doi:10.1016/j.jmb.2003.09.073 J. Mol. Biol. (2004) 335, 193–207

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Direct Phage to Intrabody Screening (DPIS): Demonstration by Isolation of Cytosolic Intrabodies Against the TES1 Site of Epstein Barr Virus Latent Membrane Protein 1 (LMP1) that Block NF-κB Transactivation

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The expression of intracellular antibodies (intrabodies) in eukaryotic cells has provided a powerful tool to manipulate microbial and cellular signaling pathways in a highly precise manner. However, there have been several technical issues that have restricted their more widespread use. In particular, single-chain antibodies (sFv) have been reported to fold poorly in the reducing environment of the cytoplasm and as such there has been a reluctance to use sFv-phage libraries as a source of intrabodies unless a pre-selection step to identify these rare sFvs from natural libraries or libraries of engineering sFvs that could fold properly in the absence of disulfide bonds were used. Here, we investigated whether target specific sFvs that are isolated from a 15 billion member non-immune human sFv-phage display library could be directly screened in pools as intrabodies without prior knowledge of their individual identity or purity within pools of antigen-specific sFvs. As the target, we used a synthetic transformation effector site 1 (TES1) polypeptide comprising the membrane-most proximal 34 amino acid residues of the carboxy-terminal cytoplasmic tail of the oncogenic latent membrane protein 1 (LMP1) of Epstein Barr virus, which serves as a docking site for adapter proteins of the tumor necrosis factor (TNF) receptor (TNFR)-associated factor (TRAF) family. Anti-TES1 sFvs, initially identified by phage ELISA screens, were grouped into pools according to the absorbance reading of the antigen-specific phage ELISA assays and then transferred as pools into eukaryotic expression vectors and expressed as cytoplasmic intrabodies. Using the pooling strategy, there was no loss of individual anti-TES1 sFvs in the transfer from prokaryotic to eukaryotic expression vectors. In addition, the initial assignments into sFv pools based on phage ELISA readings allowed the segregation of individual anti-TES1 sFvs into discrete or minimally overlapping intrabody pools. Further assessment of the biological activity of the anti-TES1 intrabody pools demonstrated that they were all able to selectively block F-LMP1-induced NFκB activity that was mediated through the TES1-site and to bind LMP1 protein with high efficiency. This direct phage to intrabody screening (DPIS) strategy should allow investigators to bypass much of the *in vitro* sFv characterization that is often not predictive of *in vivo* intrabody

Abbreviations used: TES1, transformation effector site 1; LMP1, latent membrane protein 1; TNFR, tumor necrosis factor receptor; TRAF, tumor necrosis-associated factor; DPIS, direct phage to intrabody screening; ELISA, enzyme-linked immunosorbent assay; PDI, protein disulfide isomerase; EBV, Epstein Barr virus; BSA, bovine serum albumin; MPBST, milk phosphate-buffered saline.

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function and provide a more efficient use of large native and synthetic sFv phage libraries already in existence to identify intrabodies that are active *in vivo*.

Keywords: intracellular antibodies; high-throughput screen; functional genomics; cancer; signalling

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Introduction

Antibodies have an extraordinary capacity to bind a diverse array of cognate antigens with high affinity and exquisite specificity. In addition to their extensive use *in vitro* in diagnostic and basic research applications, monoclonal antibodies are proving to be among the most safe and highly efficacious agents to treat a number of autoimmune, inflammatory and malignant diseases.[1] Recently, the use of antibodies as discovery tools and gene therapeutic agents has been greatly extended through their intracellular expression as intrabodies, where they have been shown to be capable of inhibiting the expression of specific microbial and cellular genes and in some cases even supplying gain-of-function properties.[2]

Intrabodies in the form of single-chain variable fragments (sFv) are among the most robust molecular techniques that are currently available for inhibiting microbial and cellular target protein function. This is achieved, in part, by the incorporation of short polypeptide sub-cellular trafficking signals to the N or C terminus of the intrabodies which allows them to be expressed at high concentrations in the very sub-cellular compartments where a target protein is located. Numerous examples of nuclear, cytoplasmic, mitochondria and ER-directed intrabodies have been reported.[3–8] Upon interaction with the target, intrabodies are unique in their ability to modulate target protein function and/or achieve phenotypic/functional knockout by several different mechanisms including accelerating target protein degradation and sequestering the target protein in a non-physiologic sub-cellular compartment. Other mechanisms of intrabody-mediated gene inactivation are more dependent on the actual epitope to which the intrabody is directed, such as binding to the catalytic site on a target protein or to epitopes that are involved in critical protein–protein, protein–DNA or protein–RNA interactions. In this way, intrabodies have several advantages over gene inactivation strategies that globally disrupt target gene transcription (e.g. antisense, ribozymes, siRNAs) and generally do not dissect these types of molecular interactions.[9,10]

