Regulation of cell-surface major histocompatibility complex class I expression by the endopeptidase EC3.4.24.15 (thimet oligopeptidase)

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INTRODUCTION

The Achilles heel of the CTL (cytotoxic T-lymphocyte) immune response is the dependence on cell-surface MHC I (major histocompatibility complex class I) molecules for presentation of peptides. MHC I peptide production is initiated by the processing of predominantly intracellular proteins by cytosolic proteasomes [1]. Peptides produced from the proteasome undergo trimming by aminopeptidases and must survive the assault of cytosolic peptidases, before binding to the transporter associated with antigen processing (TAP) [2–4]. TAP then transports these surviving peptides into the ER (endoplasmic reticulum), where specific peptides are deemed appropriate for MHC I presentation, on their ability to bind to MHC I [5,6]. In the ER, a complex of MHC I-associated proteins loads a peptide into the peptide-binding pocket of an MHC I molecule. The trimolecular MHC I complex, composed of the α chain, β2-microglobulin and peptide, then traffics through the secretory pathway via the Golgi complex and settles in the cell membrane, exposing the peptide for recognition by specific CTLs. It should be noted that the MHC I complex generally does not reach the cell surface without peptide loaded in its peptide-binding site [7].

A recent report demonstrated that endopeptidase EP24.15 [thimet oligopeptidase (TOP); EC 3.4.24.15], traditionally classified as a neuropeptide-processing enzyme, is the primary endopeptidase responsible for degrading MHC I peptides in cell extracts [8]. This introduced the possibility that manipulation of EP24.15 expression may induce a change in cell-surface expression of MHC I in vivo.

The major thrust of the work on EP24.15 has been on the functions of EP24.15 as a neuropeptide-processing enzyme in the neural and endocrine systems. EP24.15 is a ubiquitously expressed zinc metallopeptidase found at comparatively higher levels in the brain, pituitary and testes than in lymphoid tissues [9]. The functions of EP24.15 have far-reaching influences across organ systems. In the central nervous system, the secreted form of EP24.15 is responsible for degrading and processing neuropeptides and substrates, such as neuropeptide-Y and glutathione (GnRH) (gonadotropin-releasing hormone).

In addition to its role in the neural and endocrine systems, EP24.15 may also have an important role in the immune system. Early reports proposed that EP24.15 may act as a chaperone-like molecule, binding specific MHC I peptides in the cytosol, thereby protecting them from degradation by cytosolic peptidases [10,11]. In a contrasting report, EP24.15 was shown to efficiently degrade MHC I peptides in cell extracts [8].

The peptide substrates favoured by EP24.15 are 8–13 amino acids long, whereas peptides which bind MHC I are 8–10 amino acids long [12–15]. Substrates larger than 17 amino acids are not readily cleaved by EP24.15 [14,16], except in the case of the amyloid precursor protein [17,18]. EP24.15 favours cleavage of peptide substrates at the C-terminus of hydrophobic residues [19]. EP24.15 has been detected as cytosolic, nuclear, extracellular, and membrane-bound [20,21].

Endopeptidase EP24.15 (EC 3.4.24.15; thimet oligopeptidase), traditionally classified as a neuropeptide-processing enzyme, degrades well-known MHC I (major histocompatibility complex class I) peptides in cell extracts. In the present study, we determine the contribution of EP24.15 in vivo to the surface expression of MHC I on intact cells. CTLs (cytotoxic T-lymphocytes) recognize a vast array of peptides presented in the context of MHC I cell-surface molecules. Stable retroviral overexpression of EP24.15 induces a dramatic, long-term inhibition of surface MHC I. In contrast, overexpression of a mutant EP24.15, which is expressed, but is enzymically inactive, does not affect the surface MHC I expression level. We observed no difference in the effect of EP24.15 on the expression of different classes of MHC I. IFN-γ (interferon-γ) treatment re-established MHC I expression on these EP24.15-overexpressing cells, and also induced EP24.15 cytosolic protein expression and enzyme activity. To our knowledge, this is the first demonstration of cytokine-induced EP24.15 expression and activity. Conversely, stable retroviral silencing of endogenous EP24.15 by RNA interference induced a striking, long-term increase in surface MHC I. Subcellular fractionation and enzyme-activity experiments localized the vast majority of EP24.15 protein expression and function to the cytosol. Therefore we introduce a novel function of the cytosolic form of EP24.15. EP24.15 activity in the extracellular space is significant for neuropeptide processing, and in the present paper, we demonstrate that EP24.15 activity in the cytosol may be significant for regulation of MHC I cell-surface expression.

Key words: cytosol, endopeptidase 24.15 (EP24.15), major histocompatibility complex class I (MHC I), metallopeptidase, neuropeptide, thimet oligopeptidase (TOP).
membrane-associated and secreted forms; the functions of EP24.15 in these subcellular compartments are not fully understood. Since EP24.15 has been classically defined as a neuropeptide-processing enzyme, its activity has been attributed to the secreted form in the extracellular milieu; however, in neuroendocrine cells, generally >80% of EP24.15 is localized to the cytosol [20].

Viruses have evolved mechanisms to exploit this vulnerability of requiring MHC I surface expression for CTL activation. Many viruses, such as pox viruses, herpes viruses and HIV, encode ‘immuno-evasins’, proteins which modulate the host immune response as a protective mechanism to escape host cellular immunity [21]. Many immuno-evasins function to decrease or inhibit MHC I surface expression, in an effort to escape presentation of virally derived antigenic peptides by MHC I. Thus exogenous pathogens possess the ability to manipulate cellular MHC I surface expression by modulating endogenous factors, which in turn regulate MHC I antigen presentation and processing. Peptidases such as EP24.15, which may modulate MHC I surface expression in intact cells, could potentially serve as proteins which exert evolutionary pressure on intracellular pathogens.

In the present paper, we investigate the role of EP24.15 as a potentially important endogenous regulator of MHC I surface expression on intact cells.

