Selection of antibodies for intracellular function using a two-hybrid *in vivo* system

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Expression of antibodies inside cells has been used successfully to ablate protein function. This finding suggests that the technology should have an impact on disease treatment and in functional genomics where proteins of unknown function are predicted from genomic sequences. A major hindrance is the paucity of antibodies that function in eukaryotic cells, presumably because the antibodies fold incorrectly in the cytoplasm. To overcome this problem, we have developed an *in vivo* assay for functional intracellular antibodies using a two-hybrid approach. In this assay, antibody, as single-chain Fv (scFv) linked to a transcriptional transactivation domain, can interact with a target antigen, linked to a LexA-DNA binding domain, and thereby activate a reporter gene. We find that several characterized antibodies can bind their target antigen in eukaryotic cells in this two-hybrid format, and we have been able to isolate intracellular binders from among sets of scFv that can bind antigen *in vitro*. Furthermore, we show a model selection in which a single scFv was isolated from a mixture of half a million clones, indicating that this is a robust procedure that should facilitate capture of antibody specificities from complex mixtures. The approach can provide the basis for *de novo* selection of intracellular scFv from libraries, such as those made from spleen RNA after immunization with antigen, for intracellular analysis of protein function based only on genomic or cDNA sequences.

intrabodies | single-chain Fv | therapy | functional genomics

Intracellular antibodies have been demonstrated to function in antigen recognition in cells of higher organisms. This interaction can inhibit the function of proteins in the cytoplasm, the nucleus, or the secretory pathway (reviewed in refs. 1 and 2). This efficacy has been demonstrated for viral resistance in plants (3), and several applications of intracellular antibodies binding to HIV viral proteins (4, 5) and to oncogene products (6, 7) have been reported. The latter is an important area because enforced expression of oncogenes, usually encoding intracellular proteins, often occurs in tumor cells after chromosomal translocations (8). These proteins therefore are important potential therapeutic targets (9), which could be inactivated by binding with intracellular antibodies. Finally, the international efforts at whole genome sequencing will produce massive numbers of potential gene sequences that encode proteins about which nothing is known. Functional genomics is an approach to ascertain the function of this plethora of proteins, and intracellular antibodies promise to be an important tool in this endeavor as a conceptually simple approach to a phenotypic knockout of protein function.

Simple approaches of deriving antibodies that function in cells therefore are necessary if their use is to have any impact on the large number of protein targets. When antibodies are expressed in the cell cytoplasm, folding and stability problems often occur, resulting in low expression levels and limited half-life of antibody domains. These problems most likely are caused by the reducing environment of the cell cytoplasm (10), which hinders the formation of the intrachain disulfide bond of the V_H and V_L domains (11, 12) and is important for the stability of the folded protein. However, some single-chain Fv (scFv) have been shown to tolerate the absence of this bond (13, 14), depending on the particular primary sequence. No rules or consistent predictions can yet be made, however, about those antibodies that will tolerate the cell cytoplasm conditions. Although the scFv format (15, 16) is the most successful form for intracellular expression, not all mAbs can be made as scFv and maintain function in cells.

There is a need, therefore, to obtain antibody fragments that will fold and are stable and soluble under conditions of intracellular expression. Approaches might include modification of the V_H and V_L sequences to replace the need for disulfide bonds to stabilize the scFv with either higher intrinsic stability (17) or with differing intrachain stabilizing factors. Another possible method would be the use of entirely different structural design for ligand binding (18). Such rational approaches may lead to success in individual cases but may not be easily generally applied. A more general strategy would be to apply selection to the derivation of intracellular antigen-binding antibody fragments, which would allow isolation based on binding efficacy *in vivo*. To this aim, we have exploited the two-hybrid system (19) to monitor intracellular antigen-antibody interactions via reporter gene activation, using both yeast and mammalian cells. In these experiments, we have demonstrated that only certain antibody fragments can efficiently interact with their antigen *in vivo*. The use of reporter assays to detect antibody-antigen interaction *in vivo* thus provides a strategy to select those individual antigen-specific scFv that can function in cells.

Materials and Methods

Strains and Plasmids. The genotype of the *Saccharomyces cerevisiae* reporter strain L40 (Invitrogen) is MATa his3Δ200 trp1–901 leu2–3, 112 ade2 LYS::(lexAop)4-HIS URA3::(lexAop)8-*LacZ* GAL4 (20).

LexA fusion baits were prepared in the plasmid pBTM116 (5.5 kb) (kindly provided by Marijane Russel, Invitrogen) (21).

For AMCVp41/BTM116 (pLexA-AMCVp41), the AMCV *p41* gene was amplified by reverse transcription–PCR from artichoke mottle crinkle virus (AMCV) cDNA (provided by E. Benvenuto, ENEA, Rome) and was inserted into *Eco*RI–*Bam*HI sites of pBTM116.

