

Dephosphorylation reduces passage of ovalbumin antigen through intestinal epithelial Caco-2 cell monolayers

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**Takeshi Matsubara^{1,*}, Yuka Akiyama¹,
Kenzi Oshima¹, Tetsuya Okajima²,
Daita Nadano¹ and Tsukasa Matsuda^{1,†}**

¹Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-8601; and ²Department of Biochemistry II, Graduate School of Medicine, Nagoya University, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan

[†]Tsukasa Matsuda, Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan. Tel: +81-52-789-4129, Fax: +81-52-789-4128, email: tmatsuda@agr.nagoya-u.ac.jp

*Present address: Takeshi Matsubara, Nutritional Science Institute, Morinaga Milk Industry, Co., Ltd, 5-1-83 Higashihara, Zama 252-8583, Japan.

Allergenic potential of food proteins is associated with stability to gastric and pancreatic digestive enzymes. However, much attention has not been focused on intracellular digestion of protein antigens during the passage through intestinal epithelia. We report here the degradation and survival of a bis-phosphorylated protein, ovalbumin (OVA), in the course of passage through Caco-2 cell monolayers cultured on porous membrane. SDS–PAGE in combination with phosphoprotein staining showed that OVA, which had passed through the cell layers, was almost intact in its polypeptide chain but partly dephosphorylated. By contrast, quantitative analysis using ELISA indicated that complete dephosphorylation in advance by an alkaline phosphatase markedly reduced the OVA passage. The reduced passage was restored in the presence of cathepsin inhibitors, leupeptin and pepstatin-A. Moreover, the complete dephosphorylation increased susceptibility of OVA to *in vitro* digestion with cathepsin B, which cleaved near an OVA phosphorylation site, Ser345. The susceptibility of OVA to lysosomal proteases may affect its passage through the intestinal epithelia, leading to determination of allergic sensitization and elicitation in egg allergy.

Keywords: cathepsin/egg allergy/gastrointestinal absorption/intracellular digestion.

Abbreviations: CIAP, calf intestinal alkaline phosphatase; LY, Lucifer yellow; OVA, ovalbumin; POD, peroxidase; TEER, transepithelial electrical resistance.

significant amount of proteins, which had escaped from hydrolysis by gastric and pancreatic proteases, reach mucosal epithelia of digestive tracts and pass through the epithelium mainly by the epithelial cell transcytosis, *i.e.* apical pinocytosis/endocytosis and basolateral exocytosis (2). Among such proteins absorbed through intestinal epithelium, food proteins are generally recognized as innocuous antigens by the underlying mucosal immune system, leading normally to induction of immunological unresponsiveness (tolerance) and secretory IgA response, but occasionally to allergic sensitization and symptom provocation (3).

Amounts of intact proteins detected in blood and lymph after oral administration depend on the dose of proteins (4, 5). Inhibitors to pancreatic proteases suppress luminal degradation of the orally administered proteins and peptides, resulting in the increase in intestinal absorption of the undigested proteins (6, 7). On the other hand, few studies have been reported so far on the intracellular proteolysis affecting the transcytotic passage of protein antigens through intestinal epithelia. In culture systems *in vitro* using human intestinal epithelial cell lines and mammalian intestinal biopsies, after cellular uptake of radiolabeled proteins, about 10% of the internalized proteins were escaped from intracellular digestion and conveyed as almost intact forms to the basolateral side of the epithelia, whereas remaining 90% were estimated to be degraded intracellularly (2, 8–10).

Ovalbumin (OVA) belonging to the family of serine-protease inhibitors (serpins) (11) is a bis-phosphorylated protein with a molecular mass of 45 kDa (12) and one of the major food allergen (13) in egg white. In our recent study using a mouse model, after gastrointestinal administration, almost intact OVA but not its fragments were detected in peripheral blood, despite that a considerable amount of OVA antigenic fragments with various molecular masses were present in gastrointestinal lumen (5).

In this study, partial dephosphorylation was detected in two OVA antigen preparations, which had passed through mouse intestinal epithelia and human intestinal Caco-2 cell monolayers as *in vivo* (5) and *in vitro* (4, 14) models, respectively. Then, the effect of complete dephosphorylation on the OVA antigen passage was investigated from the viewpoints of intracellular digestion and survival. Moreover, OVA susceptibility to lysosomal proteases and a possible target site of the protease were investigated. Relationship between the phosphorylation site and the target region of initial proteolysis was discussed in terms of intracellular digestion and survival of OVA antigens taken up by intestinal epithelial Caco-2 cells.

Gastrointestinal absorption of intact and biological active proteins is known as a normal physiological event in not only neonatal but also adult animals including humans (reviewed in (1)). A small but

Materials and Methods

Materials

OVA (Grade V), pepstatin A, bovine cathepsins B and D, porcine pepsin, bovine chymotrypsin, subtilisin Carlsberg and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma (St Louis, MO, USA). IgG fractions of rabbit anti-OVA both non-labelled and biotinylated were obtained from Rockland Immunochemicals (Gilbertsville, PA, USA). Horseradish-peroxidase (POD)-conjugated streptavidin and calf intestinal alkaline phosphatase (CIAP) were purchased from GE Healthcare and Invitrogen (Carlsbad, CA, USA), respectively. The monoclonal antibody, 1B2/5E5, was provided by Dr Mizumachi (National Institute of Livestock and Glassland Science, Tsukuba).