EXPERIMENTAL

Vectors and cells

For EP24.15- or inactive mutant, Glu502→Gln (E502Q)-expressing cells, the IRES (internal ribosome entry site) on the pMXI bicistronic vector was originally derived from encephalomyocarditis virus (Clontech, Palo Alto, CA, U.S.A.). The IRES and MCS (multiple-cloning site) were excised from Herogen Spits LZRSpBMN-linker IRES-EGFP (enhanced green fluorescent protein) and subcloned into pMX, which was subsequently named pMXLEGFP. The efficacy of these constructs was tested by subcloning IL-10R (interleukin-10 receptor) upstream of the IRES. Subsequently, retroviruses were produced from these constructs in BOSC23 cells and transduced into BaF3 cells. IL-10R and EGFP expression were then detected by FL1 and FL2 fluorescence respectively. For EP24.15-silenced cells, pSUPER.retro vector was used (Oligoengine, Seattle, WA, U.S.A.). Bing amphotropic envelope-expression packaging cells, BOSC23 ecotropic envelope-expression packaging cells and L929 mouse fibroblast cells [American Type Culture Collection (ATCC), Manassas, VA, U.S.A.] were maintained in complete medium [Dulbecco’s modified Eagle’s medium with 4 mM L-glutamine, 1.5 g/l sodium bicarbonate and 4.5 g/l glucose, supplemented with 10% (v/v) foetal bovine serum (Atlanta Biologicals, Norcross, GA, U.S.A.)] with 100 units/ml penicillin and 100 µg/ml streptomycin.

Antibodies

PE (phycocerythrin)-conjugated mouse anti-(mouse H-2K^d^) (H-2K^d^ is a type of cell-surface MHC I protein) (clone 36-7-5) and biotin-conjugated mouse anti-(mouse H-2D^d^) (H-2D^d^ is another type of MHC I molecule) (clone 15-5-5) antibodies (Pharmingen/BD Biosciences, San Diego, CA, U.S.A.) were used to detect MHC I staining. Total cell-surface H-2D^d^-biotinylation was performed using the FluoReporter cell-surface biotinylation kit (Molecular Probes, Eugene, OR, U.S.A.) and was followed by staining with PE-streptavidin (Pharmingen/BD Biosciences). Antibodies for Western immunoblotting were: anti-EP24.15 (Ab#35; 1:2000), affinity-purified against recombinant EP24.15 protein [22,23], anti-lactate dehydrogenase (1:750; Rockland Immunocytochemicals, Gilbertsville, PA, U.S.A.), goat anti-rabbit secondary horseradish peroxidase-conjugated IgG antibody and ECL® (enhanced chemiluminescence) detection kit (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Cloning

To produce cell lines that expressed a given construct, the mouse EP24.15-His_s insert [24], the mouse EP24.15.E502Q-His_s mutant insert [25] and the full-length mouse SOCS-1 (suppressor of cytokine signalling) cDNA insert were separately subcloned into the pMXLEGFP vector. To produce EP24.15-silenced cells, potential oligonucleotides were first searched using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) against the non-redundant mouse gene database to insure uniqueness. All four 64 nt oligonucleotide pairs were annealed and ligated into the pSUPER.retro vector, which contains a puromycin-resistance gene. The set of oligonucleotides with the greatest attenuation of activity was 5’-GATCCCATGACTATCGAAATACCCCT AAAGATTAGGCAGGTAG-3’ (forward); 5’-GCTTCTCTTCGAGGTAGGAAG-3’ (reverse). A depiction of the production of siRNA for EP24.15-silenced cells is provided (see Figure 6A).

Retroviral transduction

Amphotropic producer cells were grown to 80% confluence in 100 mm plates, then transfected with 10 µg of appropriate construct using 60 µl of LIPOFECTAMINE™ 2000 in Optimem (Invitrogen, Carlsbad, CA, U.S.A.). Amphotropic cells were cultured for 2 days in Optimem at 37°C with 5% CO_2. Supernatant was harvested, 8 µg/ml polybrene (Sigma, St. Louis, MO, U.S.A.) was added, the mixture was centrifuged and filtered (0.45 µm), and was added to ecotropic producer cells plated at 50% confluence in Optimem. Ecotropic cells were then grown for 2 days at 37°C with 5% CO_2 in complete medium, sorted for highest 10% EGFP positivity, then grown to confluence in complete media at 37°C with 5% CO_2. Supernatant was similarly harvested, polybrene added, centrifuged, filtered and added to target L929 cells plated at 30% confluence in complete medium. L929 cells were cultured for 3 days before analysis (EP24.15-overexpressing cells) or puromycin selection (EP24.15-silenced cells). Transduction efficiency of EP24.15-overexpressing cells was confirmed by EGFP positivity by FL2 fluorescence. Transduction efficiency of EP24.15-silenced cells was confirmed by puromycin resistance (10 µg/ml), and was then tested for enzyme activity as well as the presence of protein by Western immunoblotting.

Flow cytometry and FACS

All cells were analysed using an EPICS ELITE flow cytometer ( Coulter Electronics, Miami, FL, U.S.A.). EP24.15-overexpressing (EGFP:EP24.15) or inactive EP24.15-overexpressing (EGFP:E502Q) cells were FACS-purified for the highest 1% EGFP positivity as detected by FL2 fluorescence. All cells were sorted using a MoFlo high-speed cell sorter (DakoCyto, Fort Collins, CO, U.S.A.).

