Irreversible loss of membrane-binding activity of *Listeria*-derived cytolysins in non-acidic conditions: a distinct difference from allied cytolysins produced by other Gram-positive bacteria

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Listeriolysin O (LLO), a member of the cholesterol-dependent cytolysin (CDC) family, is a major virulence factor of *Listeria monocytogenes* and contributes to bacterial escape from intracellular killing of macrophages. LLO is activated under weakly acidic conditions; however, the molecular mechanism of this pH-dependent expression of cytolytic activity of LLO is poorly understood. In this study, CDCs including LLO, ivanolysin O (ILO), seeligeriolysin O (LSO), pneumolysin (PLY), streptolysin O (SLO) and perfringolysin O (PFO) were prepared as recombinant proteins and examined for their functional changes after treatment under various pH conditions. Haemolytic and membrane cholesterol-binding activities were not affected in PLY, SLO and PFO at any pH examined. By contrast, all the *Listeria*-derived cytolysins, LLO, ILO and LSO, were active only at an acidic pH and rapidly inactivated under neutral or alkaline conditions. Once inactivated, LLO could not be reactivated even by a downward pH shift. The hydrophobicity of LLO treated at neutral or alkaline pH was increased. These data suggested that the pH-dependent loss of cytolytic activity appeared to be due to irreversible structural changes of domain 4 that resulted in the loss of target membrane cholesterol binding.

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INTRODUCTION

Listeria monocytogenes is a Gram-positive facultatively intracellular bacterium able to survive in phagocytic cells such as macrophages in infected hosts. The molecular mechanisms by which *L. monocytogenes* evades the intracellular killing system of macrophages have been extensively studied. Listeriolysin O (LLO) is a major virulence factor of *L. monocytogenes* and is required for bacterial escape from the phagosomal compartment to the cytoplasmic space in host macrophages (Cossart *et al.*, 1989; Gaillard *et al.*, 1986; Portnoy *et al.*, 1988). LLO is a member of the cholesterol-dependent cytolysin (CDC)

family, known to bind cholesterol molecules in the cytoplasmic membrane of mammalian cells and to oligomerize to pore-forming units resulting in the lysis of the membrane (Mengaud *et al.*, 1987). CDCs are produced by many Gram-positive bacteria including *L. monocytogenes* (LLO), *L. ivanovii* (ivanolysin O; ILO), *L. seeligeri* (seeligeriolysin O; LSO), *Clostridium perfringens* (perfringolysin O; PFO), *Streptococcus pyogenes* (streptolysin O; SLO), and *Streptococcus pneumoniae* (pneumolysin; PLY).

In respect of the pH sensitivity of the cytolytic activity of LLO, it has been shown that purified LLO from bacterial culture is active at a weakly acidic pH but inactive at a neutral or weakly alkaline pH (Geoffroy *et al.*, 1987; Portnoy *et al.*, 1992). Such pH-dependent activity appears to be unique to LLO produced by *L. monocytogenes*; it is not observed in other CDCs produced by extracellular bacteria such as PFO, SLO or PLY, all of which have been shown to be stable in a pH range of 5.0–8.0 (Kehoe *et al.*,

Abbreviations: CDC, cholesterol-dependent cytolysin; HU, haemolytic unit; ILO, ivanolysin O; LDH, lactate dehydrogenase; LLO, listeriolysin; LSO, seeligeriolysin O; PFO, perfringolysin O; PLY, pneumolysin; SLO, streptolysin O; SRBC, sheep red blood cell; TNS, 2-(*p*-toluidinyl)naphthalene-6-sulfonic acid, sodium salt.

1987; Walker *et al.*, 1987; Tweten, 1988). This unique property of LLO may be essential for the intracellular parasitism of *L. monocytogenes*. Activation of LLO by acidification of phagosomes containing *L. monocytogenes* is an important step in bacterial escape because the inhibition of phagosomal acidification by H^+ -ATPase inhibitors, bafilomycin A₁ or concanamycin A, results in the prevention of bacterial escape into the cytoplasm (Beauregard *et al.*, 1997; O'Connell *et al.*, 2005). Also, functional inactivation at a neutral pH is likely essential for intracellular parasitism of this bacterium. A recombinant strain of *L. monocytogenes* expressing PFO is reported to be incapable of intracellular replication and cell-to-cell spread, presumably by cytolysis of infected macrophages without inactivation of PFO inside the cytosolic space after escape from the phagosome (Jones & Portnoy, 1994). This suggests that the optimized regulation of pH-dependent cytolytic activity is essential for the intracellular parasitism of *L. monocytogenes*.

