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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

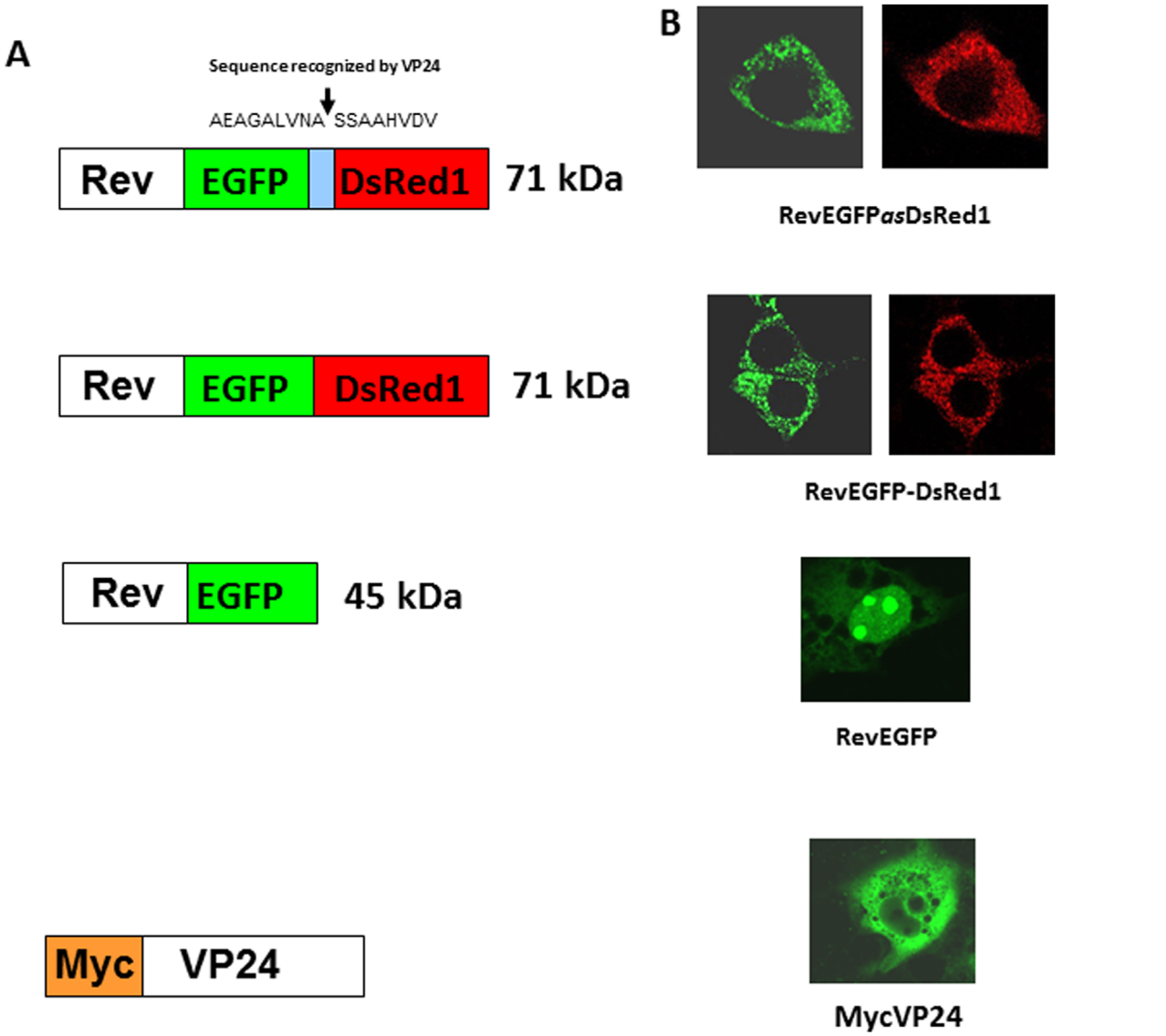

Fig. 1. Schematic representations of Rev fusion proteins, protease fusion protein and localization of these constructs in COS-7 cells. (A) RevEGFP*as*DsRed1consists of Rev, enhanced green fluorescent protein (EGFP) and red fluorescent protein (DsRed1). Between the EGFP and DsRed1 the HSV-1 VP24 recognition 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).