BMC Immunology

BioMed Central

Research article

Open Access

Fluorescent derivatization of a protease antigen to track antigen uptake and processing in human cell lines

Namrata S Patil*, David L Wong, Katherine D Collier and Hugh C McDonald

Address: Genencor International Inc., 925 Page Mill Road, Palo Alto, CA 94304, USA

Email: Namrata S Patil* - npatil@genencor.com; David L Wong - dwong@genencor.com; Katherine D Collier - kcollier@genencor.com; Hugh C McDonald - hmcdonald@genencor.com

* Corresponding author

Published: 28 June 2004

BMC Immunology 2004, **5**:12 doi:10.1186/1471-2172-5-12

Received: 04 May 2004
Accepted: 28 June 2004

This article is available from: http://www.biomedcentral.com/1471-2172/5/12

Abstract

Background: We have devised a simple and efficient fluorescence-based method to track antigen uptake and processing in human B lymphoblastoid cells (B-LCL). Fluorescein labelled subtilisin was used to optimize antigen uptake conditions and identify processed peptides from human cell lines.

Results: Fluorescein labelled subtilisin conjugates had 0.06 to 2 moles of fluorescein per subtilisin molecule. High performance liquid chromatography and mass spectrometry (NanoESI-LC/MS/MS) analysis identified fluorescein conjugated to K141, K256, and the N terminus. Conjugates retained antigenic specificity to subtilisin specific antibodies and could be processed by whole cell extracts into low molecular weight fragments at pH 5.2. Maximal antigen uptake and processing occurred when PMSF (phenylmethylsulfonyl fluoride) inhibited subtilisin conjugate was incubated with cells at 100–200 μg/ml for 16 to 24 hr. Once optimal uptake conditions were established, processed subtilisin peptides were isolated and identified from human cell lines.

Conclusion: Our studies show that FITC-conjugation provides an efficient tool to track the uptake and processing of this protease antigen and to facilitate identification of processed antigenic peptides from human cell lines.

Background

Antigen presentation by MHC class I and class II molecules is a key aspect of an effective immune response against infections, cancer, certain allergic responses and autoimmune disease. Several good reviews cover capture and processing of exogenous antigens [1,2]. The detection and identification of antigen-derived peptides is important to understand the mechanisms involved in the immune response and to be able to modulate it. Though some studies have employed synthetic peptides to detect peptide epitopes, this approach is unable to identify naturally processed and presented peptides. Some of the early methods employed for identification of naturally processed endogenous epitopes were HPLC and Edman degradation, which led to the identification of hen egg lysozyme (HEL) 52–61 on the MHC of murine B lymphoma cells expressing HEL [3]. Hunt et al. showed the power of joining immunological procedures and mass spectrometry to identify self-peptides presented on cell surfaces in picomole or less concentrations from mouse and human cell lines [4-6]. Using mass spectrometry, Vignali et al. identified 16 peptides from HEL, which contained the minimal HEL 52–61 epitope as the single immunodominant epitope in a nested set of peptides [4]. Using tandem mass spectrometry and functional analysis with SEQUEST software, Dongre, et al. identified 128 self-peptide sequences displayed by murine MHC class II molecules [7].

In the case of exogenous antigens such as ovalbumin [8], tetanus toxoid [9], Goodpasture antigen [10] and diabetes autoantigen [11], many investigators have used a modified delivery system to identify class II MHC peptides. Studies on exogenous class II peptides are limited as a result of requiring large amounts of purified antigen with over 10^9 cells, and the target peptides appearing in picomole quantities or less [12], often present as a subset of nested peptides thus diluting the mass spectrometric signal and rendering detection difficult.

Previous studies from our laboratory have identified class II epitopes from the subtilisin antigen, using synthetic subtilisin peptides with human dendritic cells and naive CD4+ T cells derived from the same donor [13]. In order to identify naturally processed epitopes, we have developed an efficient method of tracking protein in the cell using fluorescein-conjugated antigen. Fluorescent proteins have been used to track microvascular permeability [14], to study intestinal epithelial cell uptake [15], to study substrate processing [16,17] and to track proteins on cells with flow cytometry [18,19]. With the availability of the latest mass spectrometric methods, we investigated the use of fluorescein to study antigen uptake and processing. We describe here the use of FITC conjugated subtilisin in antigen uptake experiments and methods to track the processing of subtilisin both *in vitro* and *in vivo* in human cell lines.

Results

Preparation and characterization of FITC-subtilisin

FITC-labelled subtilisin (FITC-subtilisin) was prepared by derivatizing amino groups on the PMSF-inactivated subtilisin, using 10-fold molar excess of FITC. FITC-subtilisin was purified and assayed for relative fluorescent units (RFU) per microgram protein. The RFU signal was linear from 0.1 to 100 RFU/well and conjugates ranged from 0.6 to 22 RFU/μg protein, corresponding to 0.06 to 2.1 moles of FITC per mole of protein. Conjugates with low ratios of FITC to subtilisin were obtained by increasing the concentration of enzyme in the reaction. Conjugates were TCA-precipitable and showed 89–93% of the fluorescein signal in the pellets. Generally 2 moles or less of FITC were coupled per mole of subtilisin.

