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Determination of Intracellular Prolyl/Glycyl Proteases in Intact Living Human Cells and Protoporphyrin IX Production as a Reporter System

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Summary

The determination of enzyme activity or inhibition in intact living cells is a problem in the development of inhibitors for intracellular proteases. The production of fluorescent protoporphyrin IX (PpIX) from the nonfluorescent (N)-Gly/Pro-5-aminolevulinic acid (ALA) substrates was used to evaluate the prolyl/glycyl-specific dipeptidylpeptidase IV (DPPIV)-like and prolyloligopeptidase (POP)-like activities of human cells. The results demonstrated that whereas POP-like activity could be attributed to the actual POP, the DPPIV-like activity could be related to actual DPPIV only in one colon cell line. In the other breast and colon cell lines, DPPIV-like activity was intracellular and displayed by other prolyl-specific aminopeptidases. Our experiments also demonstrated the involvement of gylcyl-specific proteases in the processing of ALA precursors. These observations have important consequences for the development and evaluation of selective inhibitors for these enzymes.

Introduction

Proteases are major targets in the treatment of human diseases. However, although there has been much success with drugs developed for inhibiting extracellular proteases (either secreted or inserted in the outer cell membrane), the inhibition of intracellular proteases, and the evaluation of this inhibition, in intact living cells is difficult to achieve due to the limitation of membrane permeability of protease substrates or inhibitors. It is becoming increasingly accepted that intracellular proteases are important targets for drug development; thus, it is necessary to develop tools for evaluating the effects of inhibitors in the intracellular environment of these enzymes. Most present approaches, however, involve cell lysis, thus losing important information on the state of living cells.

Many biologically active peptides are protected from general proteolytic degradation by evolutionarily conserved Pro. This protection is provided due to conformational constraints imposed by the Pro residue, pointing to the biological importance of this amino acid. Therefore, inhibitors of prolyl-specific peptidases have a high potential for drug discovery. Of the approximately 400 known human proteases, only a few Pro-specific proteases have been described [1–6]. Dipeptidylpeptidase IV (DPPIV)/CD26 (EC 3.4.14.5.) is a widely expressed dimeric type II integral membrane protein releasing X-Pro dipeptides from the free N-terminal sequence of peptides. Nearly all peptides and proteins bearing an X-P sequence are potential substrates for DPPIV [4], and inhibitors for this enzyme are under clinical evaluation for the treatment of type 2 diabetes. Fibroblast activation protease α (FAPα)/seprase is a dimeric type II integral membrane prolyl dipeptidase found in remodeling tissue, with an enzymatic activity comparable to DPPIV. Quiescent cell proline dipeptidase (QPP)/DPPII/DPP7 (EC 3.4.14.2) is a homodimeric glycoprotein located in intracellular vesicles, with specificity and serine protease characteristics identical to DPPIV [7]. DPP8 and DPP9 are recently described monomeric, ubiquitously expressed, soluble, and cytosolic DPPIV-like enzymes of roughly 100 kDa, without a transmembrane domain [8, 9]. Of the Pro-specific DPP family, only DPP8 and DPP9 are exclusively cytoplasmic, and only DPPIV, FAPα, DPP8, and DPP9 have enzymatic activities. All these enzymes need a free N-terminal aminoacid. Prolyl oligopeptidase (POP) (EC 3.4.21.26.) is an intracellular, much conserved, and widely distributed soluble and cytosolic post-prolyl-endopeptidase of 80 kDa [5]. The interest in this enzyme has increased since it has been demonstrated that POP inhibitors ameliorate cognitive disorders [1–6]. The 3D structure and kinetic characteristics of DPPIV and POP have been determined [5]. From this information, several families of inhibitors have been synthesized and evaluated on purified enzymes [10–12] and their effects determined in a few biological models, suggesting the inhibition of families of enzymes with similar activity.