For K-ras/BTM116 (pLexA-K-Ras), the *K-Ras B* gene was amplified by PCR from pGem3Z-k-ras (provided by G. Vecchio, University of Naples, Italy) and cloned into *Bam*HI–*Pst*I sites of pBTM116.

For Syk/BTM116 (pLexA-Syk), the *Syk* gene was amplified by PCR from *Syk* cDNA (22) and inserted into *Eco*RI–*Bam*HI sites of pBTM116.

For β-galactosidase (β-gal)/BTM116 (pLexA-β-gal), the *lacZ*

Abbreviations: scFv, single-chain Fv; AMCV, artichoke mottle crinkle virus; β-gal, β-galactosidase; CHO, Chinese hamster ovary; CAT, chloramphenicol acetyltransferase.

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gene was amplified by PCR from pBtβ-gal7 (23) and inserted into *Bam*HI–*Pst*I sites of pBTM116.

For HIV-1 IN/BTM116 (pLexA-HIV-1IN33), the HIV-1 *IN33* gene was amplified by PCR from pRP1012 (5) and inserted into *Bam*HI–*Pst*I sites of pBTM116.

ScFv-VP16 fusions were constructed in pVP16* (provided by M. Russel, Invitrogen), a modification of pVP16 (24) with a polylinker upstream of the VP16 acidic activation domain, to generate in-frame protein fusions to VP16, with two simian virus 40 large T antigen nuclear localization sequences.

For pscFvF8-VP16*, the *scFvF8* DNA (3) was PCR-amplified from pGEMscFv(F8) (provided by E. Benvenuto) and inserted into *Sfi*I–*Not*I sites of pVP16*.

pscFvY13-VP16* was subcloned as *Sfi*I–*Not*I fragments from pHENY13 (derived from p3JF4; ref. 25) into pVP16*. pscFvPM163-VP16* and pscFvPM163R4-VP16 were subcloned as *Nco*I–*Not*I fragments from pPM163 and pPM163R4 (12) (provided by P. Martineau, Institut Pasteur, Paris) into pCANTAB6 and subcloned again as *Sfi*I–*Not*I fragments from pCANTAB6scFvPM163 and pCANTAB6scFvPM163R4, respectively, into pVP16*.

pscFvV6C11-VP16*, pscFvG6G2-VP16, and pscFvG4G11-VP16 were subcloned as *Nco*I–*Not*I fragments from pscFvexpV6C11, pscFvexpG6G2, and pscFvexpG4G11 (provided by Piona Dariavach, Institut de Genetique Moleculaire, Montpellier, France), respectively, into pCANTAB6 and subcloned again as *Sfi*I–*Not*I fragments from pCANTAB6scFvV6C11, pCANTAB6scFvG6G2, and pCANTAB6scFvG4G11, respectively, into pVP16*.

For pscFvIN33-VP16*, scFvIN33 (5) (provided by R. Pomerantz, Jefferson Medical College, Philadelphia) was PCR-amplified from pNLVP16 and inserted into *Sfi*I–*Not*I sites of VP16*.

For a model selection, the ''Vaughan'' scFv library (26) (provided by Cambridge Antibody Technology, U.K.) was challenged against HIV-1 reverse transcriptase (27). After three cycles, *Sfi*I–*Not*I fragments derived from the polyclonal population of pCANTAB-6-polyαRT were inserted into pVP16* vector.

pM-β-gal was constructed by subcloning the *lacZ Sfi*I–*Pac*I fragment of pN3neo2TK-1 (23) into *Sma*I-cut pM1 (28). ScFvR4 DNA was PCR-amplified from pPM163R4 (12) and subcloned into pNLVP16 (*Eco*RI digested) or pNLVP16 (*Nde*I digested) to create pNL-ScFvR4-VP16 and pNLVP16-ScFvR4 in which the scFv was, respectively, N or C terminal to VP16. pM-scFvR4 and pNLVP16-β-gal were constructed by subcloning the ScFvR4 PCR product and β-gal *Sfi*I–*Pac*I fragment into the pM1 and pNLVP16 vectors, respectively. The *Eco*RI–*Bam*HI AMCVp41 DNA fragment from AMCVp41/BTM116 was subcloned into pM1 (*Eco*RI digested) to give pM1-AMCV. The ScFvF8 DNA was amplified from pGEM-ScFvF8 by PCR and cloned into pNLVP16 (*Eco*RI digested) to create pNLScFvF8-VP16.

All clones were sequenced to confirm in-frame fusion of the inserts with the Gal4 binding domain or the VP16 in the vectors.