Subcellular fractionation and differential centrifugation

Cells were fractionated as previously described [26]. Briefly, L929 cells were grown on 100 mm plates to 80–90% confluence. The entire procedure was carried out on ice. Cells were washed
once using TBSS (25 mM Tris/HCl, pH 7.4, 4.5 mM KCl, 137 mM NaCl, 0.7 mM Na,HPO, and 1.6 mM NaSO), and were then scraped with a rubber cell scraper using homogenization buffer containing protease inhibitors (20 mM Heps, pH 7.5, 0.35 M sucrose, 1 mM EDTA, 30 µg/ml PMSF, 2 µg/ml leupeptin and 16 µg/ml aprotinin). The cells were pelleted by centrifugation at 700 g, and were resuspended in 10 vol. of homogenization buffer with protease inhibitors. Cells were passed through a 22-gauge needle until a single cell suspension was achieved. The cell suspension was then homogenized by 15 passes through a Balch cell cracker (ball-bearing homogenizer, EMBL, Heidelberg, Germany) [27] using an 18 µm clearance. Cell debris and nuclei were removed by centrifugation at 700 g for 5 min at 4C. The resulting post-nuclear supernatant was then centrifuged at 100,000 g for 60 min. The resultant cytosolic supernatant was designated S100, whereas the pellet containing organelles and membranes was designated P100.

**Assay for EP24.15 activity**

EP24.15 activity was determined with QFS [quenched fluorescent substrate: Mca-Pro-Leu-Gly-Pro-D-Lys(2,4-dinitrophenyl)], where Mca is 7-methoxycoumarin-4-acetyl], as described previously [25]. Briefly, culture medium (40 µl) and subcellular fractions (5 µl) were incubated at 37 °C with 8.8 µM QFS in reaction buffer (25 mM Tris/HCl, pH 7.5, 125 mM NaCl and 0.3 mM dithiothreitol) in a final volume of 635 µl. Activity was also assayed by a related enzyme, neurolysin (EP24.16; EC 3.4.24.16), which possesses some QFS hydrolysis activity. Specific inhibitors of both enzymes were also included in the assay to delineate the relative contributions of each enzyme towards total QFS hydrolysis. These included the transition-state inhibitor cFP-AAF-pAB {N-[1-(R,S)-carboxy-2-phenylpropyl]-Ala-Ala-Phe-p-aminobenzoate} (28 µM) [19] which predominantly inhibits EP24.15 activity, and the dipeptide Pro-Ile (4 mM), a selective inhibitor of EP24.16 activity. Reactions were terminated after 30 min with 115 µl of 0.5M sodium formate, pH 3.5. Mca-Pro-Leu product was measured at room temperature (25 °C) on a PerkinElmer LS-50B fluorescence spectrophotometer (South Plainfield, NJ, U.S.A.) at excitation and emission wavelengths of 328 and 393 nm respectively. Enzyme activities were determined from a standard curve of the Mca-Pro-Leu product standard prepared under identical assay conditions. Activity was expressed [after correction for non-specific background (approx. 1.5 %) and EP24.16 (10–15 %)] as pmol of Mca-Pro-Leu product/min per ng of protein. Suitable negative controls were included in each assay. Total substrate hydrolysis was <8 %. Statistical significance of the results was determined using Student’s two-tailed t test to compare sample cultures with the control culture.

Kinetic determinations of QFS with recombinant mouse EP24.15 was determined under discontinuous assay conditions as described previously [24]. All determinations were performed using two independent protein preparations. EP24.15 (10 ng) was incubated at 37 °C with various concentrations of QFS (4.4–22.0 µM) in a final volume of 635 µl.

Kinetic determinations of the ovalbumin octapeptide, OVA-8 (Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu), as a substrate for EP24.15 was determined under discontinuous assay conditions by quantification of substrate product peaks [the cleaved Ser-Ile-Ile-Asn-Phe and Glu-Lys-Leu peptides as determined by MS (results not shown)] via HPLC, as described previously [24,25]. Briefly, samples were eluted at 1 ml/min from a Delta-Pak C-18 (3.9 mm × 300 mm) reversed-phase column (Waters, Milford, CT, U.S.A.) by a linear gradient (solvent A, 0.08 % trifluoroacetic acid; solvent B, 0.08 % trifluoroacetic acid/70 % acetonitrile) from 5 to 85 % solvent B over 27 min with flow being maintained at 85 % solvent B for a further 3 min. EP24.15 (100–330 ng) was incubated at 37 °C with varying concentrations of peptide substrate (25 µM–1.75 mM).

Total substrate hydrolysis was less than 10 %. Kinetic parameters (Km, Vmax, kcat and kcat/Km) were evaluated using the double-reciprocal plot method of Lineweaver and Burk [28].

**SDS/PAGE and Western immunoblotting**

SDS/PAGE was conducted, under reducing conditions, in a 15 cm × 15 cm electrophoresis system using 8 % (w/v) polyacrylamide gels as previously described [20], with the ability to effect high-resolution separation of bands < 2 kDa in mass. Western immunoblotting was as described previously [23].

**RESULTS**

**EP24.15 overexpression suppresses surface MHC I expression**

We first investigated whether EP24.15 expression in intact cells has the ability to change surface MHC I levels. If EP24.15 activity results in sufficient deprivation of precursor MHC I peptides in the cytosol, then overexpression of EP24.15 should presumably decrease surface MHC I levels. EP24.15 with a C-terminal His6 tag (EP24.15–His6) was ligated in a bicistronic retroviral IRES vector with EGFP for detection of transduced cells (Figure 1A). EGFP fluorescence intensity, as detected by flow cytometric analysis (Figure 1B) and fluorescence microscopy (overlaid with phase contrast) (Figure 1C), is proportional to EP24.15–His6 expression and was thus used to detect the degree of EP24.15–His6 expression in transduced cells. Control cells were transduced with either vector alone or an inactive mutant form of EP24.15. The inactive mutant was used to determine whether or not changes in surface MHC I expression could be specifically attributed to enzyme function of wild-type EP24.15, rather than to non-specific effects of protein expression. The inactive form of EP24.15 used in these experiments was the EP24.15.E502Q mutant (residue 502 is one of the zinc-co-ordinating residues), whose proper expression, proper folding and lack of enzyme activity have been previously published by our group [25].

