Rapid assay to detect possible natural substrates of proteases in living cells

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Proteolysis is a regulatory step in many physiological processes, but which proteases in what cellular sites are involved in activation or degradation of which peptides is not well known. We developed a rapid assay consisting of living cells and fluorogenic protease substrates to determine which bioactive peptides are possible natural substrates of a specific protease with the multifunctional or moonlighting protein CD26/dipeptidyl peptidase IV (DPPIV) as a model. CD26/DPPIV catalyzes cleavage of peptides from the amino terminus of peptides with proline at the penultimate position. Many biologically active peptides, such as β -casomorphin₁₋₅, contain proline in the penultimate position. We incubated living Jurkat cells, which are T cells that lack CD26/DPPIV, and CD26/DPPIV-transfected Jurkat cells in the presence of the fluorogenic substrate [Ala-Pro]²-cresyl violet (Magic Red) and β -casomorphin₁₋₅. Fluorescent cresyl violet was generated by CD26/DPPIV-transfected Jurkat cells but not by wild-type Jurkat cells with a K_m of 3.7 μ M. β -Casomorphin₁₋₅ appeared to be a possible natural substrate of CD26/DPPIV, because it inhibited production of fluorescence competitively (K_i = 60 μ M). The assay using living cells and a fluorogenic protease substrate is an efficient system to determine whether specific peptides are possible natural substrates of a particular protease.

INTRODUCTION

Proteolytic activation or degradation of bioactive peptides is a regulatory step in many biological processes, but it is not yet exactly known which peptides are cleaved by which proteases in intact complex cellular systems. For the understanding of the regulation of (local) levels of bioactive peptides in tissues or organs and the development of therapeutic strategies in relation to these peptides, it is essential to know which proteases are involved and in what location in a living organism (1).

The ectoprotease CD26/dipeptidyl peptidase IV (DPPIV) cleaves off terminal dipeptides at the amino terminus of a peptide with proline as the penultimate amino acid (2). Many biologically active peptides such as neuropeptides, hormones, cytokines, and chemokines (3–5) contain this proline at the penultimate position as a regulatory element in proteolytic processing. Therefore, proline-specific proteases such as CD26/DPPIV are likely to play a role in modulating activity of these bioactive peptides, but which are actually cleaved by DPPIV in vivo is unknown (5). Sec-

ond, CD26/DPPIV has been considered for a long time to be the only membrane-bound protease specific for proline in the penultimate position at the amino terminus. However, in recent years, a series of CD26/DPPIV activity and/or structure homologues (DASH) has been described that are either ectoenzymes at the cell membrane or intracellular proteases (2,5). Third, complex formation of CD26/DPPIV with other proteins can modulate the accessibility of the active site, and this may differ for the different isoforms of CD26/DPPIV (6–8).

These aspects make it difficult to predict which natural substrates are processed by which isoforms of CD26/DPPIV in vivo (5), but demonstrate that the role of proteolysis in the regulation of the activity of bioactive peptides is complex. Understanding proteolysis-based regulation mechanisms in vivo is still in its infancy and is only lately receiving the attention that is needed in this era of functional proteomics (9). Therefore, we have investigated whether a relatively simple assay could be designed for detection of possible natural substrates of a specific protease, with the example of CD26/DPPIV.

Recently, we have established that two fluorogenic synthetic substrates, [Ala-Pro]²-cresyl violet (Magic Red) and Ala-Pro-rhodamine 110, can be used to determine CD26/DPPIV-like activity in living cells (10). It was shown that selectivity of the cresyl violet-based substrate was high for DPPIV activity, whereas the rhodamine 110-based substrate was also cleaved by other proteases, most likely DASH, as was predicted by Lorey et al. (11,12). These differences in selectivity are likely due to the fact that the leaving group in a substrate can affect interactions between the active site of an enzyme and the substrate and show that not only the amino acid sequence determines the reactivity (7,13). The two fluorogenic substrates have been used in combination with living Jurkat cells, which are T cells that lack CD26/DPPIV, and CD26/DPPIV-transfected Jurkat cells (14,15) to investigate whether β -casomorphin₁₋₅ (16,17) is a possible natural substrate of CD26/DPPIV. This strategy should be applicable to any protease and any possible natural substrate to elucidate which substrate is preferentially recognized by which protease.

