Original Article

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Substrate specificity of human granzyme 3: Analyses of the P3-P2-P1 triplet using fluorescence resonance energy transfer substrate libraries

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Summary **Granzyme 3 (Gr3) is known as a tryptase-type member of the granzyme family and exists in the granules of immunocompetent cells. Granule proteases including granzymes, are transported into the cytoplasm of tumor cells or virus-infected cells by perforin function, degrade cytoplasmic or nuclear proteins and subsequently cause the death of the target cells. Recently, although several substrates of Gr3 *in vivo* have been reported, these hydrolyzed sites were unclear or lacked consistency. Our previous study investigated the optimal amino acid triplet (P3-P2-P1) as a substrate for Gr3 using a limited combination of amino acids at the P2 and P3 positions. In the present study, new fluorescence resonance energy transfer (FRET) substrate libraries to screen P2 and P3 positions were synthesized, respectively. Using these substrate libraries, the optimal amino acid triplet was shown to be Tyr-Phe-Arg as a substrate for human Gr3. Moreover, kinetic analyses also showed that the synthetic substrate FRETS-YFR had the lowest K_m value for human Gr3. A substantial number of membrane proteins possessed the triplet Tyr-Phe-Arg and some of them might be *in vivo* substrates for Gr3. The results might also be a great help for preparing specific inhibitors to manipulate Gr3 activity both *in vitro* and *in vivo*.**

Keywords: Granzyme, serine protease, cytotoxic T lymphocytes

1. Introduction

Granzyme 3 (Gr3; also called granzyme K) is known as a member of the protease family named granzymes, and exists in the cytotoxic granules of cytotoxic cells in the immune-system (*1,2*). Proteases in the granules of NK cells and cytotoxic T-lymphocytes are introduced into their target cells, such as virus-infected cells and tumor cells, *via* a perforin-mediated mechanism, then hydrolyze cytosolic and/or nuclear proteins, and kill the cells (*3*). Five kinds of granzymes have been identified in humans. Two of them, granzyme A and Gr3, are known to show tryptase-type specificity and to cleave the peptide bond on the carboxylic side of a basic amino acid, Lys or Arg (*4*). Recently, some *in vitro* and *in vivo* substrates of Gr3 have been reported, however, some of their cleavage sites are unclear and the proposed amino acid sequences of the hydrolysis site differ among reports (*4-8*).

In order to clarify the *in vivo* function of Gr3 and to develop specific inhibitors for the manipulation of enzymatic activity *in vivo*, analysis of the *in vitro* specificity of Gr3 is being attempted.

Fluorescence resonance energy transfer (FRET) is a phenomenon in which electronic excitation energy can be efficiently transferred between a fluorescent energy donor and a suitable energy acceptor. FRET is often applied to determine the distance between a donor fluorophore and an acceptor fluorophore on the basis of intramolecular FRET (*9*). The FRET substrate (FRETS) contains a fluorescent 2-(*N*-methylamino) benzoyl (NMA) group as a donor fluorophore and a 2,4-dinitrophenyl (DNP) group as an acceptor in the molecule. When an enzyme cleaves any peptide bond between NMA and DNP in the substrate, the fluorescence at 440 nm with excitation at 340 nm increases in proportion to the release of the NMA fluorophore from the internal DNP quencher (*10*). The

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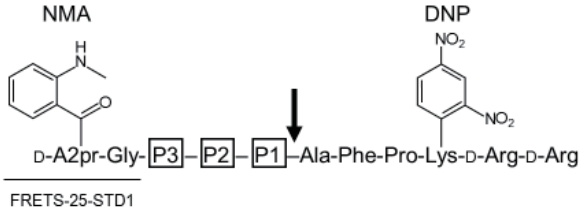
FRET substrate allows us to determine the enzymatic hydrolysis of a peptide bond between amino acids.

We previously investigated the optimal amino acid triplet (P3-P2-P1) as a substrate for Gr3 using FRETS libraries. As the P1 residue, Arg, is better than Lys by Gr3. Out of 25 P3-P2 combinations, Phe-Tyr was the sequence that reacted the most with Gr3 (*11*); however, the result was obtained using a substrate library with limited amino acid sequences. Therefore, in order to elucidate the optimal amino acid sequence of Gr3, new peptide substrate libraries were prepared in the present study. Using the new libraries, the optimal triplet was defined.



