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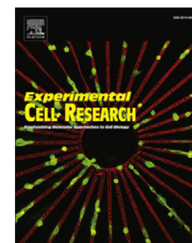

Research Article

The use of fluorescent intrabodies to detect endogenous gankyrin in living cancer cells

Anne-Sophie Rinaldi[a,1], Guillaume Freund[a], Dominique Desplancq[a], Annie-Paule Sibler[a], Mireille Baltzinger[a], Natacha Rochel[b], Yves Mély[c], Pascal Didier[c,2], Etienne Weiss[a,*,2]

[a]Ecole Supérieure de Biotechnologie de Strasbourg, UMR 7242, CNRS/Université de Strasbourg, boulevard Sébastien Brant, 67412 Illkirch, France

[b]Institut de Génétique et de Biologie Moléculaire et Cellulaire, UMR 7104, CNRS/INSERM/Université de Strasbourg, rue Laurent Fries, 67404 Illkirch, France

[c]Faculté de Pharmacie, UMR 7213, CNRS/Université de Strasbourg, route du Rhin, 67401 Illkirch, France

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ABSTRACT

Expression of antibody fragments in mammalian cells (intrabodies) is used to probe the target protein or interfere with its biological function. We previously described the *in vitro* characterisation of a single-chain Fv (scFv) antibody fragment (F5) isolated from an intrabody library that binds to the oncoprotein gankyrin (GK) in solution. Here, we have isolated several other scFvs that interact with GK in the presence of F5 and tested whether they allow, when fused to fluorescent proteins, to detect by FRET endogenous GK in living cells. The binding of pairs of scFvs to GK was analysed by gel filtration and the ability of each scFv to mediate nuclear import/export of GK was determined. Binding between scFv-EGFP and RFP-labelled GK in living cells was detected by fluorescence lifetime imaging microscopy (FLIM). After co-transfection of two scFvs fused to EGFP and RFP, respectively, which form a tri-molecular complex with GK *in vitro*, FRET signal was measured. This system allowed us to observe that GK is monomeric and distributed throughout the cytoplasm and nucleus of several cancer cell lines. Our results show that pairs of fluorescently labelled intrabodies can be monitored by FLIM–FRET microscopy and that this technique allows the detection of lowly expressed endogenous proteins in single living cells.

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Abbreviations: ANK, ankyrin repeat; CDR, complementarity-determining region; DAPI, 4′,6′-diamidino-2 phenyleindole; EGFP, enhanced green fluorescent protein; FLIM, fluorescence lifetime imaging microscopy; FRET, Förster resonant energy transfer; GK, gankyrin; GST, gluthatione-*S* transferase; LMB, leptomycin B; NES, nuclear export signal, NLS, nuclear localization signal; PLA, proximity ligation assay; RFP, red fluorescent protein; S6CT, S6 ATPase C-terminal domain; scFv, single-chain Fv; VH, variable heavy chain domain; VL, variable light chain domain.

*Corresponding author.
E-mail address: eweiss@unistra.fr (E. Weiss).

[1] Present address: Institute for Microbiological Research J-M Wiame, Université Libre de Bruxelles, avenue E. Gryson, Brussels, Belgium.

[2] Contributed equally to the supervision of this work.

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Introduction

Genetically encoded fluorescent proteins (FPs) have revolutionised the ability to study protein localization and dynamics in living cells. They enable the construction of fluorescent tools that are produced by the cell transcriptional and translational machineries and are well tolerated by most cell types [1]. Fluorescent fusion proteins are the most widely used application of FPs, since they allow specific proteins to be tracked in cells using time-lapse microscopy [2,3] or, more recently, a fluorescence-activated cell sorter [4]. While this technique is useful for imaging of proteins that have a distinct localization or show an aggregation pattern, this approach is not suitable for monitoring endogenous proteins expressed at low levels and is uninformative if transient overexpression leads to protein accumulation up to levels which affect its localisation. Fluorescent antibodies that bind specifically to the target may offer a means of studying endogenous proteins in living cells; however a technique to deliver these molecules to cells efficiently is still being developed [5].

The advances in recombinant antibodies technologies have allowed cloned antibody variable domains to be stably expressed in mammalian cells either as single-domain antibodies, such as camelid variable heavy chain domains (V_HH;) [6,7], or as single chain Fv antibody fragments (scFvs) constituted by the assembly of the variable heavy (VH) and light (VL) chain connected by a flexible linker [8–13]. These so-called intrabodies can be easily fused to FPs to allow the recognized antigen to be imaged, for instance, by fluorescence microscopy [14–17]. Although this technique overcomes some of the limitations associated with overexpressing fluorescent fusion proteins, it is difficult to determine if the fluorescence of FP-labelled intrabodies reflects binding specifically to the protein target in the intracellular environment. Indeed, it is likely that a large portion of the overexpressed intrabodies remain free in the cytosol especially when the antigen is expressed at low levels, with the results that fluorescence does not necessarily reflect the localisation of the antigen.