MATERIALS AND METHODS

Jurkat Cell Lines

Jurkat cells (clone E6-1; ATCC, Manassas, VA, USA), which lack CD26/DPPIV expression, were used as well as Jurkat cells transfected with CD26/DPPIV (14,15). This model system enables the determination of selectivity of substrate cleavage by DPPIV activity. Clone E6-1 was cultured in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, Walkerville, MD, USA), supplemented with 10% fetal calf serum, whereas the CD26/DPPIV transfectants were grown in Dulbecco's modified medium supplemented with 10% fetal calf serum, containing 1 mM glutamine and Geneticin G418 (1 mg/mL; Invitrogen, Carlsbad, CA, USA) to maintain the selection for the CD26/DPPIV construct (14,15).

Analysis of DPPIV Activity

Living Jurkat cells and transfected Jurkat cells were harvested, and their DPPIV

activity was determined in 4×10^6 cells at 37°C by means of fluorospectrometry as described previously (10) using 0–40 μM [Ala-Pro]²-cresyl violet (Magic Red; Enzyme Systems Products and Prototek, Livermore, CA, USA) (10,18) or 0–20 μM Ala-Pro-rhodamine 110 (Molecular Probes, Eugene, OR, USA) (19,20) in the presence of 0–200 μM β -casomorphin₁₋₅ (Sigma, St. Louis, MO, USA) in a final volume of 1200 μL under continuous stirring. Magic Red substrate is fluorescent by itself, but fluorescence is shifted to a longer wavelength when the amino acids are cleaved off from the leaving group, cresyl violet (10). The second substrate does not fluoresce by itself, but rhodamine 110 becomes fluorescent when the amino acids are removed (19,20). Excitation of cresyl violet was performed at 591 nm with a slit width of 10 nm, and emission was detected at 628 nm with a slit width of 10 nm (13). Rhodamine 110 was excited at 485 nm, and emission was detected at >535 nm (13). In order to correct for spontaneous breakdown of the synthetic substrates, reaction rates were determined as described in Boonacker et

al. (10). Furthermore, increase in fluorescence was measured during 4 min in 96-well plates on a Victor² 1420 multilabel counter (Wallac, Turku, Finland) using ELISA plates. First, wells were filled with a droplet of a synthetic substrate solution and a droplet of a solution of β -casomorphin₁₋₅ in opposite corners of the wells, in order to avoid mixing of the substrates before incubation. Phosphate-buffered saline (PBS) was added to the wells, and the 96-well plate was placed on a preheated stage of the photospectrometer at 37°C. The final volume in the wells was 200 μL and contained 0–20 μM Ala-Pro-rhodamine 110 and 0–200 μM β -casomorphin₁₋₅. Then, 0.67×10^6 cells were added to obtain the same concentration of cells as was used in incubations in cuvettes. Measurements were made every 5 s. Before and between measurements, the plate was shaken for 1 s. All reactions were performed in duplicate. In order to reduce the time needed for measurements, plates were only filled with 40 or 50 samples maximally. Linear regression was used to analyze the slope of the increase in fluorescence over time.

The increase in fluorescence per unit incubation time was plotted against substrate concentrations. A hyperbolic curve was fitted to the data with the use of a curve fitting program

(MacCurveFit; Apple, Cupertino, CA, USA), and maximum velocity (V_{max}), Michaelis constant (K_m), and inhibitor constant (K_i) values were calculated for living Jurkat cells and CD26/DPPIV-transfected Jurkat cells.

Statistics were performed, where appropriate, using one-way analysis of variance (ANOVA) with a Dunnett's post test (GraphPad[®] Instat version 3.00 for Windows[®]95; GraphPad, San Diego, CA, USA). Differences were considered significant when $P < 0.05$.