Among bacterial species belonging to the genus *Listeria*, *L. ivanovii* and *L. seeligeri* also produce CDC family proteins, ivanolysin O (ILO) and seeligeriolysin O (LSO), respectively (Gormley *et al.*, 1989; Leimeister-Wachter & Chakraborty, 1989). It is reported that a recombinant strain of *L. monocytogenes* producing ILO is also virulent and capable of phagosomal escape and intracellular replication (Frehel *et al.*, 2003). While the amino acid at position 461 of CDCs produced by the genus *Listeria*, including LLO, ILO and LSO, is leucine, that of other members of the CDC family such as PFO, SLO, and PLV is threonine. It has been demonstrated that the single residue replacement of leucine to threonine at position 461 of LLO resulted in the loss of pH dependency (Glomski *et al.*, 2002). These observations suggest the molecular evolution of cytolysins that enable the intracellular parasitism of the genus *Listeria*. However, it is not yet clear whether all *Listeria*-derived CDCs are the same as LLO in terms of pH sensitivity. Even the mechanisms of pH-sensitive inactivation of LLO are not fully understood.

In this study, a panel of CDCs were prepared as recombinant proteins. These included three *Listeria*-derived cytolysins (LLO, ILO, LSO) and three allied cytolysins derived from other Gram-positive bacteria (PLV, PFO and SLO). They were examined for differences in pH-dependent cytolytic activity, membrane-binding activity and cholesterol binding. We found that all *Listeria*-derived cytolysins are pH sensitive and, by using a domain 4 preparation, that the irreversible inactivation of the cytolytic activity of LLO is due to a loss of binding to the target membrane through alteration of domain 4.

METHODS

Bacterial strains, plasmids, media and cell culture. *Escherichia coli* SG13009 (Qiagen) harbouring a pREP4 plasmid, which contains *lacI* and kanamycin-resistant genes, was used as the host cell as previously reported (Kohda *et al.*, 2002). As an expression vector, we used pQE-31 (Qiagen), which is designed to place a hexahistidine tag

(His-tag) at the N terminus of the protein of interest. In order to express the recombinant protein, *E. coli* harbouring a recombinant plasmid was grown in tryptic soy broth (Difco) containing 100 µg ampicillin ml⁻¹ and 25 µg kanamycin ml⁻¹. A J774.1 mouse macrophage cell line maintained in our laboratory was used in the binding assay. Cells were maintained in RPMI 1640 medium (Gibco-BRL, Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco) and 5 µg gentamicin ml⁻¹.

Reagents. All reagents used in this study were obtained from Nacalai Tesque or Wako Pure Chemical Industries.

Construction and purification of recombinant cytolysins. The His-tagged recombinant cytolysins used in this study are listed in Table 1. PCR products were constructed using genomic DNA as the template. PCR fragments were then ligated into pQE-31 vectors (Qiagen). A recombinant plasmid harbouring a gene coding for each recombinant protein was electroporated into *E. coli* M15 or SG13009. Expression of the His-tagged recombinant cytolysin gene was induced by incubation with 1 mM isopropyl-β-D-thiogalactopyranoside (Nacalai) at 25 °C for 2–6 h. Cells were harvested by centrifugation at 6000 g for 15 min, suspended in lysis buffer [50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, 20 mM imidazole, 1 mg lysozyme ml⁻¹, 200 U DNase I], and disrupted by vortexing with 0.1 mm zirconia-silica beads. The soluble fraction was collected by centrifugation at 20 000 g for 30 min, and His-tagged recombinant cytolysins were purified with a nickel nitrilotriacetic acid column (Qiagen) according to the manufacturer's instructions. Contaminating lipopolysaccharide (LPS) was removed using Detoxi-Gel endotoxin-removing gel (Pierce Chemical). The level of LPS was determined by a *Limulus* colour KY test (Wako). Protein concentration was measured with a protein assay reagent (Nacalai), and purity determined by Coomassie brilliant blue staining and Western blotting after SDS-PAGE. A monoclonal anti-His-tag antibody (Qiagen) was used for Western blot analysis.

