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Proteolytic activity assayed by subcellular localization switching of a substrate

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ABSTRACT

An approach to assay proteolytic activity *in vivo* by altering the subcellular localization of a labelled substrate was demonstrated. The assay included a protein shuttling between different cellular compartments and a site-specific recombinant protease. The shuttle protein used was the human immunodeficiency virus type 1 (HIV-1) Rev protein tandemly fused to the enhanced green fluorescent protein (EGFP) and the red fluorescent protein (RFP), while the protease was the site-specific protease VP24 from the herpes simplex virus type 1 (HSV-1). The fluorescent proteins in the Rev fusion protein were separated by a cleavage site specific for the VP24 protease. When co-expressed in COS-7 cells proteolysis was observed by fluorescence microscopy as a shift from a predominantly cytoplasmic localization of the fusion protein RevEGFP to a nuclear localization while the RFP part of the fusion protein remained in the cytoplasm. The cleavage of the fusion protein by VP24 was confirmed by Western blot analysis. The activity of VP24, when tagged N-terminally by the Myc-epitope, was found to be comparable to VP24. These results demonstrates that the activity and localization of a recombinantly expressed protease can be assessed by protease-mediated cleavage of fusion proteins containing a specific protease cleavage site.

1. Introduction

Proteases play an important role in many biochemical pathways such as blood coagulation, complement activation, metamorphosis and digestion [1]. They have been the focus of both biological and disease-related studies, including ones involving apoptosis, Alzheimer's disease, cancer and viral infections. This has made them attractive targets for drug development [2–5], as the inactivation or inhibition of a specific protease can block either cellular or disease-related processes [6]. Both for monitoring the activity of a target protease and for drug development purposes there are benefits by carrying out testing in a living cell that represents a complex biological system. Accordingly, there has been significant interest in developing new technologies for monitoring the activity of a target protease inside a living cell. Different technologies for monitoring the activity of a target protease have been developed and assayed, for instance ones involving fluorescence cross-analysis, dimerization-dependent fluorescent protein exchange or translocation of a fluorescent biosensor after proteolytic cleavage [7–12]. Here, we present an assay to monitor recombinant protease activity *in vivo* by combining an altered subcellular distribution with fluorescence-based separation of a substrate in the form of a co-expressed shuttle protein fused to dual-colour fluorescence.

The system included two components: (1) a shuttle protein fused to two different fluorescent proteins separated by a protease-cleavable linker and (2) a cognate protease carrying an epitope detectable by an antibody. The two proteins were co-expressed in cells, and cleavage of the fusion protein was observed as separation of the two fluorescent markers by immunofluorescence microscopy.

In the current implementation of the *in vivo* protease assay, the herpes simplex virus type 1 (HSV-1) protease (VP24) was chosen

Abbreviations: CLSM, confocal laser scanning microscopy; EGFP, enhanced green fluorescent protein; RFP, red fluorescent protein; NLS, nuclear localization signal; NOS, nucleolar localization signal; NES, nuclear export signal; HIV, human immunodeficiency virus type 1; HSV-1, herpes simplex virus type 1; HTLV-1, human T-cell leukaemia virus type 1

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as the site-specific protease. The HSV-1 protease catalytic domain (VP24) is contained within the first 247 amino acid of the 635-amino acid precursor protein, and it is released from the precursor through cleavage by the HSV-1 protease itself [13,14]. The cleavage of precursor protease occurs between the Ala and Ser residues at amino acids 247/248 and 610/611 [15]. The HSV-1 protease is characterized as a serine-protease, but unlike serine digestive enzymes, the HSV-1 viral protease is a highly selective catalyst [16,17]. Furthermore, a temperature-sensitive mutant version of this protease has been identified, which can allow for a further level of control of the system [18]. As a shuttle protein the HIV-1 Rev was chosen, since there is extensive documentation of Rev trafficking inside cells [19,20]. Rev shuttles between the nuclear and cytoplasmic compartments by means of its nuclear (NLS) and nucleolar localization (NOS) signals and the leucine-rich nuclear export signal (NES) [21–24].