RESULTS

Intact living CD26/DPPIV-transfected Jurkat cells cleaved [Ala-Pro]²-cresyl violet with a K_m of 3.7 μM and Ala-Pro-rhodamine 110 with a K_m of 7.5 μM (Figure 1). The K_m value of cleavage of [Ala-Pro]²-cresyl violet is approximately twice lower than that of Ala-Pro-rhodamine 110, indicating a similar cleavage rate, because [Ala-Pro]²-cresyl violet contains two Ala-Pro dipeptides per molecule, and Ala-Pro-rhodamine 110 contains only one (10).

Figure 2 shows an example of the detection of DPPIV-like activity by the use of Ala-Pro-rhodamine 110 substrate in the presence of various concentrations of β -casomorphin₁₋₅ (0–200 μM) in a microwell plate. β -Casomorphin₁₋₅ inhibited generation of fluorescence due to hydrolysis of fluorogenic Ala-

Figure 1. Relation between increase in fluorescence per unit time (V) and concentration of fluorogenic substrates to detect dipeptidyl peptidase IV (DPPIV)-like activity in intact living CD26/DPPIV-transfected Jurkat cells. (A) [Ala-Pro]²-cresyl violet ([A-P]²-CV) and (B) Ala-Pro-rhodamine 110 (A-P-R110).

Figure 2. Example of reaction rate data of dipeptidyl peptidase IV (DPPIV)-like activity in CD26/DPPIV-transfected Jurkat cells as demonstrated in 32 wells of a 96-well plate using the fluorogenic substrate Ala-Pro-rhodamine 110 (Ala-Pro-R110) in the presence or absence of various concentrations (0–200 μM) of β -casomorphin₁₋₅ in living CD26/DPPIV-transfected Jurkat cells. Incubations were performed in duplicate, and fluorescence was measured in time during 240 s at 37°C using 485 nm excitation light, whereas emission was measured at >535 nm.

Pro-rhodamine 110 in a dose-dependent manner. It indicates that both compounds are recognized as substrate for the same enzyme(s). Because a microwell plate reader was not available with excitation and emission characteristics to specifically detect cresyl violet, we were unable to perform similar experiments in microwell plates with [Ala-Pro]²-cresyl violet. Therefore, effects of β -casomorphin₁₋₅ on cleavage of [Ala-Pro]²-cresyl violet (Figure 3) were compared with those on cleavage of Ala-Pro-rhodamine 110 (Figure 4) in a fluorospectrometer. The Dixon plots show that β -casomorphin₁₋₅ inhibits cleavage of both synthetic substrates competitively. K_i values of inhibition by β -casomorphin₁₋₅ of [Ala-Pro]²-cresyl violet cleavage (60 μ M) were approximately 2-fold lower than those of Ala-Pro-rhodamine 110 cleavage (150 μ M) in a similar manner as the K_m values. Therefore, it is concluded that the affinity for both fluorogenic substrates and the interference by β -casomorphin₁₋₅ are similar in living CD26/DPPIV-transfected Jurkat cells.

DISCUSSION

The present study shows that the question of which bioactive peptides are cleaved by which proteases *in vivo* can be addressed with a living cell system that expresses a specific protease and a fluorogenic substrate that is

cleaved selectively by that protease. It appeared that β -casomorphin₁₋₅ is recognized preferentially by CD26/DPPIV but also by other DASH proteases. The combinational use of Jurkat cells and CD26/DPPIV-transfected Jurkat cells allows direct comparison of cleavage of substrates by cells that lack DPPIV activity and the same cells that have been transfected with the gene that codes for that protease.

It is shown with our living cell system that [Ala-Pro]²-cresyl violet, also known by its commercial name Magic Red (10), is selectively cleaved by CD26/DPPIV with a K_m of 3.7 μ M. β -Casomorphin₁₋₅ inhibits cleavage of [Ala-Pro]²-cresyl violet competitively with a K_i of 60 μ M. These findings indicate that β -casomorphin₁₋₅ is recognized by the active site of CD26/DPPIV. Parallel studies using the substrate Ala-Pro-rhodamine 110 showed that that substrate is less specific and other DASH proteases also cleave it (10).