FITC-subtilisin showed 10% enzyme activity compared to the unmodified subtilisin, as tested on the synthetic substrate, sucAAPF-pNA [20], as a result of residual PMSF bound to FITC-subtilisin (data not shown).

Identification of FITC-labelled sites on subtilisin using LC-MS

LC-MS was employed to confirm FITC modification of subtilisin. A deconvoluted LC-MS mass spectrometry profile of the modified protein showed free subtilisin (27,476 Da) and FITC-subtilisin with one (27,865 Da) and two moles of FITC (28, 255 Da) (Figure 1). To localize the amino groups on subtilisin modified by FITC, TCA precipitated FITC-subtilisin was hydrolysed with trypsin, which cleaves preferentially at lysine residues. Figure 2 shows identification of subtilisin peptide 137–145 that contained the FITC-modification at the lysine 141 residue. Using fragmentation software analysis, the N-terminus and sites K256 and K141 were also identified as FITC-modification sites.

FITC-subtilisin is immunoreactive and degraded by human B-LCL extracts

To ensure that the overall structure of subtilisin was unchanged, we bound FITC-subtilisin with anti-subtilisin antibody and tracked immunoadsorption using the fluorescein label. Relative fluorescent units showed over 40% of the FITC-subtilisin bound to rabbit polyclonal anti-subtilisin antibody (Table 1). We have routinely used this antibody to track unmodified subtilisin in western blots (data not shown). As a positive control, a mouse anti-FITC monoclonal antibody was used to immunoprecipitate the FITC-subtilisin. No significant binding was observed to normal rabbit gamma globulin control.

To determine whether FITC-subtilisin would be processed by cellular enzymes used in the antigen processing machinery, FITC-subtilisin was incubated with whole cell extracts from Preiss cells. To track processing, low molecular weight fragments were separated with a centrifugal filtration device and relative fluorescent units in the flow-through were measured. Control samples contained PMSF to retard autolysis of subtilisin and prevent processing by the cellular enzymes. Cell extracts from Preiss cells generated significant amounts of low molecular weight peptides at pH 5.3, the pH of endocytic compartments within the cell, compared to controls (See Table 2). At pH 7, however, autolysis of FITC-subtilisin was comparable to the hydrolysis by the cell extracts. Similar results were obtained with whole cell extracts from cell line 8.1.6. These results showed that FITC-subtilisin was susceptible to hydrolysis *in vitro* by mammalian cellular enzymes and could be used to track and detect peptides generated from antigen processing.

Establishing optimal conditions for antigen uptake and processing in human B-LCL

Once we established that FITC-subtilisin was comparable in overall structure to unmodified subtilisin and capable of being processed *in vitro*, we tracked uptake and processing *in vivo* in cells. FITC-subtilisin was co-incubated with Preiss cells for 24 hours, the cells were lysed in acid and RFU were analyzed in the low molecular weight fractions of the cell lysates (See Table 3).