In the approach described here, Rev was fused to EGFP and DsRed1 separated by one of the cleavage sites (*as*) recognized by VP24 protease (AEAGALVN*AS*SAAHVDV), while the VP24 protease was fused N-terminally with a Myc epitope for antibody detection. Expressed alone the RevEGFP*as*DsRed1 fusion protein localized predominantly to the cytoplasm in COS-7 cells. When co-expressed with the VP24 protease, cleavage was demonstrated both by cytoplasmic localization of DsRed1 and nucleolar and nuclear accumulation of RevEGFP. The cleavage was confirmed by Western blot analysis.

2. Materials and methods

2.1. Plasmids construction

An overview of the different proteins expressed from the recombinant plasmids created are shown in Fig. 1A. The plasmid pcRev encoding wild-type Rev was kindly provided by Drs. M. Malim and B. Cullen [21]. To construct the plasmid pRevDsRed1, a

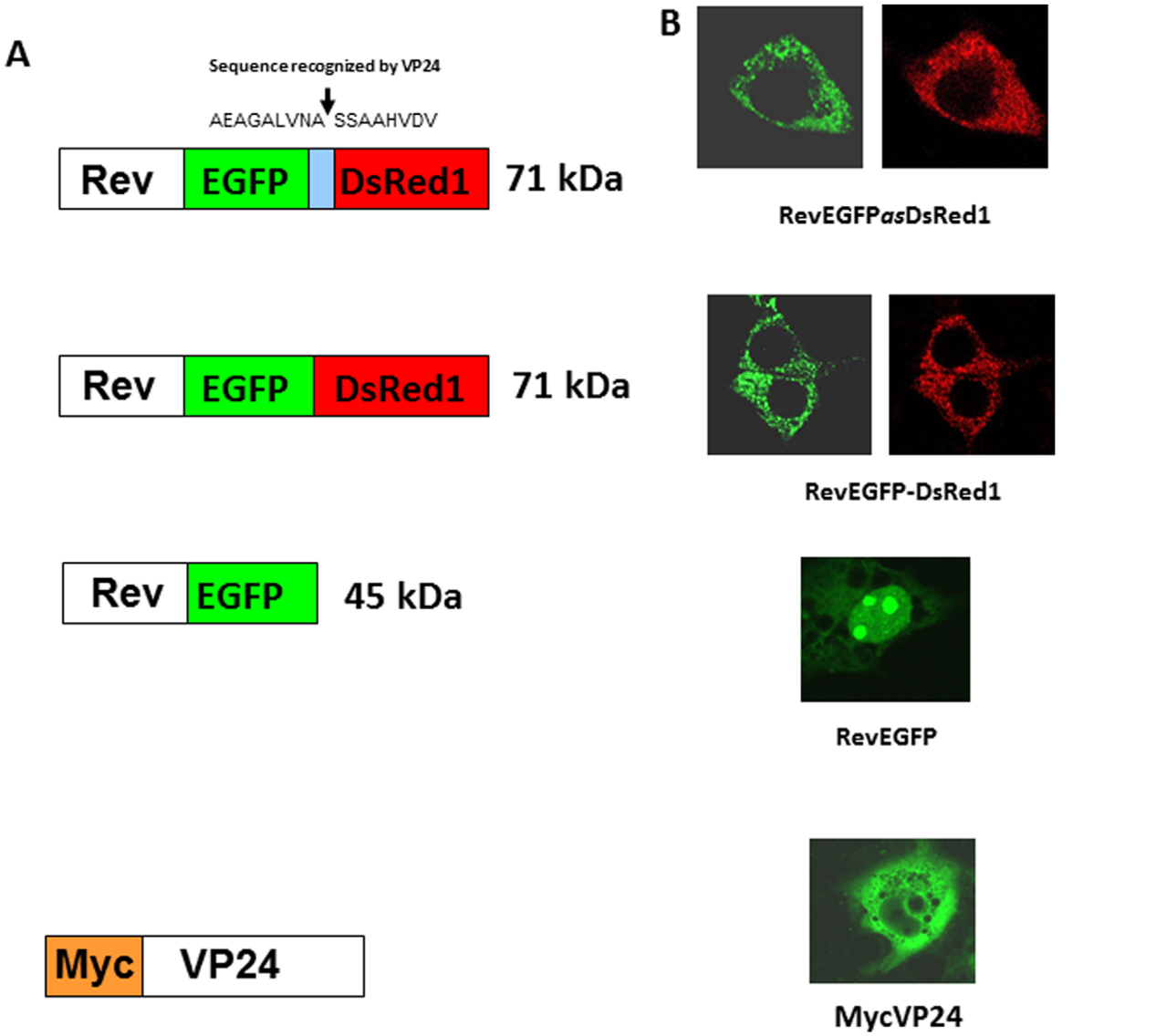

Fig. 1. Schematic representations of Rev fusion proteins, protease fusion protein and localization of these constructs in COS-7 cells. (A) RevEGFP*as*DsRed1consists of Rev, enhanced green fluorescent protein (EGFP) and red fluorescent protein (DsRed1). Between the EGFP and DsRed1 the HSV-1 VP24 recognition sequence AEAGALVNASSAAHVDV was inserted. RevEGFP-DsRed1 represents the fusion protein without the HSV-1 optimal recognition sequence while RevEGFP is the fusion protein without DsRed1. The HSV-1 VP24 protein is N-terminally tagged with the Myc epitope. The molecular sizes are indicated. (B) The intracellular steady state localization of RevEGFP*as*DsRed1, RevEGFP-DsRed1, RevEGFP and MycVP24 in COS-7 cells. The anti-Myc Mab 9E10 was used for immunofluorescent detection of MycVP24. The cells were fixed 48 h post-transfection and analysed by confocal laser scanning microscopy (CSLM).

