK2, formerly named as casein kinase II) in vitro.\(^8\) However, the physiological functions of LFs and LFcins in the inflammatory response to microbial infections remain to be elucidated.

CK2 is ubiquitous, cAMP-, cGMP- and Ca\(^{2+}\)-independent multifunctional serine/threonine protein kinase found in the cytoplasm, nuclei and mitochondria of all eukaryotic cells.\(^9\)–\(^11\) CK2 plays important roles in the regulation of DNA replication and transcription through specific phosphorylation of various mediators and regulatory factors involved in cell differentiation and cell proliferation.\(^11\)–\(^13\) Also, it has been reported that (i) CK2 is implicated in the regulation of protein synthesis through its specific phosphorylation of 60S acidic ribosomal proteins (P0, P1, P2)\(^14\) and eukaryotic initiation factors (eIFs), such as eIF-2, eIF-4E and eIF-5, in mammalian cells\(^15\)–\(^17\); and (ii) the phosphorylation of 60S acidic ribosomal proteins is required for the reconstitution of ribosomal subunits. In addition, we recently reported that (i) CK2 is associated with the 60S acidic ribosomal proteins prepared from porcine liver\(^18\) and plant cells\(^19\); (ii) poly-arginine (poly-Arg) markedly stimulates the CK2-mediated phosphorylation of 60S acidic ribosomal proteins (P0, P1, P2) as well as Hsp90 (p98) in vitro\(^18\),\(^19\); and (iii) other functional proteins with oligo-Arg clusters, such as salmine A1 (salmon sperm DNA-binding basic protein),\(^20\) histone H2B (sea urchin sperm)\(^20\) and Rev of human immunodeficiency virus type 1 (HIV-1),\(^21\) act as potent CK2 activators in vitro. The CK2 fraction (Superdex fraction) prepared from porcine liver is a convenient material for an in vitro assay of CK2 activity, because 60S acidic ribosomal proteins and Hsp90 in the fraction are effectively phosphorylated by incubation with \([\gamma-32P]ATP\) in the presence of a suitable CK2 activator.\(^10\) Using the Superdex fraction, the stimulatory effects of two LFcins (hLFcin and bLFcin) on CK2 activity (phosphorylation of 60S acidic ribosomal proteins and Hsp90) were investigated in vitro.

Here, we describe (i) the stimulatory effects of two LFcins on CK2 activity in vitro; (ii) phosphorylation of hLFcin by A-kinase in vitro; and (iii) the effect of hLFcin phosphorylation by A-kinase on CK2 activity in vitro.

MATERIALS AND METHODS

Chemicals \([\gamma-32P]ATP\) (3000 Ci/mmol), HiTrap Heparin and Superdex 200 prep grade columns were obtained from Amersham Pharmacia Biotech, Inc. (Uppsala, Sweden); purified A-kinase (bovine heart), dithiothreitol (DTT), poly-Arg (about 100 residues), salmine A1 and protamine 3a from Sigma Chemical (St. Louis, U.S.A.); diethylaminoethyl (DEAE)-cellulose (DE52) from Whatman (Maidstone, England). Synthetic bLFcin and hLFcin were obtained from Tana Laboratories (Texas, U.S.A.).

Extraction and Partial Purification of CK2 CK2 was partially purified from a 1.5 M NaCl crude extract of porcine liver by means of successive DEAE-cellulose and heparin-Sepharose column chromatographies and gel filtration on a Superdex 200 pg HPLC column, as previously reported.\(^18\) The Superdex fraction was used as a partially purified CK2 fraction containing 60S acidic ribosomal proteins [P0 (p35), P1 (p17), P2 (p15)] and Hsp90 (p98) in the present study.