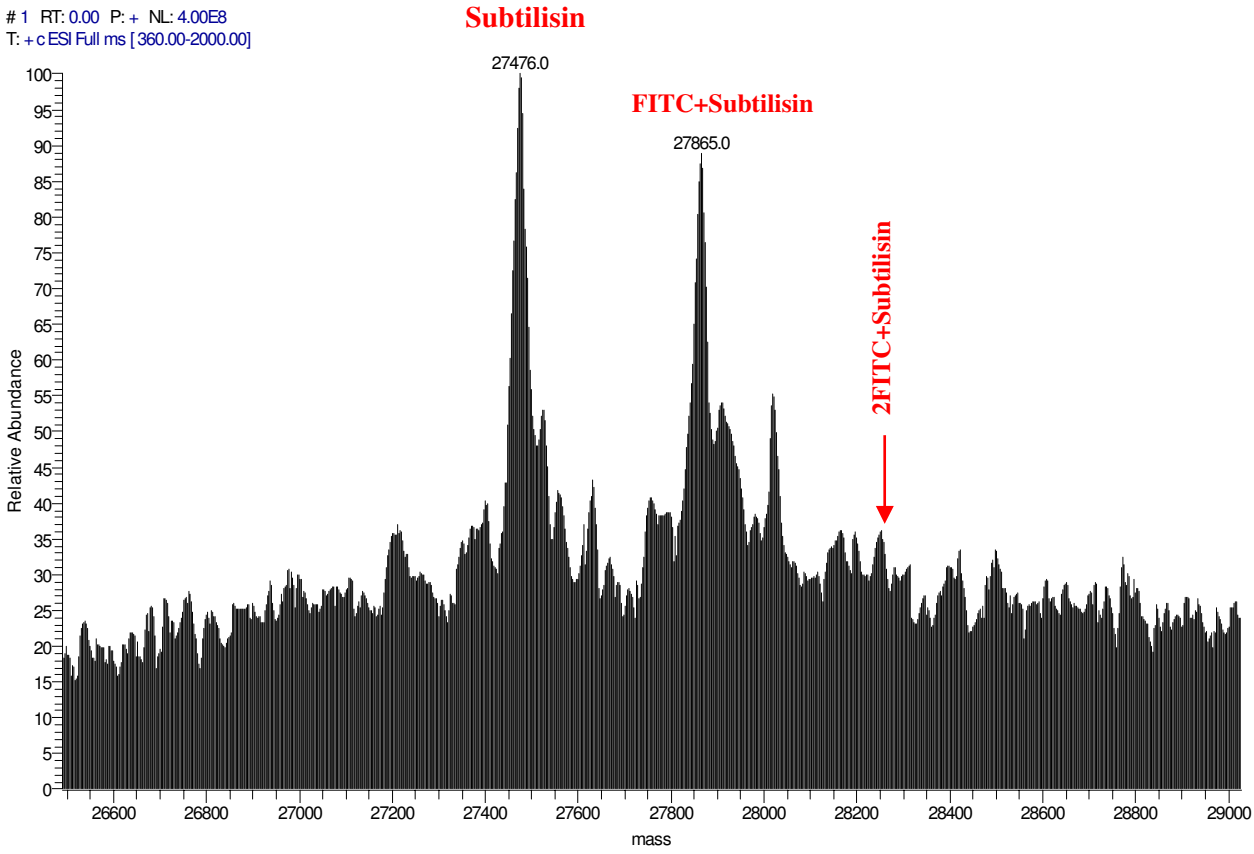

Figure 1
LC/MS Analysis of FITC-Subtilisin. The de-convoluted mass spectrum shows the un-labeled subtilisin (27,476 Da), subtilisin conjugated to one (27,865 Da) or two (28,255 Da) FITC molecules.

Using this relatively simple method, we tested several different conditions that could facilitate antigen uptake and processing. We compared uptake of enzymatically active FITC-subtilisin, TCA-precipitated FITC-subtilisin and autolyzed FITC-subtilisin in 8.1.6 cells (Table 4). Enzymatically active subtilisin degraded the cells and was not used in subsequent experiments. Significantly more TCA-precipitated subtilisin was associated with the washed cell pellet though only 0.06% RFU were recovered from the low molecular weight fraction. Similarly, incorporation of FITC-subtilisin in lysosomes and commercial carriers resulted in increased RFUs in the cell pellet but did not convert into significant increases in low molecular peptides (See Table 5). Multimers of FITC-subtilisin prepared by glutaraldehyde coupling or antibody FITC-subtilisin conjugates also failed to show increased processing. The early (2–12 hour) and late (36–48 hour) time points did not show significant increase in uptake and processing; neither did increasing antigen dose. Using greater numbers of cells as well as incubating antigen for 16 to 24 hours, however, did result in greater uptake and processing.

Identification of processed FITC-subtilisin peptides in human B-LCL cells

Based on our results of optimal conditions for uptake and processing, 4–6 × 10^9 cells were incubated with 0.2 mg/ml FITC-subtilisin (0.6 mole FITC/mole subtilisin) for 24 hours. The cell pellets were either acid treated directly as described above or processed for DR extraction; low