	P3	P2	P1
FRETS-25Arg	Phe,Ala,Val,Glu,Arg	Pro,Tyr,Lys,Ile,Asp	Arg
FRETS-F19R	Phe	19 natural amino acids excluding Cys	Arg
FRETS-19FR	19 natural amino acids excluding Cys	Phe	Arg

Figure 1. Structure of fluorescence resonance energy transfer substrates (FRETS) used in the present study. The arrow shows the desired hydrolytic site for Gr3. The structure of FRETS-25-STD1 used for the calibration standard is also shown.

2. Materials and Methods

2.1. *Materials*

FRETS-25Arg, FRETS-F19R, and FRETS-19FR were synthesized by the Peptide Institute, Inc. (Osaka, Japan). FRETS-25-STD1 (*N*-β-[2-(*N*-methylamino) benzoyl] -2,3-diaminopropionyl-glycine) was also purchased from the Peptide Institute, Inc. The micro BCA Protein Assay Reagent kit was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). *N*-α-Benzyloxycarbonyl-lysine thiobenzyl ester (BLT) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Trifluoroacetic acid (TFA) and acetonitrile (MeCN) for high performance liquid chromatography (HPLC) analysis were purchased from Kanto Chemical Co. (Tokyo, Japan). Wakosil-II 5C18 RS was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Other chemicals used were all of analytical grade.

2.2. *Recombinant human Gr3*

Recombinant human Gr3 was produced and purified as previously described (*11*). The protein concentration was determined by a bicinchoninic acid assay (BCA assay). Gr3 activity was determined as the hydrolysis of BLT. Purified protein was dissolved in 10 mM potassium phosphate buffer, pH 6.0, 1 M NaCl, supplemented with 0.1% Triton X-100 to prevent adsorption to the tubes, divided into aliquots in siliconized tubes (Sarstedt K.K., Tokyo, Japan), and stored at –80°C until use.

2.3. *FRET substrate (FRETS) libraries*

Three sets of libraries were used in this study (Figure 1). The libraries were prepared by the Peptide Institute, Inc. Based on the structure of FRETS-25Arg, which was used in the previous study, new libraries were prepared. These fluorescence resonance energy transfer substrates contain a highly fluorescent NMA group and an efficiently quenched DNP group in the molecule. When an enzyme cleaves any peptide bond between NMA and DNP in the substrate, the fluorescence at 440 nm with excitation at 340 nm increases in proportion to the release of the NMA fluorophore from the internal DNP quencher (*10*).

To analyze the specificity at the P2 position, FRETS-F19R was designed. The P1 position of the substrate sequence was fixed as Arg according to our results using the FRETS-25Xaa series, as described previously (*11*). Because the substrate carrying the Phe-Tyr-Arg (P3-P2-P1) triplet contained in the FRETS-25Arg library was hydrolyzed the fastest by Gr3 (*11*), Phe was employed at the P3 position. The P1 and P3 positions were fixed with Arg and Phe, respectively, and each of the 19 amino acids excluding Cys was incorporated in the P2 position in the FRETS-F19R library.

Based on the results obtained with FRETS-F19R, FRETS-19FR was designed to determine the specificity at the P3 position. The P1 and P2 positions were fixed with Arg and Phe, respectively. Each of the 19 amino acids excluding Cys was incorporated at the P3 position.

FRETS-YFR, FRETS-FFR, FRETS-WFR, and FRETS-VFR were isolated from the FRETS-19FR library using reversed-phase HPLC and used for kinetic analyses. The concentration of the purified substrate was determined using FRETS-25-STD1 as a reference standard.

2.4. *Enzyme activity with FRET substrates*

To 100 nM of purified Gr3 in 0.2 M Tris-HCl buffer (pH 8.0), 0.1 mM (final) FRET substrate was added and incubated for up to 15 min at 37°C. The enzymatic reactions were stopped with the addition of a 9-fold volume of 0.2% TFA. After filtration through a 0.45 μm membrane filter, a 100 μL aliquot was injected into HPLC. HPLC conditions used were the same as described previously (*11*). Hydrolytic activity was

determined from the peak area of the hydrolyzed product.

The amino-acid sequences of the separated products were identified according to the product description sheets of the libraries. In addition, some amino acid sequences were verified using the combination of absorption spectrometry and fluorescence analysis. The products containing Tyr were identified by detection with absorbance at 280 nm and fluorescence at 440 nm with excitation at 275 nm, which occurred by fluorescence energy transfer from Tyr to NMA. Similarly, a Trp-containing product was confirmed by detection of fluorescence at 440 nm with excitation at 295 nm.