As indicated by EGFP expression, flow cytometric analysis indicated varying degrees of transduction efficiency (Figure 1B). A similar variation in transduction efficiency was observed when producing control cell lines. Total transduced EP24.15–His6–overexpressing cells were denoted ‘total EGFP:24.15 cells’, and included all cells which were retrovirally transduced with the EP24.15–His6 construct. Similarly, total vector-transduced control cells were denoted ‘total EGFP:control cells’, and total enzymically inactive E502Q–His6–overexpressing cells were denoted ‘total EGFP:E502Q cells’.

Total EGFP:24.15 cells, total EGFP:control cells and total EGFP:E502Q cells were stained with PE-anti-H-2K, antibody, and were analysed using two-colour flow cytometry. With increasing EGFP, both total EGFP:control cells and total EGFP:E502Q cells maintain a tight distribution of MHC I (H-2K) surface expression. This indicates that, despite the heterogeneity of transduction efficiency, the MHC I surface expression is the same on all total EGFP:control cells (Figure 2A) and on all total EGFP:E502Q cells (Figure 2C). In contrast, with increasing EGFP, total EGFP:24.15 cells gradually decrease MHC I surface expression (Figure 2E). This indicates that only the highest
EP24.15-expressing cells decrease surface MHC I expression, and that the majority of total EGFP.24.15 cells demonstrate the same surface MHC I as that on total EGFP.E502Q cells. In addition, total EGFP.control cells maintained identical levels of MHC I with those found on non-transduced cells (see Figures 5A and 5B), indicating that the vector itself does not affect MHC I expression.

To isolate a homogeneous population of the highest 1% of EP24.15–His8-expressing cells, total EGFP.24.15 cells were FACS-sorted for the highest 1% of EGFP-expressing cells; these cells were denoted ‘post-sorted EGFP.24.15 cells’. Total EGFP.control cells sorted for the highest 1% of EGFP-expressing cells were denoted ‘post-sorted EGFP.control cells’, and total EGFP.E502Q cells sorted for the highest 1% of EGFP-expressing cells were denoted ‘post-sorted EGFP.E502Q cells’. Post-sorted EGFP.24.15 cells, post-sorted EGFP.E502Q cells and post-sorted EGFP.control cells were then stained with PE–anti-H-2Kk antibody and were analysed by flow cytometry. Strikingly, post-sorted EGFP.24.15 cells demonstrate a marked decrease in MHC I surface expression (Figure 2F) compared with MHC I surface expression on post-sorted mutant EGFP.E502Q cells (Figure 2D) or on post-sorted EGFP.control cells (Figure 2B). The dramatic decrease of MHC I surface expression on post-sorted EGFP.24.15
These data indicate that the EP24.15–His8 protein demonstrates suppress MHC I expression on the cell surface (Figure 2E). Results demonstrate that an approx. 4–5-fold increase in EP24.15 expression (compared with endogenous levels) is required to achieve full repression on H-2Kk or H-2Dk expression on the cell surface. Our results indicate that EP24.15 overexpression does not appear to differentially regulate classes of MHC I.

To ensure that these results were not vector-dependent, we performed identical experiments using a different retroviral construct with a neomycin-resistance gene in place of the EGFP. In this case, transduced cells were selected with 1 mg/ml neomycin (instead of by EGFP fluorescence). Control cells were transduced with vector alone, and were similarly selected for neomycin resistance. The same decrease in MHC I expression was noted upon EP24.15 expression in neomycin-resistant cells (results not shown). Therefore, a decrease in surface MHC I occurs in EP24.15-overexpressing cells, regardless of the retroviral vector used.

Western blot analysis demonstrates abundant expression of both the EGFP.E502Q protein and the EGFP.24.15 protein, as indicated by the upper bands on the gel (Figure 3A, lane 3) and (Figure 3A, lane 4) respectively. Next, we quantitatively determined the fold up-regulation of cytosolic EP24.15 activity that could successfully induce repression of MHC I surface expression. Since all approx. EP24.15 is present in the cytosol of L929 cells, the EP24.15 enzyme activity assay was performed on S100 supernatants from non-transduced cells, post-sorted EGFP.control cells, post-sorted EGFP.E502Q cells and post-sorted EGFP.24.15 cells, to determine cytosolic EP24.15 activity (Figure 3B). To quantify the degree of EP24.15 activity required to inhibit MHC I surface expression, hydrolysis of QFS was measured [25]. EP24.15 enzyme activity in post-sorted EGFP.24.15 cells was found to be approx. 4.5-fold greater than EP24.15 activity in post-sorted EGFP.control cells or in non-transduced cells, and approx. 4-fold greater than EP24.15 activity in post-sorted EGFP.E502Q cells (Figure 3B). The increases in EP24.15 activity had no effect on cell viability, as post-sorted EGFP.24.15 and non-transduced cells showed >98% cell viability by Trypan Blue dye exclusion (results not shown). These results demonstrate that an approx. 4–5-fold increase in EP24.15 expression (compared with endogenous levels) is required to suppress MHC I expression on the cell surface (Figure 2E).

These data indicate that the EP24.15–His8 protein demonstrates the same subcellular expression pattern and activity as endogenous EP24.15, and also that, since the overwhelming majority of EP24.15 is present in the cytosol in L929 cells, it appears that the cytosolic form of EP24.15 may be most relevant to modulation of MHC I surface levels.