Animal experiments

Mice (6- to 7-week-old female ddY) were purchased from Japan SLC (Hamamatsu, Japan) and maintained as described (5), according to the Nagoya University guidelines for animal study. Research plan of this study was approved by the Committee on Animal Experiments of Graduate School of Bioagricultural Sciences, Nagoya University. Oral administration of OVA (50 mg per mouse), collection of blood at peak time (20 min after gavage) and immunoprecipitation of OVA from blood were done as described previously (5).

ELISA and immunoblotting

The two-site ELISA, SDS-PAGE, 2D PAGE and immunoblotting were described previously (5). Briefly, ELISA plates were coated with rabbit anti-OVA IgG fraction, incubated with sample and standard OVA solutions and then with biotinylated rabbit anti-OVA IgG. The plates were finally incubated with POD-conjugated streptavidin. IEF using ReadyStrip™ (linear pH 3–6 IPG Strips, Bio-Rad) was performed using the PROTEAN IEF Cell (Bio-Rad) according to the manufacturer's instruction.

Cell culture

Caco-2 cells from the American Type Culture Collection (Rockville, MD, USA) were maintained in DMEM supplemented with 10% heat inactivated fetal calf serum, 1% non-essential amino acids solution (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin and grown under humidified 5% CO₂ and 95% air at 37°C. For all studies, Caco-2 cells were seeded at 1.0×10^5 cells/cm² on to Transwell inserts (24 well) with a pore diameter of 0.4 µm (Corning) pre-coated with Type-I collagen (Nitta Gelatin). Transepithelial electrical resistance (TEER) was monitored using a Millicell electrical resistance system (Millipore). The monolayers of the cells with stable TEER higher than 300 Ω cm² were used for passage experiments on Days 18–21 of culture.

Assay for OVA passage through Caco-2 cell monolayers

The cell monolayers were rinsed with Hank's balanced salt solution (HBSS) containing Ca²⁺ and Mg²⁺ supplemented with 10 mM HEPES (pH 7.4). For the equilibration, the cells were incubated with HBSS for 50 min in CO₂ incubator at 37°C. The apical side fluid (100 µl) was replaced with pre-warmed OVA solution (2 mg/ml) containing a paracellular passage marker, Lucifer Yellow (LY), (400 µg/ml) in HBSS. After incubation for 3 h, the basolateral solutions were collected. For immunoblotting, proteins in the collected solution were recovered by trichloroacetic acid (TCA) precipitation. The cells cultured on the inserts were washed four times with ice-cold PBS and lysed with lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM phenylmethanesulfonyl fluoride, 40 µM leupeptin and 15 µM pepstatin-A. After centrifugation, supernatants were collected as cell lysates. Concentrations of LY in basolateral solutions were measured using FluoroScan II (LabSystems) (excitation, 485 nm; emission, 538 nm).

To determine the effect of protease inhibitors on the OVA passage, the cell monolayer passage experiments were performed in the presence of a cysteine protease inhibitor, leupeptin at 0.5 or 2.5 mM, or an aspartic protease inhibitor, pepstatin-A, at 5 or 25 µM.

Enzymatic dephosphorylation and phosphoprotein staining

OVA (40 mg) with or without CIAP (0.2 mg) were dissolved in 0.5 ml of 40 mM glycine/NaOH buffer (pH 9.5) containing 4 mM MgCl₂ and incubated for 18 h at 37°C. The reaction mixture was adjusted to pH 7.4 and dialyzed against HBSS. OVA preparations separated in SDS-PAGE gel were stained with Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen) according to manufacturer's instruction.

Protease treatment

OVA preparations treated with and without CIAP were digested with cathepsin B or D as described (15) with some modification of final concentrations of OVA and enzymes, *i.e.* OVA (0.4 mg/ml for cathepsin B digestion and 2.5 mg/ml for cathepsin D digestion), cathepsin B (0.4 U/ml) and cathepsin D (50 µg/ml). Digestive reactions were also performed in the presence of leupeptin (50 µM) and pepstatin-A (50 µM) as inhibitors for cathepsins B and D, respectively.

For pepsin digestion, OVA (0.4 mg/ml) in diluted HCl (pH 2.0) was incubated with pepsin (20 µg/ml) at 37°C for ~120 min. For chymotrypsin digestion, OVA (1 mg/ml) dissolved in 100 mM Tris-HCl (pH 7.5), 20 mM CaCl₂ and 0.05% Triton X-100 were incubated with chymotrypsin (2 and 10 µg/ml for low and high ratios, respectively) and incubated at 37°C for ~120 min. For subtilisin digestion, OVA (1 mg/ml) in PBS was incubated with subtilisin Carlsberg (5 µg/ml) at 37°C for 120 min.