Although intrabodies provide a highly refined approach to the analysis and manipulation of microbial and cellular pathways, there have been several technical issues that have limited their full development and more general use. For example, a number of investigations have focused on the claim that many intrabodies fold poorly in the reducing environment of the cytoplasm[11] and there has been considerable effort in engineering sFvs to achieve more stabilized frameworks that could fold properly in the absence of intradomain disulfide bonds.[12–17] However, there are also numerous examples of ER-directed intrabodies that do not achieve phenotypic knockout of their target proteins despite being expressed in their natural compartment with the protein disulfide isomerase (PDI) and other folding chaperones.[18,19] Therefore, other parameters such as target sequestration, epitope accessibility, post-translational modifications of the target protein and ineffective competition with endogenous proteins also appear to play an important role. In addition, we and others have reported that intracellular half-life is a better predictor of intrabody activity than is the *in vitro* binding affinity of the sFv.[20,21] In fact many sFvs that have high-affinity *in vitro* binding properties behave poorly as ER-directed or cytosolic intrabodies.[22–24] As a result, choosing an sFv as an intrabody based on higher affinity alone is a poor predictor of function *in vivo*.[20,21,23]

In recent years, filamentous phage,[25–30] ribosome,[31] yeast,[32] *Escherichia coli*[33] and other sFv display libraries of considerable size[34] and derived from human IgG repertoires have become increasingly available. The large sizes of these libraries has allowed the isolation of numerous sFvs against diverse targets. These sFvs share the common property of having broader epitope diversity and higher affinities compared to the sFvs that were recovered from the smaller libraries that were initially generated.[35] It is likely that these libraries are rich in sFvs with different framework structures, affinities and epitope specificities that could function as intrabodies. However, a limitation to their broader use in high-throughput functional genomic screens has been finding a way to couple the two technologies so that, for example, phage panning and functional intrabody screens are more closely linked. In this regard several selection methods that attempt to streamline these processes have been reported.[36–40]

Here, we describe studies that were aimed at determining whether target-specific sFvs that are isolated from a 15 billion member non-immune human sFv-phage display library could be directly screened in pools as intrabodies without prior knowledge of their individual identity or purity within pools of antigen specific sFvs. This strategy would allow an investigator to bypass much of

the *in vitro* sFv characterization that is often not predictive of *in vivo* intrabody function. As a model we chose a target of considerable medical importance, the oncogenic latent membrane protein 1 (LMP1) of Epstein Barr virus (EBV).[41–43] We isolated sFv phage after panning against a transformation effector site 1 (TES1) polypeptide, that comprises the membrane-most proximal 34 amino acid residues of the carboxy-terminal cytoplasmic tail of LMP1 and serves as a docking site for adapter proteins of the tumor necrosis factor (TNF) receptor (TNFR)-associated factor (TRAF) family. Using this strategy, we demonstrate that these anti-TES1 intrabodies can bind LMP1 intracellularly and block NFκB activity that is mediated through this site.[44–46] This direct phage to intrabody screening (DPIS) strategy should allow the more efficient use of large native and synthetic sFv phage libraries already in existence to identify intrabodies that are active *in vivo*.

Results

Selection of sFvs against the CTAR1/TES1 site of LMP1

EBV LMP-1 resembles a classical oncogene in its ability to transform rodent fibroblast cell lines and drive the immortalization of primary human B lymphocytes *in vitro*. LMP1 is a 386 amino acid transmembrane protein comprising a 24 amino acid N-terminal cytoplasmic tail, six hydrophobic membrane-spanning domains, and a 200 amino acid cytoplasmic C terminus. The short N-terminal cytoplasmic domain is responsible for the correct orientation of LMP1 in the plasma membrane but is dispensable for B-cell transformation.[47] The six membrane-spanning domains promote the oligomerization of LMP1 molecules, a function necessary for the transduction of oncogenic signals from the C-terminal cytoplasmic portion of the protein. Two domains have been identified within the C-terminal cytoplasmic sequences of LMP1 as being important for B-lymphocyte growth transformation and phenotypic changes in a variety of cell types, CTAR1/TES1 and CTAR2/TES2.[46,48,49] CTAR1/TES1 (C terminus activation region 1/transformation effector site 1) comprises the membrane-most proximal 34 amino acids (amino acid residues 196–231) and contains a $P^{204} \times Q^{206} \times T^{208}D^{209}$ motif which serves as a docking site for adapter proteins of the tumor necrosis factor (TNF) receptor (TNFR)-associated factor (TRAF) family such as TRAF1, TRAF2, TRAF3, and TRAF5.[44,50–53]

A human non-immune sFv phage display library consisting of 1.5×10^{10} members was used as the source of high-affinity human sFvs for these studies (unpublished results). A biotinylated 44-mer polypeptide corresponding to the CTAR1/TES1 site of LMP1 (TES1 polypeptide) was synthesized and added to the phage during the panning procedures at decreasing concentrations starting at 500 nM (rounds 1 and 2), 250 nM (round 3) and 100 nM (rounds 4 and 5). Bound phage were captured using streptavidin alternating with avidin-coated agarose beads in each subsequent round of panning. After five rounds of panning, 36/96 selected individual phages were positive in a phage ELISA assay for their ability to bind to biotinylated LMP1 that was captured on a streptavidin-coated plate. Specificity of binding was confirmed using uncoated ELISA plates and plates coated directly with biotinylated TES1, streptavidin or BSA alone (data not shown).