Therefore, it is necessary to develop methods that will allow researchers to differentiate between the activities of the cytoplasmic and membrane forms of these enzymes in order to asses the cellular effects of inhibitors. We have previously shown that pseudopeptide derivatives of 5-aminolevulinic acid (ALA) are substrates for selected proteases that release free ALA from the derivatives; the free ALA is metabolized by living cells to the fluorescent protoporphyrin IX (PpIX) retained inside cells [13, 14]. Thus, intracellular accumulation of the fluorescent PpIX following administration to cells of nonfluorescent peptide derivatives of ALA is an indicator of the cellular expression and/or activity of these proteases. Specifically, we have shown that Pro/Gly derivatives can be substrates for cellular proteases. We reasoned that such substrates can be used as a potential reporting system for the kinetic determination of the cellular level of proteolytic activity in living intact cells. In this article, we validate such a concept using prolyl/glycyl-specific amino dipeptidase-like (DPPIV-

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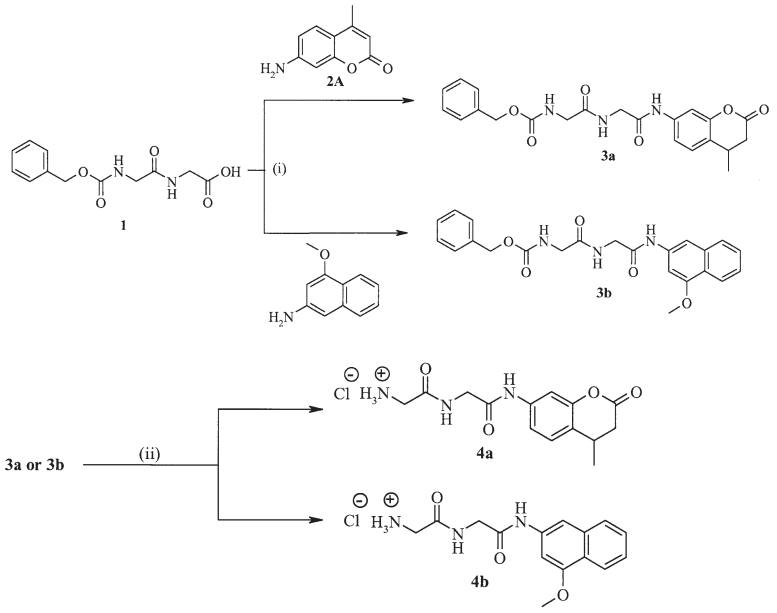


Figure 1. Scheme: Chemical Syntheses

Reagents: (i) P_2O_5, $PO(OC_2H_5)_2$, NEt_3, 100°C, 2 hr; (ii) HCl/MeOH 1.25 M, H_2, MeOH, Pd/C, RT, 4 hr.

like) and endopeptidase-like (POP-like) activities in human breast and colon cancer cells.

Results and Discussion

To evaluate cellular enzymatic activities, most methods rely on fluorogenic substrates, such as amido-4-methylcoumarin derivatives in lysates of tissues or cells. These approaches allow for determining overall levels of enzymatic activity and inhibition, but do not provide information on enzyme activity, or residual activity, in a living cell. To some degree, these drawbacks can be overcome by using histoenzymography on tissue sections or fixed cell layers, such as the red precipitates obtained following the release of methoxy-2-naphthylamine from peptide substrates in the presence of a diazotating reagent [13]. However, for most intracellular proteases, these assays cannot generally be performed in nonpermeabilized living cells. We have previously shown [13, 14] that peptide-ALA derivatives are first internalized into cells using an ALA transport system; then, ALA is proteolitically released from its peptide carrier to produce the fluorescent PpIX via the heme synthetic pathway. Thus, the production of PpIX from ALA precursor derivatives may be used as a reporter system for the kinetic determination of the cytoplasmic processing of a peptide by proteolytic enzymes. Here we compared actual PpIX production in human carcinoma cells exposed to Gly/Pro-containing dipeptide-ALA derivatives with proteolytic activity obtained using standard substrates in intact living cells or in cell extracts.