2.5. *Kinetic analyses*

For determination of the kinetic parameters, Gr3 (5 nM) was incubated with 16 to 80 μM of each FRET substrate, FRETS-YFR, FRETS-FFR, FRETS-WFR or FRETS-VFR, for 15 min at 37ºC. Then a 9-fold volume of 0.2% TFA was added to stop the reaction. After filtration through a 0.45 μm membrane filter, a 100 μL aliquot was injected into HPLC. HPLC conditions used were as follows. The column was Wakosil-II 5C18 RS (4.6 I.D. × 150 mm) and was maintained at 45°C. Gradient elution was performed with mobile phase A (0.1% TFA in H_2O) and mobile phase B (0.1% TFA in MeCN). The flow-rate was set at 1.0 mL/min and the mobile phase composition was changed from 0% to 44% B over a period of 22 min. The products were detected by absorbance at 254 nm or fluorescence at 440 nm with excitation at 340 nm. The hydrolysis rate was determined as an increase of the peak area of cleaved products. Michaelis constants (K_m), the maximum velocities (V_{max}), k_{cat} values and k_{cat}/K_m values were calculated using Lineweaver-Burk plots (*12*).

2.6. *The protein sequence library search*

The proteins possessing YFR tripeptide, the amino acid sequence specifically decomposed by Gr3, were searched in the NCBI transcript sequence database using the gene and transcript searching system called GGRNA ver.1 (*http://ggrna.dbcls.jp/v1/*) (*13*). This system can quickly retrieve the information on the transcription product registered into the NCBI database according to the existence of short amino acid sequences, such as tripeptides. Multiple transcripts from a single gene and transcripts that are not identified at a protein level were excluded before analysis.

3. Results

3.1. *Determination of the P2 position using FRETS-F19R*

To determine the optimal amino acid at the P2 position, the FRETS-F19R library was prepared (Figure 1). After reaction with Gr3, three major hydrolyzed products were observed (Figure 2). The peaks increased in a time-dependent manner (Figure 2 inset). By comparing the data with those of unhydrolyzed substrates and of the tryptic digest of FRETS-F19R, peak 1 to 3 were identified as Phe-Phe-Arg, Phe-Tyr-Arg, and Phe-Trp-Arg, respectively. FRETS hydrolyzed products were monitored under two conditions, absorption of Phe residue at 254 nm and fluorescence of NMA group at 440 nm with excitation at 340 nm. For the most abundant peak (peak 1), the ratio of absorbance at 254 nm to fluorescence at 440/340 nm was higher than that of other peaks (data not shown). This result showed that peak 1 was confirmed as the Phe-Phe-Arg product which contained more Phe residues than others. The retention time of peak 2 was the same as Phe-Tyr-Arg in the hydrolyzed product of FRETS-25Arg under the same HPLC conditions; therefore, peak 2 was confirmed as Phe-Tyr-Arg. The third peak, eluted around 82 min, was identified as Phe-Trp-Arg according to the data provided by the manufacturer. In addition, when fluorescence at 440 nm was monitored with excitation at 295 nm, the height of peak 3 was markedly higher than other peaks (data not shown). This is due to the fluorescence energy transfer between Trp and NMA residues. First, Trp residues emit fluorescence at 340 nm by excitation at 295 nm, and the NMA group emits fluorescence at 440 nm by the emission of Trp residue at 340 nm. Thus, the products carrying the Trp residue showed fluorescence at 440 nm and excitation at 295 nm.

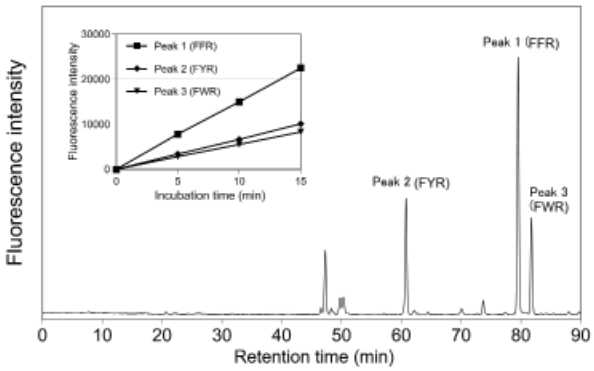


Figure 2. HPLC chromatogram of the hydrolyzed products of FRETS-F19R by Gr3. FRETS-F19R was incubated with Gr3 for 15 min at 37ºC. Inset shows the peak area of peak 1 (FFR), peak 2 (FYR), and peak 3 (FWR). The amino acid sequence of these products is shown as P3-P2-P1 in one letter notation.

