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Role of Furin in Delivery of a CTL Epitope of an Anthrax Toxin-Fusion Protein

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***Abstract*: Anthrax toxin lethal factor (LF) in combination with anthrax toxin protective antigen (PA) was endocytosed and translocated to the cytosol of mammalian cells. Residues 1–255 of anthrax toxin lethal factor (LFn) was fused to a cytotoxic T lymphocyte (CTL) epitope of an influenza virus. For processing the toxins, PA must be cleaved into a 63-kDa fragment (PA63) by furin, which is a subtilisin-like processing endoprotease expressed by many eukaryotic cells. To test the ability of cells treated with the LFn fusion protein plus PA to deliver the epitope, CTL assay was performed. Two types of cell lines were identified, one was able to deliver CTL epitope while the other failed to efficiently deliver the epitope. To further elucidate the differences between these cells, the role of furin in these cells was examined. Disruption of the furin gene reduced its ability to deliver the CTL epitope. Furin expression in cells capable of efficiently delivering CTL epitope was quantitatively higher than in cells unable to deliver the epitope. The results suggest that furin plays a critical role in delivery of the CTL epitope of LFn fusion protein.**

***Key words*: Furin, Anthrax toxin, CTL**

To deliver a cytotoxic T lymphocyte (CTL) epitope to CTL, bacterial toxins, such as anthrax toxin (2, 9) and pertussis toxin (7) have been employed as delivery vehicles in the form of fusion proteins. Anthrax toxin (20, 23) is composed of three proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF). PA binds to the surface of host cells, where it is cleaved by furin (16) into its active form of PA63, a carboxyl-terminal 63-kDa fragment. LF or EF then binds to PA63, and the protein complex is internalized by receptor-mediated endocytosis (20). After endosomal acidification, PA directs the translocation of either LF or EF into the cytoplasm of host cells, where EF expresses its adenylate cyclase activity, and LF expresses a yet undefined activity inducing the overproduction of cytokines in macrophage target cells (14).

Furin is a subtilisin-like, Ca^{2+}-dependent, processing endoprotease expressed at low levels by many eukaryotic cells (4, 26) and is localized primarily to the Golgi membrane (6, 22). Furin cleaves precursor proteins such as pro-von Willebrand factor and complement pro-C3 (29, 30) as well as bacterial toxins, including anthrax toxin PA, *Pseudomonas* exotoxin A (PE) and diphtheria toxin (DT), which must be proteolytically processed for activity toward mammalian cells (11, 13). Thus, furin plays an important role in the processing of various proproteins.

Leppla et al (1) reported that residues 1–254 of LF are capable of cellular uptake of fused proteins. Subsequently, several groups reported that the amino-terminal 254 or 255 residues free of carboxy-terminal toxic domain of LF were fused to specific CTL epitopes (2, 3, 8, 9) and the fusion protein in combination with PA could deliver the epitope to CTL both *in vitro* as well as *in vivo*.

In the present study, residues 1–255 of anthrax toxin lethal factor (LFn) was fused to a CTL epitope (aa 518–526) of an A/PR/8 influenza virus hemagglutinin (HA) (18). We examined the ability of several cells treated with the fusion protein in combination with PA to deliver the epitope to CTL. Two types of cell lines were recognized: one was able to deliver CTL epitope, while the other could not deliver the epitope efficiently. Furin as mentioned above is thought to play an important role in processing a complex of LF and PA63. Therefore, we

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Abbreviations: Ag, antigen; CTL, cytotoxic T lymphocyte; EF, edema factor; HA, hemagglutinin; LF, lethal factor; PA, protective antigen; PBS, phosphate buffered saline; LFn, residues 1–255 of anthrax toxin lethal factor; RT-PCR, reverse transcription-polymerase chain reaction; TBS, tris-buffered saline.

examined the role of furin in delivery of the CTL epitope fused to LFn in both type of cells. Disruption of the furin gene reduced the ability of cells to deliver the CTL epitope. In addition, the expression level of furin of cells capable of efficiently delivering CTL epitope was higher than that of cells that failed to deliver the epitope.