At each time point, 20 µl of reaction solution was sampled, mixed with 5× Laemmli SDS-PAGE sample buffer and boiled for 10 min.

Statistical analysis

Data were expressed as the mean $\pm$ SD. Differences between two groups were analysed by unpaired *t*-test.

Results

OVA antigen was dephosphorylated partially during passage through mouse intestinal epithelia and the Caco-2 cell monolayers

In our previous study (5), the OVA antigen, recovered from mouse peripheral blood, showed its isoelectric point shifted slightly to a basic side, suggesting loss of negative charge. In this study, the OVA antigen recovered from the blood samples was found to be positive to the phosphoprotein staining, but the staining intensity was weaker than those of native OVA (Fig. 1A). To clarify whether OVA antigen was dephosphorylated within intestinal epithelial cells, the phosphorylation of OVA antigen, which had passed through the Caco-2 cell monolayer, was examined (Fig. 1B). The relative phosphorylation level decreased to about one-half after the passage through the cell monolayers. These observations made us presume that some OVA antigens are dephosphorylated prior to intracellular proteolytic degradation during passage through intestinal epithelia and others escaped from such enzymatic degradation are conveyed in an intact form to the basolateral side.

A 40-kDa as well as the 45-kDa OVA fragments were detected in mouse blood, whereas only the 45-kDa OVA in the basolateral culture medium of Caco-2 cells. The 40-kDa fragment may be generated by digestion with intestinal elastase, because OVA was reported to be a good substrate of pancreatic elastase and cleaved specifically at the peptide bond between Ala352 and Ser353, resulting in the production of a 40-kDa OVA fragment (16).

In vitro dephosphorylation reduced negative charge but not antigenic reactivity of OVA

In the native PAGE, the dephosphorylated OVA migrated more slowly than native one and gave a few bands. In 2D PAGE, both of native and dephosphorylated OVAs exhibited three major horizontally lined spots with different pIs (spots 2–4 and 4–6 for native and dephosphorylated OVA antigens, respectively), while the blood OVA (5) also gave three major spots 3–5 (Fig. 1C). The complete dephosphorylation, as revealed by the negative phosphoprotein staining (Fig. 1A and B), caused horizontal shift of the OVA spots by two spot units to the basic side, while the blood OVA exhibited the shift of one spot unit. To evaluate any changes caused by dephosphorylation in antigenic reactivity of OVA, binding activity to specific antibodies was examined using two-site ELISA (Fig. 1D). Two regression lines (ELISA value versus protein concentration) of native and dephosphorylated OVAs were almost the same.

Complete dephosphorylation markedly reduced the OVA passage through the cell monolayers

After the dephosphorylation, the OVA passage through the cell layers markedly decreased by

approximately 70% ($P < 0.01$), while the OVA retained inside the cell layers also decreased moderately (Fig. 2A). Such significant reduction in the OVA passage was reproducible in the five independent experiments using four to six Caco-2 cell monolayers each, though there were some variations in the absolute amount of OVA among the independent experiments using a freshly thawed batch of Caco-2 cells (data not shown). LY as a control molecule for cellular passages showed no difference between the cell monolayers incubated with native and dephosphorylated OVA antigens (Fig. 2A).

OVA antigens in the basolateral culture medium were concentrated by TCA precipitation and analysed by immunoblotting using a polyclonal antibody with reactivity to diverse OVA proteolytic fragments (~ 10 – 40 kDa) (5). Both native and dephosphorylated OVAs recovered from the medium showed the molecular size of ~ 45 kDa, which was almost identical to their corresponding standard OVAs, and no other bands corresponding to degradation products were detected (Fig. 2B, left). Similarly, the OVA antigens in the cell lysates were almost intact but only a small amount of degraded fragments were detected (Fig. 2B, right).

Inhibitors to lysosomal proteases restored the OVA passage through the cell monolayer

The dephosphorylation-induced remarkable reduction of OVA passage suggested that dephosphorylation affected susceptibility of the OVA polypeptide to lysosomal proteases involved in intracellular degradation. Indeed, leupeptin (used as an inhibitor of cysteine proteases such as cathepsins B and L) increased both the passage of native and dephosphorylated OVA antigens in a concentration-dependent manner. The passage of dephosphorylated OVA antigen was restored to a level comparable to native OVA in the presence of 2.5 mM leupeptin (Fig. 3A). Pepstatin A (an inhibitor of aspartyl proteases such as cathepsins D and E) also increased the passage of both of the native and dephosphorylated OVA antigens (Fig. 3B). However, the pepstatin A-dependent increase in the dephosphorylated OVA passage was statistically significant but much smaller than the leupeptin-dependent increase and, therefore, it might not be relevant biologically.