The synthesis and characterization of the N-protected and N-deprotected dipeptide derivatives of 5-ALA has been previously described [13, 14]. To synthesize (see the Supplemental Data available with this article online) the 4-methyl-coumaryl-7-amide (AMC) and 4-methoxy-β-naphtylamide (β-NA) Gly derivatives, which are not commercially available, we used a modification of the phosphorus pentoxide method of Schramm and Wissman [15, 16], in which the amine is activated as a phosphoramide monoethyl ester and then coupled with a carboxylic acid to yield a carboxamide (Figure 1, Table S1). An initial attempt, using cyclohexylcarbodiimide as the coupling agent, failed to yield any product (data not shown).

First we determined whether human surgical samples and cells in culture demonstrated DPPIV-like and POP-like activities. Histological slides of frozen human surgical tissue were exposed to substrates specific for DPPIV-like (Gly-Pro-β-NA) or POP-like (Z-Gly-Pro-β-NA) peptidases (Figure 2A; DPPIV-like activity in nontumoral and tumoral human colon). In normal human colon, DPPIV-like activity was expressed along, but not at the tips of, the crypts. In human colon cancer, only a few tumoral cells expressed this activity, suggesting heterogeneous expression of DPPIV-like activity by human cancer. POP-like and post-Gly-cleaving activities were never found using such an approach (data not shown). To study these activities in more detail, we selected two human colon (SW480 and HT-29) and two human breast (T47D and MCF-7) carcinoma cell lines. Human colon and breast carcinoma cells expressed the mRNAs for DPPIV, FAPα, and POP (Figure 2B), as observed using RT-PCR and pairs of primers (Table S2) selective for POP, DPPIV, and FAPα. Extracts of human colon and breast carcinoma cells hydrolyzed the fluorogenic Z-Gly-Pro-AMC (POP-like) and Gly-Pro-AMC (DPPIV-like) substrates (Figure 2C). Z-Gly-Gly-AMC was never hydrolyzed, whereas Gly-Gly-AMC was hydrolyzed at very low levels only by SW480 and T47D cell extracts (Figure 2C). Only HT-29 colon carcinoma cells expressed DPPIV-like activity using zymography of cells extracts (Figure 2D). POP-like activity was never detected using zymography, suggesting that the zymography process inactivates this enzyme. Western blotting experiments