Production Media and Protocols. Yeast was grown at 30°C (or at 20°C, when appropriate) for 72–144 h, in rich medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose, and 0.1% mg/ml adenine buffered at pH 5.8) or in synthetic minimal YC (0.12% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.1% succinic acid, 0.6% NaOH, 2% glucose and, as required, 2% agar) medium containing 0.075% amino acid supplements (lacking Trp, Leu, Ura, Lys, and His; 0.1% each of adenine sulfate, Arg, Cys, Thr; 0.05% each of Asp, Ile, Met, Phe, Pro, Ser, and Tyr) buffered at pH 5.8. When necessary, 0.01% each of Trp, Ura, Lys, and Leu and 0.005% His were supplemented to the media.

The plasmids were transformed into L40 yeast strain by using lithium acetate transformation protocol (29). Positive clones were selected by using auxotrophic markers for both plasmids and for lysine and histidine prototropy. Histidine-positive colonies and controls were lysed in liquid nitrogen and assayed for β-gal activity on filters as described (30).

Analysis of the Intrachain Disulfide Bonds. Yeast protein extracts were prepared as described (31); samples were subjected to SDS/PAGE (12% acrylamide) in the presence or absence of β-mercaptoethanol (11) and transferred onto nitrocellulose membranes (Schleicher & Schuell). Filters were incubated with the mAb anti-myc antibody 9E10 (32), followed by anti-mouse-peroxidase conjugate (Dako).

Mammalian Cell Transfection. Chinese hamster ovary (CHO) cells were grown in α minimal essential medium (GIBCO/BRL) with 10% FCS, penicillin, and streptomycin. CHO cells were transfected with Lipofectin (GIBCO/BRL) and 5 μg of each plasmid, together with 5 μg of pE5C-chloramphenicol acetyltransferase (CAT) reporter (33). pBSpt DNA was added where necessary to compensate for unequal DNA input in each transfection. Cells were harvested 48 h after transfection, and CAT assay was performed as described (33). Each transfection was repeated twice and a PhosphorImager (Molecular Dynamics) was used for quantification of the signals in the CAT assay.

Results

The Antibody-Antigen Two-Hybrid System. We have adapted the eukaryotic two-hybrid assay (19) to monitor the interaction of scFv fragments with their corresponding antigens, under conditions of intracellular expression. The procedure is outlined in Fig. 1. Expression vectors were obtained by cloning the sequences coding for target antigen at the 3′ end of the DNA binding domain of the *Escherichia coli* protein LexA (21) or cloning scFv fragments sequences at the 5′ end of the activation domain of the herpes virus 1 VP16 transcription factor (20) (Fig. 1*a*, vectors 1 and 2). These vectors are cotransfected in yeast cells lacking histidine production (i.e., cannot grow in the absence of histidine) but that carry the *HIS3* gene under the control of a minimal promoter with LexA DNA recognition site (Fig. 1*b*). If the scFv antibody fragment binds to the antigen target *in vivo*, a complex is formed that can bind to the *HIS3* promoter (via the LexA part of the LexA-antigen fusion) and activate transcription (via the scFv is linked to the VP16 transcriptional activation domain). This activity will restore histidine independent growth of the yeast (Fig. 1*b*). In addition, the yeast cells carry a *lacZ* gene controlled by a minimal promoter also with a LexA DNA binding site. Thus β-gal production (measured by activation of β-galactosidase and blue colonies) is also a consequence of antibody-antigen interaction in the transfected yeast cells. Finally, two nuclear localization signals are located on the scFv-VP16 fusion product, whereas the antigen bait has none. Therefore, the interaction between the antigen and the scFv must occur in the cytoplasm, before the complex is translocated to the nucleus and activates transcription.

As a model system, we used the scFv fragment F8, recognizing the p41 coat protein of the AMCV, previously shown to protect transgenic *Nicotiana benthamiana* plants from viral attack when intracellularly expressed (3). Coexpression of the LexA-AMCV bait (AMCVp41/BTM116) and the scFvF8-VP16 fusion protein in yeast leads to an efficient growth of the cells in the absence of histidine, because of HIS3 activation and shows a high level of β-gal activity (Fig. 2, row 4). The transfection of the bait alone, of the bait with the VP16 vector, or of an unrelated antigen fusion (LexA-lamin) with the scFvF8 fusion (Fig. 2, rows 1, 2, and 3, respectively) did not result in activation of the *lacZ* gene. These results demonstrate that the scFvF8 is able to specifically