Materials and Methods

Construction, expression, and purification of fusion protein. Expression and purification of an anthrax toxin-fusion protein was performed using pET System (Novagen, Madison, Wisc., U.S.A.). DNA fragments encoding LFn-HA518–526 (IYSTVASSL) (18) were constructed by polymerase chain reaction (PCR). LFn-HA518–526 was amplified with an upstream primer which included an *Nde*I site and sequence homologous to the 5′ end of the LF gene. The downstream primer was homologous to the sequence encoding the last six amino acids of LFn and included (downstream of the homology) a sequence encoding the HA518–526 epitope, stop codons, and a *Bam*HI site. The genomic DNA of *Bacillus anthracis*, kindly provided by Dr. Uchida (National Institute of Animal Health, Ibaraki, Japan), was used as the template. The amplified fragment was digested with *Nde*I and *Bam*HI and ligated into compatible sites within the multiple cloning region of the expression vector pET15b (Novagen). The ligation product was used to transform *Escherichia coli* JM109 (Toyobo, Osaka, Japan). For each clone, the plasmid DNA was amplified, purified, and screened for the appropriate insert by restriction analysis.

Recombinant proteins expressed in pET15b contain a His6 tag at the amino terminus of the protein. This tag allows for a one-step affinity purification of the expressed protein using an Ni^{2+}-charged column. Cultures of BL-21/pET15b (LFn-HA518–526) were grown in Luria broth containing ampicillin (50 μg/ml) to an optical density at 600 nm of 0.6 to 0.8, and protein expression was induced by the addition of 1 mM IPTG (isopropyl-[β]-D-thiogalactopyranoside) for approximately 3 hr. Cells were then pelleted and disrupted by sonication. The sonicate was centrifuged, and the supernatant was passed over an equilibrated Ni^{2+}-charged column. The bound fusion protein was removed with 0.5 M imidazole according to the instructions provided by the manufacturer (Novagen). The eluted protein was then equilibrated in 20 mM Tris-HCl, pH 7.5. After determination of the protein concentration, the sample was frozen at −20 C.

Protective antigen protein. Native PA and the mutant PA dFF defective for translocation (27) were kindly provided by Dr. Leppla (NIH, Bethesda).

CTL clone B7B7. $CD8^+$ CTL clone B7B7 used in this study was established and maintained as described previously (19). CTL clone B7B7 was stimulated weekly with 50 μg/ml mitomycin C-pretreated BALB/c spleen cells, which were pulsed with a synthetic peptide, residues 518–526 of the HA2 subunit of A/PR/8 virus HA (18) in the presence of 10% Con A-induced rat IL-2 and 5×10^{-5} M 2-ME. The synthetic peptide was used as an antigen (Ag) in all experiments described in this study.

CTL assay. $P388D_1$ cells ($H\text{-}2^d$), P815 cells ($H\text{-}2^d$) and furin-deficient $P388D_1$ cells as described below were used as target cells. The target cells were preincubated with LFn-HA518–526 (50 ng/ml)+PA (100 ng/ml), LFn-HA518–526+mutant PA (100 ng/ml), PA, mutant PA, LFn-HA518–526 or HA peptide (1 μg/ml) for 12 hr. In one experiment, 100 μM chloroquine (Sigma, St. Louis, Mo., U.S.A.) was added to the preincubation of target cells with LFn-HA518–526+PA and HA peptide. The pretreated target cells were incubated with 250 μCi $Na_2{}^{51}CrO_4$ (ICN, Irvine, Calif., U.S.A.) at 37 C for 2 hr. After triplicate washing, target cells were further incubated at 37 C for 1 hr. After double washing, target cells (1×10^4) were placed into a 96-well round-bottom microtiter plate with CTL clone B7B7. The cells were incubated for 6 hr at the indicated E/T ratio. At the end of the incubation period, the supernatant fluid was harvested and ^{51}Cr was measured by using a γ-counter. Specific lysis was determined as follows: percent specific lysis = [(release by CTL − spontaneous release) / (maximum release − spontaneous release)] × 100.