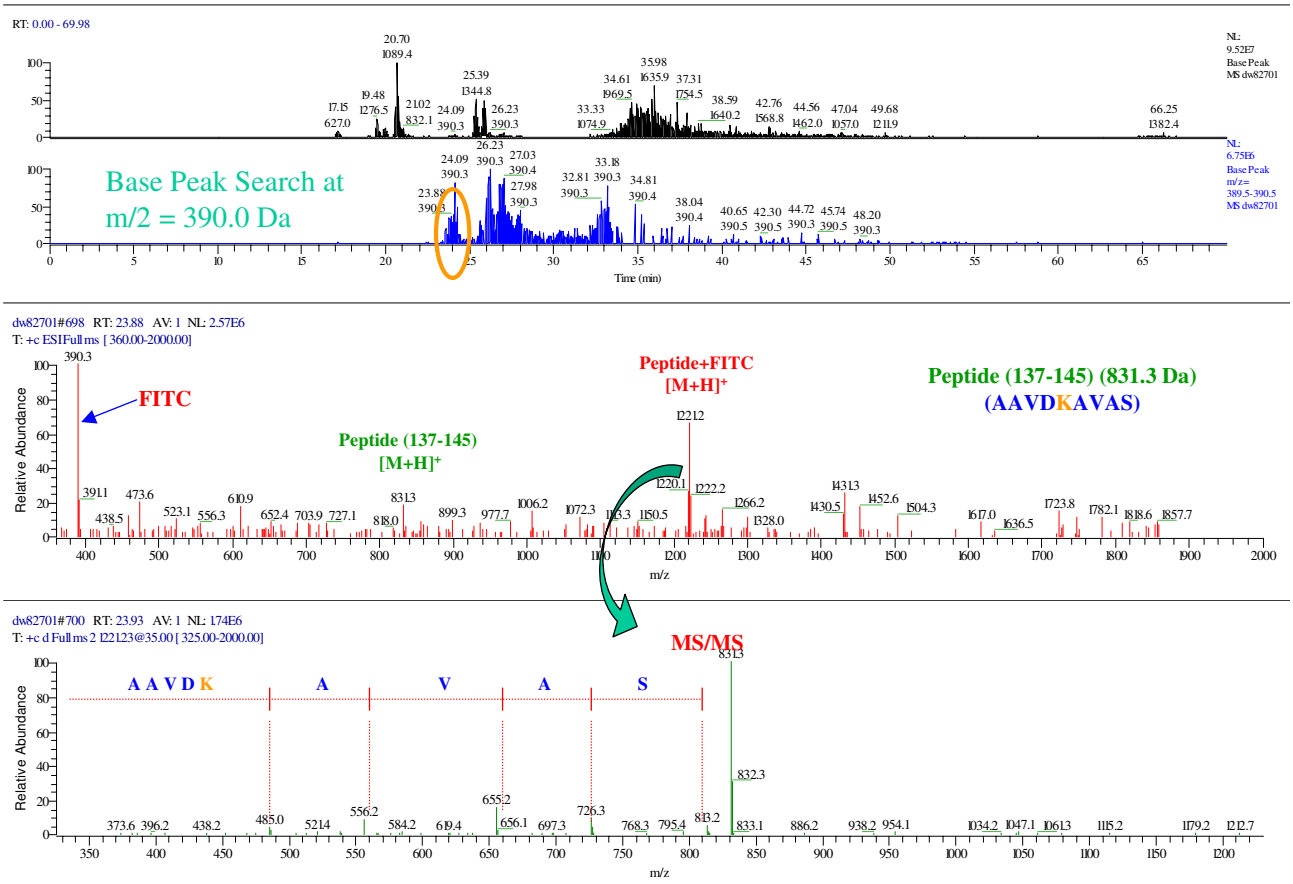

Figure 2
LC/MS/MS analysis of tryptic digested FITC-Subtilisin. Top panel: Full base peak MS spectrum and base peak search spectrum for FITC molecule at m/z = 390.0 Da. Middle panel: A FITC-subtilisin peptide (137–145, 1221.2 Da) was identified at RT = 23.88 min. Bottom panel: Subsequent MS/MS fragmentation on the same peptide molecule confirmed its sequence.

molecular weight fractions were analyzed for RFUs and then by mass spectrometry. For DR-extraction, the cells were washed and lysed and loaded on a HLA-DR immunoaffinity column. The column flow-through had significant RFU. This was fractionated and the low molecular weight fraction was concentrated. HLA-DR molecules purified by the immunoaffinity column were isolated and DR-bound peptides were acid-eluted from these molecules. Half as many RFUs were detected from the DR-eluted peptides (See Table 5). Both samples were analyzed by LC-MS spectroscopy. A subtilisin peptide was identified from the flow-through sample.

Subtilisin peptide 114–126 found in the flow-through was confirmed by spiking a synthetic version of this peptide in control cell extracts and comparison of the synthetic peptide with the unknown peptide by retention time, m/z charge, and fragmentation pattern. Overall, three subtilisin regions were identified from cellular extracts of cells incubated with antigen but not from control cell extracts not incubated with antigen, by ms/ms fragmentation in multiple experiments (See Table 6). Nested sets of peptides from these regions were consistently observed in two or more experiments. However, peptides were not detected in the DR-eluted fraction of the extracts.

To test whether DR associated peptides could be detected in other cell lines, KG-1 cells (dendritic-like cell line derived from monocytes) were incubated with FITC-sub-

Table 1: Binding of FITC-Subtilisin to Immuno-adsorbants[a]

RFU	Anti-Subtilisin	Anti-FITC	Control Antibody
Initial RFU	244	244	244
Bound	113	80	6
Eluted	14.6	5.6	1.4

[a]12 μg of FITC-subtilisin (20.3 RFU/ug protein) were mixed with 0.1 ml of each immunoadsorbent in triplicate, in a total volume of 1 ml of Dulbecco's PBS containing 0.05% BSA and 2 mM PMSF, overnight at 4°C for 18 hr.

Table 2: In Vitro hydrolysis of FITC-Subtilisin to < 10 K by Preiss Cell Extracts[a]

Cell Fraction	pH 5.2	pH 5.2	pH 7.7	pH 7.7
Cell Extract	+	-	+	-
Initial RFU	100	100	100	100
< 10 K Fraction	44	0.46	27	19

[a]Preiss cell extracts (50 μl) were adjusted to pH 5.2 with 0.1 M sodium acetate or to pH 7.7 with 0.1 M sodium bicarbonate in a total volume of 1 ml and incubated with 15 μg of PMSF inactivated FITC-subtilisin (22 RFU/μg protein) at 37°C for 4 hr. The contents of each tube were centrifuged on a Centriprep YM-10 concentrator and the percentage of fluorescent activity recovered in the low molecular weight fraction calculated.