Treatment of recombinant CDCs at acidic, neutral or alkaline pH. The concentrated preparations of recombinant CDCs (>20 µM) were diluted to 200 nM with 20 mM phosphate buffer adjusted to each desired pH. The CDC samples at different pHs were incubated on ice or a heat block (EYELA) adjusted to the test temperature for 30 min, then rapidly cooled down and kept on ice until assays.

Haemolytic and cytolytic activities of recombinant CDCs. The haemolytic activity of CDCs was determined by the level of haemoglobin released from a 0.5% suspension of sheep red blood cells (SRBCs). Briefly, CDCs (200 nM) that had been treated at various pHs in the presence or absence of 10 µg cholesterol ml⁻¹ were serially diluted with PBS (pH 7.4). These were mixed with 50 µl of 1% SRBCs (total volume: 100 µl of 0.5% SRBC) and then incubated at room temperature for 30 min. After centrifugation (800 g, 10 min, 4 °C), the supernatant was collected and the haemoglobin absorbance measured at 415 nm. One haemolytic unit (HU) was defined as the amount of recombinant protein required for 50% haemoglobin release from SRBCs in the reaction mixture. The relative haemolytic activity was indicated as the HUs per 1 mg recombinant protein. The cytolytic activity to a mouse monocyte cell line J774.1 was determined by measuring the level of lactate dehydrogenase (LDH) release. Cells were suspended in RPMI 1640 culture medium and plated at 1.0×10^5 cells per well in a 48-well flat-bottomed tissue culture plate. After incubation at 37 °C overnight, cells were washed and incubated with various doses of LLOs for 6 h. Culture supernatants were collected and the titre of LDH activity measured with an LDH cytotoxicity detection kit (Takara Shuzo).

Conformational analysis of CDCs. Conformational changes of CDCs were examined by fluorescence analysis as described for a previously reported method (Qa'Dan *et al.*, 2000, 2001). Briefly,

2-(*p*-toluidinyl)naphthalene-6-sulfonic acid, sodium salt (TNS) (Molecular Probes) solution (15 mM) was prepared in a phosphate buffer. TNS solution was added to CDCs (200 nM) after treatment at various pHs in a final volume of 200 µl (final concentration of TNS was 150 µM) and incubated at 37 °C for 20 min. Each sample was analysed for its fluorescence intensity with an ARVO sx 1420 Multilabel Counter (Wallac) with excitation at 366 nm and emission at 440 nm.

Binding activity of recombinant CDCs to the cytoplasmic membrane. To examine the activity of recombinant CDCs in binding to cell-surface membranes, recombinant CDCs were treated at pH 6.0, 7.0 or 8.0 at 4 or 37 °C for 30 min and incubated with SRBCs or J774.1 cells at 4 °C for 15 min. Cells were then recovered by centrifugation (500 g, 5 min, 4 °C). After two washings with ice-cold PBS, cells were resuspended in 2 × SDS sample buffer. The amount of CDCs bound to cells was detected by Western blot analysis using monoclonal anti-His-tag antibody or polyclonal anti-LLO antibody (Nomura *et al.*, 2002).

Binding activity of CDCs to cholesterol immobilized on PVDF membrane. To quantitatively detect the binding activity to cholesterol, an assay with a PVDF membrane was carried out. Briefly, 2 µg cholesterol dissolved in a chloroform/ethanol (1:1) solution was plated in a 96-well filtration plate with Immobilon-P membrane (Millipore) at the bottom and allowed to dry overnight. The cholesterol-coated wells were treated with blocking buffer consisting of 4% heat-inactivated fetal bovine serum in PBS for 1 h, and 100 µl of 2 nM CDCs in blocking buffer was added to each well. After 1 h incubation, wells were washed four times with blocking buffer and sequentially treated with monoclonal anti-His-tag antibody and horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG) (Zymed Laboratories). The binding of CDCs to cholesterol was determined quantitatively by the addition of 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) in phosphate/citrate buffer (pH 5.0) containing 0.01% H₂O₂ and measurement of the absorbance at 450 nm after termination of the reaction with 100 µl of 0.18 M H₂SO₄.

Statistical analysis. Statistical significance of the data was determined by Student's *t* test, with a *P* value of less than 0.05 considered significant.