Furin-deficient $P388D_1$ cells. For constructing furin-gene disruption vector, plasmid pmFur (15) containing murine furin, kindly provided by Dr. Nakayama of University of Tsukuba, Japan, was digested with *Bam*HI, the 924-bp fragment removed, and ligated with neomycin-resistant gene fragment. The ligated DNA was used to transform chemically competent *E. coli* JM109. DNA extracted from the transformants was screened by restriction enzyme digestion to identify the desired recombinant plasmids. One of these plasmids was designated pDFur. This clone was amplified overnight in 500 ml of LB with ampicillin at a concentration of 100 μg/ml, and plasmid DNA was purified using Qiagen plasmid kit (QIAGEN Inc., Valencia, Calif., U.S.A.), according to the instructions provided by the manufacturer. pDFur was introduced into $P388D_1$ cells by Gene pulser (Bio-Rad, Hercules, Calif., U.S.A.). pDFur (10 μg) was incubated on ice with $P388D_1$ for 10 min prior to pulse of 200 V at 960 μF. After electroporation, cells were allowed to recover for 10 min on ice, then transferred to media and grown for 24 hr before the addition of G418 to select stable transfectants. G418-resistant cells were

used as furin-deficient P388D$_1$ cells.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of furin mRNA. Total RNA of P388D$_1$, furin-deficient P388D$_1$ and P815 cells was isolated as described previously (12). One μg of total RNA from each sample was used for reverse transcription using Takara RNA PCR kit (Takara Shuzo Co., Ohtsu, Japan). The protocols recommended by the manufacturer were strictly followed for both reverse transcription and PCR. For detection of furin in P388D$_1$ and furin-deficient P388D$_1$ cells, the sequences of specific primers were: mouse furin sense, 5′-CTGCTGATGGGTTTAATGATTGGG-3′; mouse furin antisense, 5′-TCAAAGGGCGCTCTGGTCTTTGAT-3′. In the case of P388D$_1$ and P815 cells, the sequences of specific primers were: mouse furin sense, 5′-GCTGGTATCATTGCTCTCACC-3′; mouse furin antisense, 5′-TTCAACAATGCACTTCCGC-3′. In both experiments, primers of G3PDH (Clontech Laboratories, Inc., Palo Alto, Calif., U.S.A.) were used as a control.

Flow cytometry. P815 cells and P388D$_1$ cells (1×10^6 cells) were fixed with methanol for 10 min at −20 C and washed twice with cold phosphate buffered saline (PBS). Before treatment with the antibody, cells were preincubated in 10 μg of 2.4G2 (PharMingen, San Diego, Calif., U.S.A.), which reacts with murine Fc receptors. Cells were incubated in 1 μg of rabbit anti-furin peptide Ab (IgG) (22) in a volume of 20 μl and as control 1 μg of normal rabbit IgG (Inter-Cell Technologies, Inc., Hopewell, N.J., U.S.A.) for 45 min on ice, and washed twice with cold PBS. Cells were incubated with goat FITC-conjugated anti-rabbit IgG antibody (ICN Pharmaceuticals, Inc., Aurora, Ohio, U.S.A.) at an appropriate dose for 45 min on ice. After washing the cells twice with cold PBS, furin antigen of the cells was analyzed with a CytoAce (JASCO Corp., Tokyo).

Western blot. Western blot was performed as described previously (17). Briefly, P388D$_1$ and P815 cells were washed twice with PBS and denatured by boiling in equal amounts of reducing 2×SDS sample buffer (0.1 M Tris-HCl (pH 6.8), 0.1 M DTT, 0.2%(w/v) bromophenol blue, 4%(w/v) SDS, and 20%(v/v) glycerol), and 1×10^5 cell equivalents were loaded per lane for P388D$_1$ and P815. After separation on a 10% SDS-PAGE gel, proteins were transferred to nitrocellulose membrane by electroblotting using a solid plate tank transfer apparatus (Bio-Rad, Richmond, Calif., U.S.A.). Nonspecific binding was inhibited by incubation of the membrane in 10% skim milk in tris-buffered saline (TBS) (10 mM Tris (pH 7.6) and 0.15 M NaCl) containing 0.1% Tween 20 (TBS-T) for 1 hr at room temperature. The membrane was rinsed in TBS-T and incubated with the rabbit anti-furin peptide Ab (22) (1: 1,000 dilution) in 1% skim milk in TBS-T. After 1 hr of incubation at room temperature, the membrane was incubated for 1 hr with peroxidase-conjugated rabbit anti-goat immunoglobulin (Cappel Research, Durham, N.C., U.S.A.) (1: 10,000 dilution) in 1 % skim milk in TBS-T at room temperature and developed with an enhanced chemiluminescence system (Amersham, Arlington Heights, Ill., U.S.A.).