Table 3: Tracking FITC-Subtilisin in an antigen uptake experiment with acid extraction[a]

Cell Fraction	RFUs	Protein (μg)	% Recovered
Conjugate added	140,000	15555	100%
Washed cells	750	83	0.53
Cell extract	261	29	0.18
< 10 K fraction	26	2.9	0.018

[a]5×10^8 8.1.6 cells were resuspended in 300 ml of complete media, mixed with 15.5 mg of fluorescein-subtilisin and incubated for at 37°C for 24 hr in a roller bottle.

Table 4: Summary of Treatments to Increase Exogenous Uptake of FITC-Subtilisin

	Treatment	Uptake and Processing
1.	Active Subtilisin	Cell Lysis
2.	TCA-precipitated FITC-subtilisin	Uptake, no increase in processing
3.	Autolysed FITC-subtilisin	Uptake, no increase in processing
4.	FITC-subtilisin antibody conjugate	Uptake, no increase in processing
5.	FITC-subtilisin crosslinked with glutaraldehyde	Uptake, no increase in processing
6.	Carriers (BioPorter, Chariot, TAT peptide) + FITC-subtilisin	Uptake, no increase in processing
7.	Liposomes + FITC-subtilisin	Uptake, no increase in processing
8.	Alpha-2-macroglobulin + FITC-subtilisin	No increase
9.	IFN gamma + FITC-subtilisin	10–25% increase in processing
10.	Pulse 2 hr, 12 hr, 24 hr, 36 hr, 48 hr	24 hr best
11.	Antigen dose 100–900 μg/mL	100–200 μg/mL best

Table 5: Tracking FITC-Subtilisin in a large scale antigen uptake experiment with immuno-affinity purification[a]

Fraction	RFUs	Protein (μg)	% Recovered
Pulsed Cells	133800	223000	100%
Washed Cells	1358	22630	1
Cell lysate	558	930	0.4
Immunoaffinity column flow-through	343	571	0.20
< 10 K flow-through fraction	79.2	132	0.06
DR-eluted peptides	31	51	0.02

[a]8.5×10^9 8.1.6 cells were mixed with 223 mg of conjugate (0.6 RFU/ug) in 1600 ml of complete media and incubated at 37°C for 22 hr.

Table 6: Subtilisin Peptides Identified from Subtilisin Antigen Uptake in Human Cells

Cells	HLA	Peptide Source	Subtilisin peptide	Peptide sequence	Theory (Da)	Found (Da)	Ion match
8.1.6	DR3	Cell extract	114–126	AIANNMDVINMSL	1405.7	1406.1	10/24
Preiss	DR4	Cell extract	108–122	INGIEWAIANNMDVI	1672.8	1671.6	9/28
8.1.6	DR3	Cell extract	43–53	KVAGGASMVPS	1003.5	1004.3	10/20
KG-1	DR11, DR14	DR-bound	70–84 *	GTVAALNNSIGVLGV	1384.6	1384.78	12/28

* Identified by Q-TOF analysis. The others were identified by LC-MS analysis.

tilisin and HLA-DR was extracted from the cell lysates. These cells are more proficient at antigen uptake than B cell lines though less amenable to large-scale cultures. DR-bound peptides were eluted and analyzed on the Q-TOF and a subtilisin peptide was identified (Table 6) in a single experiment. This peptide corresponded to a region of subtilisin, 70–84 that had been identified as an epitope in another study (unpublished data).

Discussion

The use of fluorescent conjugates to investigate immunological processes has a long history. This study exemplifies their use in tracking antigen to identify optimal conditions for antigen uptake and processing.

Subtilisin is one of the most extensively studied of all bacterial proteins, due to its commercial importance [20,21]. Our laboratory is interested in its potential role as an antigen [13]. In order to identify naturally processed peptides from subtilisin, we devised a simple way to label, stabilize, measure, and track the protein in cell studies. We demonstrate the preparation and characterization of FITC-subtilisin conjugates, and analysis of antigen taken up and processed by human cell lines.

We have shown that subtilisin can be labelled with FITC with generally two or fewer moles of FITC per mole of protein. These conjugates retain antigenicity and up to 30% of their enzymatic activity on small substrates. These conjugates can be degraded by cellular extracts of mammalian cells at pH 5.3 showing the FITC label does not prevent hydrolysis. We show that the fluorescein residues on FITC-subtilisin are present on the N terminus and two other lysine residues. Though the final processed peptides may contain these fluorescein residues, they are present in minute amounts in the low molecular weight fractions, precluding RFU detection within the sensitivity of our current detection systems.