Detection of Polypeptides Phosphorylated by CK2 in Vitro Protein phosphorylation by CK2 was carried out in standard reaction mixtures (50 \(\mu\)l) comprising 40 mM Tris–HCl (pH 7.6), 2 mM DTT, 3 mM Mn\(^{2+}\), 20 \(\mu\)M \([\gamma-32P]ATP\) (1000 cpm/\(\mu\)mol) and the indicated amounts of partially purified CK2 fraction (Superdex fraction). After incubation for the indicated periods at 30°C, \(^{32}\)P-labeled polypeptides [60S acidic ribosomal proteins (p35, p17, p15) and Hsp90 (p98)] in the reaction mixtures were detected by autoradiography.

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RESULTS AND DISCUSSION

As reported previously, 18 60S acidic ribosomal proteins (p35, p17, p15) and Hsp90 (p98) were detected as phosphorylated polypeptides when the Superdex fraction (partially purified CK2 fraction) was incubated with 20 μM [γ-32P]ATP (1000 cpm/pmol) in the presence of poly-Arg (a CK2 activator) (Fig. 2A). Under the same experimental conditions, the stimulatory effect of hLFcin, containing an Arg-rich region near the N-terminus (Fig. 1), on CK2 activity (phosphorylation of 60S acidic ribosomal proteins and Hsp90) was examined in vitro. hLFcin stimulated CK2 activity in a dose-dependent manner (Fig. 2). The stimulatory effect of hLFcin on CK2 activity at 0.5 μg was similar to the level (approx. 5-fold) observed with poly-Arg (Fig. 2). The stimulatory effect of hLFcin on CK2 activity was about half that of hLFcin (data not shown). These results show that hLFcin functions in vitro as a potent CK2 activator, as previously shown in the marked stimulation of CK2 activity by other CK2 activators, such as salmine A1, 20 sperm histone H2B 20 and HIV-1 Rev. 21 Taken together, these results presented here and our previous observations 20,21 suggest that an Arg-rich region of hLFcin may be responsible for the in vitro activation of CK2.

The marked stimulation of CK2 activity by synthetic basic polypeptides (poly-Arg and poly-Lys) and DNA-binding basic proteins (sperm protamine 3a, salmine A1 and histone H2B) is well-known as a unique property of CK2,20) distinguishing it from those of other protein kinases, such as cAMP-dependent protein kinase (A-kinase) and Ca2+- and phospholipid-dependent protein kinase (C-kinase).22,23) The Arg-rich regions of these potent CK2 activators interact with an acidic cluster (at positions 55—64) in the N-terminal domain of the β-subunit of CK2. This interaction induces conformational changes in CK2, increasing the accessibility of the protein substrates to the catalytic site (at positions 154—163) of the α-subunit of CK2.24)

hLFcin contains two potential phosphorylation sites (Ser-6, Ser-42) for A-kinase and a site (Ser-13) for CK2, whereas bLFcin has not their potential phosphorylation sites (Fig. 1). Therefore, the substrate activity of hLFcin for these two protein kinases in vitro was tested. Under the given experimental conditions, hLFcin was phosphorylated by A-kinase time-dependently, reaching a plateau within 120 min (Fig. 3A). However, no phosphorylation of hLFcin by CK2 was detected (data not shown). To phosphorylate completely hLFcin by A-kinase in vitro, hLFcin was incubated for 120 min at 30°C with A-kinase in the presence of 20 μM ATP. After full phosphorylation of the hLFcin by A-kinase, the Superdex fraction and 20 μM [γ-32P]ATP were added to the reaction mixtures, and then further incubated for 60 min at 30°C. The autoradiogram (Fig. 3B) detected 60S acidic ribosomal proteins (P0, P1, P2) and Hsp90 (p98) as phosphorylated polypeptides on the incubation with unphosphorylated hLFcin (UP-hLFcin, lane 2) or phosphorylated hLFcin (P-hLFcin, lane 3). The stimulatory effect of hLFcin on CK2 activity was significantly lower than that of UP-hLFcin (lane 2, Fig. 3B). These results suggest that the stimulatory effect of hLFcin on CK2 activity is reduced by its phosphorylation with A-kinase in vitro. Phosphorylation of hLFcin at Ser-6 by A-kinase may alter an ionic strength of an Arg-rich region of hLFcin in its interaction with the β-subunit of CK2. It is therefore concluded that, at the cellular level, (i) hLFcin may function as a potent CK2 activator; and (ii) the stimulatory effect of

Fig. 1. Amino Acid Sequences of hLFcin and bLFcin

The gray box (□) indicates two potential phosphorylation sites (Ser-6, Ser-42) on hLFcin for A-kinase and the open box (□) indicates a potential phosphorylation site (Ser-13) for CK2. The lines indicate the disulfide bonds.