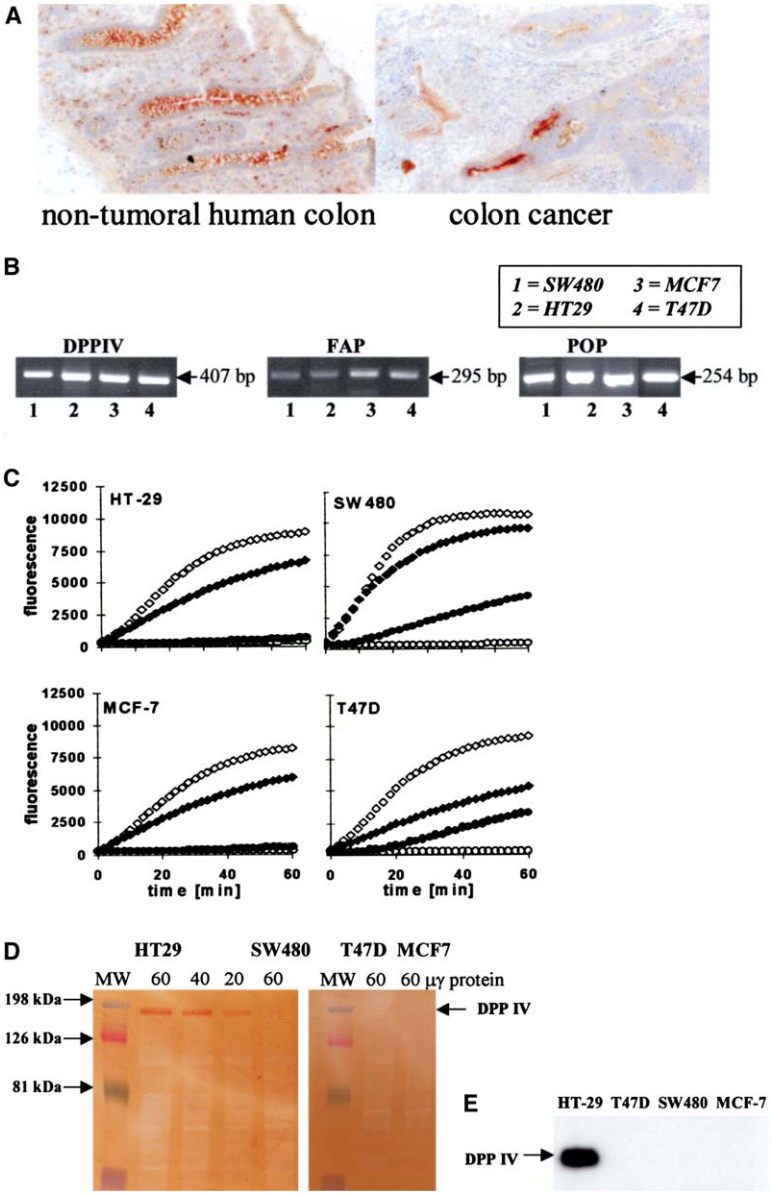


Figure 2. Determination of Prolyl-Specific Peptidases in Human Tissue and Cells

(A) Histoenzymography: Frozen histological slides of either human normal colon or colon cancer were exposed to Gly-Pro-β-Methoxy-naphthylamide and Fast Blue B. Cells expressing the enzyme activity are localized by a red precipitate.

(B) RT-PCR: RNA was extracted from SW480, HT-29, T47D, and MCF-7 cells and subjected to RT-PCR amplification using primers shown in Table S2.

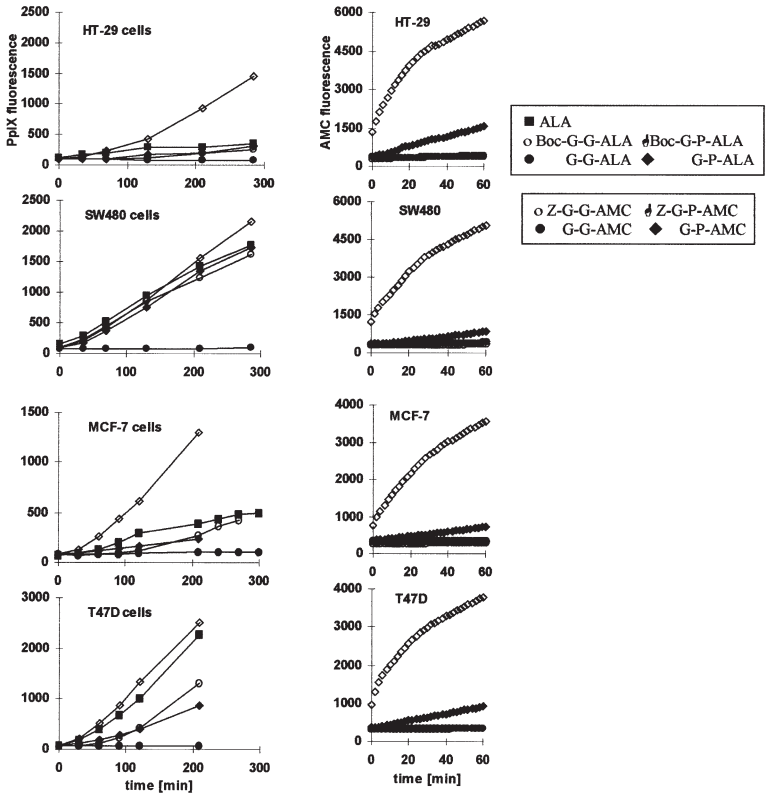
(C) Enzymatic activities: Human carcinoma cell extracts were exposed to AMC substrates and increase in fluorescence was recorded. Open circles, Z-Gly-Gly-AMC; open diamonds, Z-Gly-Pro-AMC; closed circles, Gly-Gly-AMC; closed diamonds, Gly-Pro-AMC.