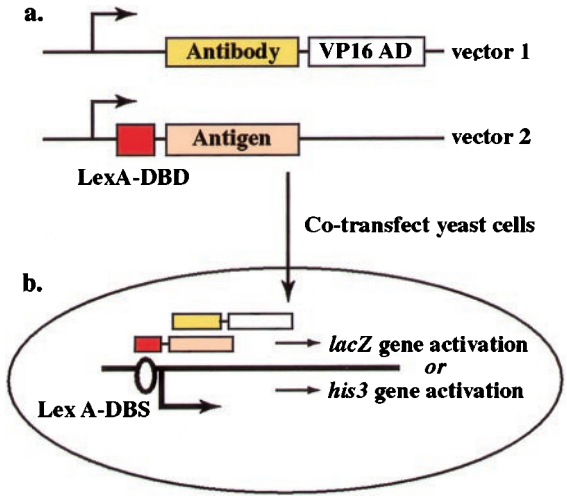

Fig. 1. Diagram of antibody-antigen *in vivo* interaction assay. The two-hybrid system of Fields and Song (19) was adapted to detect antibody-antigen interaction *in vivo*. (*a*) Yeast expression constructs were prepared encoding either (*i*) an antibody fragment, in the form of scFv, linked to the VP16 transcriptional activation domain (AD) or (*ii*) the LexA DNA binding domain (DBD) linked to an target antigen sequence. (*b*) These constructs are cotransfected into yeast cells unable to synthesis histidine and carrying either the histidine (*his*) gene or the *lacZ* gene controlled by a minimal transcription promoter with a LexA DNA binding site (DBS). If antibody-antigen interaction occurs *in vivo*, the resulting complex can bind to the LexA DBS upstream of *his* or *lacZ* genes and transcription of these genes occurs (the VP16 activation domain is thus brought close to the DNA transcription start site and can recruit accessory factors needed for transcription). The transcriptional activation of the *his* gene facilitates growth of yeast on media lacking histidine or and activation of the *lacZ* gene produces β-gal, which can be assayed with 5-bromo-4-chloro-3-indolyl β-D-galactoside to yield blue yeast colonies. Neither feature of the transfected yeast will occur if the antibody fragment does not functional inside cells.

interact with its corresponding p41 antigen under the intracellular conditions of this assay.

The Yeast Antibody-Antigen Two-Hybrid System Can Distinguish Intracellular Binders. The general use of the yeast cell to detect antigen-scFv interaction was assessed with a panel of scFv

Fig. 2. AMCV antigen-scFv F8 antibody interactions in the yeast two-hybrid system. L40 yeast cells (auxotrophic for Trp and Leu and with *HIS3* and *lacZ* reporter genes for VP16-dependent transcriptional activation) were cotransfected with a LexA-AMCVp41 antigen bait vector (AMCVp41/BTM116) and scFvF8-VP16 fusion vectors or the VP16 vector alone. Yeast colonies were grown on agar and appear slightly pink. Interaction of antigen bait and ScFvF8-VP16 fusion causes growth on his−plates because of HIS3 activation and blue color in 5-bromo-4-chloro-3-indolyl β-D-galactoside substrate. β-gal assays of L40 yeast were transfected with the following vectors. Row 1: AMCVp41/BTM116 alone. Row 2: AMCVp41/BTM116 + VP16. Row 3: Lamin/BTM116 + scFvF8/VP16. Row 4: AMCVp41/BTM116 + scFvF8/VP16. The expression level of LexA, LexA-Lamin, LexA-AMCVp41, and F8-VP16 proteins was assessed by Western blot analysis using a polyclonal antibody anti-LexA protein (InVitrogen) for bait constructs and mAb 9E10 (32) (recognizing the myc-tag) for F8-VP16 (data not shown). β-gal activation was observed only when the LexA-AMCVp41 bait was coexpressed with scFvF8.