Complete dephosphorylation increased susceptibility of OVA to cathepsin B

Minor differences in the proteolysis between the two OVA antigens were observed in the cathepsin-B digest (Fig. 4A, upper panels). The shift to 40-kDa-truncated OVA and production of 35- and 26-kDa fragments were induced relatively faster in the digest of dephosphorylated OVA. Such effects of dephosphorylation on the susceptibility to cathepsin B were observed more notably in the co-presence of cathepsin D, though the dephosphorylation did not affect the OVA digestibility to cathepsin D itself. In the digest of dephosphorylated OVA, a 40-kDa fragment was produced more rapidly and after 5 h digestion intact OVA antigen disappeared. The shift from intact OVA to the 40-kDa fragment by cathepsin B was suppressed not only by leupeptin but also by pepstatin (see the

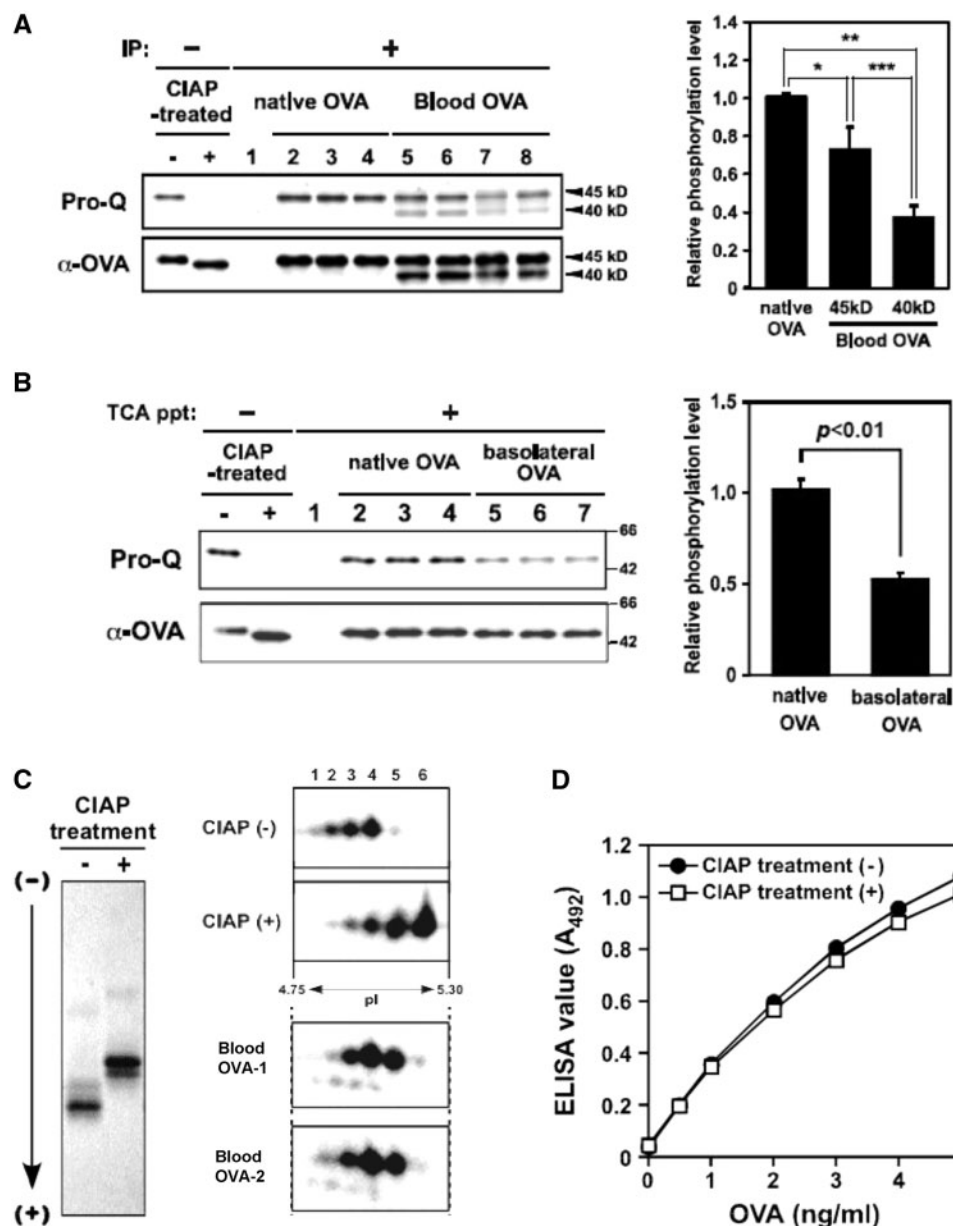


Fig. 1 *In vivo* and *in vitro* dephosphorylation of OVA antigen. (A) Peripheral blood was collected from four mice (lanes 5–8) after intragastric administration of OVA and serum samples were prepared. A certain volume (~0.5 ml) of the serum samples containing OVA (90 ng as estimated by ELISA) was immunoprecipitated followed by SDS–PAGE and phosphoprotein staining. Normal mouse sera with (lanes 2–4) and without (lane 1) addition of native OVA (90 ng) were analysed as controls for immunoprecipitation and phosphoprotein staining. Native and dephosphorylated (CIAP-treated) OVAs were included as controls for phosphoprotein staining. Staining intensity of each band was analysed by densitometric quantification. The phosphorylation level of each stained band was represented as a ratio of the phosphoprotein staining to immunoblotting of each band. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.01$. (B) OVA (90 ng as estimated by ELISA) in the basolateral solution of Caco-2 cell monolayers (lanes 5–7) was collected by TCA precipitation, followed by SDS–PAGE and phosphoprotein staining or immunoblotting. TCA precipitates of HBSS with (lanes 2–4) and without (lane 1) addition of native OVA (90 ng), in addition to native and dephosphorylated OVAs were also analysed as controls. The data analysis and representation were the same as in A. (C) Native (CIAP-) and dephosphorylated (CIAP+) OVA were analysed by native PAGE (left). In addition to these two OVA samples, two samples of blood OVA were analysed by 2D PAGE followed by immunoblotting (right). In the 2D PAGE, spots of OVA were numbered as 1–6 in order from acidic to basic sides. (D) Native (closed circles) and dephosphorylated (open squares) OVA were analysed by ELISA. Means of ELISA values in triplicate were plotted against biochemically determined protein concentration (~5 ng/ml).