polymerase chain reaction (PCR) was performed using primer pairs 5′GA**A GAT CT**A TGG CAG GAA GAA GCG GAG AC-3′ and 5′-CCG **GAA TTC** GTT CTT TAG TTC CTG ACT CCA-3′ with pcRev as a template. The PCR product was cloned into *EcoRI*- and *BglII*-digested pDsRed1-N1 (Clontech). The restriction sites, marked in bold, were introduced in the primers. To construct the plasmid pRevEGFP, a polymerase chain reaction (PCR) was performed using the primer pairs 5′GA*A* **GAT CT**A TGG CAG GAA GAA GCG GAG AC-3′ and 5‘-CCG **GAA TTC** GTT CTT TAG TTC CTG ACT CCA-3‘ with pcRev as a template. The restriction sites were introduced in the primers. The PCR was cloned into *EcoRI*- and *BglII*-digested pEGFP-N1 (Clontech). To construct pbsGas (pBluescript vector), PCR was conducted using the primers 5‘-CGG **GGT ACC** GAT GGT GAG CAA GGG CGA-3′ and 5′-TCC ***CCC CGG G***CC ACG TCC ACG TGG GCG GCG CTG CTG GCG TTC ACC AGG GCG CCG GCC TCG GCC TTG TAC AGC TCG TCC ATG-3′ with pEGFP-N1 (Clontech) as a template. The sequence encoding amino acids AEAGALVNASSAAHVDV representing the VP24 recognition sequence is underlined. The PCR product was treated with kinase and cloned into *SmaI*-digested pBlueScript (-) vector. To construct pRev*Gas*R, pbs*Gas* was digested with *KpnI* and *XmaI* and cloned into *KpnI*- and *XmaI*-digested pRevDsRed1. The protein expressed from the plasmid pRevGasR was called RevEGFP*as*DsRed1. A control plasmid lacking this optimal protease recognition site was named pRevGR, and was constructed using the primers 5′CGG **GGT ACC** GAT GGT GAG CAA GGG CGA-3′ and 5′-TCC C**CC CGG G**CC TTC TCG CTG GCC TGC AGG TAC TTG TAC AGC TCG TCC AT-3′ with pEGFP-N1 as a template and cloned into *KpnI*- and *XmaI*-digested pRevDsRed1. The restriction sites were introduced in the primers and the sequence encoding the amino acids YLQASEK representing a short VP24 recognition site is underlined. The protein expressed from the plasmid pRevGR was called RevEGFP-DsRed1.