RESULTS

Different sensitivity of recombinant CDCs to neutral or alkaline pH

We first prepared full-size CDCs including LLO, ILO, LSO, PLY, SLO and PFO (Table 1) by using a large-scale

protein purification system in *E. coli*. All CDCs showed strong cytolytic activity against SRBCs (200 000–600 000 HU mg⁻¹). The cytolytic activity was inhibited by pretreatment with a small amount of cholesterol (data not shown). It is well known that the haemolytic activity of native CDCs purified from culture supernatant is activated by treatment with reducing agents such as DTT. However, CDCs purified from the *E. coli* expression system showed strong haemolytic activity even in the absence of treatment with the reducing agents (data not shown). CDCs adjusted to 200 nM with a phosphate buffer of acidic (6.0), neutral (7.0) or alkaline (8.0) pH were incubated at 4 or 37 °C for 30 min and haemolytic activity then determined. The strong haemolytic activities of PLY, SLO and PFO showed no change at all in the pH conditions tested (Fig. 1). In contrast to this stable expression of activity of these three CDCs derived from bacteria other than *Listeria*, a significant reduction in the haemolytic activity was observed for LLO, ILO and LSO after treatment at pH 7.0 or 8.0 at 37 °C (Fig. 1). The reduction of haemolytic activity observed at pH > 7.0 at 37 °C was not observed when treatment was undertaken at 4 °C. Thus, the CDCs produced by species of *Listeria* were the only ones sensitive to neutral or alkaline pH. They were inactivated in a short period at 37 °C but not at 4 °C.

Irreversible functional alteration of LLO after treatment at alkaline pH

LLO was first treated at pH 7.4 for 30 min at 37 °C, and the pH of inactivated LLO was then adjusted to 5.4, 6.4 or 7.4 by addition of phosphate buffer followed by incubation for a further 30 min at 37 °C. However, once LLO was inactivated at pH 7.4, a pH shift or the addition of DTT could not restore the cytolytic activity (Fig. 2a). LLO treated under reducing conditions at pH 7.4 was also inactivated (Fig. 2b).

Conformational change of LLO after treatment at alkaline pH

To determine whether the irreversible inactivation of LLO at a neutral or alkaline pH is due to proteolytic degradation or not, we compared the changes of molecular size by Western blot analysis after non-reducing SDS-PAGE.

Table 1. List of recombinant CDCs used in this study

Cytolysin name	Mol. mass (kDa)	Origin	Reference
LLO listeriolysin O	55.8	<i>Listeria monocytogenes</i> EGD	Kohda <i>et al.</i> (2002)
LLO C484S	55.8	LLO C484S	This study
LLO domain 4	13.2	LLO 416–529	Kohda <i>et al.</i> (2002)
ILO ivanolysin O	56.0	<i>Listeria ivanovii</i> ATCC 19119	Kimoto <i>et al.</i> (2003)
LSO seeligeriolysin O	56.3	<i>Listeria seeligeri</i> ATCC 35967	Ito <i>et al.</i> (2001)
PLY pneumolysin	52.8	<i>Streptococcus pneumoniae</i> IID553	Baba <i>et al.</i> (2001)
PLY domain 4	13.0	PLY 360–471	Baba <i>et al.</i> (2001)
SLO streptolysin O	60.2	<i>Streptococcus pyogenes</i> KY011	Watanabe <i>et al.</i> (2006)
PFO perfringolysin O	52.5	<i>Clostridium perfringens</i> NCTC8239	This study

Fig. 1. Temperature and pH dependency of cytolytic activity in various CDC proteins. Proteins were diluted in phosphate buffer adjusted to pH 6.0, 7.0 or 8.0 and treated at 4 (white bars) or 37 °C (black bars) for 30 min. The cytolytic activities of the CDCs were measured by haemolysis assay using SRBCs. The data are the mean $\pm$ SE for three determinations. Significance of difference was calculated with Student's *t* test. **P* < 0.05.

Western blot analysis did not demonstrate any degradation products of LLO after treatment at pH 5.4, 6.4 or 7.4 at 37 °C (Fig. 3a). Western blot analysis revealed an additional band of a size approximately equivalent to that of the LLO dimer. However, the band at around 115 kDa could not be detected when treatment was done in the presence of DTT, a reducing agent (Fig. 3b). The most probable interpretation of this finding was that LLO formed a dimer by a disulfide bond with the unique cysteine residue located at position 484. To address this possibility, we constructed LLO C484S, in which cysteine was replaced with serine. The above interpretation was

verified by the absence of a higher molecular mass in LLO C484S even after treatment at pH 7.4 at 37 °C (Fig. 3c). The cytolytic activity of LLO C484S treated at alkaline pH was also reduced (Fig. 3d). Next, we examined the hydrophobicity of LLO and PLY using fluorescence analysis. The hydrophobicity of PLY did not change after treatment at pH 6.0, 7.0 or 8.0 at 37 °C, as compared with treatment at 4 °C. By contrast, there was a significant change in the hydrophobicity of LLO after treatment at pH 7.0 or 8.0 at 37 °C (Fig. 3e).