Next, recombinant mouse EP24.15 was employed to determine kinetics for the hydrolysis of a classic MHC I peptide, OVA-8, and for the hydrolysis of the ideal EP24.15 QFS. The $k_{\text{cat}}$ of QFS was $13.5 \pm 4.1 \mu \text{M}^{-1} \text{s}^{-1}$ and the $V_{\text{max}}$ was $1220 \pm 331 \mu \text{M}$, whereas the $V_{\text{max}}$ of OVA-8 was $72.7 \pm 10.1 \text{pmol/min per ng of protein}$ and the $V_{\text{max}}$ of OVA-8 was $13.5 \pm 3.3 \text{pmol/min per ng of protein}$. The $K_{\text{m}}$ of QFS and OVA-8 was $0.88 \pm 0.06$ and $1.25 \pm 0.11 \text{ s}^{-1}$ respectively. The specificity constants ($k_{\text{cat}}/K_{\text{m}}$) of QFS and OVA-8 as substrates for EP24.15 were $6.5 \times 10^{5} \text{ M}^{-1} \text{s}^{-1}$ and $0.14 \times 10^{4} \text{ M}^{-1} \text{s}^{-1}$ respectively. These substrates behaved differently towards EP24.15 with the ideal fluorescent substrate appearing to be more efficiently degraded.

**Subcellular localization and activity of EP24.15 are concurrent in the cytosol**

As EP24.15 overexpression induces repression of surface MHC I, we sought to determine in which subcellular compartment(s) (relevant to MHC I antigen processing) EP24.15 was present. We reasoned that if EP24.15 overexpression was mediating a decrease in surface MHC I by depleting the pool of precursor MHC I peptides (found in the cytosol) as propounded in a previous report, then EP24.15 should be detected in cytosolic protein fractions [8]. A specialized method of homogenization (Balch cell cracking) [27] was performed to preserve intact organelles during homogenization, to minimize contamination of proteins from the membrane protein fraction to the cytosolic protein fraction. Differential centrifugation and examination of S100 supernatant and P100 pellet protein fractions provided respectively, cytosolic and membrane/organellar protein fractions of non-transduced cells, post-sorted EGFP.control cells, post-sorted EGFP.E502Q cells and post-sorted EGFP.24.15 cells. Results are expressed as enzyme activity means ± S.D. for two individual determinations performed in triplicate.

**Figure 3 Protein expression and activity of engineered cell lines**

(A) Western immunoblot of cytosolic and membrane protein fractions. Proteins (30 µg/lane) were separated by SDS/PAGE and immunoblotted. The blot was probed with affinity-purified antibody against EP24.15. (B) Cytosolic EP24.15 enzyme activity was measured by the QFS assay, comparing non-transduced cells, post-sorted EGFP.control cells, post-sorted EGFP.E502Q cells and post-sorted EGFP.24.15 cells. Results are expressed as enzyme activity means ± S.D. for two individual determinations performed in triplicate.
EGFP.control cells demonstrate the same level of endogenous EP24.15, indicating that, as expected, the vector itself does not influence EP24.15 expression (Figure 4, upper blot). In the cytosolic fraction of post-sorted EGFP.24.15 cells, two closely juxtaposed bands are present: the upper band correlates with expression of the transduced EP24.15–His8 insert, and the lower band correlates with expression of endogenous EP24.15. EGFP.24.15 cells exhibit the same amount of endogenous EP24.15 as that in EGFP.control cells, but EGFP.24.15 cells also express the EP24.15–His8 insert (Figure 4, upper blot). In the membrane protein fraction, EP24.15 is barely detected in non-transduced cells or in post-sorted EGFP.control cells (Figure 4, upper blot). In EGFP.24.15 cells, a residual amount of EP24.15 was detected; it is difficult to discern, however, whether this species derives from endogenous EP24.15 or from expressed EP24.15–His8 (Figure 4, upper blot). Regardless, even with over-exposure of the film to accentuate the membrane protein content, the amount of endogenous EP24.15 and of expressed EP24.15–His8 in membrane protein fractions was nominal.

To examine the purity of the S100 and P100 fractions, we examined the presence of a cytosolic marker, lactate dehydrogenase, in the membrane fraction (Figure 4, lower blot). The results demonstrate a lack of cytosolic protein contamination in the membrane protein fraction. Therefore, in post-sorted EGFP.24.15 cells, it is apparent that the overwhelming majority of both endogenous EP24.15 and expressed EP24.15–His8 was found in the cytosol.

**IFN-γ (interferon-γ) re-establishes MHC I surface expression in EP24.15-overexpressing cells**

If MHC I surface expression is decreased by EP24.15 overexpression, what exogenous soluble factors or cytokines could potentially mediate a change in EP24.15 cytosolic activity? We investigated whether IFN-γ (a potent inducer of surface MHC I) could induce surface MHC I expression on post-sorted EGFP.24.15 cells. IFN-γ treatment (10 ng/ml for 24 h) of non-transduced cells and post-sorted EGFP.control cells induced the expected increase in MHC I expression (Figures 5A and 5B). Strikingly, IFN-γ induced an increase in MHC I expression in post-sorted EGFP.24.15 cells (Figure 5C). Control cells for IFN-γ treatment were SOCS-1-overexpressing (EGFP.SOCs-1) cells, which do not respond to IFN-γ receptor signalling (Figure 5D) [29].

**IFN-γ induces EP24.15 expression and enzyme activity**

After demonstrating that IFN-γ was able to re-establish MHC I surface expression in post-sorted EGFP.24.15 cells, we sought to determine whether or not this increase in MHC I was mediated via
the manipulation of intracellular EP24.15 expression and activity. We reasoned that if the mechanism by which IFN-γ increases MHC I expression relies on manipulation of EP24.15, then presumably IFN-γ treatment of cells should induce a decrease in EP24.15 expression and activity. Western immunoblotting (Figure 5E) and EP24.15 enzyme-activity assays (Figures 5F and 5G) were performed to determine whether or not EP24.15 expression and activity was influenced by IFN-γ treatment. Since IFN-γ is cytotoxic at high concentrations and over an extended time of treatment, we generated a ‘kill curve’ using increasing concentrations of IFN-γ. In this way, we established a concentration of IFN-γ and time of treatment that caused the least cytotoxicity to avoid confounding data due to differences in cell viability between samples. Treatment of cells with 10 ng/ml IFN-γ for 24 h was chosen based on maintenance of >98% cell viability (n = 3) for the duration of the experiment. Cell viability was assessed by Trypan Blue dye exclusion (results not shown).