The plasmid pJK58 encoding VP24 (kindly provided by Dr V. Preston) was here named pVP24 [25]. The protein expressed from the plasmid pVP24 was called VP24. The VP24 gene in pVP24, flanked by *BglII* sites, was digested with *BglII* and cloned into *BamHI*-digested pBluescript II SK + vector (pbsVP24). To construct pcDNA3.1-MycVP24, PCR was performed using the primers 5′CCG **GAA TTC** GCA CCA TGG AAC AAA AAC TCA TCT CAG AAG AGG ATC TGA TGG CAG CCG ATG CCC C-3′ and 5′-ATT TGC GGC CGC TCA CGC CTG GAG GTA GGT-3′ with pVP24 as template. The PCR product was digested with *EcoRI* and *BamHI* together with a *BamHI*- and *NotI*-digested pbsVP24 cloned together into *EcoRI*- and *NotI*-digested pcDNA3.1+(Invitrogen). The restriction site *EcoRI* was included in the primer, while the *BamHI* restriction site was present in the pVP24 sequence. To make pcMycVP24, pcDNA3.1-MycVP24 was digested with *NcoI* and *NotI* and cloned into *NcoI*- and *NotI*-digested pCMV/Myc/cyto (Invitrogen). This recombinant vector construct has been described before [26]. The protein expressed from the plasmid pcMycVP24 was called MycVP24. All the constructs were verified by sequencing.

2.2. Cell culture and transfection

COS-7 cells were maintained in Dulbecco's Eagle medium supplemented with 5% fetal bovine serum, Glutamine and Gentamycin. A total of 3 μg of plasmid DNA were used in transfection experiments with COS-7 cells at 80% cell density in 6-wells plate using the Lipofectamine 2000 procedure in accordance with Gibco BRL's instructions. For testing VP24 protease activity the amount of Rev plasmid was 0.5 μg, while protease plasmids varied from 1 to 2 μg.

2.3. Immunofluorescence

Immunofluorescence microscopy was performed as described previously [27]. Briefly, cells were fixed in 4% formaldehyde on ice for 5 min and then treated with methanol at 20 °C overnight. Monoclonal antibody (Mab) 9E10 against the Myc-epitope was used to detct Myc-tagged VP24 [28]. The secondary antibody used for immunofluorescence was fluorescein isothiocyanate (FITC) labelled anti-mouse IgG1. The cells were examined with confocal laser scanning microscopy (CLSM) (LEICA, TCS-SP).

2.4. Western blot analysis and antibodies

Transfected COS-7 cells in 35 mm wells were prepared as described previously [27]. For detection of Rev the anti-Rev Mab 8E7 cell culture medium was diluted 1:100 [29]. The *anti*-Myc Mab 9E10 was diluted 1:200 and used for immunofluorescent detection of MycVP24. Secondary POD-conjugated anti-mouse was diluted 1:2000 (Amersham). The membranes were developed using the ECL detection system.

3. Results

The methodology described here involves two components: (i) a substrate protein fused to two different fluorescent proteins separated by a linker cleavable by a highly sequence-specific protease and (ii) the cognate protease fused to the Myc epitope. Two different versions of fusions constructs were made: (I) pRevGasR where the EGFP and the DsRed1 were separated by a linker encoding the cleavage site for VP24 and (II) pRevGR without this optimal recognition sequence (Fig. 1A). When RevEGFP*as*DsRed1 was expressed in COS-7 cells a predominantly cytosolic distribution was observed (Fig. 1B). This is in contrast to the more nucleolar localization of RevEGFP shown here (Fig. 1B) and previously [30,31]. In order to detect VP24, a Myc epitope was fused to the N-terminal (Fig. 1A) and cytoplasmic localization was evident when MycVP24 was expressed in COS-7 cells (Fig. 1B). To visualise the subcellular localization of the cleaved products, transfected cells were analysed by laser confocal microscopy (Fig. 2). In COS-7 cells transfected with pRev*Gas*R alone a strong cytoplasmic fluorescence was observed with an aggregated appearance of the fusion protein (Fig. 2A–C). However, in COS-7 cells transfected with pRev*Gas*R together with pcMycVP24, a change in intracellular distribution as a strong green signal in both the nuclei and nucleoli similar to RevEGFP, while the red signal was found in the cytoplasm (Fig. 2D–F).

The Rev fusion proteins RevEGFP*as*DsRed1 and RevEGFP-DsRed1 was detected by the anti-Rev Mab 8E7 when analysing expressing COS-7 cells by Western blot. Fig. 3A shows RevEGFP after cleavage of the fusion protein RevEGFP*as*DsRed1 by VP24 (lane 1) and that this cleavage did not take place when VP24 was omitted (lane 2). Lane 3 shows the migration of pRevEGFP. Fig. 3B shows the same experiment as in A performed with the fusion protein without the cleavage site for VP24. In Fig. 3C the experiment was performed with the Myc-tagged VP24 showing that the activity was retained after adding the Myc epitope N-terminally to the VP24 sequence.