right most lane and the third lane from the right in the upper right two panels). No significant difference between the native and dephosphorylated OVA antigens was observed in the peptic and chymotryptic digests (Fig. 4A, lower panels). A 22-kDa fragment was produced as a stable degradation intermediate in the chymotrypsin digest.

Cathepsin B cleavage sites on production of the 40-kDa-truncated OVA were estimated using a monoclonal antibody (1B2/5E5) specific for Ile335–Ser345 of OVA (17) and the OVA subtilisin digest known as plakalbumin (a nicked form of OVA) (18) (Fig. 4B). The OVA sequence corresponding to a limited-proteolysis target region conserved among the

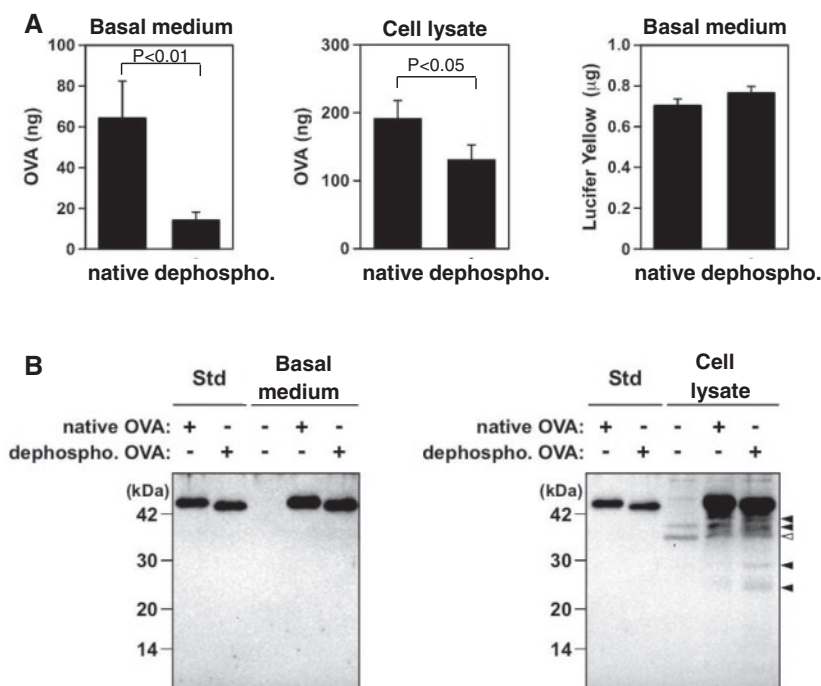


Fig. 2 Effect of dephosphorylation on OVA passage through the Caco-2 cell monolayers. Concentrations of native and dephosphorylated OVA in basolateral solutions (A, left) and cell lysates (A, middle) were determined by ELISA and shown as weight (ng) per well (600 and 100 μ l of the basolateral solution and the cell lysate, respectively). LY in basolateral solutions was determined for comparison (A, right). Each value represents the mean $\pm$ SD of six wells. OVA antigens in the basolateral solutions (basolateral side) were recovered by TCA precipitation. The precipitates (B, left) and the cell lysates (intracellular) (B, right) containing 4 and 15 ng of OVA antigen as determined by ELISA, respectively, were subjected to SDS-PAGE followed by immunoblotting. As controls, the basolateral solution and the cell lysate of Caco-2 cell monolayer cultured without OVA were used. Native and dephosphorylated OVA (4 ng) were subjected directly to SDS-PAGE as standards (Std). Closed and open arrowheads represent OVA fragments and a cell-derived non-specific band, respectively.

serpin-family proteins (19) is shown along with the 1B2/5E5 epitope, the phosphorylation site and the subtilisin cleavage sites (18) (Fig. 4C). A 3D image of OVA showed that the limited proteolysis target region including the phosphorylation site, S345, and a speculated subtilisin cleavage site, D351, formed a polypeptide loop protruding from the OVA globular molecule (Fig. 4D, left). In the SDS-PAGE/immunoblotting, the OVA cathepsin B digest produced an $\sim$ 40-kDa fragment with a molecular mass slightly lower than that of the subtilisin digest and was negative to the 1B2/5E5 immunostaining. By contrast, the OVA subtilisin digest retaining the epitope was positive to the 1B2/5E5.