Grouping of anti-TES1 sFvs into pools based on initial ELISA readings

To develop a method that would aid in intrabody screening prior to DNA sequencing and identification of the individual clones, an ELISA-based screening system was set up to allow pooling of individual clones based on the absorbance of the ELISA screens. Control experiments with circa 20 clones showed that the phage titers varied no more than twofold between individual clones. The 36 positive clones were divided into four pools (pool I, ten clones, $A < 0.5$; pool II, seven clones, A 0.5–1.0; pool III, eight clones, A 1.0–1.5; and pool IV, 11 clones, $A > 1.5$). Fingerprinting analysis, performed using *Bst*NI, demonstrated that the clones could be classified into four groups although there was not a strict correlation between the ELISA readings and the fingerprinting pattern in each pool (see Discussion).

The sFv genes from the four pools were removed from pFARBER by *Sfi*1 and *Not*1 digestion and cloned as separate pools into a *Sfi*1/*Not*1-digested eukaryotic expression vector that allows cytoplasmic intrabodies to be expressed with a carboxy-terminal HA tag. To ensure that all sFv genes from the four pFARBER pools were represented in the four intrabody pools, three times that number of colonies from each transformation were picked to make the intrabody pool (e.g. 30 individual colonies were randomly chosen for pool I, 21 colonies for pool II, etc.) after confirming 100% transformation efficiency for each pool. The results of the DNA sequence analysis of the individual clones that were used to make the pFARBER and intrabody pools are described below.

Inhibition of LMP1 or LMP1-TES1-induced NFκB activation induced by anti-TES1 intrabody pools

LMP1 is known to activate NFκB.[46,50,52] To investigate the effects of anti-TES1 intrabody pools on LMP1-mediated NFκB activation, 293 cells were co-transfected with varying concentrations of either wild-type F-LMP1 or F-LMP1-TES1 expressing plasmids and an NF-κB-dependent promoter luciferase reporter plasmid to establish a reporter assay. As can be seen in Figure 1A, wild-type

F-LMP1 induced high-level NFκB activation whereas F-LMP1-TES1 expression vector induced a lower level of NFκB activity as reported.[46] The F-LMP1-TES2 plasmid induced about 70% of NF-κB activity compared to wild-type F-LMP1 (data not shown).

Next, the ability of the anti-TES1 intrabody pools to inhibit NFκB activation by wild-type F-LMP1 or F-LMP1-TES1 was investigated. As can be seen in Figure 1B, each of the anti-TES1 intrabody pools was able to inhibit NFκB activity induced by wild-type F-LMP1 by about 60% (range 57–65%) while the control anti-Tat intrabody had no inhibitory activity. This degree of inhibition is consistent