These results showed that the protein translated from pRev*Gas*R was cleaved by MycVP24. Moreover, the results demonstrated that the sequences added N-terminally to VP24 did not affect the catalytic activity of the protease. Interestingly, when the Myc-epitope was placed C-terminally in fusion with VP24, protease activity was lost, underscoring the need for suitable activity tests for recombinantly expressed proteins before conclusions regarding function can be made [32].

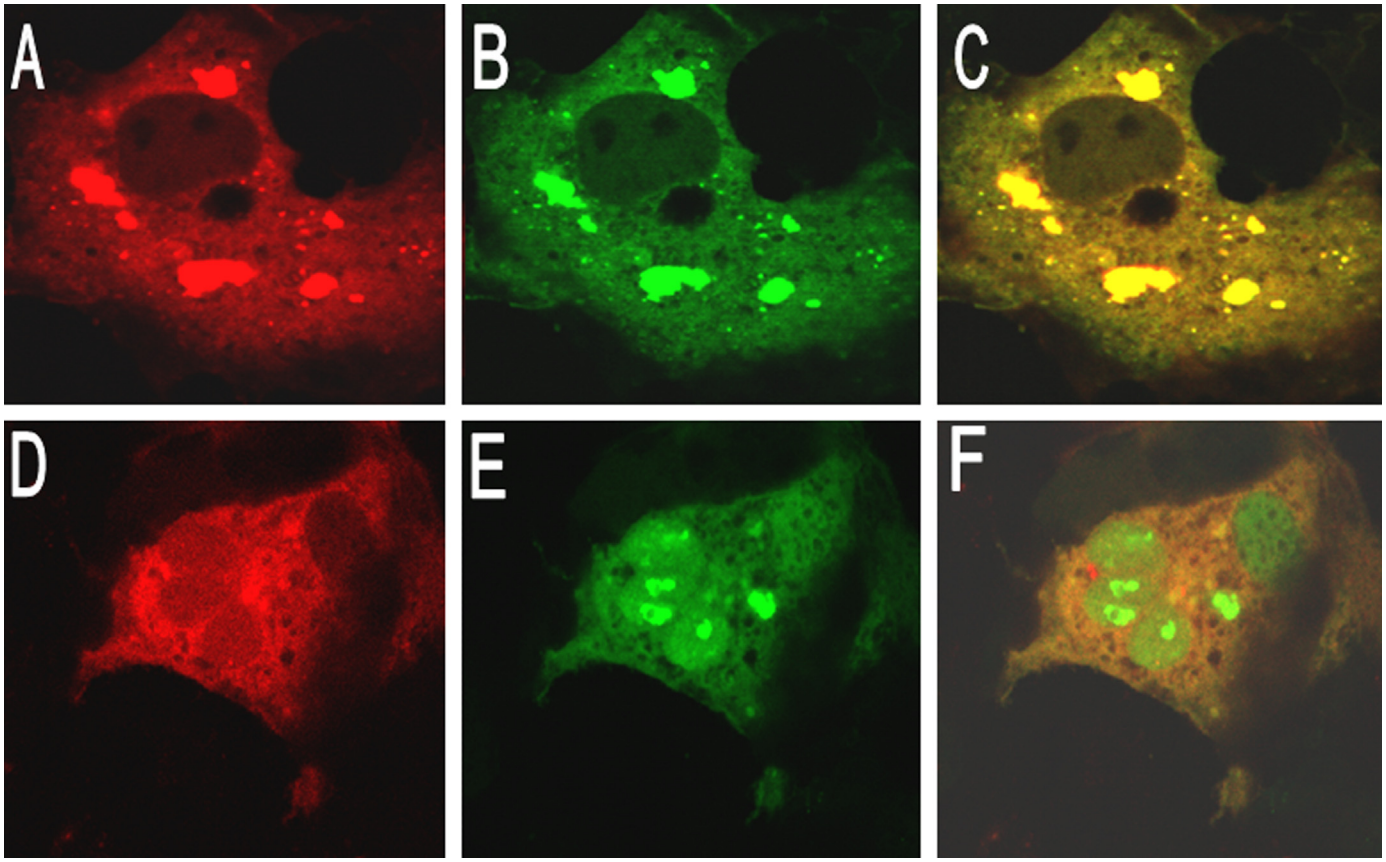

Fig. 2. Protease activity assayed by changes in localization of RevEGFP*as*DsRed1 after transfection with pRevG*as*R without or with pcMycVP24 in COS-7 cells. Panels A–C show cells after transfection with pRevG*as*R, panels D-F show cells after cotransfection with pRevG*as*R and pcMycVP24. The left, middle and right columns show images in red, green and merged channels, respectively. The cells were analysed by confocal laser scanning microscopy (CSLM). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