Mass spectrometry analysis of the low molecular weight fraction showed detectable and identifiable subtilisin peptides when the specific RFU/μl of sample was 0.5 or more. Thus, using the 10 K fraction, if 50 RFU are isolated after peptide elution, assuming an average mw of 1500 daltons, this signa1 represents about 55 ng of peptides. The 55 ng peptides when prepared for mass spectrometry analysis would be in the picomole to subpicomole range, which is consistent with current mass spectrometry detection limits and with the calibrated sensitivity of our LC-MS system at 0.5–1.0 picomoles of peptide spiked into cell extracts without antigen. Our detection limits are also comparable to reports in the literature where self-peptides presented by class II MHC molecules induced by a measles vaccine virus infection were present at 0.5 pmol and identified [22].

Our results show that although cell associated RFUs following extensive washes could be indicative of impressive uptake responses; these did not translate to processing of antigen into low molecule weight peptides in the cell

extracts or into peptides bound to MHC molecules. We believe the high RFUs associated with cells may be non-specific adherence of the antigen to the cell membranes. The B-LCL cells endocytosed very minute amounts of antigen (less than 1% of incubated antigen), of which only 10–20% were low molecular weight peptides of less than 10,000 daltons. In conclusion, despite testing several different conditions, none increased antigen uptake significantly in the B-LCL cells, consistent with the low efficiency of non-receptor mediated antigen uptake by B cells [9,23].

Our peptide analysis results from multiple experiments shows three distinctive regions of the subtilisin molecule that are detected in the cellular extracts by mass spectrometry. The one DR-bound peptide detected is from a different region of the molecule and corresponds to a previously identified subtilisin epitope (unpublished data). This DR-bound peptide was analyzed on the Q-TOF, which is more sensitive than the LC-MS system. The peptides detected in the cellular extracts by LC-MS could be peptides that are processing intermediates of peptides to be bound and presented on class I or class II molecules or simply regions of the protein more stable in the presence of cellular degrading enzymes. Our data do not distinguish between these possibilities.

Conclusions

In conclusion, we have demonstrated that FITC conjugation can be used successfully to track antigen uptake and processing and detect processed peptides in the cellular extracts and DR-bound peptides in human cells. This method is simple, efficient, and relatively inexpensive. Thus it would be useful to study the uptake and processing of several other antigens. Improved mass spectrometry methods to detect the presence of MHC bound naturally processed peptides would further enhance the utility of the described method. Our future studies will focus on studying uptake and processing of other FITC labelled antigens and improving mass spectrometry methods to detect the presence of other MHC bound naturally processed peptides.

Methods

Enzyme

Subtilisin was a single amino acid mutant (BPN' Y217L) prepared from *Bacillus amyloliquefaciens* (Swissprot accession number P00782) and purified by crystallization. It was formulated in 50% glycerol at 55 mg/ml and stored at -17°C, where it was stable. Subtilisin activity was measured in a kinetic assay with the synthetic substrate sucAAPF-pNA as previously described [20].

Preparation of FITC-subtilisin conjugates

Two milliliters of enzyme were desalted over a Sephadex G-25 column (PD-10 Sulpeco, Bellefonte, FA) equilibrated in 100 mM sodium carbonate, pH 9 at 4°C. Phenylmethyl sulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO) dissolved at 100 mM in absolute ethanol was added to 2 mM FITC mixed isomer (Sigma Chemical Company, St. Louis, MO) dissolved at 2 mg/ml in 100 mM sodium carbonate buffer. In an ice bath, 0.5 ml of FITC solution was added to 2 ml of enzyme containing 5 to 50 mg/ml and the reaction allowed to proceed in the dark for 90 minutes and then an additional 0.25 ml of FITC solution was added for 30 min. FITC conjugated subtilisin (FITC-subtilisin) was isolated by Sephadex G-25 chromatography equilibrated in Dulbecco's PBS, pH 7.1 at 4°C. The collected void volume containing FITC-subtilisin was brought to 2 mM PMSF, covered in aluminum foil, and stored in aliquots at -17°C.

Characterization of FITC-subtilisin conjugates

Protein concentration was measured using the BCA Protein Assay (Pierce, Rockford, IL) adapted to microtiter plates using a bovine serum albumin standard. Relative fluorescent units (RFU)/μg protein were determined on an fmax Fluorescence Microplate Reader (Molecular Devices Corp. Sunnyvale, CA), with 485 nm excitation and a 535 nm emission filters. Experimental samples were assayed in triplicate by dilution in Dulbecco's PBS to RFU values of 50 or less. Blank values were corrected if they were 1 RFU or more. Comparisons between experiments used conjugates with comparable RFU/protein activities, generally between 20 and 22 RFU/μg protein. Reproducible differences of 25% or more were considered significant.

Cells

EBV-transformed B lymphoblastoid cell lines 8.1.6, and Preiss, were a kind gift from Dr. Elizabeth D. Mellins, Stanford Univ., CA [24]. KG-1 cells were obtained from ATCC [21]. Both cell types were grown by culture in RPMI 1640 1X (MOD) media with 25 mM Hepes buffer (Mediatech, Herndon, VA), Heat-inactivated fetal bovine serum (HyClone, Logan, Utah) was added to 10 % along with 55 μM 2-ME, 100 μg/ml streptomycin, 100 U/ml of penicillin, and 0.29 mg/ml glutamine (Life Technologies, Grand Island, *NY*). Large quantities of cells were grown in 2 L roller bottle culture flasks (Corning Inc., Corning, *NY*).

