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Research paper

Design and selection of an intrabody library produced de-novo for the non-structural protein NSP5 of rotavirus

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Abstract

Intracellular antibodies or intrabodies have great potential in protein knockout strategies for intracellular antigens. We applied the Intracellular Antibody Capture Technology for the direct selection in yeast of a mouse scFv library (V_L–V_H format) constructed from animals immunised with recombinant non-structural protein NSP5 of Rotavirus. We selected five different intracellular antibodies (ICAbs), which specifically recognize Δ2, an NSP5 deletion mutant used as bait. The anti-NSP5 ICAbs were well expressed both in yeast and mammalian cells as cytoplasmic or nuclear-tagged forms. By immunofluorescence and co-immunoprecipitation assays we characterised the intracellular interaction of the five anti-NSP5 ICAbs with the co-expressed antigens.

Keywords: Intrabodies; scFv library; IACT; NSP5; Rotavirus

1. Introduction

Intracellular antibodies (ICAbs) or intrabodies have great potential as tools for neutralisation or phenotypic knockout of intracellular proteins (Cattaneo and Biocca, 1997; Lobato and Rabbitts, 2003; Wheeler et al., 2003). Numerous functional studies have illustrated that some recombinant antibodies expressed in the cytoplasm of cells are able to fold and assemble correctly, maintaining their selective-binding properties against their antigens (Ags) (Biocca et al., 1990, 1994; Cattaneo and Biocca, 1999). The single chain Fv format (scFv) of V region antibody fragments (Bird et al., 1988) has been shown to be particularly suitable for intracellular expression (Marasco et al., 1993; Tavladoraki et al., 1993). In the reducing cytoplasmic environment many antibodies are unable to fold properly, resulting in non-functional

Abbreviations: Ig, Immunoglobulin; V_L, light-chain variable domain; V_H, heavy-chain variable domains; ICAb, intracellular antibody; scFv, single chain Fv; VLS, viroplasm-like structure; Ab, antibody; Ag, antigen.

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molecules with low expression levels and short half-lives. The main cause of the improper folding is lack of intra-chain disulphide bonds in the light- and heavy-chain variable domains (V_L and V_H, respectively) of the scFv (Biocca et al., 1994; Proba et al., 1998; Worn and Pluckthun, 1998). However, in spite of the adverse conditions, some scFvs retain their ability to assemble and exhibit antigen specificity in the non-natural nuclear and cytoplasmic compartments (Proba et al., 1997, 1998). At present, it is not possible to predict whether a specific scFv will correctly fold in the cytoplasm of a cell. The developing database VIDA (Validated Intrabody Database) collects sequences of validated intracellular antibodies for the identification of consensus sequences to allow prediction of competent ICAbs (Visintin et al., 2004a,b).

The Intracellular Antibody Capture Technology (IACT) (Visintin et al., 2002) was developed to select competent intracellular antibodies using the yeast two-hybrid system (Visintin et al., 1999, 2002; Tse et al., 2002a,b; Tanaka and Rabbitts, 2003). Initial applications of this technology were based on the use of scFv phage-display libraries enriched in vitro on immobilised protein antigen, prior to selection in yeast. Here we report the construction and selection of a hyper-immune scFv library for fast and direct selection of ICAbs in yeast using IACT, without any in vitro pre-selection. As antigen, we chose the rotavirus non-structural protein NSP5, which is expressed at early times post-infection exclusively in the cytoplasm of infected cells (Eichwald et al., 2004). NSP5 is a phosphoprotein that interacts with other viral proteins (NSP2, VP1 and VP2) (Afrikanova et al., 1996; Blackhall et al., 1997; Fabbretti et al., 1999; Eichwald et al., 2002) and localises in characteristic cytoplasmic viral structures, called viroplasms (Kattoura et al., 1994; Afrikanova et al., 1998; Fabbretti et al., 1999; Eichwald et al., 2002; Berois et al., 2003) where viral replication occurs.

The aim of the present work was to obtain specific ICAbs for use as molecular tools to knock out rotaviral proteins in vivo during virus replication, because of the lack of a reverse genetic system in rotavirus. We report the selection and the characterisation of five different scFvs that were able to interact specifically with antigen in the cytoplasm of transfected mammalian cells.

2. Materials and methods

2.1. Plasmids

pBTM116 (Visintin et al., 1999) vector was used to cloned NSP5 and its deletion mutants. PCR amplification of NSP5 c-DNA (Afrikanova et al., 1996) was performed using primers 5′-CGGAATTCATGTCTC-TCAGCATTG-3′ and 5′-GCGGGATCCTTA CAAATCTTC GATC-3′. The product was digested with *Eco*RI/*Bam*HI and subsequently cloned in pBMT116. The Δ2 mutant was cloned with the same strategy. pVP16/D was generated by cloning a linker into *Sfi*I/*Xho*I restriction sites of the pVP16* (Visintin et al., 1999). The linker was annealed from synthesised oligonucleotides 5′-CGCGCATACAGCTAGCGTT-3′ and 5′-TCGAAACGCTAGCT GTATG-3′, which introduces *Bss*HII/*Nhe*I restriction sites into the construct. The construct was further modified with the fragment encoding the trans-activation domain (aa 401 to 479) of the Herpes simplex virus type I. pVP16/D-scFv vector was generated by cloning a human V_L-linker-V_H (Whitlow et al., 1993) fragment into the *Bss*HI/*Nhe*I sites of the pVP16/D vector. For scFv expression in mammalian cells, the V_L-linker-V_H cassettes were subcloned into pcDNA3 (Invitrogen®) or pEGFP-N1 (Clontech®) vectors. The scFv fragments in pcDNA3 vectors were tagged with two nuclear localisation signals (NLS) of SV40 L-antigen (Persic et al., 1997) using annealed oligonucleotides 5′-C T A G T G A T C C A A A A A A G A A G A G A-AGGTAGATCCAA AAAAGAAGAGAAAGGTAG-3′ and 5′-CTAGCTACCTTTCTCTTCTTTTTTGGAT CTACCTTTCTCTTCTTTTTTGG ATCA-3′ inserted into the *Nhe*I site. R4(NLS) is a human scFv anti-β-galactosidase (Martineau et al., 1998).

2.2. Construction of the immunised scFv library

RNA was extracted (RNAzol B kit TEL-TEST, INC) from the spleen of mice immunised with GST-NSP5 protein following a standard protocol (Eichwald et al., 2002). 1 μg of RNA was used for RT-PCR using random hexamers. Amplification of Ig repertoires was performed with murine degenerated primers (Orlandi et al., 1989) for the heavy and light variable regions, separately. Each set of degenerated oligonucleotides was divided in three groups for the V_L and V_H PCR