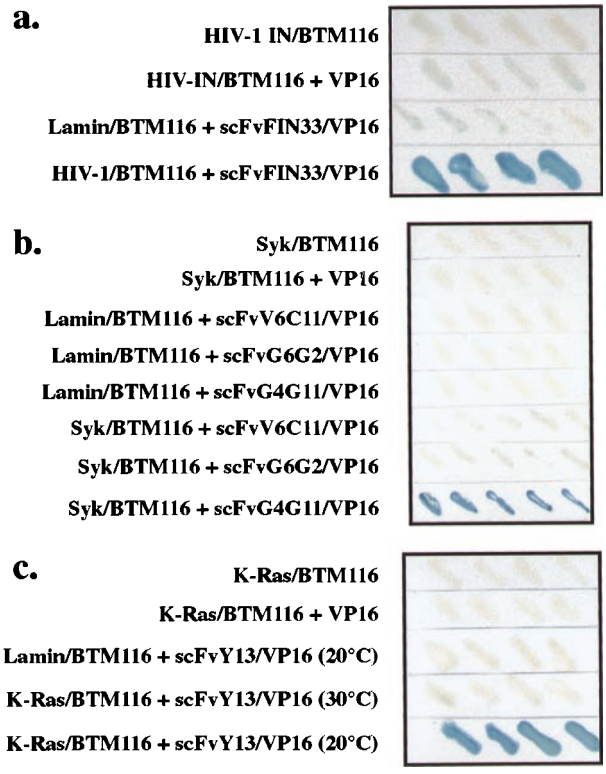

Fig. 3. Intracellular interaction of scFv with target antigens in yeast. L40 yeast were cotransfected with Lex A-antigen DNA-BD (baits) in the pBTM116 vector and scFv fusions with the VP16 transcriptional transactivation domain in the pVP16 vector. Yeast were streaked and grown on his− medium and scored for β-gal activity. (*a*) Transfection of HIV-1 integrase HIV-1 IN/BTM116 alone (row 1), with the pVP16 vector (row 2), or with the anti-integrase scFvIN33/VP16 (row 4). In row 3 scFvIN33/VP16 was cotransfected with the nonrelevant Lamin/BTM116 bait. β-gal activation was observed only in row 4. (*b*) Transfection of Syk bait Syk/BTM116 alone (row 1) or with pVP16 (row 2). In rows 3–5 three anti-Syk scFv-VP16 clones (scFvV6C11, G6G2, and G4G11-VP16) were cotransfected with a nonrelevant antigen bait (Lamin/BTM116), and in rows 6–8 the same three anti-Syk scFv-VP16 clones were cotransfected with the Syk/BTM116 bait. β-gal activation was observed only in row 8. (*c*) Transfection of K-ras bait K-ras/BTM116 alone (row 1) or with pVP16 (row 2). In row 3, the anti-ras scFv clone scFvY13/VP16 was cotransfected with the nonrelevant Lamin/BTM116 bait at 20°C. Rows 4 and 5 refer to clones cotransfected with K-ras/BTM116 and scFvY13/VP16 grown at 30°C and 20°C, respectively. β-gal activation was observed only in row 5 (20°C).

derived either from mAbs or from phage display antibody libraries, and some of which had been shown to have biological activity when intracellularly expressed *in vivo*. We have tested scFv binding to HIV-1 integrase, the tyrosine kinase Syk, and p21-ras (Fig. 3). All bait clones used in these assays were individually assessed for auto-activation of the *HIS3* and *lacZ* genes, with and without the empty pVP16 vector, and each was tested by cotransfection with another bait encoding a lamin antigen, and growth was detected in his− medium (data not shown). The anti-HIV integrase scFv IN33 was derived from a mAb and was expressed in human cells leading to a specific neutralization of HIV-1 integrase activity (5). When the HIV-1 IN/BTM116 bait was expressed with the scFvIN33/VP16 fusion in L40 yeast, we observed a high level of β-gal activity (Fig. 3*a*, row 4), whereas expression of the scFvIN33-VP16 fusion with a lamin bait (Fig. 3*a*, row 3), of the bait alone or of the HIV-1 IN/BTM116 bait with the VP16 vector alone (Fig. 3*a*, rows 1 and 2, respectively) did not result in any activation.

To determine whether scFv selected from phage display libraries *in vitro* could function in the antibody-antigen two-

hybrid assay, we tested three phage-derived scFv fragments (scFvV6C11/VP16, scFvG4G11/VP16, and scFvG6G2/VP16) recognizing the tyrosine kinase Syk protein, and which react with Syk in ELISA, immunoprecipitation, immunofluorescence, or Western blot (P. Dariavach, personal communication). Only one of these, scFvG4G11/VP16, shows a positive interaction with the LexA-Syk bait (Syk/BTM116) (Fig. 3*b*, rows 6–8). The specificity of scFvG4G11 interaction with the syk/BTM116 bait was established with the Lamin bait (Fig. 3*b*, row 5). These data extend the notion that *in vitro* selection of scFv from phage display libraries is an inadequate criterion for their subsequent use as interacting intracellular antibodies. The inference that scFv selected purely for their ability to bind antigen *in vitro* (e.g., in Western blot assays) may not necessarily function correctly as intracellular antibodies was endorsed by the observation that no intracellular binders were found among two anti-BCR, two anti-ABL, or eight anti-MLL scFv selected from phage display libraries (E.T. and T.H.R., data not shown), despite their having specific binding to antigen in Western blotting analyses.

The conclusion that individual antibodies may not possess binding properties *in vivo* was re-enforced by our findings with a panel of scFv directed against the signal transduction protein p21 ras. There is a significant inhibition of signal transduction processes involving the activation of p21-ras when the anti-ras Y13–259 scFv is expressed in cells (6, 7, 34). When scFv fragment Y13–259 was fused to the VP16 activation domain (scFvY13/VP16) and expressed in yeast cells, together with the K-ras protein fused to LexA (K-ras/BTM116), it failed to induce growth in the absence of histidine or β-gal activity at 30°C (Fig. 3*c*, row 4), but did so when the same cells were cultured at 20°C (Fig. 3*c*, row 5). This interaction was specific for the K-ras antigen (Fig. 3*c*, row 3) and the K-ras bait does not activate the reporter genes on its own, at 20°C (Fig. 3*c*, rows 1 and 2), or 30°C (not shown). A number of other scFv fragments against the p21-ras protein (35), isolated from two different phage display libraries, also were tested in this system. The binding affinity of each of these scFv fragments for p21-ras, determined by surface plasmon resonance, was in the range of 5 to 800 nM. However, only two of a total of 12 distinct scFv showed any effect on the K-ras antigen, even when cells were grown at 20°C (data not shown). Thus, clearly not all scFv fragments isolated from a phage display library are able to bind their antigen when intracellularly expressed in the two-hybrid system.