Curiously, Western immunoblotting results showed an increase in endogenous EP24.15 expression in both IFN-γ-treated post-sorted EGFP.control cells and IFN-γ-treated post-sorted EGFP.24.15 cells (Figure 5E). We then performed enzyme-activity assays on each of these populations. Consistent with protein expression results, IFN-γ treatment of post-sorted EGFP.control cells induced a 1.85-fold increase in EP24.15 enzyme activity (Figure 5F), and IFN-γ treatment of post-sorted EGFP.24.15 cells induced a 2.60-fold increase in EP24.15 enzyme activity (Figure 5G). Since IFN-γ treatment increases both EP24.15 protein expression and enzyme activity, we surmise that the increase of surface MHC I on post-sorted EGFP.24.15 cells (Figure 5C) is probably mediated via mechanisms other than those involving EP24.15. To our knowledge, this is the first demonstration of cytokine-induced changes in EP24.15 expression and activity.

Stable siRNA (short interfering RNA) silencing of endogenous EP24.15 mediates an increase in surface MHC I

As EP24.15 overexpression induces a decrease in MHC I surface expression, we wished to investigate whether silencing of endogenous EP24.15 could analogously mediate an increase in cell-surface MHC I. A retroviral construct for stable siRNA-silencing of EP24.15 in mouse fibroblast L929 cells was generated. Four different pairs (forward and reverse) of 64 nt oligonucleotides were designed, each with a 2 nt UU overhang and with a different 19 nt sense (and antisense) sequence. After transduction into L929 cells, the 64-mer folds upon itself at the complementary sense and antisense sites to form a hairpin. The 9 nt spacer in between the sense and antisense sequences is then cleaved by Dicer, the enzyme digesting the double-stranded (ds) DNA to generate the active 21 nt dsRNA which mediates stable silencing of EP24.15 (Figure 6A). The vector itself contained a puromycin-resistance gene.

Each of four 64-mer sequences was ligated in the siRNA retroviral vector, separately transduced into L929 cells, and selected with 10 µg/ml puromycin. Western immunoblot analysis of cytosolic fractions from each siRNA line was probed with EP24.15 affinity-purified antibody to assess which 64 nt oligonucleotide was most effective in decreasing endogenous expression of EP24.15 (Figure 6B). The 64 nt sequence demonstrating the most potent capacity to diminish endogenous EP24.15 (siRNA.24.15 #1) was utilized for all subsequent siRNA experiments (Figure 6B). Transduced cells were denoted ‘siRNA.24.15 cells’. Control cells were transduced with the siRNA vector and were selected similarly with puromycin; control cells were denoted ‘siRNA.control cells’.

Before analysis, puromycin treatment of siRNA.24.15 cells and siRNA.control cells was stopped for 7 days (to avoid artifacts in results due to puromycin treatment). Cells were then stained with PE–anti-H-2Kk antibody and analysed by flow cytometry for surface MHC I staining. Our results indicate that siRNA.24.15 cells demonstrate a significant increase in MHC I expression compared to MHC I expression in siRNA.control cells (Figure 6C). As expected, siRNA.control cells maintained similar levels of MHC I as non-transduced cells (results not shown). Therefore, silencing of endogenous EP24.15 is able to increase MHC I surface levels.

To assess whether or not siRNA.24.15 cells increased surface expression of both types of MHC I (H-2Kk and H-2Dk), siRNA.24.15 cells and siRNA.control cells were then stained with biotinylated anti-H-2Dk antibody. siRNA.24.15 cells manifested increased H-2Dk, similar to that seen with H-2Kk (results not shown). These results suggest that when endogenous EP24.15 is silenced, cell-surface expression of different classes of MHC I proteins are similarly increased.

We then sought to determine the quantitative repression of endogenous EP24.15 in siRNA.24.15 cells. What degree of silencing of EP24.15 is required to induce a notable increase in surface MHC I relative to MHC I expression on siRNA.control or non-transduced cells? We determined by EP24.15 enzyme-activity assay that the siRNA.24.15 population exhibits approx. 20% of the EP24.15 activity found in siRNA.control cells or non-transduced cells (Figure 6D). It is notable, then, that incomplete silencing of EP24.15 is sufficient to induce a significant change in MHC I surface expression.

To summarize, it is apparent that increased EP24.15 activity in post-sorted EGFP.24.15 cells induces repression of MHC I surface expression, and likewise that decreased EP24.15 activity in siRNA.24.15 cells induces facilitated MHC I surface expression. We questioned, however, whether or not, over time, post-sorted EGFP.24.15 cells possessed the capacity to adapt and overcome the increased cytosolic activity of EP24.15, and analogously, whether siRNA.24.15 cells would adapt and overcome the decreased cytosolic activity of EP24.15. In post-sorted EGFP.24.15 cells, it was found that under the stipulation that an approx. 5-fold increase in EP24.15 expression was maintained, MHC I expression remained at the same diminished levels after 60 days (results not shown). In siRNA.24.15 cells, under the stipulation that an 80% decrease in EP24.15 expression was maintained, surface MHC I remained at the same elevated level after 60 days (results not shown). These results suggest that cells are not able to adapt and compensate for changes in cytosolic EP24.15 in order to maintain normal or homoeostatic levels of MHC I.