Pulsing and harvesting cells

Cells were cultured to 1–1.2 × 10^6/ml and resuspended in 400 ml of complete medium to a concentration between 0.5–1 × 10^7 per ml and 150–250 μg/mL PMSF inactivated FITC-subtilisin. Cells were cultured at 37°C for 2 hr and then diluted 1:2 in complete medium. After 20 hrs cells were washed extensively in Dulbecco's PBS.

Acid lysis of cells

Washed cell pellets were lysed by adding 0.2 M HCL to a final concentration of 1×10^8 cells/ ml and shaken vigorously for 45 min at 4°C. Cells were centrifuged at 15,000 g for 30 min in a Sorvall RC-5B Centrifuge (Sorvall Centrifuges, Newtown, CT) and the supernatant was concentrated in an Amicon Centriprep YM-10 filter device (Millipore Inc., Billerica, MA) at 3000 g. The fraction less than 10 K was collected and frozen at -17°C, or lyophilized and stored at -17°C. To remove salt and reduce detergent from low molecular weight fractions, acid cell extracts and detergent lysates were purified on 100 mg, 3 ml Bond Elut-C18 columns (Varian, Harbor City, CA). The FITC associated fractions were eluted in 1 ml of 50% acetonitrile. Eluted peptides were lyophilized and stored at -17°C until analyzed.

DR purification

For affinity purification of HLA-DR molecules, washed cells were resuspended in lysis buffer (0.1 % Nonidet P-40, 50 mM Tris-HCL pH 8.5, 0.15 mM NaCl) at 1×10^8 cells /ml following the procedure of Gorga et al. [25]. Briefly, cells were lysed using15 lbs nitrogen pressure at 4°C in a 35 ml Parr implosion device (Parr Inst. Co., Moline, IL), and homogenized 30X in a 100 ml Dounce Homogenizer (Kontes Glass Co., Vineland, NJ). Extracts were cleared of debris and nuclei by centrifugation at 10,000 × g for 1 hr at 4°C and the supernatant fluid containing solubilized HLA DR molecules was purified by affinity chromatography as described [9]. The eluate was separated into a low molecular weight (10 K) fraction as described above and frozen at -17°C.

Preparation of immunoadsorbents

Subtilisin and purified polyclonal antibody to subtilisin were coupled to cyanogen bromide-activated Sepharose (Sigma Chemical Co., St. Louis, MO) as described [26]. Mouse monoclonal antibody against FITC was purchased from Sigma Chemical Co. and immobilized similarly.

Peptide synthesis, HPLC, mass spectrometry, MS data analysis

Subtilisin peptides 70–84 and 114–126 were synthesized (Applied Biosystems, 4331 Peptide Synthesizer, Foster City, CA) in-house and purified to 90%. All MS and MS/MS data were acquired using the Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) coupled to the LCQ Classic Ion Trap Mass Spectrometer (ThermoFinnigan, San Jose, CA) or the CapLC system with the Q-TOF Ultima API MS (Micromass, Milford, MA). The HPLC gradient was programmed from 0 to 70% B over 70 minutes. Solvent A: 0.1% formic acid (FA) in water and solvent B: 0.1% FA in acetonitrile. Data processing was performed using TurboSequest and Xcalibur software (ThermoFinnigan) or the Masslynx program (Micromass).

Authors' contributions

HCM did the fluorescent derivatization and characterization work. DLW performed the mass spectrometry analysis. NSP and KDC did the DR bound-peptide identification experiments. NSP and HCM wrote the manuscript, all authors read and approved it.

Acknowledgements

We would like to thank Fiona Harding for her guidance, Roopa Ghirnikar for her assistance in editing the manuscript and Meng Heng for generously sharing the purified subtilisin protein.