Intracellular Interaction in a Mammalian Antibody-Antigen Two-Hybrid System. Our experiments in yeast show that antibody-antigen interaction can assemble the cooperative transcription complex necessary to transcribe the *HIS3* or *lacZ* genes, providing the means to determine whether a particular antibody has the potential for use *in vivo* as an intracellular antibody, in higher organisms, particularly mammalian cells. Therefore we evaluated the antibody-antigen two-hybrid assay system in CHO cells. Antigen fusions were made with the Gal4 DNA binding domain within the vector pM1 and scFv fusions with pNLVP16 (28) and cotransfections were performed in CHO cells with a CAT reporter vector. We tested scFv that bind to AMCVp41, HIV-1 integrase, K-ras, and β-gal. By contrast with the results obtained in yeast, only the latter activated the CAT reporter when coexpressed with the appropriate bait (the latter could not be used in yeast, because the β-gal bait required was not suitable). When CHO cells were cotransfected with the CAT reporter with the pM-β-gal bait and the pNLS-scFvR4-VP16 vectors [encoding a fusion between the anti-β-gal ScFvR4 (12) and the VP16 transcriptional transactivation domain] about 60-fold increase in CAT activity was detected (Fig. 4*a*, lane 1 compared with control lane 2). This finding indicates that the interaction of ScFv with the β-gal antigen in the CHO cells was sufficient to activate CAT transcription in a specific manner (see controls in Fig. 4*a*, lanes 3–6). In addition, the fusion of the ScFvR4 with the GAL4 DNA binding domain and of β-gal with the VP16 activation domain retained the ability to activate the CAT reporter, albeit with about 50% lower level (Fig. 4*a*, lane 7). The lower efficiency may result from structural constraints, as a construct expressing an ScFvR4 linked at the C terminus of the VP16 activation domain also showed reduced CAT activation (Fig. 4*a*, lane 10). The quantitative data from two independent experiments are reported in Fig. 4*b*.

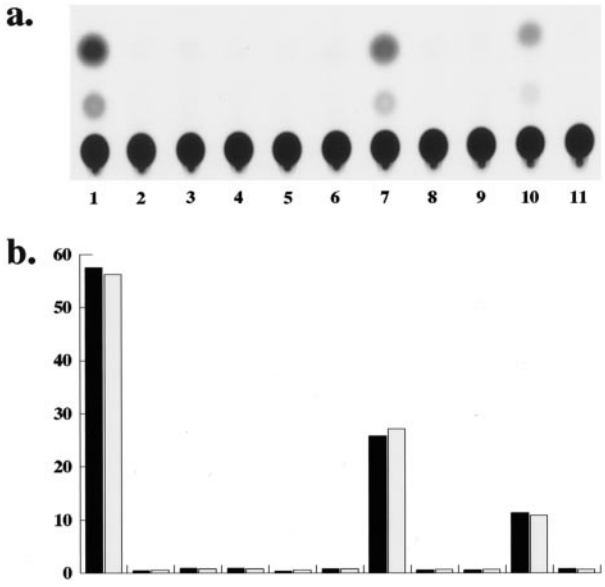

Fig. 4. Mammalian antibody-antigen two-hybrid CAT assay. CAT reporter clone (pE5C-CAT) was transfected into CHO cells with combinations of pM1 or pNLVP16 derivatives and CAT activity was scored by TLC (*a*) or PhosphoImager analysis (*b*). Two independent transfections were performed for each vector combination. Each transfection had pE5C-CAT reporter together with: lane 1, pM-β-gal + pNLscFvR4-VP16; lane 2, pE5C-CAT reporter alone; lane 3, pM-β-gal; lane 4, pNLscFvR4-VP16; lane 5:,pMβ-gal + pNLscFvF8-VP16; lane 6, pNLscFv-VP16 + pM1-AMCV; lane 7, pM-scFvR4 + pNLVP16-β-gal; lane 8, pM-scFvR4; lane 9, pNLVP16-β-gal; lane 10, pM-β-gal + pNLVP16-scFvR4; and lane 11, pNLVP16-scFvR4.