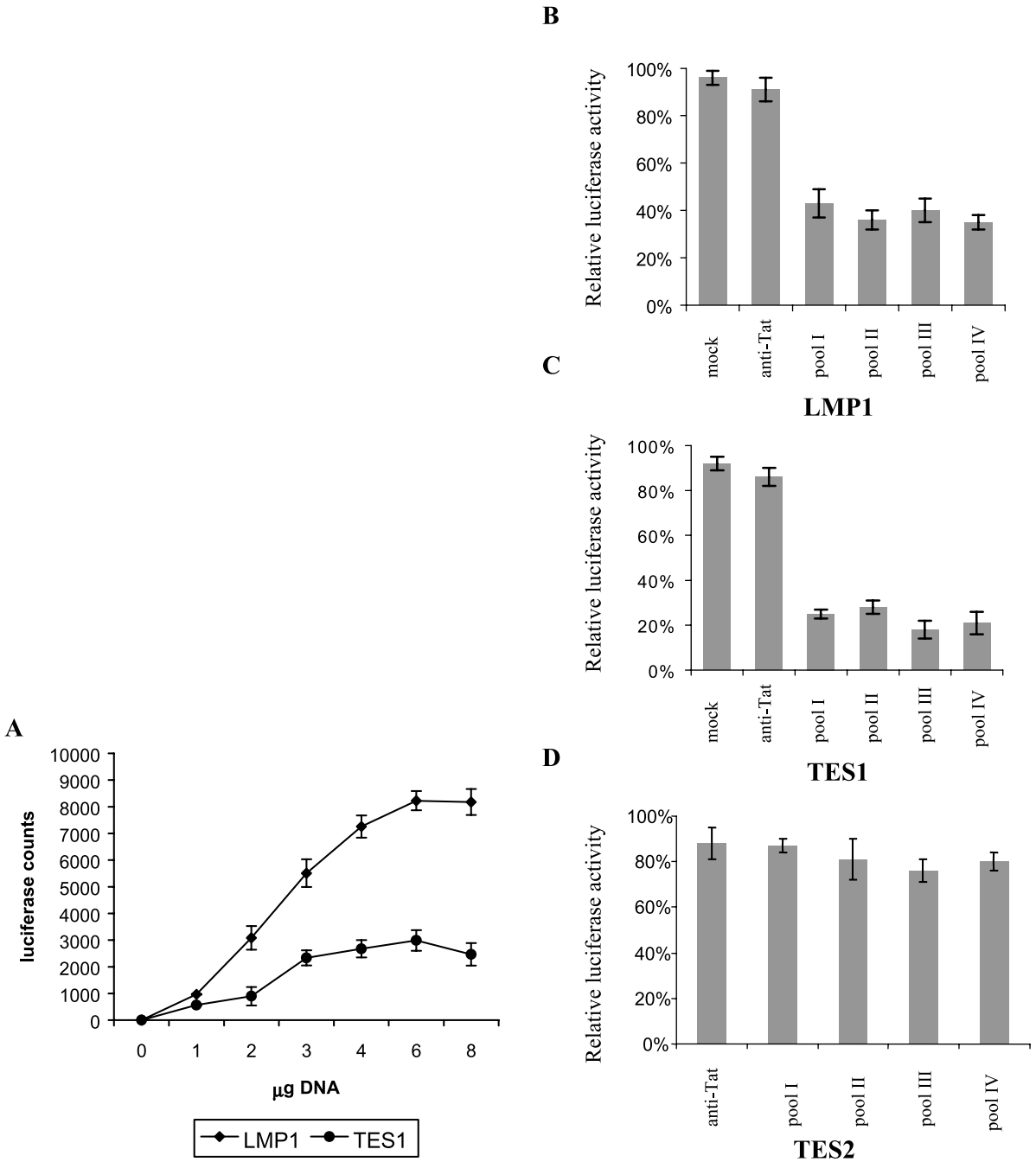

Figure 1. Inhibition of wild-type F-LMP1 and F-LMP1-TES1 induced NFκB activation by anti-LMP1-TES1 intrabody pools. The 293 cells were transiently transfected with the 3 × NFκB luciferase reporter plasmid (3 × -κB-L), F-LMP1 or F-LMP1-ΔTES1/ΔTES2 mutants, the different anti-TES intrabody pools or control intrabody and β-galactosidase plasmids to normalize transfection efficiency. At 24 hours post-infection cells were lysed in luciferase lysis buffer. Luciferase and β-galactosidase assays were performed on cleared lysates. A, Dose-response curve for induction of NFκB-dependent luciferase activity by wild-type F-LMP1 (filled symbols) and F-LMP1-TES1 (open symbols). B, C and D, Inhibition of NFκB activity by anti-TES1 intrabody pools induced by wild-type F-LMP1 (B), F-LMP1-TES1 (C), F-LMP1-TES2 (D). Values represent mean ± S.D. of four experiments.

with the expected contribution of the TES1 site to the total activity of F-LMP1, as shown in Figure 1A. As shown in Figure 1C when similar experiments were performed with the F-LMP1-TES1 plasmid, the anti-TES1 intrabodies showed a markedly greater (mean 78%, range 72–85%) inhibition of NFκB activity. The specificity of the inhibition by the anti-TES1 intrabodies is demonstrated in Figure 1D, which shows that the anti-TES1 intrabodies do not inhibit NFκB activity when the F-LMP-TES2 plasmid that has a ΔTES1 site deletion was used. Thus, the anti-TES1 intrabody pools can selectively inhibit NFκB activity that is mediated through the TES1 site of F-LMP1.

Direct physiochemical association between anti-LMP1 intrabodies and F-LMP1 in mammalian cells

We next examined whether the cytosolic anti-TES1 intrabodies were functionally active in binding to the TES1 epitope of LMP1 in mammalian cells. F-LMP1 and the different anti-LMP1 intrabody pools were transiently co-transfected in 293 cells and evaluated in co-IP-Western blot experiments. As can be seen in Figure 2, anti-Flag Mab was able to specifically co-immunoprecipitate a circa 30 kDa band in the four pools (lanes 1–4) that corresponds to the molecular mass of the HA-tagged anti-LMP1 intrabodies. This co-immunoprecipitation was specific, since the intrabodies were not precipitated with anti-Flag antibody when F-LMP1 was not included in the transfection (lane 6). When the intrabody pools (I–IV) were mixed and immunoprecipitated directly with anti-HA Mab (lane 5) a similar amount of intrabody was precipitated as was co-immunprecipitated when F-LMP1 was included in the transfection (compare lane 5 with lanes 1–4) thus providing evidence that the anti-TES1 binding to F-LMP1 is efficient. From these combined studies we conclude that the anti-TES1 intrabody pools provide a valid screening strategy of candidate intrabodies for further biochemical and functional studies.