Results

Lysis of Target Cells Treated with LFn-HA518–526 Plus PA by CTL Clone B7B7

To examine whether the CTL epitope of LFn-HA518-526 can be delivered to CTL, we performed CTL assay using two different cell lines. Target cells were preincubated with combinations of LFn-HA518–526, PA, mutant PA, and the HA-peptide for 12 hr prior to incubation with CTL clone B7B7. As shown in Fig. 1A, the level of lysis of P388D$_1$ cells treated with LFn-HA518–526 plus PA was 56% at an E/T ratio of 10, which was almost similar to that of P388D$_1$ cells treated with the HA-peptide. P388D$_1$ cells treated with LFn-HA518–526 plus mutant PA were not significantly lysed. Neither untreated P815 cells nor P815 cells treated with LFn-HA518–526, PA, or mutant PA alone were lysed. In contrast, the level of lysis of P815 cells treated with LFn-HA518–526 plus PA by CTL clone was approximately 10% (Fig. 1B), which was much lower than that of P388D$_1$ cells. P815 cells treated with LFn-HA518–526 plus mutant PA were not lysed. Neither untreated P815 cells nor P815 cells treated with LFn-HA518–526, PA, or

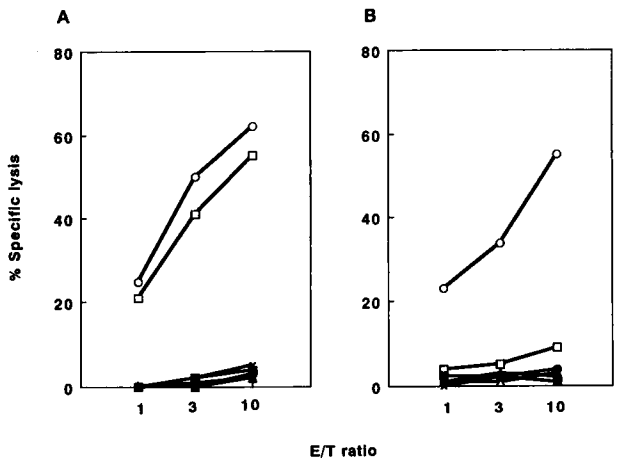

Fig. 1. Lysis of target cells treated with LFn-HA518–526 plus PA by CTL clone B7B7. P388D$_1$ cells (A) and P815 cells (B) were preincubated with LFn-HA518–526+PA (□), LFn-HA518–526+mutant PA (■), PA (△), mutant PA (▲), LFn-HA518–526 (●), HA peptide (○) or control medium (×) for 12 hr and then labeled with ^{51}Cr as described in "Materials and Methods."

mutant PA alone were lysed. These results show there exist differences in the level of lysis of target cells treated with LFn-HA518–526 in combination with PA.

Inhibitory Effect of Chloroquine on Target Lysis

To confirm that LFn-HA518–526 is intracellularly processed, the effect of chloroquine on the lysis of target cells treated with LFn-HA518–526 plus PA was examined, since the lysosomotropic agent chloroquine was reported to block the ability of PA to mediate the action of LF (10). P388D$_1$ cells were preincubated with LFn-HA518–526 plus PA or HA peptide in the presence of chloroquine (100 μM) for 12 hr and then labeled with ^{51}Cr. As shown in Fig. 2, chloroquine inhibited by approximately 60% the lysis by CTL clone B7B7 of target cells treated with LFn-HA518–526 plus PA. However, chloroquine failed to inhibit the lysis of HA-peptide treated target cells. The results indicate that CTL epitope of LFn-HA518–526 is processed through endosomal compartment and expressed at the target surface.

Disruption of Furin Inhibits Delivery of CTL Epitope

Furin is required for processing LFn-HA518–526 plus PA (4, 16). To confirm that P388D$_1$ utilizes furin for processing, P388D$_1$ cells were transfected with furin-gene disruption vector, and thereby furin-deficient P388D$_1$ cells were grown. As shown in Fig. 3A , the level of lysis of furin-deficient P388D$_1$ treated with LFn-HA518–526 plus PA was approximately half of that of normal P388D$_1$ cells. In contrast, the level of lysis of HA-peptide treated furin-deficient P388D$_1$ cells was comparable to that of normal P388D$_1$ cells (Fig. 3B). Neither untreated furin-deficient P388D$_1$ cells nor untreated P388D$_1$ cells were significantly lysed (Fig. 3C). To further confirm the disruption of furin in furin-deficient P388D$_1$ cells, RT-PCR was performed to analyze furin mRNA. Figure 4 showed that PCR product (812 bp) specific for furin was amplified in normal P388D$_1$ cells, whereas the corresponding PCR product in furin-deficient P388D$_1$ cells was not detected. The same level of PCR products (983 bp) for G3PDH was observed in both cells. These results indicate that furin of P388D$_1$ cells functions in the processing of LFn-HA518–526 plus PA.