3.2. *Determination of the P3 position by FRETS-19FR*

The FRETS-19FR library for screening the optimal amino acid at the P3 position was prepared, in which P1 and P2 positions were fixed as Arg and Phe, respectively, and each of the 19 natural amino acids

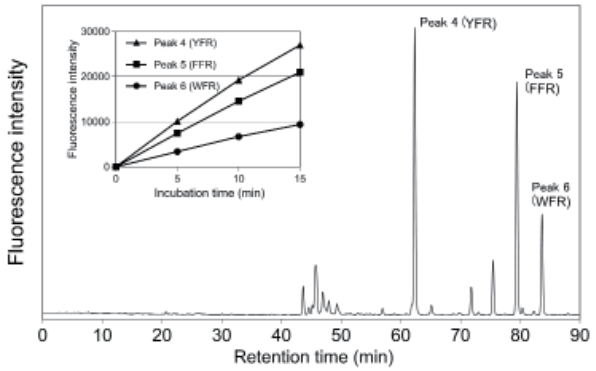


Figure 3. HPLC chromatogram of the hydrolyzed products of FRETS-19FR by Gr3. FRETS-19FR was incubated with Gr3 for 15 min at 37ºC. Inset shows the peak area of peak 4 (YFR), peak 5 (FFR), and peak 6 (WFR).

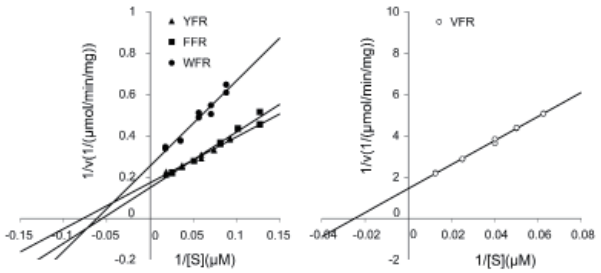


Figure 4. The Lineweaver-Burk plot of the hydrolysis of FRET substrates by Gr3. Gr3 was incubated with 16-80 μM of each FRET substrate for 15 min at 37ºC. The hydrolyzed product was analyzed by HPLC. The results of FRET-VFR were separate from others, which are shown in a separate panel.

Table 1. The K_m, k_{cat}, and k_{cat}/K_m values for hydrolysis of FRET substrates by Gr3

Substrates	K_m (μM)	K_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}•M^{-1})
FRETS-VFR	37.7	0.31	8.23×10^3
FRETS-FFR	18.1	3.14	1.74×10^5
FRETS-WFR	16.3	1.84	1.13×10^5
FRETS-YFR	13.0	2.72	2.11×10^5

excluding Cys appeared at the P3 position (Figure 1).

In the same way as FRETS-F19R, FRETS-19FR was hydrolyzed by Gr3 and analyzed by HPLC. Three major peaks, peaks 4 to 6, were identified as Tyr-Phe-Arg, Phe-Phe-Arg, and Trp-Phe-Arg, respectively (Figure 3).

The highest peak, peak 4, was identified as the Tyr-Phe-Arg product according to the analysis data of unhydrolyzed FRETS-19FR. To confirm that peak 4 is a Tyr-carrying product, the HPLC analysis condition was changed; the fluorescence at 440 nm was monitored with excitation at 275 nm instead of 340 nm. Under this condition, peak 4 became higher than other peaks (data not shown). This result confirmed that peak 4 was a Tyr-Phe-Arg product, in which the fluorescence of Tyr residue at around 320 nm upon excitation at 275 nm acted as the excitation light for the NMA group. Retention time of peak 5 in FRETS-19FR hydrolyzed products was the same as Phe-Phe-Arg, peak 1 in Figure 2; therefore, peak 5 was confirmed as Phe-Phe-Arg. Peak 6 showed strong fluorescence at 440 nm with excitation at 295 nm, which indicated that the Trp-containing product was eluted at peak 6 (data not shown); thus, peak 6 was confirmed as Trp-Phe-Arg.

All three peaks increased with time and peak 4 (Tyr-Phe-Arg) was the highest (Figure 3 inset). These results indicated that Tyr-Phe-Arg was the most suitable substrate sequence for Gr3 in the FRETS-19FR library.