Structural analysis of the individual anti-LMP1-TES1 sFvs

To further validate the use of the intrabody pooling strategy described above, DNA sequence analysis was performed on the 36 individual anti-TES1 pFARBER phage clones that were used to make the four anti-TES1 intrabody pools. Complete DNA sequence analysis was obtained on 31 of the 36 pFARBER clones, which represented five unique anti-TES1 sFvs and were designated A3H5, B10E7, H11F6, B10B7 and B2A4 Clone B1G1 was found be a pseudo-gene with an intact VH gene (VH5 family) and an open reading frame (ORF) of correct size for a light chain but it did not match any immunoglobulin consensus sequence. Because of the latter, the functional properties of B1G1 were not further evaluated. Of the remaining five clones, the complete DNA sequence could not be obtained.

The DNA sequence analysis and predicted amino acid alignments of the five anti-TES1 sFv genes is shown in Table 1.[54] Four of the five VH genes were members of the VH3 family (A3H5, B10E7, H11F6 and B10B7). The heavy chains from clones B10E7, B10B7 and H11F6 were identical except for the latter, which had a single silent nucleotide change. B2H4 was the only member of the VH1 gene family that was seen. The VH of A3H5 had single amino acid changes in FR1, FR2 and CDR3 and two changes in CDR2 compared to the other three VH3 family members. These three VH3 genes were paired with four VL genes of the Vλ6 family, which had multiple amino acid differences throughout their FR and CDR regions. The light chain of clone B2A4 was again different (Vλ2 family) compared to the other four light chains.

Validation of the grouping strategy to create sFv pools for rapid intrabody cloning and functional screening

The frequency distribution of the five sFvs and B1G1 pseudo-gene among the original 36

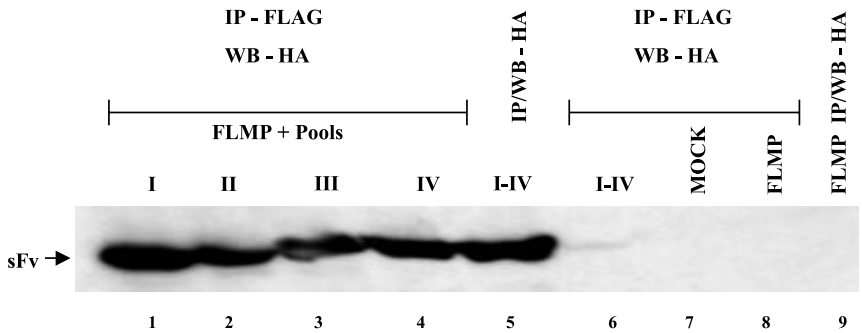

Figure 2. Co-immunoprecipitation of F-LMP1 and anti-LMP1-TES1 intrabodies. The 293 cells were transfected with F-LMP1 and/or anti-TES1 intrabody pools I–IV as indicated. After 24 hours cells were disrupted in 1% Brij 58 buffer and the cell lysates were incubated with anti-Flag affinity matrix (SIGMA) (lanes 1–4 and 6–8). SFvs co-precipitating with F-LMP1 were analyzed by Western blot with anti-HA polyclonal antibody (BabCO). In lanes 5 and 9, the cell lysates were treated with anti-HA Sepharose beads.

Table 1. Amino acid sequence analysis of anti-TES1 V_H and V_L genes

A. *V_H genes*

Sample	VH1 FR1	VH CDR1	VH FR2	VH CDR2
	1 2 3 12345678901234567890123456789O	1ab2345	4 67890123456789	5 6 012abc3456789012345
A3H5 VH3	QVQLVQSGGGLVQPGGSLRLSCSASGFTFN	SYAMH--	WVRQAPGKGPEHVS	AISS--DGGSTYYAESVKG
B10E7 VH3	QVQLVQSGGGLVQPGGSLRLSCSASGFTFS	SYAMH--	WVRQAPGKGLEYVS	AISS--NGGSTYYADSVKG
H11F6 VH3	QVQLVQSGGGLVQPGGSLRLSCSASGFTFS	SYAMH--	WVRQAPGKGLEYVS	AISS--NGGSTYYADSVKG
B10B7 VH3	QVQLVQSGGGLVQPGGSLRLSCSASGFTFS	SYAMH--	WVRQAPGKGLEYVS	AISS--NGGSTYYADSVKG
B2A4 VH1	EVQLVQSGAEVKKPGASVKVSCKASGYIFT	KYDIT--	WVRQAPGQGLEWMA	WINP--NSGNTGYAQKFKG

Sample	VH FR3	VH CDR3	VH FR4
	7 8 9 6789012345678901 2abc345678901234	0 567890abcdefghijk12	1 34567890123
A3H5	RFTISRDNSKNTLYLQMSSLRAEDTAVYYCVK	PAYDSSGYYRHFF----DY	WGQGTLVTVSS
B10E7	RFTISRDNSKNTLYLQMSSLRAEDTAVYYCVK	PAYDSSGYYRHYF----DY	WGQGTLVTVSS
H11F6	RFTISRDNSKNTLYLQMSSLRAEDTAVYYCVK	PAYDSSGYYRHYF----DY	WGQGTLVTVSS
B10B7	RFTISRDNSKNTLYLQMSSLRAEDTAVYYCVK	PAYDSSGYYRHYF----DY	WGQGTLVTVSS
B2A4	RVTITRDTSINTAYMEMTGLTSDDTAVYFCAR	EEKTSGNWALQ-------V	WGQGTTVTVSS