Discussion

Complete dephosphorylation of OVA markedly reduced the amount of OVA released into the basolateral culture medium of the Caco-2 cell monolayers. Increase in the OVA passage in the presence of protease inhibitors in addition to decrease in the OVA resistance to cathepsin B by complete dephosphorylation suggest that the dephosphorylation affects OVA susceptibility to lysosomal proteases and reduces the proportion of intact OVA after intracellular digestion during the passage through cell monolayers. In addition to the previously reported result that $\sim$ 80% of OVA molecules are bis-phosphorylated (19), almost no

changes after the complete dephosphorylation in the number (three major spots) of OVA pI isoforms (Fig. 1C) indicated that such an isoelectric heterogeneity of native OVA is not generated by differential phosphorylation. Moreover, the horizontal shift of the three OVA spots by two spot units to the basic side without marked changes in the heterogeneity might indicate that the pI shift is caused by complete removal of the two phosphoryl groups. Similarly, the pI shift of blood OVA by one spot unit might indicate that OVA taken up across intestinal epithelia still retains one phosphoryl group, *e.g.* at Ser345 as discussed later.

The presence of partially dephosphorylated OVA in the mouse blood and the cell culture medium may indicate that complete or site-specific dephosphorylation of bis-phosphorylated protein, OVA, is crucial for the proteolysis susceptibility. Although there may be some other explanation, such as effect of dephosphorylation on the cellular uptake through electrostatic or enzyme (alkaline phosphatase)-substrate interactions of OVA with the intestinal epithelial cell surface, it could be concluded that the dephosphorylation affected, at least in part, the intracellular digestion of OVA antigen and subsequent release of undigested OVA to the basolateral side of epithelial cells. The dose-dependent increase by protease inhibitors in OVA passage across the Caco-2 cell monolayers suggested that the OVA passage was linked to the susceptibility to lysosomal

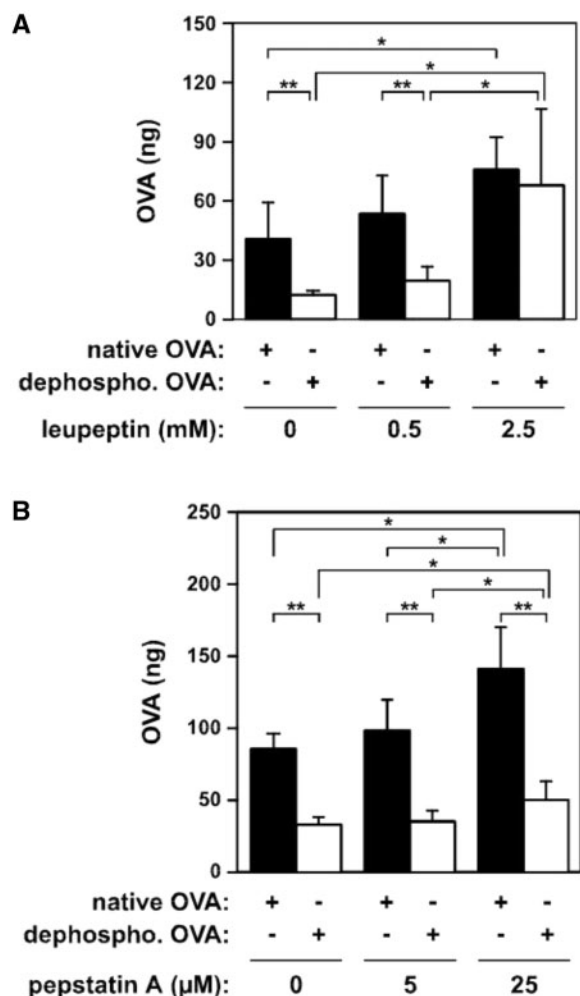


Fig. 3 Increase in OVA passage through the Caco-2 cell monolayers in the presence of protease inhibitors. Passage of native (closed bars) and dephosphorylated (open bars) OVA through the cell monolayers was analysed in the absence and presence of protease inhibitors, 0.5 and 2.5 mM leupeptin (A); 5 and 25 μM pepstatin A (B). The OVA concentration in the basolateral solutions was determined by ELISA and shown as the protein weight (ng) per well as in Fig. 3. Each value represents the mean $\pm$ SD ($n=4-6$). * $P<0.05$, ** $P<0.01$.

proteases, such as cathepsins B and D, which in fact hydrolyzed OVA *in vitro*. According to a gene expression database (RefExa, LSBM) and literatures (20, 21), cathepsins B and D are estimated to be relatively abundant in Caco-2 cells, while some other proteases such as cathepsins E and L are also expressed at relatively lower levels (about one-seventh or lower) in Caco-2 cells. Therefore, contribution of the minor proteases other than cathepsins B and D could not be ruled out.