(D) Zymography: Cells were extracted in PBS-Triton and subjected to electrophoresis, then exposed to Gly-Pro-β-methoxy-naphthylamide and Fast Blue B.

(E) Western blotting: Cells were extracted in PBS-Triton and subjected to electrophoresis, then exposed to anti-human DPPIV antibody.

demonstrated that only HT-29 cells expressed actual DPPIV protein (Figure 2E), corresponding to the zymography experiments. As expected, FAPα was not expressed by all cancer cell lines, but by primary human fibroblasts in culture used as positive control cells (results not shown). We performed gel filtration experiments using SW480, HT-29, T47D, and MCF-7 cell extracts (data not shown). In all cells, DPPIV-like activities eluted at approximately 200 kDa and POP-like activity at approximately 80 kDa, which corresponded to the expected value of the molecular weight for these families of enzymes.

We then compared the AMC-hydrolyzing activities in intact cells (Figure 3, right panels) with PpIX production (Figure 3, left panels) from precursors with similar amino acid substitution. Only Z-Gly-Pro-AMC (POP-like activity) was rapidly hydrolyzed by all intact cells, whereas cell extracts (Figure 2C) hydrolyzed Gly-Pro-AMC (DPPIV-like activity) and Z-Gly-Pro-AMC with comparable equipotency. These results suggested either that POP-like enzyme(s) were expressed at the cell surface or that the substrate was rapidly transported across the cell membrane, whereas DPPIV-like activity was not accessible to a substrate added extracellularly. Z-Gly-Gly-AMC and Gly-Gly-AMC were not hydrolyzed by intact cells. Boc-Gly-Pro-ALA was hydrolyzed by all cells. SW480 and T47D cells hydrolyzed Boc-Gly-Gly-ALA and Gly-Pro-ALA more efficiently than HT-29 and MCF-7 cells. None of the cell lines hydrolyzed Gly-Gly-AMC. Thus, protease activities measured in intact cells with AMC substrates did not correlate with actual PpIX production from the ALA derivatives, whereas DPPIV-like and POP-like activities in cell extracts (as determined from data presented in Figures 1C) and PpIX production were correlated (Figure 3; for correlations and Figure S1, see the Supplemental Data). This information



correlations

cell extracts			intact cells			
GG-AMC	GP-AMC	Z-GP-AMC	GG-AMC	GP-AMC	Z-GP-AMC	
0,69	0,12	0,14	no act	-0,22	-0,06	**Boc-GG-ALAOEt**
0,08	-0,32	-0,09	no act	-0,02	0,28	**Boc-GP-ALAOEt**
0,90	***0,55***	***0,48***	no act	-0,31	-0,04	**GP-ALAOEt**
0,49	0,03	-0,33	no act	-0,05	-0,5	**GG-ALAOEt**

Figure 3. PpIX Production in Intact Human Carcinoma Cells Exposed to Pro/Gly-ALA-Derivatives. Comparison with Post-Pro/Gly-AMC Cleaving Activities in Intact Cells

Left panels: PpIX production. Cells were grown to confluence and exposed to either ALA used as a standard molecule for PpIX production, or dipeptide Gly/Pro-ALA derivatives. PpIX production was determined by continuous recording of increase in PpIX fluorescence. SW480 and HT 29 cells: closed squares, ALA (4 mM); open circles, Boc-Gly-Gly-ALA-OEt (1.35 mM); closed circles, Gly-Gly-ALA-OEt (1.65 mM); open diamonds, Boc-Gly-Pro-ALA-OEt (1.9 mM); closed diamonds, Gly-Pro-ALA-OEt (1.6 mM).

MCF-7 and T47D: closed squares, ALA (0.67 mM); open circles, Boc-Gly-Gly-ALA-OEt (0.64 mM); closed circles, Gly-Gly-ALA-OEt (0.8 mM); open diamonds, Boc-Gly-Pro-ALA-OEt (0.9 mM); closed diamonds, Gly-Pro-ALA-OEt (0.82 mM).