Membrane-binding activity of CDCs under various pH conditions

We examined whether membrane-binding activity is affected by treatment under various alkaline pH conditions. There was no significant difference in the amount of membrane-bound PLY, PFO and SLO among samples treated at different pHs (Fig. 4a). No significant difference was noted between samples of these three CDCs treated at 4 °C or 37 °C, all of which were not pH sensitive. By contrast, no binding was detected when three *Listeria*-derived CDCs were treated at pH 8.0 at 37 °C, and a similar level of membrane binding was observed after treatment at pH 6.0 or 7.0, and even at pH 8.0 at 4 °C (Fig. 4a). In the next experiment, we studied the membrane binding of LLO using the J774.1 mouse macrophage cell line, as macrophages are the representative target cell of the intracellular parasitism of *L. monocytogenes*. Compared to the intensity of the band of LLO bound at an acidic pH to the macrophage membrane, a significant decrease was observed when LLO was pretreated at pH 7.4 at 37 °C. There was no detectable band of membrane-bound LLO when treated at pH 7.9 at 37 °C (Fig. 4b). We also measured the activity of LDH released from damaged J774.1 cells. In the case of treatment at 4 °C, LLO showed a high cytolytic activity against J774.1 cells (Fig. 4c). By contrast, the LDH-releasing activity of LLO was reduced after treatment at pH 7.4–7.9 at 37 °C.

Fig. 2. Irreversible inactivation of LLO by treatment at neutral or weakly alkaline pH. (a) After pH 7.4 treatment of LLO at 37 °C for 30 min (1st), the pH was readjusted to 5.4, 6.4 or 7.4 with or without DTT and incubation continued at 37 °C for a further 30 min (2nd). After the second treatment, the cytolytic activity of LLO was assessed by haemolysis assay. (b) LLO was treated at pH 7.4 with or without DTT at 4 or 37 °C for 30 min. Cytolytic activity of LLO was examined by haemolysis assay. Representative results of three similar experiments are shown and data are the mean $\pm$ SE for three determinations. Significance of difference was calculated by Student's *t* test. **P* < 0.05.

Fig. 3. Structural analysis of CDCs after treatment in neutral or alkaline pH conditions. (a) After pH 7.4 treatment of LLO at 37 °C for 30 min (1st), the pH was readjusted to 5.4, 6.4 or 7.4 with or without DTT and incubation continued at 37 °C for a further 30 min (2nd). After the second treatment, the molecular size of inactivated LLO was analysed by Western blotting using a polyclonal anti-LLO antibody. (b) LLO was treated at pH 7.4 in the absence or presence of DTT at 4 or 37 °C for 30 min, and the molecular size was confirmed by Western blot analysis. (c, d) LLO C484S was treated at pH 5.4, 6.4, 7.4 or 8.0 at 4 °C (white bars) or at 37 °C (black bars) for 30 min, and the molecular size was confirmed by Western blot analysis. The cytolytic activity of LLO C484S was measured by haemolysis assay using SRBCs. The data are the mean $\pm$ SE for three determinations. Significance of difference was calculated by Student's *t* test. **P*<0.05. (e) LLO or PLY was treated at pH 6.0, 7.0 or 8.0 at 4 or 37 °C for 30 min, and the hydrophobicity then analysed by TNS fluorescence assay. Representative results of two similar experiments are shown and data are the mean $\pm$ SE for three determinations. Significance of difference was calculated by Student's *t* test. **P*<0.05.

Cholesterol-binding activity of CDCs treated at various pHs

It is well known that most of the CDC family proteins bind to cholesterol in the cell surface membrane (Alouf, 1999). We examined whether the cholesterol-binding activity of CDCs is affected by treatment at various pHs. Six CDCs were treated at pH 6.0, 7.0 or 8.0, and then added onto a PVDF well coated with varying amounts of cholesterol. The amount of CDCs bound to the cholesterol well was then measured by quantitative ELISA using anti-His-tag antibody. No significant difference was observed among the binding of PFO, SLO and PLY, all of which showed no functional reduction at any pH. By contrast, a significant reduction was noted in the binding activity of LLO, ILO and LSO to cholesterol on the PVDF well after treatment at pH 7.0 or 8.0 at 37 °C (Fig. 5). These findings were consistent with the results of the changes in cell-binding activity obtained in the preceding experiment.