B. *V_L genes*

Sample	VL FR1	VL CDR1	VL FR2	VL CDR2
	1 2 123456789012345678901 23	3 45678901abcdef234	4 567890123456789	5 0123456
A3H5 V 6	NFMLTQPHSVSESPGKTVTISCT	RSGGSIASN-----YVQ	WYQQRPGSSPTTVIY	EDNQRPS
B10E7 V 6	NFMLTQPHSVSESPGKTVTISCT	RSSGNIANN-----YVQ	WYQQRPGSVPTPVIY	EDNQRPS
H11F6 V 6	NFMLTQPHSVSESPGKTVTISCT	RSSGSLANH-----YVQ	WYQQRPGSSPTTVIY	EDNQRPS
B10B7 V 6	NFMLTQPHSMSESPGKTVTISCT	RSSGSIASN-----YVQ	WYQQRPGSVPTTLIY	EDKQRPS
B2A4 V 2	QSALTQPASVSGSPGQSITISCT	GTSSDLGGHN----FVS	WYQQHPGKAPKLLIY	DVFNRPS

Sample	VL FR3	VL CDR3	VL FR4
	6 7 8 789012345ab67890123456789012345678	9 9012345abcdef67	0 890123456a7
A3H5	GVPDRFSGSIDGSSNSASLTISGLQPEDEADYYC	QSYDSGNR-----YV	FGPGTTVTVLG
B10E7	GVPDRFSGSIDRSSNSASLIISGLETDDTADYYC	QSYDAGNR-----LV	FGGGTKLTVLG
H11F6	GVPDRFSGSIDSSSNSASLTISGLKTEDEADYYC	QSYDSSNH-----VV	FGGGTKLTVLG
B10B7	GVPDRFSGSIDGSSNSASLTISGLKTEDEADYYC	QSYDSSNR-----VI	FGGGTKVTVLG
B2A4	GVSSRFSGS—KSGTSASLAISGLQSEDEADYYC	AAWDDSLNGF---WV	FGGGTKLTVLG

Numbering as defined by Chothia & Lesk.[54] Shaded areas represent different amino acid residues for the four highly related antibody genes.

pFARBER clones and the intrabody pools is shown in Figure 3 and Table 2. For the intrabody pools, a total of $(n - 1) \times 3$ clones from each pool were subjected to DNA sequencing (total = 96 clones), where n is the number of colonies assigned to the individual pFARBER pools. Complete DNA sequence was obtained for eight of nine colonies from pFARBER sFv pool I, which contained five copies of A3H5, three copies of B2A4 and one copy of B1G1 and this same pattern, albeit different absolute ratios, was seen in intrabody pool I. pFARBER pool II contained three copies of A3H5, one clone of H11F6 and one clone of B1G1, complete DNA sequence was not obtained for two clones. An analysis of intrabody pool II again showed a majority of clone A3H5 and one clone each of H11F6 and B1G1 but there were no clones of B2A4 as was seen in pool I. The pFARBER pool III had four colonies of clone B10E7, which was not seen in pools I and II, and three colonies of H11F6, complete DNA sequence was not obtained for one clone. These same clones were found in near-equal frequency in intrabody pool III. Finally, pFARBER pool IV had eight colonies of clone B10E7, and one colony each of clone B10B7 and H11F6, and complete DNA sequence was not obtained for one clone. There was also loss of clone A3H5 in pool III. The analysis of intrabody pool IV showed a predominance of clone B10E7 and fewer clones of H11F6 and B10B7 as expected. Thus, the original pFARBER pooling strategy based on ELISA readings resulted in relative enrichments of different sFvs in each pool and these enrichments were largely maintained in the intrabody pools. Importantly, there was no loss of sFv clones in moving from pFARBER to intrabody expression vectors.

Inhibition of LMP1 or LMP1-TES1-induced NFκB activation induced by the individual anti-TES1 intrabodies

We next evaluated the individual anti-TES1 intrabodies for their ability to inhibit NFκB