Model Selection of scFv from a Large Mixture in the Yeast Antibody-Antigen System. The ability of only some antigen-specific scFv to bind antigen intracellularly suggests that folding and stability of antibodies in the cell cytoplasm is not constant and therefore a selection strategy is needed to isolate the subset of scFv that can bind inside cells. The two-hybrid assay for intracellular antigen-antibody interactions should allow the isolation of antibody domains that tolerate the absence of the intra-chain disulfide bond, in the reducing environment of the cytoplasm. The anti-AMCV scFvF8 is able to fold sufficiently well to bind antigen in plant cells (3) and in the two-hybrid interaction shown here (Fig. 2). We therefore investigated the redox state of the scFvF8/VP16 fusion in yeast cells with a gel mobility assay (11) (Fig. 5). No difference was seen in the mobility of the scFvF8-VP16 protein when reduced or unreduced, as compared with a clear gel shift between the oxidized and the reduced forms of a scFv expressed in the endoplasmic reticulum, where the disulfide bond is formed (Fig. 5). Thus, the scFvF8 made in yeast does not seem to have S-S bonds, yet does retain antigen binding. Therefore we considered scFvF8 as an ideal molecule with which to perform model selections *in vivo* to show that the yeast antibody-antigen system should be applicable to antibody selection strategies.

A model selection was carried out by using the AMCVp41/

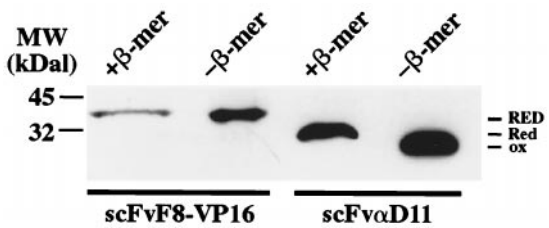

Fig. 5. Redox state of scFv fragments. Western blot analysis of scFvF8-VP16 (expressed in the cytoplasm of L40 yeast cells) and of a secreted scFv fragment (scFvαD11, expressed as a secreted protein in the endoplasmic reticulum of insect cells). Samples were prepared in SDS/PAGE loading buffer containing (+ β-mer) or not (− β-mer) β-mercaptoethanol. After blotting, the scFv fragments were detected with the anti-myc antibody 9E10. The bars at the right of the lanes indicate the molecular mass gel shift between the oxidized (ox) and reduced (red) forms of the scFvαD11. ScFvF8-VP16 fusion protein does not show a shift in electrophoretic mobility, between reducing and nonreducing conditions, indicating that it does not form disulfide bonds in the yeast cytoplasm (RED).

BTM116 bait with the scFvF8-VP16 fusion diluted with DNA from a library encoding nonrelevant scFv-VP16 fusion proteins (Fig. 6). A plasmid DNA mixture was made with one part scFvF8 added to 5×10^5 parts scFv library. The mixture was cotransformed into yeast cells with the AMCVp41/BTM116 bait, and colonies were isolated on histidine-deficient plates. After 3 days, 84 colonies had grown from one-fortieth of the transfection mix. These were picked and replated on a grid plate (without histidine) and these colonies were screened for β-gal activity. In Fig. 6*a*, the results with 48 of these clones are shown, of which 22 developed a strong blue color, indicating that the yeast contain scFvF8-VP16 binding to the AMCV bait DNA binding protein. This conclusion was confirmed by analysis of the plasmid DNA isolated from 10 colonies from which a strong blue color was found. This plasmid DNA was first compared by their restriction pattern after *Bst*N1 digestion and each yielded a pattern identical with scFVF8 staring material. Direct sequencing of plasmid DNA recovered from yeast colonies confirmed that all 10 β-gal-positive clones were scFvF8 (data not shown). These data show that selecting from a dilution of 1 in half a million can be achieved and therefore selections at even higher dilutions are feasible by appropriately scaling up the number of yeast colonies transfected.