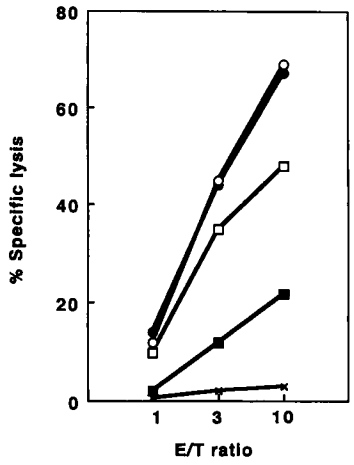

Fig. 2. Inhibitory effect of chloroquine on lysis of target cells treated with LFn-HA518–526 plus PA by CTL clone B7B7. P388D$_1$ cells were preincubated with LFn-HA518–526 plus PA (□), HA peptide (○) or control medium (×) in the presence (closed symbols) or absence (open symbols) of chloroquine (100 mM) for 12 hr and then labeled with ^{51}Cr for 2 hr.

Analysis of Furin mRNA by RT-PCR

As described above, the level of lysis of P815 treated with LFn-HA518–526 plus PA was significantly lower than that of P388D$_1$ with the same treatment. To elucidate the reason for the difference in the target lysis, we examined the expression level of furin, which can cleave PA into a 20-kDa fragment (PA20) and a 63-kDa frag-

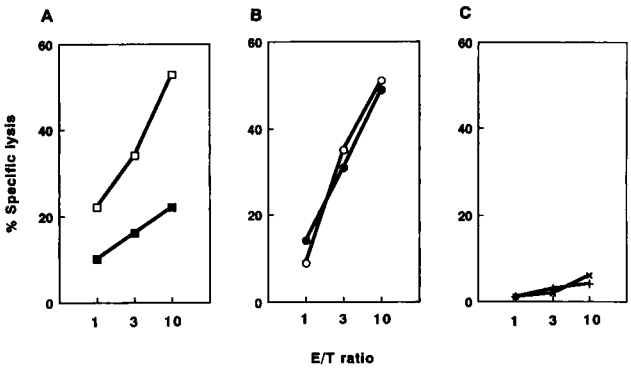

Fig. 3. Resistance of furin-deficient P388D$_1$ to CTL clone lysis. Normal P388D$_1$ (□,○,×) and furin-deficient P388D$_1$ (■,●,+) were preincubated with LFn-HA518–526 plus PA (A), HA peptide (B) or control medium (C) for 12 hr and then labeled with ^{51}Cr for 2 hr.

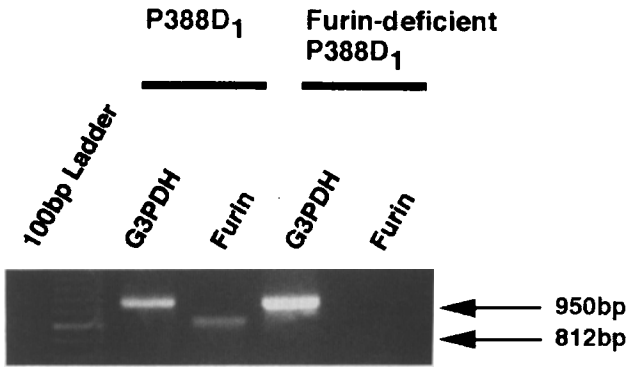

Fig. 4. RT-PCR analysis of furin mRNA in normal P388D$_1$ and furin-deficient P388D$_1$. Total RNA was extracted and performed by RT-PCR using primers specific to furin. G3PDH was used as a control.