Right panels: AMC-cleaving activities. Colon (HT-29, SW480) or breast (MCF-7, T47D) carcinoma cells were grown to confluence and intact living cells were exposed to dipeptide Gly/Pro-AMC substrates. AMC release was determined by continuous recording of increase in AMC fluorescence. Open circles, Z-Gly-Gly-AMC; open diamonds, Z-Gly-Pro-AMC; closed circles, Gly-Gly-AMC; closed diamonds, Gly-Pro-AMC.

Correlations: Comparison of the correlation coefficients (R) between enzymatic activities measured by hydrolysis of AMC derivatives (Δfluorescence AMC/20 min) in intact living cells (intact cells), in cell extracts (cell extracts) and PpIX production measured as PPIX fluorescence at 300 min (Figure S1). For calculation of the correlation coefficients, the initial enzymatic rates (Δfluorescence AMC/20 min) either in intact living cells or in cell extracts, and PPIX production (PPIX fluorescence at 300 min) were correlated for each cell line studied.

suggests that the proteolytic activities involved in the hydrolysis of precursors located intracellularly rather than at the cell surface, and that Gly-hydrolyzing activities participate in the proteolytic processes and are predictive of intracellular proteolysis of Gly/Pro substrates.

Finally, from the data presented in Figure 3 (left panels), we calculated the overall kinetic enzymatic characteristics ("overall K_M ") (Table S3) of PpIX production in the four cell lines exposed to the various (N)-Gly/Pro-ALA precursors (Figure 3). Overall K_M was determined using an adaptation of the Lineweaver and Burke double-reciprocal plot for the determination of enzymatic kinetic constants. Cells were exposed to increasing concentrations of ALA or the various Gly/Pro-ALA derivatives and steady-state fluorescence increase was used to draw double-reciprocal plots of 1/increase in fluorescence versus 1/concentration of ALA derivative added to cells and K_M values calculated from the plots. The results showed that, whereas the amount of PpIX produced by cells may be different, the overall K_M values were generally comparable for one given precursor, suggesting that the amount of PpIX production depended only on the cellular internalization of the precursor and/or hydrolytic release of ALA by the appropriate protease; cellular PpIX synthesis from proteolytically released ALA was comparable in all cells.

In conclusion, our results suggest that POP-like activity corresponds to actual intracellular POP. Both Z-Gly-Pro-AMC and Z-Gly-Pro-ALA precursors provided identical information, indicating that they can be rapidly transported across the cell membrane. The identity of the DPPIV-like activities is more difficult to ascertain. DPPIV-like activity can be attributed to actual DPPIV only in HT-29 cells, because only these cells expressed DPPIV mRNA, protein (measured by western blotting), activity (measured by fluorometry and zymography), and molecular weight (determined by gel filtration) corresponding to DPPIV. Because FAPα protein was not expressed in these cells, this enzyme can be excluded; gel filtration excluded DPP8 and DPP9, which have lower molecular weights than DPPIV or FAPα. Interestingly, our experiments also demonstrated, particularly in SW480 and T47D cells, the involvement of post-Gly-cleaving activities in the cellular processing of ALA precursors that were not detected using AMC precursors.

Significance

Inhibitors for post-Pro-cleaving peptidases, such as DPPIV-like and POP-like peptidases, have been developed as potential therapeutically useful drugs in the treatment of various human diseases. However, the observation that ubiquitously expressed enzymes in various subcellular locations and with comparable enzymatic activities may be inhibited by these peptidases raises the question of the exact site, mode of action, and biological target(s) of these inhibitors. We demonstrate here the potential of Gly/Pro-ALA-derivatives as a reporter system to evaluate the activity and inhibition of post-Pro and post-Gly peptidases in intact, living human cells. Importantly, our results indicate that although cell membrane DPPIV was the main enzyme postulated in the processing of bioactive peptides and the target of inhibitor development, it is necessary to consider intracellular activity of peptidases with comparable specificity and kinetic characteristics in the evaluation of such inhibitors. Our results with ALA derivatives do not suggest the involvement of activities other than those of POP enzyme; however, DPPIV-like peptidases other than actual DPPIV were mainly involved in the processing of these Pro drugs.