References

1. Watts C: **Capture and processing of exogenous antigens for presentation on MHC molecules.** *Annu Rev Immunol* 1997, **15:**821-850.
2. Solheim JC: **Antigen processing and presentation protocols.** *Methods in Molecular Biology Volume 156*. Totowa NJ, Humana Press; 2001.
3. Nelson CA, Roof RW, McCourt DW, Unanue ER: **Identification of the naturally processed form of hen egg white lysozyme bound to the murine major histocompatibility complex class II molecule I-Ak.** *Proc Natl Acad Sci U S A* 1992, **89:**7380-7383.
4. Vignali DA, Urban RG, Chicz RM, Strominger JL: **Minute quantities of a single immunodominant foreign epitope are presented as large nested sets by major histocompatibility complex class II molecules.** *Eur J Immunol* 1993, **23:**1602-1607.
5. Hunt DF, Michel H, Dickinson TA, Shabanowitz J, Cox AL, Sakaguchi K, Appella E, Grey HM, Sette A: **Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-Ad.** *Science* 1992, **256:**1817-1820.
6. Hunt DF, Henderson RA, Shabanowitz J, Sakaguchi K, Michel H, Sevilir N, Cox AL, Appella E, Engelhard VH: **Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry.** *Science* 1992, **255:**1261-1263.
7. Dongre AR, Kovats S, deRoos P, McCormack AL, Nakagawa T, Paharkova-Vatchkova V, Eng J, Caldwell H, Yates J. R., 3rd, Rudensky AY: **In vivo MHC class II presentation of cytosolic proteins revealed by rapid automated tandem mass spectrometry and functional analyses.** *Eur J Immunol* 2001, **31:**1485-1494.
8. Rodriguez GM, Diment S: **Destructive proteolysis by cysteine proteases in antigen presentation of ovalbumin.** *Eur J Immunol* 1995, **25:**1823-1827.
9. Moore SE, Schofield JP, Watts C: **Low abundance of naturally processed T-cell determinants following antigen pulsing of antigen-specific human B-cell lines.** *Immunology* 1995, **85:**523-530.
10. Phelps RG, Turner AN, Rees AJ: **Direct identification of naturally processed autoantigen-derived peptides bound to HLA-DR15.** *J Biol Chem* 1996, **271:**18549-18553.
11. Peakman M, Stevens EJ, Lohmann T, Narendran P, Dromey J, Alexander A, Tomlinson AJ, Trucco M, Gorga JC, Chicz RM: **Naturally processed and presented epitopes of the islet cell autoantigen IA-2 eluted from HLA-DR4.** *J Clin Invest* 1999, **104:**1449-1457.
12. Velazquez C, DiPaolo R, Unanue ER: **Quantitation of lysozyme peptides bound to class II MHC molecules indicates very large differences in levels of presentation.** *J Immunol* 2001, **166:**5488-5494.
13. Stickler MM, Estell DA, Harding FA: **CD4+ T-cell epitope determination using unexposed human donor peripheral blood mononuclear cells.** *J Immunother* 2000, **23:**654-660.
14. Harris NR, Whitt SP, Zilberberg J, Alexander JS, Rumbaut RE: **Extravascular transport of fluorescently labeled albumins in the rat mesentery.** *Microcirculation* 2002, **9:**177-187.
15. So AL, Small G, Sperber K, Becker K, Oei E, Tyorkin M, Mayer L: **Factors affecting antigen uptake by human intestinal epithelial cell lines.** *Dig Dis Sci* 2000, **45:**1130-1137.
16. Thompson VF, Saldana S, Cong J, Goll DE: **A BODIPY fluorescent microplate assay for measuring activity of calpains and other proteases.** *Anal Biochem* 2000, **279:**170-178.

17. Evans KL, Crowder J, Miller ES: **Subtilisins of Bacillus spp. hydrolyze keratin and allow growth on feathers.** *Can J Microbiol* 2000, **46:**1004-1011.
18. Hentz NG, Richardson JM, Sportsman JR, Daijo J, Sittampalam GS: **Synthesis and characterization of insulin-fluorescein derivatives for bioanalytical applications.** *Anal Chem* 1997, **69:**4994-5000.
19. Buranda T, Lopez GP, Simons P, Pastuszyn A, Sklar LA: **Detection of epitope-tagged proteins in flow cytometry: fluorescence resonance energy transfer-based assays on beads with femtomole resolution.** *Anal Biochem* 2001, **298:**151-162.
20. Wells JA, Estell DA: **Subtilisin--an enzyme designed to be engineered.** *Trends Biochem Sci* 1988, **13:**291-297.
21. Harwood CR: **Bacillus subtilis and its relatives: molecular biological and industrial workhorses.** *Trends Biotechnol* 1992, **10:**247-256.
22. Ovsyannikova IG, Johnson KL, Naylor S, Poland GA: **Isolation and rapid identification of an abundant self-peptide from class II HLA-DRB1*0401 alleles induced by measles vaccine virus infection.** *J Immunol Methods* 2000, **246:**1-12.
23. Stockinger B: **Capacity of antigen uptake by B cells, fibroblasts or macrophages determines efficiency of presentation of a soluble self antigen (C5) to T lymphocytes.** *Eur J Immunol* 1992, **22:**1271-1278.
24. Mellins E, Woelfel M, Pious D: **Importance of HLA-DQ and -DP restriction elements in T-cell responses to soluble antigens: mutational analysis.** *Hum Immunol* 1987, **18:**211-223.
25. Gorga JC, Horejsi V, Johnson DR, Raghupathy R, Strominger JL: **Purification and characterization of class II histocompatibility antigens from a homozygous human B cell line.** *J Biol Chem* 1987, **262:**16087-16094.
26. Hochleitner EO, Gorny MK, Zolla-Pazner S, Tomer KB: **Mass spectrometric characterization of a discontinuous epitope of the HIV envelope protein HIV-gp120 recognized by the human monoclonal antibody 1331A.** *J Immunol* 2000, **164:**4156-4161.