3.3. *Kinetic analysis using FRETS-YFR, FRETS-FFR, FRETS-WFR, and FRETS-VFR*

To analyze the reactivity of Gr3 more precisely, kinetic analyses were carried out using isolated FRET substrates. Four kinds of FRET substrates, FRETS-YFR, FRETS-FFR, FRETS-WFR, and FRETS-VFR, carrying Tyr-Phe-Arg, Phe-Phe-Arg, Trp-Phe-Arg, and Val-Phe-Arg, respectively, were isolated from FRETS-19FR using HPLC.

Using these purified substrates, Gr3 activity was assayed and Lineweaver-Burk plots were drawn (Figure 4). From these plots, the kinetic parameters for the four substrates were calculated (Table 1). Among the four, FRETS-YFR showed the minimum K_m value of around 13.0 μM and the others were in the following order, WFR < FFR << VFR. On the other hand, FRETS-FFR showed the maximum k_{cat} value and the others were in the following order FRETS-YFR > WFR >> VFR.

When calculating the k_{cat}/K_m values, the order was FRETS-YFR > FFR > WFR >> VFR (Table 1), which coincided with the order of the peak heights of the hydrolyzed FRETS-19FR (Figure 3). These results indicated that the Tyr-Phe-Arg (P3-P2-P1) triplet was the most suitable substrate for human Gr3, under both purified and mixed conditions.

3.4. *The proteins potentially hydrolyzed by Gr3*

As a result of the library search using GGRNA, the number of human proteins that possess YFR tripeptide, the amino acid sequence specifically hydrolyzed by Gr3 were 769. Out of the 769 kinds of proteins, 222 proteins are membrane bound proteins.

4. Discussion

From the results using FRETS-F19R, aromatic amino acids were considered more favorable at the P2 position for Gr3 substrate; however, the Phe-Phe-Arg substrate was hydrolyzed much faster than the other two (Phe-Tyr-Arg and Phe-Trp-Arg) and the selectivity of the P2 site amino acid by Gr3 seemed to be high.

Based upon the results with FRETS-F19R, another substrate library, FRETS-19FR, was prepared for the

analysis of P3 position selectivity.

Similar to the P2 position, Gr3 was shown to prefer aromatic amino acid at P3 position; however, the difference between the fastest Tyr-Phe-Arg and the second Phe-Phe-Arg was smaller than the case of the P2 position, indicating that the stringency of the amino acid specificity at P3 position was lower than at P2 position.

The results described above were obtained using a substrate library, mixtures of the substrates. In order to declare substrate specificity more precisely, kinetic analyses using purified substrates were needed. When comparing the kinetic parameters, Tyr-Phe-Arg substrate showed the smallest K_m value, indicating that human Gr3 showed the highest affinity for the triplet (Table 1). In addition, the k_{cat}/K_m values obtained from the kinetic study coincided well with the results of the hydrolysis pattern of the FRETS-19FR library. The Tyr-Phe-Arg substrate proved to be the most favorable for human Gr3 also with purified FRETS-YFR in addition to the substrate library mixture.

Mahrus and Craik (*5*) reported substrate specificity analysis of several granzymes using positional scanning synthetic combinatorial libraries (PS-SCL) of ACC substrates. Their results indicated that Tyr, Arg, Phe, and Lys were the most reactive with human GrK (equal to Gr3) for P4, P3, P2, and P1 positions, respectively. Based on the results, they concluded that the Tyr-Arg-Phe-Lys (P4-P3-P2-P1) quartet was the most suitable for human Gr3. Compared with our present results, only the P2 position was consistent. In particular, the difference of the P1 position was evident. This discrepancy might be due to differences in the structure of the carboxyl side of the P1 position. The FRET substrate used in the present study has an alanine residue at the P1' position, whereas the ACC substrate has a substituted coumarin group. This difference might affect the substrate recognition and/or enzymatic reaction.

Synthetic substrates, those that have *p*-nitroanilide (pNA) or 4-methylcoumarinamide (MCA) as chromogenic groups, were hydrolyzed much slower by Gr3 than by trypsin. Moreover, some of them were hardly hydrolyzed by Gr3 (*11*). These results suggested that an aromatic ring of pNA and MCA substrates might reduce the binding of these substrates to the active site of Gr3. In contrast, FRETS have oligopeptide structures in the potentially hydrolyzed position. Therefore, FRETS were suitable for analyzing substrate specificity and also for searching for the physiological substrates, because the *in vivo* targets of Gr3 are considered to be proteins.