Experimental Procedures

Syntheses

Procedures for the preparation and characterization of the 7-amino-4-methylcoumarin and 4-methoxy-β-naphtylamine ALA peptide analogs are outlined in Figure 1 and described in detail in the Supplemental Data.

Biological Approaches: Cells and Culture Conditions

Human colon HT29 and SW480 or breast T47D and MCF-7 carcinoma cell lines were from the ATCC (American Type Culture Collection, Manassas, VA), and treated as previously described [13, 14]. Human primary fibroblasts were obtained from cultures of human surgical specimens using the explant technique.

RT-PCR Experiments

Total RNA from confluent cells was prepared using Trizol reagent (GIBCO-BRL, Basel, Switzerland) and RT-PCR for DPPIV, FAP, and POP was performed according to standard procedures and specific primers, the sequence and size of transcripts of which are described in Table S2.

PpIX Fluorescence Measurements

Evaluation of PpIX production in living cells by continuous fluorescence recording was essentially performed as previously described [13, 14]. Experiments were repeated at least twice and were performed in sextuple wells. Means of results ± SD were calculated. Correlation coefficients were calculated using a two-tailed paired Student's t test.

Peptidase Activities

Peptidase activities were determined using AMC substrates (commercially available from Bachem, Bubendorf, Switzerland, or custom synthesized) either in intact living cells in DMEM medium without phenol red and FCS (GIBCO), or in cell extracts obtained in PBS-0.1% Triton X-100 (Fluka, Buchs, Switzerland). Increase in fluorescence was continuously recorded for 30–60 min at 37°C in a fluorescence multiwell plate reader (CytoFluor Series 4000; PerSeptive Biosystems, Framingham, MA; λ_{ex}: 360 nm; λ_{em}: 460 nm). Experiments were repeated at least twice and were performed in sextuple wells. Means of results ± SD or correlation coefficients were calculated.

In Situ Enzymatic Activity Determination—Cyto/Histoenzymography

The experiments were essentially performed as previously described [13] using peptide-β-methoxynaphthyl-amide (β-NA) derivatives (Bachem or custom synthesized) using 5 μm sections of frozen tissue. The presence of enzymatic activity is visualized as a red precipitate.

Zymography and Western Blotting Experiments

Cells were extracted in 0.05 M Tris, 0.15 M NaCl, 2 mM EDTA, 0.01% Triton X-100 (pH 7.2). Protein content was determined using the BCA kit (Pierce, Socochim, Switzerland), after which 60, 40, or 20 μg for zymography, or 30 μg for Western blotting, of protein was separated on a 7.5% acrylamide gel containing 0.1% SDS, without prior denaturation and without β-mercaptoethanol, and then transferred onto a nitrocellulose membrane (Scleicher and Schuell, Dassel, Germany). For the zymography experiments, membranes were exposed to either Z-Gly-Pro-β-methoxy-naphthylamide or Gly-Pro-β-methoxy-naphthylamide (2.2 mM final concentration) and 2.3 mM Fast Blue B (Fluka) for 5 hr in a humidified chamber at 37°C. For the Western blotting experiments, membranes were exposed to the anti-human DPPIV monoclonal antibody (clone 2A6, eBioscience, San Diego, CA) or anti-human FAP (BenderMedSystems, Inc., Burlingame, CA; Code BMS168) and peroxydase-anti-mouse secondary antibody and ECL detection (Amersham Biosciences, Little Chalfont, UK) were performed.

Supplemental Data

Supplemental Data, including additional Experimental Procedures, three tables, and one figure, are available at http://www.chembiol.com/cgi/content/full/12/8/867/DC1/.

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