Membrane- and cholesterol-binding activity of domain 4 of LLO

The reduction in the haemolytic and the membrane- and cholesterol-binding activities of LLO at alkaline pH strongly suggested the involvement of domain 4, the C-terminus portion of LLO, in the pH-dependent functional inactivation. This was because domain 4 is responsible for

the membrane-binding activity of CDCs (Shimada *et al.*, 2002) and the leucine residue at position 461 in domain 4 is implicated in the pH-dependent cytolytic activity of LLO (Glomski *et al.*, 2002). To confirm this possibility, we constructed domain 4 preparations of LLO and PLY as representatives of pH-sensitive and pH-insensitive CDCs, respectively. Again, after treatment of the domain 4 proteins at different pHs, the binding activities to cholesterol were examined using quantitative ELISA on a PVDF well (Fig. 6). The cholesterol-binding activity of domain 4 of PLY was not reduced even under alkaline pH conditions. By contrast, the cholesterol-binding activity of domain 4 of LLO was significantly reduced when treated at 37 °C at pH 7.0 or 8.0 (Fig. 6). These results clearly indicated that treatment of LLO at neutral or alkaline pHs targets primarily the domain 4 portion of LLO leading to the functional reduction of all the cytolytic activity of LLO.

DISCUSSION

Until recently, CDCs had been called oxygen-labile cytolysins or thiol-activated cytolysins (TACs) because cytolysin protein purified from bacterial culture supernatant was inactivated by oxidation and reactivated by treatment with a reducing agent such as DTT, 2-mercaptoethanol or L-cysteine (Alouf, 1999). Among several similar proteins of the CDC family purified from

Fig. 4. Membrane-binding activity of CDCs after treatment in various pH conditions. (a) Each His-tagged CDC was treated at pH 6.0, 7.0 or 8.0 at 4 or 37 °C. Then, CDCs were mixed with SRBCs and incubated at 4 °C. After incubation, the SRBCs with bound CDC were recovered and washed by centrifugation with ice-cold PBS. The CDCs bound to SRBC membrane were analysed by Western blotting using monoclonal anti-His-tag antibody. (b) After treatment of LLO at pH 5.4, 6.4, 7.4 or 7.9 at 4 or 37 °C, LLO was added to J774.1 cells and incubated at 4 °C. After incubation, cells were washed by centrifugation with ice-cold PBS. The LLO bound to J774.1 cells was analysed by Western blotting using polyclonal anti-LLO antibody. (c) The LLO samples treated at pH 5.4, 6.4, 7.4 or 7.9 at 4 (○) or 37 °C (●) were mixed with J774.1 cells and incubated at 37 °C for 30 min. After incubation, culture supernatants were recovered and the amount of LDH released in the supernatant was measured by LDH-release assay reagent. Representative results of two similar experiments are shown and data are the mean $\pm$ SE for three determinations. Significance of difference was calculated by Student's *t* test. **P*<0.05.

Fig. 5. Cholesterol-binding activity of CDCs. Cholesterol (2.00 μ g per well) was immobilized on a PVDF membrane well. His-tagged CDCs treated at pH 6.0, 7.0 or 8.0 at 4 °C (white bars) or 37 °C (black bars) were placed in the cholesterol-immobilized PVDF well and incubated for 1 h. After incubation, wells were washed and the binding of CDCs to cholesterol was analysed by ELISA using anti-His-tag antibody. Representative results of two similar experiments are shown and data are the mean $\pm$ SE for three determinations. Significance of difference was calculated by Student's *t* test. **P*<0.05.