EP24.15 does not perturb total cell-surface protein expression

We next determined whether or not cell-surface expression of MHC I was selectively affected by overexpression or silencing of EP24.15, or if many cell-surface proteins were also altered. If the latter were true, it would be anticipated that a notable suppression of total cell-surface proteins should be observed on post-sorted EGFP.24.15 cells, and that an overly augmented increase in total cell-surface proteins should be observed on siRNA.24.15 cells. Since EGFP.24.15 cells express approx. 25-fold more EP24.15 than siRNA.24.15 cells do, the expression of total cell-surface proteins on post-sorted EGFP.24.15 cells was compared with that on siRNA.24.15 cells, for the largest potential contrast in results. Biotinylation of total cell-surface proteins was performed on intact cells, followed by staining with PE-streptavidin, which conjugated to the cell-surface-bound biotin. Flow cytometric analysis was then performed to identify the
Figure 6 Stable siRNA-mediated silencing of EP24.15 increases surface MHC I expression

(A) The siRNA pSUPER retroviral construct used to create stably silenced L929 cells (siRNA.24.15 cells). The 64-mer oligonucleotide insert complementarily pairs at the 19 nt sense and 19 nt antisense sites to form a hairpin. The 9 nt spacer is cleaved by Dicer to form the active 21 nt dsRNA (19 nt sequence and 2 nt UU overhang). Transduced cells were selected with 10 \( \mu \)g/ml puromycin.

(B) EP24.15 expression in non-transduced cells, siRNA-control cells (siRNA-vector-transduced cells) and four lines of siRNA.24.15 cells (siRNA.24.15 #1, #2, #3 or #4; each line was transduced with one of four different siRNA 64 nt inserts). Cytosolic protein (30 \( \mu \)g/lane) was separated by SDS/PAGE, immunoblotted, and probed with affinity-purified antibody against EP24.15. siRNA.24.15 #1 demonstrates the most potent decrease in EP24.15 expression compared with non-transduced cells or siRNA.control cells. (C) Surface H-2Kk expression on siRNA.24.15 cells and siRNA.control cells. Similar results were obtained with another MHC I surface protein, H-2Dk (results not shown). siRNA.24.15 cells demonstrate increased surface MHC I relative to siRNA.control cells. (D) Cytosolic EP24.15 activity by the QFS assay (enzyme-activity assay) was performed on siRNA.control cells (siRNA vector-transduced) and siRNA.24.15 cells [siRNA.24.15 #1-transduced, as shown in (B)]. Results are expressed as mean activity ± S.D. of two individual determinations performed in triplicate.

Fluorescence intensity of PE-streptavidin (total cell-surface-protein expression). The results illustrate that total cell-surface-protein expression is maintained at the same level on post-sorted EGFP.24.15 cells as on siRNA.24.15 cells (Figure 7). These data suggest that EP24.15 modulation selectively influences MHC I surface expression.

DISCUSSION

EP24.15 has been reported as the primary endopeptidase responsible for degrading classic MHC I peptides in cell extracts [8]. In the present paper, we investigated whether manipulation of EP24.15 would induce modulation of MHC I expression on the surface of intact cells. Our findings suggest that EP24.15 may potentially serve as an important endogenous regulator of MHC I expression on the surface of cells. Overexpression of EP24.15 in intact cells (post-sorted EGFP.24.15 cells) dramatically decreases MHC I on the cell surface (Figure 2G) and, conversely, siRNA-mediated reduction of endogenous EP24.15 (siRNA.24.15 cells) increases MHC I cell-surface levels (Figure 6C). In either post-sorted EGFP.24.15 cells or siRNA.24.15 cells, we detected no difference between surface expression of different

Figure 7 Total cell-surface protein expression on siRNA.24.15 and EGFP.24.15 cells

Total cell-surface proteins were biotinylated and quantified by detecting fluorescence intensity with PE-streptavidin by flow cytometry. EP24.15 enzyme activity was approx. 25-fold higher in EP24.15-overexpressing cells (post-sorted EGFP.24.15) than in EP24.15-silenced cells (siRNA.24.15). Total cell-surface protein expression is similar between the two cell lines.
types of MHC I (H-2K\* or H-2D\*), indicating that EP24.15 does not differentially regulate classes of MHC I. We show that an approx. 4.5-fold increase in EP24.15 expression is required for suppression of surface MHC I expression in post-sorted EGFP.24.15 cells (Figure 3B), and that an approx. 80% decrease in EP24.15 expression is sufficient for facilitation of surface MHC I expression (Figure 6D). In siRNA.24.15 cells, it is notable that incomplete silencing of EP24.15 is sufficient to induce a viable increase in MHC I surface expression.

EP24.15 has been traditionally classified as a neuropeptide-processing enzyme. As neuropeptide substrates are found in the extracellular space, the activity of EP24.15 has traditionally also been correspondingly localized to the extracellular milieu. Despite the preponderance of focus on EP24.15 activity in the extracellular space, subcellular fractionation experiments have indicated that the majority (generally > 80%) in neuroendocrine cells) of EP24.15 is localized to the cytosol potentially relevant to MHC I expression, whereas the remainder has been apportioned between the nuclear, membrane-associated and extracellular compartments [20,30].

There appears to be a disparity between the kinetic parameters of two potential substrates of EP24.15, the synthetic QFS and a representative MHC I peptide OVA-8. Though there is a large difference in the specificity constant ($k_{cat}/K_m$) and $K_m$, this compares a model synthetic substrate chosen for its ability to be cleaved by EP24.15 with a substrate occurring in the cytosol. There is a precedent for these kinetics with the key reproductive peptide, GnRH, whose processing is regulated by EP24.15. GnRH appears to be a suboptimal substrate with a $K_m < 1700 \mu M$ [24], less than that of the OVA-8 peptide. Furthermore, the specificity constants ($k_{cat}/K_m$) are comparable between GnRH and the OVA-8 peptide. So crucial is GnRH to the chemistry of the enzyme that the EP24.15-specific active-site-directed inhibitor, cFP-AAF-pAB, is designed based on the hydrophobic and spatial considerations of the GnRH peptide structure [19]. Previously OVA-8 peptide was evaluated as a substrate, and was found to be cleaved, but not as well as other proven antigenic MHC I peptides [8]. Thus, in vitro, the higher $K_m$ value for hydrolysis of the OVA-8 peptide (compared with the specifically designed synthetic QFS) may be interpreted as having generally lower affinity and selectivity, allowing some presentation of this antigen at the surface of the cell. Furthermore, complementary to the experiments presented herein, specific inhibitors of EP24.15 and immunodepletion of enzyme activity by antibodies both attenuated the effects of processing MHC I [8]. Although EP24.15 regulates MHC I presentation, this does not preclude more than one enzyme involved in antigenic peptide degradation.