By the cathepsin B treatment, the 40-kDa-truncated OVA was produced in the first stage of proteolytic degradation and existed as a stable intermediate fragment. These results suggest that dephosphorylation does not induce global conformational changes but affects locally near the cathepsin B cleavage sites of OVA molecules. This cathepsin B-induced truncation of OVA agrees well with the previously reported results that OVA was digested mainly into a 40-kDa fragment by mouse lysosomal protease preparation, and this fragmentation had markedly been inhibited

by a cathepsin B-specific inhibitor, CA074 (20). The cathepsin B cleavage site of OVA antigen is unknown at this moment. However, as noted below, it is speculated that the cathepsin B cleavage site exists near by a phosphorylation site, Ser345, of the two (22). The subtilisin cleavage sites for the production of a nicked form of OVA (Plakalbumin) were previously reported to locate within the sequence Ala346 to Val354 (18, 23) in the limited proteolysis target region conserved among the serpin-family proteins (19). In this study, the truncated OVA of the subtilisin digest was shown to retain the 1B2/5E5 monoclonal antibody epitope (Ile335–Ser345) at its C-terminus, whereas, the 1B2/5E5 epitope no longer existed in another truncated OVA with a slightly lower molecular mass in the cathepsin B digest. Very small difference in the size between the two truncated OVA antigens with and without the epitope indicates that cathepsin B cleaves OVA at a site nearby or within the epitope, which contains the Ser345. Interestingly, the OVA crystal structure indicated that the phosphorylated S345 electrostatically interacts with E196 via an unidentified metal ion, possibly via Ca^{2+} (Fig. 4D, right). This phosphoryl group-dependent intra-polypeptide bridge may locally stabilize this region and reduced flexibility of the loop structure. Thus, dephosphorylation of phosphorylated Ser345 of OVA may affect the susceptibility of this speculated cleavage site to cathepsin B. The reason remains uncertain why the co-existence of cathepsin D enhanced the cathepsin B digestion of OVA antigen and not only leupeptin but also pepstatin suppressed production of the 40-kDa cathepsin B-hydrolytic fragment. A speculation is that the cathepsin B-hydrolytic fragments of OVA exhibit feedback-inhibitory effect on cathepsin B activity and such fragments are further degraded by cathepsin D, resulting in the loss of the feedback inhibition.

A 40-kDa fragment was produced as a stable intermediate by cathepsin B digestion *in vitro* (Fig. 4A) but almost not within the cells (Fig. 2B), indicating that *in vivo* (within the cells) this 40-kDa intermediate was further degraded immediately to peptides and amino acids by the other lysosomal proteases. We speculate that the slight difference in the susceptibility to cathepsin B digestion largely affects the rate of such OVA degradation to peptides and amino acids, in other words, OVA survival from the intracellular digestion. Such difference in the degradation rate might result in the considerable difference in transcellular passage between native and dephosphorylated OVAs.

Quantitative data on transepithelial passage of intact proteins obtained in this study using Caco-2 cells as well as earlier studies using intestine organ culture (2, 8, 9) suggested that proteins taken up by enterocytes from intestinal lumen through pinocytosis or in some cases endocytosis are mostly degraded in lysosome/endosome vesicles but a part of them escape the proteolytic degradation followed by the basolateral release as intact proteins or larger fragments. The results that the intact OVA antigens and only a small amount of degradation intermediates, which were detected in the *in vitro* digests (Fig. 4), were detected either in the basolateral medium or in the cell lysate