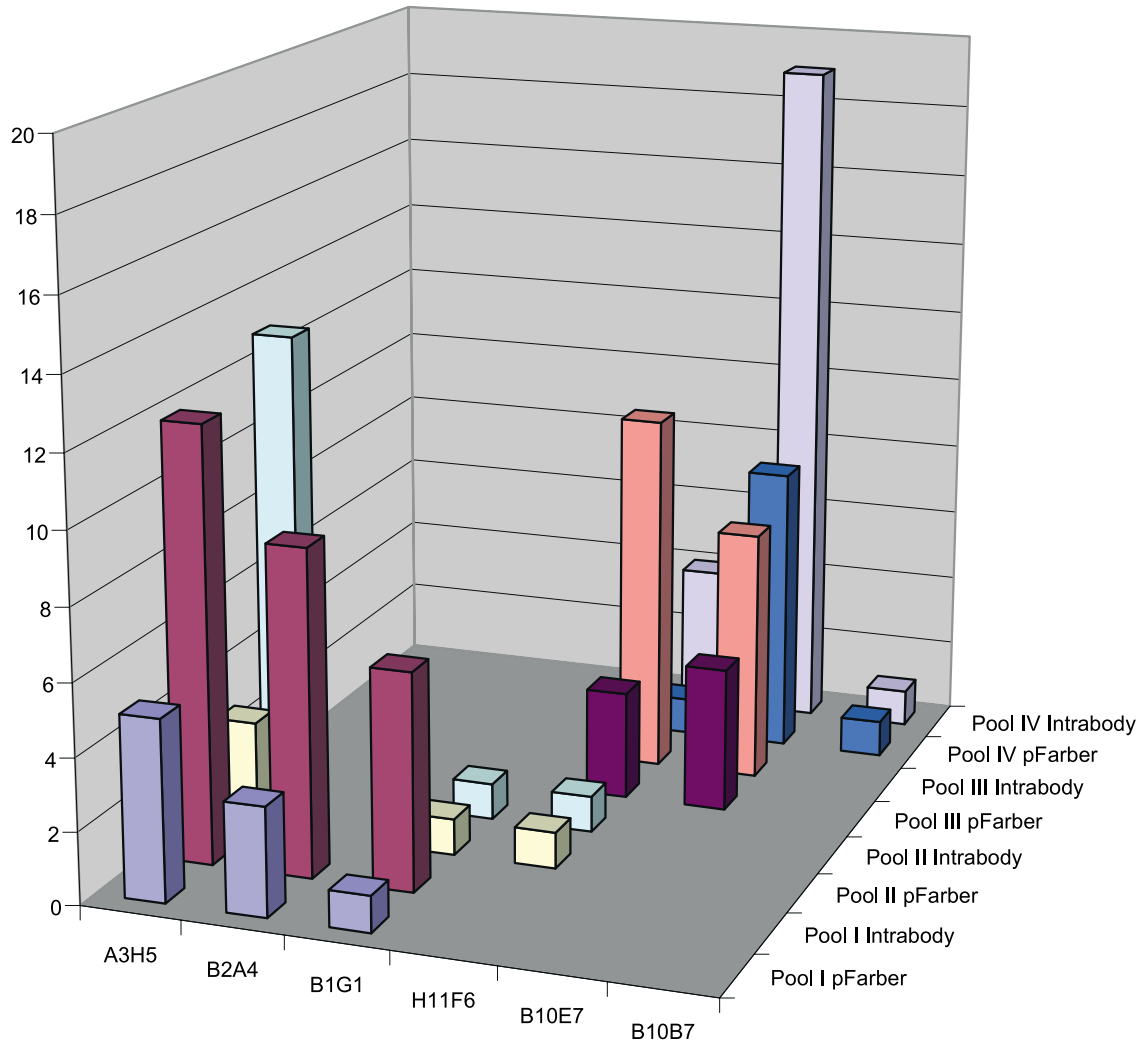

Figure 3. Frequency distribution of anti-TES1 sFvs in pFARBER and intrabody pools. Three-dimensional histogram of the five unique anti-TES1 sFvs and B1G1 pseudo-gene in the original pFARBER pools I–IV and the intrabody pools I–IV.

activation by LMP1 and LMP1-TES1 in transiently transfected 293 cells using procedures identical with those used for the intrabody pools. The results of these studies are shown in Figure 4. As can be seen in Figure 4A, each of the anti-TES1 intrabodies were able to inhibit NFκB activity induced by wild-type F-LMP1 by 27–56%. The anti-TES1 sFvs that were isolated from pools I and II (A3H5 and B2A4), and therefore had lower binding affinities by ELISA screens, also showed lower inhibitory activity *in vivo*, 27 and 40%, respectively, whereas two of the three anti-TES1 sFvs that were isolated from pools III and IV (H11F6 and B10E7) and thus had higher binding affinities by ELISA

Table 2. Distribution of individual anti-TES1 sFvs in the pFarber and intrabody pools

	Pool I		Pool II		Pool III		Pool IV	
	PFarber	Intrabody	pFarber	Intrabody	pFarber	Intrabody	pFarber	Intrabody
A3H5	5	12	3	13				
B2A4	3	9						
B1G1	1	6	1	1				
H11F6			1	1	3	10	1	4
B10E7					4	7	8	19
B10B7							1	1
N.S.	1	0	2	0	1	0	1	0

N.S., complete DNA sequence was not obtained.