Fig. 6. Cholesterol-binding activity of domain 4 of LLO or PLY. His-tagged LLO domain 4 or PLY domain 4 treated at pH 6.0, 7.0 or 8.0 at 4 °C (white bars) or 37 °C (black bars) were placed in the cholesterol-immobilized PVDF wells and incubated for 1 h. After incubation, PVDF wells were washed and the binding of CDCs to cholesterol was analysed by ELISA using anti-His-tag antibody. Representative results of two similar experiments are shown and data are the mean $\pm$ SE for three determinations. Significance of difference was calculated by Student's *t* test. * $P < 0.05$.

a bacterial culture supernatant, LLO, but not PFO, SLO, PLY or alveolysin O, was known to show cytolytic activity only under acidic pH conditions, not neutral or alkaline pH conditions (Geoffroy *et al.*, 1987). We observed that the cytolytic activity of LLO secreted by *L. monocytogenes* into RPMI medium adjusted to weakly acidic pH was stronger than that of LLO secreted into RPMI medium adjusted to neutral or alkaline pH (data not shown). Glomski *et al.* (2002) reported that the leucine residue at position 461 of LLO is responsible for the pH sensitivity and that the leucine at this position is conserved in ILO and LSO from the genus *Listeria* but not in other CDCs. These observations suggest that ILO and LSO also show a cytolytic activity dependent on an acidic pH. In the present study, we constructed these CDCs and demonstrated that the pH-dependent cytolytic activity is characteristic of CDCs derived from the genus *Listeria*, such as LLO, ILO and LSO (Fig. 1). These results strongly support the observation that leucine at position 461 in the C-terminal domain (domain 4) of LLO is responsible for the pH-dependent cytolytic activity.

Recently, Schuerch *et al.* (2005) showed that neutral or alkaline pH treatment caused LLO to form an insoluble aggregate. We also made similar observations. Although it

is true that the cytolytic activity of LLO is active only under acidic pH conditions (Beauregard *et al.*, 1997), it has not been clear whether the inactivation at pH above 7.0 is reversible. We examined whether the cytolytic activity of LLO inactivated by neutral or alkaline pH treatment was reactivated reversibly by treatment at acidic pH. We demonstrated that LLO was irreversibly inactivated by treatment at neutral or alkaline pH (Fig. 2a). Because LLO secreted in a culture medium, such as brain heart infusion (BHI) broth, requires treatment with a reducing agent for activation, it has been considered that LLO secreted from *L. monocytogenes* is reversibly inactivated by oxidation in BHI broth (at neutral pH, 37 °C). However, LLO was not protected against inactivation by alkaline pH treatment by the addition of a reducing agent (Fig. 2b). These results suggested that the treatment at neutral or alkaline pH leads to irreversible changes of the LLO molecule by some mechanism other than oxidation. We therefore examined whether the LLO structure is affected under neutral or alkaline pH conditions. As shown in Fig. 3(a, b), it was demonstrated that LLO was not digested in neutral or alkaline pH conditions. We also observed dimer formation of LLO after treatment at an alkaline pH (Fig. 3a, b) and showed that this dimer was formed by a disulfide bond through the only cysteine residue, located at position 484 (Fig. 3b, c). However, this unique cysteine residue was not responsible for the pH sensitivity, because a mutant LLO in which the cysteine residue was substituted by serine (LLO C484S) showed almost the same sensitivity to neutral or alkaline pH as wild-type LLO (Fig. 3d). These results suggested that the loss of cytolytic activity caused by neutral or alkaline pH treatment is due to an irreversible conformational change(s) but not to degradation of the protein. One possibility was a change of the hydrophobicity of the LLO protein; thus we undertook fluorescence analysis for hydrophobicity. This showed that the treatment at neutral or alkaline pH at 37 °C resulted in an increase in the hydrophobicity of LLO but not of PLY (Fig. 3e).

Our observation that pH-sensitive cytolytic activity of LLO was also temperature-dependent is consistent with a previous report (Schuerch *et al.*, 2005). The cytolytic activity of LLO could be maintained in the whole range of pHs tested if incubated at below 25 °C. However, when incubated at 37 °C, LLO exhibited cytolytic activity only under acidic pH conditions, not at neutral or alkaline pH. On incubation at above 42 °C, it was rapidly inactivated in all pH conditions. Thus, LLO was revealed to be an extremely heat-labile protein. Sampathkumar *et al.* (1999) reported that a heat-shock condition (48 °C) induces overexpression and accumulation of LLO inside *L. monocytogenes* cells without detectable levels of secretion in culture media. Their results suggest that overexpressed and accumulated LLO is quite unstable in bacterial cells or in culture supernatant at 48 °C. The cytolytic activity of LLO appears to be regulated by many environmental factors through different mechanisms. Our findings and

these observations suggest that LLO secreted from *L. monocytogenes* is stable under acidic pH conditions but is not stable and undergoes an irreversible denaturation in neutral or alkaline pH conditions at 37 °C.