In addition, in the PS-SCL method, the most suitable amino acid residue, was determined at each position individually and their combination was not analyzed. That might be the reason why their and the present results are not consistent. Their result for the P3 position is similar to our previous results that Arg residue was the second choice for P3 position after Phe out of 5 amino acids (Phe, Arg, Ala, Val, Glu) (*11*).

Another *in vitro* specificity analysis of human Gr3 was carried out using the PepChip protease array, which consisted of more than 1,000 randomized 15 amino acid polypeptides (*7*). The results indicated that Gr3 preferred polypeptides which had Arg, aromatic and hydrophobic amino acids at P1, P2, and P4 positions, respectively. The results were approximately consistent with our results.

On the other hand, several candidates for an *in vivo* substrate for Gr3 have been reported. Gr3 reportedly hydrolyzes the nuclear SET complex of the target cells in a similar manner to granzyme A (*7,14,15*). This is considered to result in the release of NM23-H1 and degradation of ssDNA. Moreover, Gr3 was investigated to degrade hnRNP K (*16*) and Bid, which related to mitochondria-mediated apoptosis of the cells (*17*).

Recently, the degradation site of several cellular proteins by human Gr3 was reported. It was investigated whether Gr3 hydrolyzed DLWK^{24}↓L and GSTK^{305}↓R of p53 protein (*6*), YVPR^{62}↓A and QQYR^{282}↓A of β-tublin (*7*) and ERER^{713}↓Q of valosin-containing protein (*8*); however, it is very difficult to find some regularity in these results. Similarity to our results from these reports is also hardly found.

Gr3 is stored in intracellular granules as an inactive precursor and transformed to its enzymatically active form by cathepsin C (*18*). The activated enzymes including Gr3 are considered to be transported into the cytoplasm of the target cells, degrade various proteins and cause the subsequent death of the target cells. To determine expression of Gr3 in activated lymphocytes, human lymphokine-activated killer (LAK) cells were prepared by incubating isolated lymphocytes from peripheral blood with IL-2 for 7 days. When the culture supernatant and cell lysates of LAK cells were analyzed using Gr3 ELISA, Gr3 was detected in culture supernatant as well as in cell lysates (data not shown). Therefore, Gr3 might function not only in the target cells but also outside of the cells, such as attacking plasma membrane proteins in blood plasma.

Human Gr3 is a positively charged protein due to its high content of Arg and Lys. Therefore, it can be considered that Gr3 tends to associate with negatively charged biomolecules such as sulfated carbohydrates and phospholipids. In fact, intracellular Gr3 was hardly extracted with physiological saline solution containing some detergents but with buffers containing 1 M NaCl (data not shown). Gr3 binds to negatively charged proteoglycans along with other granzymes when stored in cytotoxic granules in lymphocytes. Once excreted from lymphocytes, Gr3 might bind to a negatively charged cell surface and have some cytotoxic effect by hydrolyzing cell surface proteins. In addition, we considered that the sequence YFR in the membrane

bound protein is likely to be located at a boundary of the membrane surface, because the former two aromatic amino acids are buried in a hydrophobic atmosphere and the positively charged Arg acts as an anchor by interacting with polar residues of membrane phospholipids. Gr3 may hydrolyze membrane bound proteins after invading target cells.

When GGRNA was used to search human proteins that contained YFR sequence, 769 kinds of proteins were found in the NCBI protein database (*13*). Among those proteins, 222 were known to locate on a cellular membrane including nuclear and mitochondrial membranes. Therefore, degradation of membrane proteins by Gr3 might be a potential mechanism of its cytotoxic activity. The involvement of the hydrolysis of membrane bound proteins is not known in the induction of apoptosis by granzymes including granzyme A and granzyme B. Bovenschen *et al*. identified cellular proteins that could be hydrolyzed by Gr3 (*7*). However, they analyzed only cytoplasmic proteins of Jurkat cells. Therefore, membrane bound substrate proteins were not considered in their study. Whether membrane bound proteins are hydrolyzed by Gr3 in the target cells remains to be understood.

This study clearly showed the optimal substrate sequence for Gr3, Tyr-Phe-Arg, by determining the order of the hydrolysis rate. Inhibitors prepared using this sequence might be useful for distinguishing Gr3 from other granzymes *in vitro* and possibly valuable for controlling disease progression, which might involve Gr3 malfunction.

Acknowledgements

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