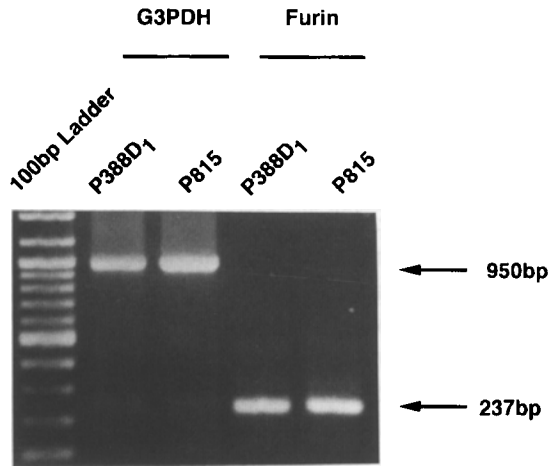

Fig. 5. Analysis of furin mRNA in P388D$_1$ and P815 cells by RT-PCR. Total RNA was extracted and performed by RT-PCR using primers specific to furin. G3PDH was used as a control.

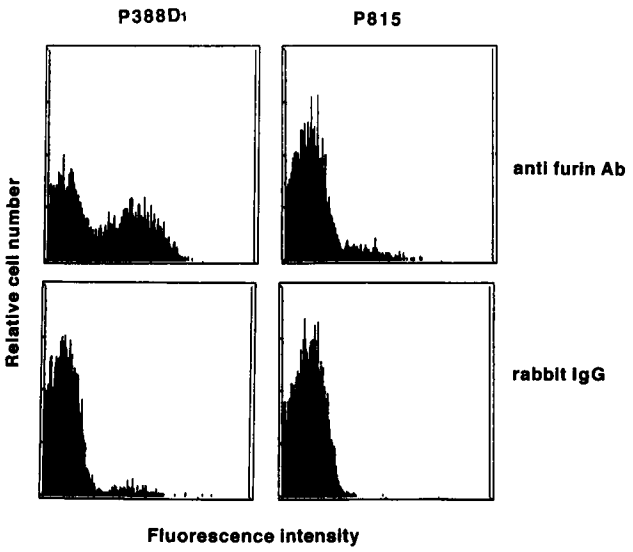

Fig. 6. Comparison of furin expression in fixed cells by flow cytometry. P388D$_1$ and P815 cells, both pretreated with methanol, were stained with rabbit anti-furin Ab or normal rabbit IgG as a control followed by a secondary FITC-labeled anti-rabbit IgG Ab, and analyzed by flow cytometry.

ment (PA63). The latter binds to LF, and the complex of PA63 and LF is subsequently processed intracellularly. Shapiro et al (26) reported the presence of differences in expression levels of furin in different cell lines. Therefore, we performed RT-PCR to analyze furin at the mRNA level in P388D$_1$ and P815 cells. As shown in Fig. 5, the PCR product (237 bp) of furin in P388D$_1$ was similar to that of P815, and the same level of PCR product (983 bp) of G3PDH as a control was found in both cells. These results show the lack of difference in the level of furin mRNA between P388D$_1$ and P815 cells.

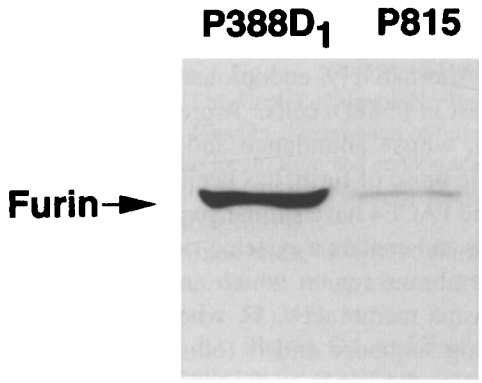

Fig. 7. Western blot analysis of furin expression in P388D$_1$ and P815 cells. Lysates from the cells were separated on a 10% SDS-PAGE gel under reducing conditions, and furin antigen was detected with anti-furin peptide Ab as described in "Materials and Methods."

Comparison of Furin Expression in P388D$_1$ and P815 Cells

Furin expression in P388D$_1$ and P815 cells was examined by flow cytometry. Initial experiments showed that most cells were nonspecifically stained. Therefore, 2.4G2, a monoclonal Ab (28), was used to prevent nonspecific binding of Ab via FcR interactions before staining with anti-furin Ab. As shown in Fig. 6, approximately 40% of P388D$_1$ cells were furin-positive, whereas hardly any furin-positive P815 cells were identified. Treatment using the same amount of rabbit IgG showed no positive cells among either kind of cell. Thus, the results of flow cytometric analysis indicate that the level of furin expression in P388D$_1$ cells is significantly higher than in P815 cells. To further confirm the level of furin expression, Western blot analysis was performed. As shown in Fig. 7, the level of furin expression in P388D$_1$ cells was severalfold higher than in P815 cells.