The K_m value of Ala-Pro-rhodamine 110 cleavage in both Jurkat cells and CD26/DPPIV-transfected Jurkat cells is 2- to 3-fold higher than the K_m value of cleavage of [Ala-Pro]²-cresyl violet, indicating that the bonds

between the dipeptide and the leaving group are cleaved at similar rates, because the first substrate contains only one dipeptide per molecule, whereas the second one contains two.

Specific detection of fluorescence of cresyl violet is not routine yet due to the rather unusual excitation and emission settings that have to be used (excitation, 591 nm; emission, 628 nm), and these settings are not available on all types of equipment, such as microplate readers, confocal laser scanning microscopes, or flow cytometers. However, microplate readers equipped with a monochromator, such as the SpectraMax[®] (Molecular Devices, Sunnyvale, CA, USA), are excellent readers to monitor the production of cresyl violet due to proteolytic activity. The only confocal microscope that is yet available with these excitation and emission settings is the recently-developed Acoustic Optical Beam Splitter (AOBS) system of Leica (Mannheim,

Figure 3. Dixon plot of reaction rates of dipeptidyl peptidase IV (DPPIV) activity in intact living CD26/DPPIV-transfected Jurkat cells in the presence of 2.5 (■), 5 (▲), 10 (▼), and 20 μ M (◆) [Ala-Pro]²-cresyl violet synthetic substrate. The plot of inverse reaction rate versus β -casomorphin₁₋₅ concentration shows that inhibition of [Ala-Pro]²-cresyl violet by β -casomorphin₁₋₅ is competitive.

Figure 4. Dixon plots based on reaction rates of dipeptidyl peptidase IV (DPPIV)-like activity in intact living Jurkat cells in the presence of various concentrations (0–200 μ M) of β -casomorphin₁₋₅ and 2.5 (■), 5 (▲), 10 (▼), and 20 μ M (◆) Ala-Pro-rhodamine 110 synthetic substrate. Inhibition of fluorescence production by β -casomorphin₁₋₅ was determined in (A) CD26/DPPIV-transfected Jurkat cells, as well as (B) Jurkat cells lacking CD26/DPPIV. The plot of inverse reaction rate versus β -casomorphin₁₋₅ concentration shows that the inhibition of cleavage of Ala-Pro-rhodamine 110 is competitive in all cases.

Germany) (10). Because we were not able to use a microplate reader equipped with a monochromator, we performed the assays in 96-well microplates with [Ala-Pro]-rhodamine 110 only as rhodamine 110 can be visualized using standard fluorescence settings (excitation, 488 nm; emission, >535 nm). Figure 2 shows the results of such an experiment, and it is clear that the assay is both [Ala-Pro-rhodamine 110]-dependent and [β -casomorphin₁₋₅]-dependent.

We selected a living cell system for the determination whether a bioactive peptide (β -casomorphin₁₋₅) is a possible natural substrate for a specific protease (CD26/DPPIV), because the activity of the protease is regulated at the posttranslational level and depends on the microenvironment of the enzyme (9,10,13,21–27). Insight in selectivity of CD26/DPPIV for specific chemokines has been performed by ranking the chemokines as substrates on the basis of turnover number (k_{cat}/K_m values (28). A huge difference was found between k_{cat}/K_m values for the different chemokines [e.g., $5 \times 10^{-6}/(M)(s)$ for stromal-derived factor 1 α (SDF-1 α) versus $0.04 \times 10^{-6}/(M)(s)$ for regulated-upon-activation normal T expressed and secreted (RANTES)]. However, the experiments were performed in vitro with purified CD26/DPPIV, and it is unknown whether their findings can be transposed directly to the in vivo situation, which is manyfold more complex. For example, glucagon₁₋₂₉ is a 29 amino acid long peptide hormone that has a half-life of 5 to 6 min in the circulation, and CD26/DPPIV is considered to be at least partly responsible for the inactivation of glucagon₁₋₂₉ (29). However, it is not known which tissues are responsible for clearance of glucagon from the circulation. One would assume that the kidneys play an important role, because CD26/DPPIV is present on the apical surface of epithelial cells of proximal tubules (30) where it can degrade glucagon₁₋₂₉ that is small enough to be filtered in the glomeruli into the primary urine. However, it is not understood why glucagon₁₋₂₉ is not degraded in the circulation (31,32), because CD26/DPPIV is present on the surface of T cells and B cells and as freely circulating soluble CD26/DPPIV. This would implicate that different cell types express different isoforms of CD26/DPPIV with different substrate

specificities, and only certain isoforms of CD26/DPPIV are responsible for degradation of glucagon₁₋₂₉ in vivo (33,34). Therefore, the need for a living cell system to elucidate proteolytic processes in vivo is obvious.