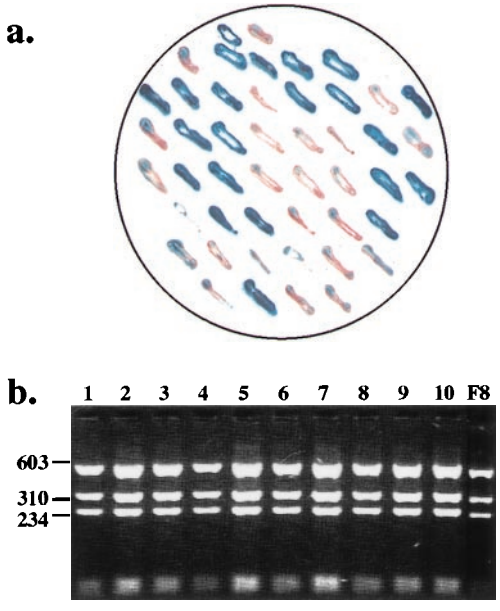

Fig. 6. Model selection of scFvF8-VP16 clone from a mixture of 500,000 scFv clones. Yeast L40 was cotransformed with the AMCVp41/BTM116 DNA-binding bait clone together with DNA of a nonrelevant, naive scFv-VP16 library into which scFv-F8-VP16 had been added at a dilution of 1 in 500,000. Yeast were plated on plates lacking histidine to select for interacting partners. Forty eight clones that grew on these plates were picked and grown on a fresh grid and the β-gal assay was performed (*a*). Ten blue clones were grown, DNA was prepared, and the fingerprint of BstN1 digestion products compared with scFv-F8-VP16. All 10 clones showed an indical pattern to this clone (*b*). DNA sequencing (not shown) confirmed that the selected clones were identical to scFv-F8-VP16. (*a*) Forty eight colonies from his− plates were restreaked and β-gal was assay carried out. Twenty two colonies (46%) showed a robust blue coloration. (*b*) Ten of these colonies were picked into liquid culture and plasmid DNA was prepared after 2 days growth. The plasmid DNA were digested with *Bst*N1 and separated on agarose in comparison with scFv-F8-VP16. Sizes are indicated from coelectrophoresis of size markers and indicated on the left.

Discussion

Intracellular use of antibody fragments to abrogate protein function has potential application to disease therapy and in functional genomic studies (1). A convenient form of antibody fragment is the scFv (15, 16), which comprises a V_H chain linked to a V_L chain. While assessing various antigen-specific scFv, we found that many do not function when expressed inside cells. This paucity of functional intracellular scFv requires selection procedures that are based on intracellular action rather than *in vitro* antigen binding alone. Selection schemes based on the phenotype conferred by the intracellular antibody have been proposed recently (12, 27). However, such phenotypic selection schemes are not general and need to be tailored for each specific application. We therefore have developed a general antibody-antigen two-hybrid assay system, in which a positive outcome (e.g., activation of His3 or *LacZ*) depends on the interaction of scFv with target antigen under intracellular conditions. We find that this requirement can be accomplished for many scFv expressed in yeast and to a lesser extent in mammalian cells. We show that most scFv selected with antigen from phage display libraries do not bind to antigen in the cytoplasm of yeast cells. Thus a selection step after the *in vitro* stage is necessary, to subdivide the "*in vitro* only" binders from those that also can bind *in vivo*.

The low number of scFv that can bind *in vivo* is probably the result of the lack of disulfide bonding in the reducing environment of the cell cytoplasm (11). The folding stability of antibody domains is contributed by many residues in the frameworks (14), with different scFv fragments having different overall stabilities. Therefore, those scFv fragments that are intrinsically more stable will tolerate the loss of intrachain disulfide bond and remain folded, whereas others will not. In addition, good intracellular expression is related to additional parameters such as solubility versus propensity to aggregate, cellular half-life, and others (2). Experimental establishment of the necessary factors for efficient interaction with antigen in the cytoplasm is thus complex and general rules may be hard to establish.

We therefore have developed an *in vivo* selection scheme for the isolation of intracellular scFv, based on their ability to bind antigen under conditions of intracellular expression. We tested a panel of scFv fragments, some of which had been used previously as intracellular antibodies. All those fragments that previously were shown to induce a biological phenotype when expressed in cells also were positive in the yeast antibody-antigen system. The anti-p21ras Y13–259 scFv is only positive at 20°C, the same temperature of the *Xenopus laevis* oocyte assay in which this scFv showed a strong intracellularly activity (6). This effect is probably because this scFv aggregates intracellularly at higher temperatures (34). A low level of positive scores was found in the mammalian version of the assay, probably because of the rela-

tively stringent requirements for activation of the CAT reporter in the cellular milieu, compared with biological effects that can be manifest with lower levels of protein expression. It is also noteworthy that not all the scFv fragments gave a positive result in the antibody-antigen system, even if their binding affinities *in vitro* were comparable to those that did function. This finding confirms the requirement for a robust selection procedure for intracellular antigen-antibody interactions.

The model selection described here, enabling one clone to be isolated from half a million different clones, demonstrates the feasibility of isolating antigen-specific scFv fragments from a large pool of scFv specificities. Thus such a selection approach obviates the need for scFv engineering such as adapting new frameworks for increased folding stability (for instance, by random or directed mutagenesis). Existing scFv phage display libraries could be used for primary *in vitro* screening followed by *in vivo* selection in yeast. Alternatively, phage display may be eliminated if immunized mouse spleen RNA is cloned directly as scFv into the yeast vectors for immediate *in vivo* screening. This system should greatly facilitate the identification of candidate antibodies for intracellular antibody applications and should lend itself for the development of cell-based high throughput screening procedures in functional genomic applications.

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