Discussion

In the present study, we demonstrated that furin plays a critical role in delivery of the CTL epitope of LFn-HA518–526 in combination with PA. The CTL epitope of LFn-HA518–526 was delivered to CTL when LFn-HA518–526 was in the presence of PA, whereas it was not delivered in other combinations, such as LFn-HA518–526 plus mutant PA and LFn-HA518–526 alone (Fig. 1). In addition, the processing of the CTL epitope was inhibited in the presence of chloroquine (Fig. 2), suggesting that the processing occurs via endosomes, where acidification occurs. As shown in Fig. 3, lysis of furin-deficient P388D$_1$ cells treated with LFn-HA518–526 in combination with PA was inhibited by approximately 50%. The incomplete inhibition suggests that a low

level of furin remained in the furin-deficient P388D$_1$ cells, although no furin mRNA was detected by RT-PCR. Alternatively, endoprotease(s) other than furin may exist in P388D$_1$ cells. A protease similar to furin, PACE4, whose abundance and substrate specificity resemble those of furin, has been described (11). Both furin and PACE4 have similar sequences in the catalytic domains and contain a cysteine-rich region. Furin has a transmembrane region which anchors it to the Golgi and plasma membranes (5), whereas PACE4 lacks an anchoring sequence and is found only intracellularly (24), raising the question of whether intracellular PACE4 can cleave PA, since PA is thought to be cleaved at the cell surface (16).

Furin is thought to be expressed by many eukaryotic cells (15, 25), whereas no furin is detected in certain cell lines (26). Figure 1 shows that there exist differences in the ability of two distinct cells to deliver the CTL epitope: P388D$_1$ cells can deliver the CTL epitope more efficiently than P815 cells. The results suggest that the level of furin expression may be variable in these cells. In fact, the levels of furin were similar as confirmed by RT-PCR (Fig. 5). Furthermore, in order to detect furin at the cellular level, flow cytometric analysis and Western blot analysis were performed. As shown in Fig. 6, the level of furin expression in P388D$_1$ cells was significantly higher than in P815 cells. In addition, Western blot analysis (Fig. 7) also showed that the level of furin expression in P388D$_1$ cells was severalfold higher than in P815 cells, which was consistent with the results of the flow cytometric analysis. However, the inconsistent results between RT-PCR and the latter two experiments may be due to the relative sensitivity of the methods employed for analysis. The sensitivity of RT-PCR would be much higher than that of flow cytometric analysis and Western blot analysis. Similarly, it has been reported that furin in COS cells is hardly ever detected by the method of immunofluorescent staining of fixed-permeabilized cells, although furin mRNA in the COS cells has been demonstrated (21, 26). The reasons for this discrepancy are also unclear. To our knowledge, there are no reports that have demonstrated the presence of furin at the cell surface. We also failed to detect furin at the cell surface of P388D$_1$ (data not shown). Furin is considered to be localized primarily to the Golgi membrane (6, 22). Consequently, after treatment of cells with methanol-fixation we were able to detect furin in P388D$_1$ cells (Fig. 6). Nevertheless, we cannot rule out the possibility that the active-site His-containing domain of furin (22), to which the anti-furin Ab used in this study binds, might not be an extracellular domain.

The present study was conducted using *in vitro* system, however, *in vivo* experiments, such as immunization, are also necessary to evaluate the anthrax toxin-mediated delivery of CTL epitopes as a vehicle for vaccine development. Indeed, we tried to induce Ag-specific CTL *in vivo* by immunization of mice with LFn-HA518–526 in combination with PA. However, so far in our experimental system, we have failed to detect significant CTL activity after immunization, although Ballard et al (3, 8) reported the *in vivo* induction of Ag-specific CTL after immunization of LFn fused to CTL epitopes plus PA. We assume that the failure to generate CTL *in vivo* may be due to the deficiency of furin expression in BALB/c mice. Studies aimed at analyzing the expression level of furin in these mice are currently in progress in our laboratories.

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