In conclusion, a simple and fast assay has been developed to determine whether or not bioactive peptides are possible natural substrates for a specific protease in a model system of living cells and synthetic fluorogenic substrates with the example of β -casomorphin₁₋₅ and CD26/DPPIV and DASH proteases.

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Direct retransformation of yeast with plasmid DNA isolated from single yeast colonies using rolling circle amplification

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We have efficiently amplified plasmid DNA from single yeast colonies using rolling circle amplification (RCA). The amplified DNA can be directly used for restriction digestion, DNA sequencing, or yeast transformation. The RCA-based high-fidelity amplification would be useful for plasmid manipulation in a variety of yeast-based systems, particularly for high-throughput analyses.

INTRODUCTION

Yeast-based systems, such as one-hybrid and two-hybrid screening, are powerful tools for studying protein-DNA and protein-protein interactions (1). However, yeast is not ideal for direct analysis of plasmid DNA. Typically, only a small quantity of plasmid DNA can be isolated from yeast cells using conventional procedures. The recovered plasmids are not sufficient for many routine molecular assays, such as restriction digestion, DNA sequencing, and subsequent yeast transformation. Therefore, further propagation of the recovered DNA in bacterial hosts becomes essential. This approach is feasible for low-throughput analyses. When performed in a high-throughput manner, the approach would, however, involve thousands of yeast and bacterial cultures, bacterial transformations, and plasmid DNA preparations from both bacteria and yeast. Such quantities of work are not practical for many research laboratories.

Rolling circle amplification (RCA) is an *in vitro* DNA amplification technique utilizing the rolling circle mechanism used by bacteria to replicate circular plasmids or viruses (2). By using random hexamer primers and ϕ 29 DNA polymerase, circular DNA templates can be amplified 10,000-fold at a constant temperature in a short time period (3). The ϕ 29 DNA polymerase has the capacity to perform displacement DNA synthesis for more than 70,000 base pairs without disso-

ciation from the template and has high proofreading activity to ensure a high fidelity of DNA amplification (4,5). It has been reported that the error rate for this enzyme is 10^{-6} – 10^{-7} (6).

Here we describe a method to amplify plasmid DNA from single yeast colonies using RCA. The amplified DNA is sufficient for restriction analysis, DNA sequencing, and retransformation of yeast cells for confirmation of the initial yeast two-hybrid interactions.

MATERIALS AND METHODS

The yeast strain CG-1945 (*MATa*, *ura3-52*, *his3-200*, *lys2-801*, *ade2-101*, *trp 1-901*, *leu2-3, 112*, *gal4-542*, *gal80-538*, *LYS 2::GAL1_{UAS}GAL1_{TATA}-HIS3*, *URA3::GAL4 17-mers*)₃-*Cyc1_{TATA}-lacZ*, *cyh^r2*) was used in this study. Transformation of the yeast cells was carried out using the Frozen-EZ Yeast Transformation II™ kit (Zymo Research, Orange, CA, USA) according to manufacturer's instructions (<http://www.zymoresearch.com/t2001-frame.html>). Yeast two-hybrid assays were performed according to the methods described (7). The primers used for DNA sequencing are listed in Table 1.

The RCA reactions were performed using the TempliPhi™ 100 Amplification kit (Amersham Biosciences, Piscataway, NJ, USA). Briefly, 1/3–1/2 of each single yeast colony was picked using a toothpick and suspended into 5 μ L of sample buffer (Amersham Biosciences). For enzymatic treatment, 2 U of zymolase (Zymo Research) were added