This study demonstrated that the activity a recombinant, site-specific protease can be tested by co-expression of a fluorescently labelled target protein containing the cleavage site. Here, this was shown both by Western blot analysis and by a changes in the subcellular localization of a Rev fusion protein representing a substrate protein and HSV-1 VP24 as the sequence-specific protease.

Rev was fused to the tandem EGFP/DsRed1 with a connecting sequence between them that included a HSV-1 VP24 recognition site. One possible disadvantage of using EGFP and DsRed1 as protein tags is their sizes and oligomerization tendency, which could affect the localization of the fusion protein. However, in this case it was an advantage since it allowed for detection of changes in cellular distribution without using inhibitors for nuclear import or export. This is apparent from the experiments shown here, where RevEGFP*as*DsRed1 was localized to the cytoplasm in contrast to the normal nuclear and nucleolar distribution of wild-type Rev and RevEGFP (Fig. 1C) [29,30,33].

Herpes Simplex virus 1 protease (VP24) was selected as a site-specific protease. The MycVP24 protein localized to the cytoplasm (Fig. 1B). We selected one of the two Ala/Ser sequences within the protease precursor protein that are known sites for autoproteolytic cleavage [15]. The sequence corresponding to the cleavage site proximal to the C terminus of the 635-residue HSV-1 precursor protein (AEAGALVNASSAAHVDV), inserted between the green and red fluorescence protein of the RevEGFP*as*DsRed1 fusion protein, was recognized by VP24 when co-expressed in COS-7 cells (Fig. 2D-F). In contrast, the fusion protein with a sequence representing the cleavage site proximal to the N-terminus of the 635-residue HSV-1 preprotease (YLQASEK), inserted between the green and red fluorescence protein of the RevEGFP-DsRed1, was not cleaved by the protease (Fig. 3B). Earlier results have shown that cleavage of synthetic peptides by HSV-1 protease, requires 5–8 residues on both sides of the scissile bond indicating that the identity and length of the amino acids flanking the P1-P1′ is critical for recognition and efficient cleavage [34]. The observation that the linker peptide YLQASEK was not digested also suggested that the potential proteolysis of cellular proteins by the HSV-1 protease was a minor problem.

The experiments shown in Fig. 2 demonstrate how a change in subcellular localization can be changed by means of a specific proteolytic cleavage. When the RevEGFP*as*DsRed1 protein was expressed in COS-7 cells in the absence of the protease, both fluorescent markers colocalize in the cytoplasm. When the cytoplasmic protease (MycVP24) is coexpressed with RevEGFP*as*DsRed1 the two fluorescent markers separated. The DsRed1 signal remained cytoplasmic after cleavage while the RevEGFP protein accumulated in the nucleus and nucleoli similar to cells transfected with pRevEGFP (Fig. 1B). These results strongly suggested that cytosolic MycVP24 cleaved the RevEGFP*as*DsRed1, allowing RevEGFP to enter nuclear/nucleolar compartments, leaving the DsRed1 protein in the cytoplasm.

The aggregation of the DsRed1 protein together with Rev oligomerization seemed to impair the nuclear import of the RevEGFP-DsRed1 fusion proteins. Later versions of the RFP protein were design to abolish the aggregation.