The mechanism of pore formation by proteins of the CDC family involves a series of complex events including recognition and binding to the target membrane, the formation of pre-pore complexes on the membrane (oligomerization), and insertion of a transmembrane β -barrel (Heuck *et al.*, 2003; Ramachandran *et al.*, 2004; Tilley *et al.*, 2005). Recently, it was reported that rapid denaturation of LLO was triggered at neutral pH by the premature unfolding of domain 3 transmembrane β -hairpins from the normal transmembrane β -barrel form (Schuerch *et al.*, 2005). In that study, it was demonstrated that the glutamic acid residue at position 247 in domain 3 was responsible for the effect under the neutral or alkaline pH conditions. As well as the involvement of domain 3 in all the steps of cytolysis, there is a possibility for the engagement of domain 4 in the pH-dependent loss of cytolytic activity, as domain 4 of CDCs first binds cholesterol of the target membrane (Heuck *et al.*, 2000). In the present study, the alkaline pH-induced loss of binding activity to SRBCs (Fig. 4a), J774.1 macrophage-like cells (Fig. 4b, c) or cholesterol molecules immobilized on PVDF wells (Fig. 5) was observed for LLO, ILO and LSO but not for PLY, PFO and SLO. It was demonstrated that at least the step of LLO binding to membrane cholesterol is affected by denaturation under neutral or alkaline pH conditions. CDCs have four functional domains, among which the C-terminal domain 4 is essential for the recognition and first binding of CDCs to target membrane (Rossjohn *et al.*, 1997; Shimada *et al.*, 2002). Several studies have suggested that the domain 4 of CDCs remains close to the membrane surface in the membrane-inserted oligomer without being embedded deeply in the bilayer (Heuck *et al.*, 2000; Ramachandran *et al.*, 2004). Furthermore, it is reported that a replacement mutation of leucine at position 461 to threonine (L461T) in domain 4 of LLO resulted in a change of the sensitivity of LLO to neutral or alkaline pH (Glomski *et al.*, 2002). These reports and the present results indicate that the function of domain 4 is affected in neutral or alkaline pH conditions. Therefore, we compared the pH sensitivity of LLO domain 4 and PLY domain 4 with respect to their binding activity to target cholesterol. The loss of binding activity to cholesterol was detected in LLO domain 4 but not in PLY domain 4 after treatment at neutral or alkaline pH (Fig. 6). These results suggest that the sensitivity of LLO to neutral and alkaline pH conditions is also due to domain 4. Treatment in neutral or alkaline pH conditions could cause conformational changes in LLO domain 4 and result in a reduced binding activity to membrane cholesterol. Exposure to neutral or alkaline pH conditions was likely to cause conformational and functional changes in various domains of the LLO molecule.

It is known that the pH of an *L. monocytogenes*-containing phagosome is neutral at first, and then gradually acidifies

(Beauregard *et al.*, 1997). Therefore, it has been considered that LLO is activated in an acidified environment in phagosomes but is not activated at neutral or weakly alkaline pH in the cytosol of host macrophages. Moreover, it is reported that *L. monocytogenes* expressing pH-insensitive CDCs such as PFO or LLO L461T exhibits a strong cytotoxicity against host cells, resulting in a reduction of intracellular replication and cell-to-cell spreading compared with wild-type *L. monocytogenes* (Jones & Portnoy, 1994; Glomski *et al.*, 2002, 2003). These findings suggest that pH- and temperature-sensitive cytolytic activity in host cells is required to optimize the intracellular parasitism and the expression of adequate virulence. The precise molecular mechanism that regulates the pH dependency of LLO needs further analysis of the detailed conformational change.

In this comparative study using various CDC proteins, it was clearly shown that the pH sensitivity is characteristic of all the *Listeria*-derived CDCs, but is not present in allied CDCs from other Gram-positive bacteria. The pH-dependent loss of the cytolytic activity in *Listeria*-derived CDCs appeared to involve an irreversible conformational change in domain 4 of LLO, affecting the binding activity to cholesterol molecules in target cell membranes of this pore-forming cytolysin. Our results have clearly shown that the pH-dependent expression of cytolytic activity of LLO of *L. monocytogenes* is due to a pH-dependent loss of function, not due to pH-dependent activation as has been previously suggested.

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