In the present paper, we propose that an important role of cytosolic EP24.15 may be to arbitrate the level of MHC I expression on the cell surface. EP24.15 is predominantly found in the brain, pituitary and testes [9], which are classically immunoprivileged sites. Such immunoprivileged locations maintain low levels of MHC I expression relative to levels of MHC I expression in lymphoid tissues, such as the spleen, lymph nodes or liver [31–33]. This implies that cytosolic EP24.15 may have an important role in endogenous MHC I regulation in tissue of up-regulated EP24.15 expression and activity. Expression of an EP24.15-like protein is not responsible for the striking attenuation of MHC I expression on the surface of the post-sorted EGFP.24.15 cells. Robust expression of a properly folded inactive 24.15 mutant with a single point mutation of the wild-type Glu502, inducing change to a catalytically inactive glutamine residue (retaining the topology and volume of glutamic acid [25]), exhibits no change in MHC I expression compared with the wild-type (Figures 2D and 3B). We hypothesize that modulation of cytosolic EP24.15 may thus serve as a potential mechanism used to regulate surface MHC I for homeostatic maintenance of immune privilege. Implications of this work in the central nervous system may be significant for neural cells with macrophage-like activity, such as microglial cells and astrocytes, which maintain higher levels of MHC I expression relative to other neural cell populations [34]. We are currently investigating whether modulations in EP24.15 are relevant to differential MHC I expression in brain tissue and neuroendocrine cells, and what changes in EP24.15 activity may be physiologically relevant.

As support that EP24.15 may serve as an important regulator of MHC I expression, our findings show that overexpression of EP24.15 is capable of inducing long-term repression of surface MHC I (Figure 2G), and, correspondingly, that decrease in EP24.15 is capable of inducing long-term facilitation of surface MHC I (Figure 6C). Producing stable lines of both EP24.15-overexpressing cells and EP24.15-silenced cells enabled us to assess whether or not cells overexpressing EP24.15 would maintain suppression of MHC I long-term, and whether or not cells with decreased endogenous levels of EP24.15 would maintain an increase in MHC I long-term; after 60 days under either condition, MHC I levels remained unchanged. Thus, in our engineered cell lines, cellular mechanisms were unable to compensate for a change in MHC I surface expression, such that ‘normal’ or homeostatic MHC I levels could once again manifest on the cell surface.

As a cytokine that could potentially mediate a change in EP24.15 cytosolic protein expression and enzyme activity, IFN-γ treatment was able to ‘overcome’ repression of surface MHC I on EP24.15-overexpressing (post-sorted EGFP.24.15) cells (Figure 5C). IFN-γ-treated cells process protein via immunoproteasomes, which function similarly to proteasomes, but possess three different protease subunits [β11 (LMP2), β5i (LMP7) and β2i (MECL-1)]. Immunoproteasomes are enriched at the cytosolic face of the ER, and generate peptides which end more commonly in hydrophobic or basic residues; this not only favours binding to MHC I, but also favours entry into the ER. Increased immunoproteasome activity both increases the quantity of cytosolic peptides and changes the quality of the cytosolic peptide repertoire [35]. Such qualitative peptide changes (i.e. the variability of peptide sequences in the repertoire), as well as an increase in the quantity of peptides profoundly contributes to the recovery of MHC I surface expression on EP24.15-overexpressing cells.

The effect of IFN-γ on EP24.15 protein expression has been reported as both inhibitory [36] and inducing no change [8]. Our results show that IFN-γ induces both EP24.15 protein expression (Figure 5E) and enzyme activity (Figures 5F and 5G) in L929 cells. Our Western blot and enzyme assay experiments accounted for cytotoxicity induced by IFN-γ treatment by using a cytokine concentration and time of treatment that maintained > 98% cell viability in all samples. It is possible that the difference in cell lines (L929 cells compared with HeLa cells) may provide the explanation for this difference in findings. An increase in EP24.15 expression and activity by IFN-γ in post-sorted EGFP.24.15 cells would be anticipated to induce a further decrease in surface MHC I expression, and thus the mechanisms by which IFN-γ increases surface MHC I probably do not involve EP24.15 modulation.

As overexpression of EP24.15 provides the capacity to repress MHC I cell-surface expression, our work suggests that EP24.15 may be an auspicious protein target for manipulation or mimicry by viruses to decrease cell-surface MHC I presentation of foreign epitopes, and thus to escape host cellular immunity. Many viruses, such as pox viruses, herpes viruses and HIV, encode immunomodulators: proteins which modulate the host immune response as a protective mechanism to promote their survival in the host.
[21]. Long-term suppression of MHC I gives credence to the possibility that EP24.15 may be an important protein candidate, which viruses may attempt to mimic in order to diminish MHC I presentation of viral epitopes on the cell surface. To underscore further the importance of EP24.15, and concurrent with our findings, similar work confirming many of our conclusions has also recently been published [37].

In addition, our data suggest that EP24.15 may serve as a potentially therapeutically significant protein, whose expression could be modulated to manipulate surface MHC I expression to clinical advantage. Possible applications include control of MHC I expression in allogeneic transplants which differ at minor and major histocompatibility antigens, optimizing MHC I expression for increased cellular immune response to immunizations of live attenuated viruses, especially those with a tendency to back-subversion of immunity: a structural perspective. Curr. Opin. Immunol. 13, 442–450


Cummins, P. M., Babon, A., Margules, E. H. and Glucksman, M. J. (1999) Zinc coordination and substrate catalysis within the neuropeptide processing enzyme endopeptidase EC 3.4.24.15: identification of active site histidine and glutamate residues. J. Biol. Chem. 274, 16003–16009