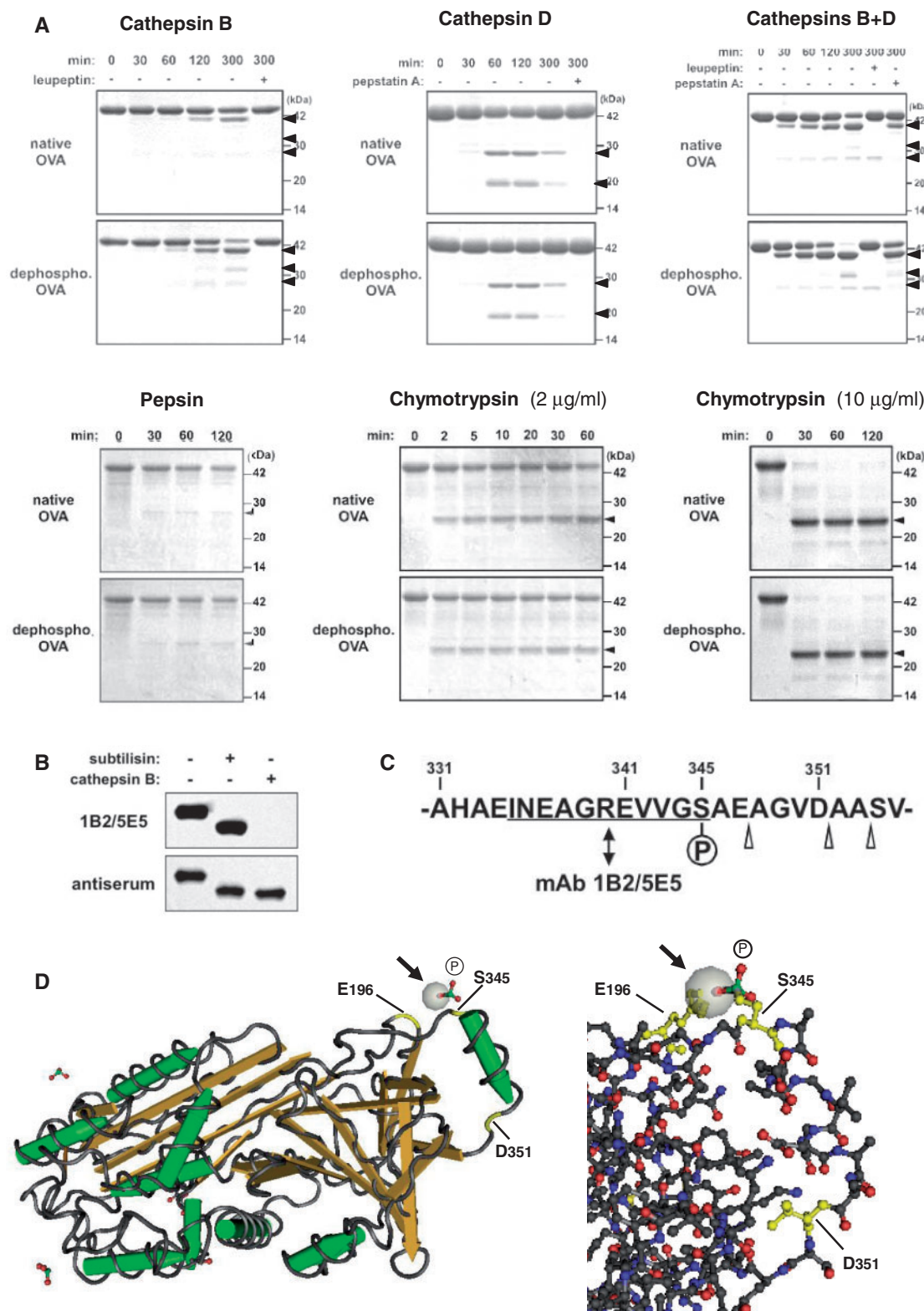


Fig. 4 Protease susceptibility of native and dephosphorylated OVA antigens. (A) Native and dephosphorylated OVA incubated as described in 'Materials and Methods' section with the protease shown above each panel were analysed by SDS-PAGE followed by CBB staining. Appropriate amounts (10–30 µg of OVA protein per lane) of the digests were applied to detect minor stained bands of OVA fragments. The OVA was also incubated with the cathepsins in the presence of 50 µM of leupeptin or pepstatin A to confirm contribution of cathepsins to the OVA degradation. Arrowheads indicate proteolytic fragments of OVA. (B) OVA was digested with subtilisin or the cathepsin B and subjected to SDS-PAGE followed by immunoblotting using the anti-OVA monoclonal antibody, 1B2/5E5, and anti-OVA antiserum. (C) A schematic representation of the OVA sequence near the phosphorylation site, Ser345, is shown together with the 1B2/5E5 epitope (underlined) and the subtilisin cleavage sites in the limited-proteolysis target region (open arrowheads). (D) The crystal structure of OVA (PDB ID: 1OVA) and its limited proteolysis target region are drawn by Cn3D (NCBI) as worms and ball-and-stick models, respectively. The phosphorylated S345 and the speculative subtilisin cleavage site, D351, are highlighted. An arrow indicates the unidentified metal ion, which is chelated between the E196 carboxyl group and the phospho-S345 phosphoryl group.

regardless the dephosphorylation suggest that once the proteolytic cleavage starts, subsequent proteolysis proceeds immediately, resulting in an almost all-or-none manner degradation of OVA antigens to peptides (smaller than ~10 kDa) and amino acids. Thus, dephosphorylation might affect the rate of degradation from intact OVA to small peptides and amino acids, but not the stability of unidentified degradation intermediates, which may be too labile against proteolysis to be detected by immunoblotting (Fig. 2B). Such intact proteins released into intestinal lamina propria would induce oral tolerance, allergic sensitization or provocation of pathological symptoms occasionally. Therefore, susceptibility or digestibility of a protein antigen to lysosomal/endosomal proteases of enterocytes, in addition to that to gastric and pancreatic proteases (24), would be an important factor for the proteins to be an oral antigen with high allergenic potency.

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Conflict of interest

None declared.

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