We recently isolated scFvs from an intrabody library that bind gankyrin (GK), a cellular protein initially found to be expressed at higher levels in hepatocellular carcinoma cells [18]. This ubiquitous protein, also termed p28(GANK) or PSMD 10, interacts with the S6 ATPase subunit of the 19S proteasome regulatory particle and binds to pRb, cyclin-dependent kinase 4/6, MDM2 and RelA, and thereby contributes to oncogenic processes [19,20]. Since GK interacts with the 26S proteasome and tumour suppressors, it is a potential drug target for liver cancers [21]. The native structures of human GK and its yeast homologue, Nas6p, have been reported [22,23]; however, it remains unclear how GK interacts with the aforementioned proteins *in cellulo* and how it regulates their functions. One reason for this is that GK cannot be easily detected with conventional antibodies in single cells. Since scFvs that are stably expressed under reducing conditions and that bind specifically to GK in solution are available, we hypothesized that if these scFvs were used to make FP-labelled intrabodies, they may enable GK to be visualised in living cancerous cells. Since no washing steps can be performed under these conditions, non-specific fluorescence from scFvs not bound to GK may be a problem. We reasoned that this could be overcome using the principle of a recently described method, the proximity ligation assay (PLA) [24], which uses two antibody recognition events for identification to enhance selectivity, and Förster resonant energy transfer (FRET) to monitor the dual binding of the fluorescent intrabodies to GK. FRET involves non-radiative energy transfer between two adjacent fluorophores, a property that has been successfully used to detect protein-protein interactions in living cells using fluorescence lifetime imaging microscopy (FLIM–-FRET) [25–28].

In this study, we describe the selection and characterisation of scFvs that bind specifically to GK in cells. Following precise epitope binning using gel filtration and structural analysis, scFvs fused to either enhanced green fluorescent protein (EGFP) or red fluorescent protein (RFP) were used in combination with each other to detect endogenous GK by FLIM–FRET analysis in living cells. GK was found to be homogeneously expressed at a constant rate in various cancerous cell lines including a hepatocellular carcinoma line. This is the first example of the detection of endogenous GK in single cells, since its low expression level means it cannot be detected by conventional antibody-based methods. The biosensing strategy described in this study, in which FRET measurement is performed with pairs of fluorescent intrabodies is likely to have many applications in live-cell imaging and may provide a means of imaging other endogenous proteins that are expressed at low levels in cells.

Materials and methods

Plasmid construction

pSV-scFv-E was generated by replacing the scFv 13R4 coding region of p513-13R4-EGFP [29] with scFv fragments amplified from pETOM-scFv vectors [18] digested with HindIII and SpeI restriction enzymes. This vector was used to generate pSV-scFv-G by replacing the EGFP tag with the gluthatione-S transferase (GST) coding region from pETM30 [18] and adding a myc tag encoded by the following oligonucleotides 5′-CTAGTGAACAAAAACTCATCTCAGAAGAGGATCT-GAATG and 5′-CTAGCATTCAGATCCTCTTCTGAGATGAGTTTTTGTTCA. To construct pSV-scFv-N-R, the coding region of RFP was amplified from pDsRed-monomerN1 (Clontech Laboratories, Mountain View, USA) and inserted into pSV-scFv-E that had been digested with SpeI and EcoRI. Myc, B10 and NLS tags were inserted into the resulting plasmid using oligonucleotides encoding the myc and B10 tags, followed by the insertion of oligonucleotides 5′-CTAGTCCTC-CAAAAAAGAAGAGAAAGGTAGAAGACCCCG and 5′–CTAGCGGG-GTCTTCTACCTTTCTCTTCTTTTTTGGA GGA into the unique SpeI site. These oligonucleotides were also used to modify pSV-scFv-E to generate pSV-scFv-NLS-E. To construct vectors containing the β-actin promoter, the unique SpeI site of pDRIVE-hβ-actin (InvivoGen, San Diego, USA) was first deleted. The resulting plasmid pβA was modified by inserting a scFv, followed by an EGFP, RFP, nuclear export signal (NES) or nuclear localization signal (NLS) tag using the unique NcoI and EcoRI restriction sites as described above for the pSV series, generating pβA-scFv-E, pβA-scFv-R, pβA-scFv-NES and pβA-scFv-NLS, respectively. The cloned NES sequence was the same as that in pscFv-NES [15].

The pSV-GK vector corresponds to pCMV4-HA-gankyrin previously described [18]. To construct pSV-GK-NLS plasmid, the coding region of GK was amplified and inserted into p513-scFv-E

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