Fig. 2. The Stimulatory Effects of hLFcin and Poly-Arg on CK2 Activity in Vitro

[A] To determine the stimulatory effects of hLFcin and poly-Arg on CK2 activity [phosphorylation of 60S acidic ribosomal proteins (p35, p17, p15) and Hsp90 (p98)], the purified CK2 fraction (Superdex fraction; approx. 1 μg) was incubated for 20 min at 30°C with 20 μM [γ-32P]ATP (1000 cpm/pmol) in the presence of hLFcin or poly-Arg. After incubation, the 32P-labeled polypeptides (p98, p35, p17, p15) were detected by autoradiography after SDS-PAGE. Lane 1, control (Superdex fraction alone); lane 2, lane 1 + poly-Arg (2.5 μg); lanes 3 to 8, lane 1 + hLFcin (13.8 ng to 4.14 μg). [B] The autoradiogram was scanned by a spectrophotometer and the phosphorylation rates of 60S acidic ribosomal proteins [P0 (p35), P1 (p17), P2 (p15)] and Hsp90 (p98) were determined. △, P0; ○, P1; □, P2; and ▽, p98 (▲, ■, ▼, ◼ poly-Arg).
hLFcin on CK2 activity may be regulated through its specific phosphorylation by A-kinase.

Since it has been demonstrated that phosphorylation of 60S acidic ribosomal proteins is required for their binding to the large ribosomal subunit and for reconstitution of active core ribosome particles, the CK2-mediated phosphorylation of 60S acidic ribosomal proteins may be implicated in translational processes at the cellular level. Indeed, the in vitro translation activity in rabbit reticulocyte lysate using XEF-1 mRNA is significantly stimulated when one of potent CK2 activators, such as hLFcin and sperm DNA-binding proteins (protamine and histone H2B), is added to the reaction mixtures (data not shown). From these indirect observations, it is possible to speculate that (i) CK2 associated with 60S acidic ribosomal proteins may be activated by potent CK2 activators including hLFcin; and (ii) the phosphorylation of 60S acidic ribosomal proteins as well as eIFs by the activated CK2 may be involved in the stimulation of protein synthesis at the cellular level. In addition, it seems likely that hLFcin may stimulate markedly the CK2-mediated phosphorylation of mediators and regulatory factors involved in activation and differentiation of neutrophils in the inflammatory response to microbial infections, as demonstrated in the CK2-mediated phosphorylation of specific proteins in lymphocytes treated with concanavalin A or interleukin 2 (T cell growth factor),11 fertilized sea urchin egg20 and HIV-1-infected cells.21

The physiological significance of the A-kinase-mediated phosphorylation of hLFcin in the regulation of CK2 activity at the cellular level remains unclear at present. For understanding the physiological significance of hLFcin on the CK2-mediated metabolic regulation and signal transduction, further analytical studies will be required to detect (i) the hLFcin-induced stimulation of the CK2-mediated phosphorylation of specific mediators and regulatory factors involved in the inflammatory response and protective mechanism during microbial infections, and (ii) direct phosphorylation of hLFcin by A-kinase in the hLFcin-sensitive cells. It is also of interest to determine the physiological interaction between hLFcin, CK2 and GL, since (i) hLFcin contains a GL-binding site7; (ii) hLFcin directly binds to the β-subunit of CK2 in vitro; and (iii) GL inhibits the hLFcin-induced stimulation of CK2 activity in vitro.28

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