The results shown here demonstrated that the *in vivo* activity of a recombinant protease can be monitored not only by band shift in

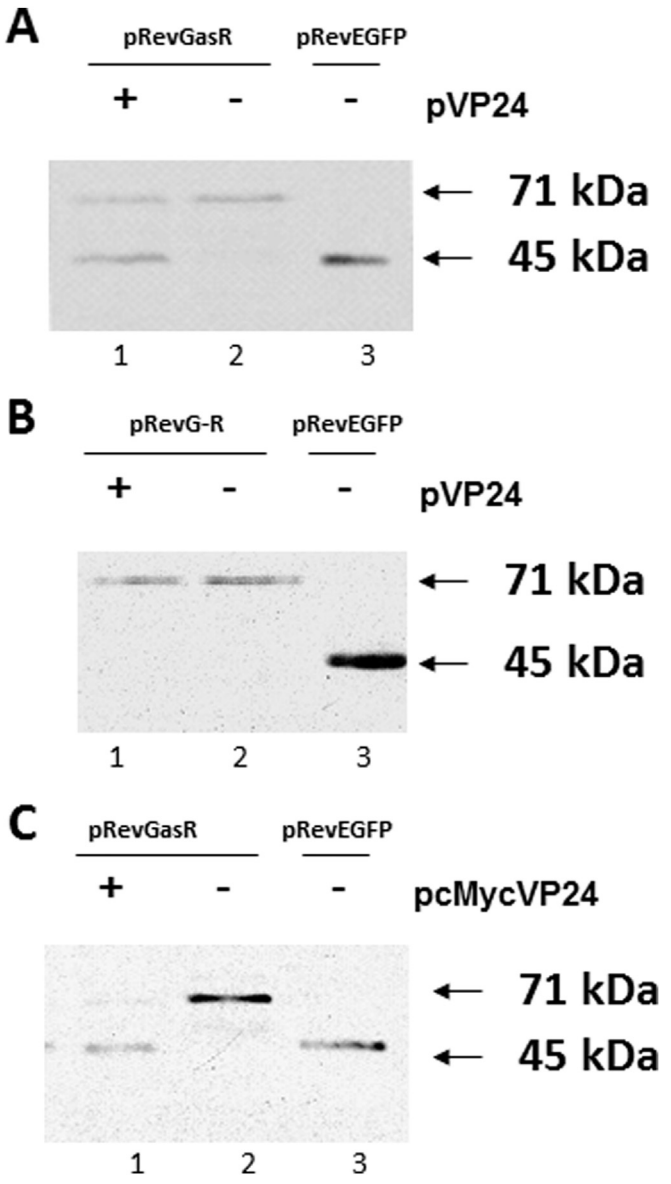

Fig. 3. Activity of HSV-1 VP24. COS-7 cells were transfected with the plasmids indicated above the lanes. A) Western blot analysis of COS-7 cells expressing Re*v*EGFP*as*DsRed1 protein in the presence (+) or absence (−) of VP24 protease. B) Western blot analysis of RevEGFP-DsRed1 proteins in the presence (+) or absence (-) of VP24. C) Western blot analysis of RevEGFP*as*DsRed1 protein in the presence (+) or absence (−) of MycVP24. The Rev fusion proteins were detected using the anti-Rev Mab 8E7. The RevEGFP in lane 3 is shown as a molecular size reference. Arrowheads: 71-kDa Rev fusion protein and the 45-kDa cleavage product.

Western blot analysis but also by a change in the subcellular localization of a double-labelled substrate shuttle protein. Thus the detection of protease activity can easily be assessed by fluorescence microscopy, even in living cells. Given that we have demonstrated the basic components of the *in vivo* protease system, we suggest that the same approach could be used to monitor *in vivo* activities of other proteases. This could be achieved by adding different cleavage recognition signals between EGFP and DsRed1 and applying them to other proteases than HSV-1 protease. HSV-1 VP24 protease also offers the use of temperature-sensitive mutants, allowing for monitoring the commencement of the protease activity in a live cell setting.

Monitoring protease activity in living cells is an important tool that enables testing in systems that more closely resemble real-life conditions. Several methods have been developed for testing proteolytic activity using autofluorescent proteins or nucleocytoplasmic transport in living cells. Here we combine the use of dual autofluorescent proteins as an imaging tool with nucleo-cytoplasmic transport of the substrate to investigate protease activity in a living cell. This gives the additional benefit of allowing for observing the activity both at the level of separation of fluorescent proteins and of changes in their location inside the cells. The method seems to be a highly promising tool for high-throughput screening for protease activity or drug discovery. Furthermore, a method using this technology is now being developed in our lab to monitor if a protein has visited a specific cellular compartment. The availability of a generalized method for monitoring visits to different subcellular compartments would be useful additions to the toolbox for both cell biology and cancer research.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.07.011.

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