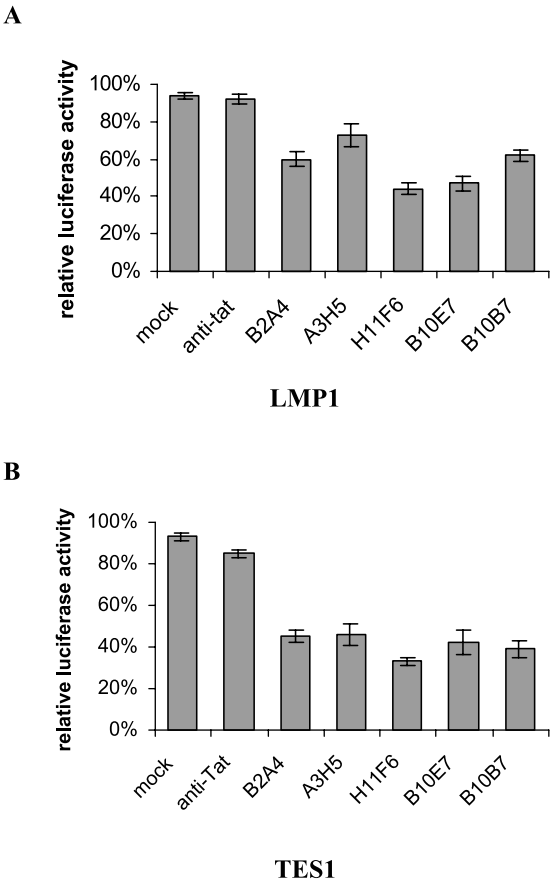

Figure 4. Inhibition of wild-type F-LMP1 and F-LMP1-TES1 induced NFκB activation by individual anti-TES1 intrabodies. The 293 cells were transiently transfected with 3 × κB-L and β-galactasidase reporter plasmids, F-LMP1 or F-LMP1/TES1/TES2 mutant and individual anti-TES1 or control anti-tat intrabody plasmids as described for Figure 1. A and B, Inhibition of NFκB activity by individual anti-LMP1-TES1 intrabodies induced by wild-type F-LMP1 (A) and F-LMP1-TES1 (B). Luciferase and β-galactosidase assays were performed on cleared lysates. Values represent mean ± SD of four experiments.

screening also had greater inhibitory activity *in vivo*, 56 and 53%, respectively. As shown in Figure 4B, this same result was seen when similar experiments were performed with the F-LMP1-TES1 plasmid. In addition, the anti-TES1 intrabodies again showed an overall greater inhibition of NFκB activity compared to the activity seen against the wild-type F-LMP1 plasmid, with inhibition ranging from about 55% for A3H5 and B2B4 to 58–67% for B10E7, B10B7 and H11F6, respectively. The overall lower inhibitory activity of the individual intrabodies (Figure 4A and B) compared to the intrabody pools (Figure 1B and C) has not been evaluated further (see Discussion). There was no inhibition of NFκB activity when the individual anti-TES1 intrabodies were tested with the F-LMP1-TES2 plasmid (results not shown).

Individual anti-TES1 intrabodies are co-immunoprecipitated with F-LMP1 in mammalian cells

We next examined whether the individual anti-TES1 intrabodies were functionally active in binding to the TES1 epitope of LMP1 in mammalian cells. F-LMP1 and the individual anti-TES1 or control intrabodies were transiently co-transfected in 293 cells and evaluated in co-IP-Western blot experiments. As can be seen in Figure 5, anti-Flag Mab was able to specifically co-immunoprecipitate each of the HA-tagged anti-LMP1 intrabodies (lanes 1–5) but not the control anti-tat intrabody (land 6). Near-equal amounts of the anti-TES1 intrabodies were co-IP'd, with the exception of the B2A4 intrabody, which showed a lower-density band. When F-LMP1 was not included in the transfection, the anti-TES1 intrabody pools (I–IV) were not co-immunoprecipitated with anti-Flag (lane 7).

Discussion

Here, a successful feasibility study is described which demonstrated that a higher throughput and direct screening strategy could be established to allow candidate sFvs against a target protein of interest to be evaluated as intrabodies without prior knowledge of their individual identity or purity within pools of antigen specific sFvs. This strategy eliminates the initial time delay in identifying unique sFvs isolated from very large sFv libraries that may not possess the desired *in vivo* biological function. It also allows DNA sequence analysis to be focused on selective clones that are recovered from the intrabody pools after their biological function has been investigated. Importantly, using the pooling approach described herein, there was no loss of individual anti-TES1 sFvs in the transfer from prokaryotic to eukaryotic expression vectors. In addition, the initial assignments into sFv pools based on phage ELISA readings allowed segregation of individual anti-TES1 sFvs into discrete or minimally overlapping intrabody pools that were active in their ability to selectively inhibit F-LMP1 induced NFκB activation that is mediated through the TES1 site and to bind LMP1 protein with high efficiency. Our pooling strategy did not rely on the often-used practice of DNA fingerprinting with *Bst*NI, which was performed but not chosen as a grouping tool in our study, since it is not a reliable unique identifer when used to screen sFv genes isolated from libraries of this size, due to the extensive somatic mutations that are present in the variable region genes. Indeed in the present study, *Bst*NI fingerprinting did not distinguish three of four anti-TES1 sFvs that were composed of VH III-λ6 gene segments as being unique (data not shown). Another finding of interest in this study was that the intrabody pools appeared to be more active than the individual intrabodies. One

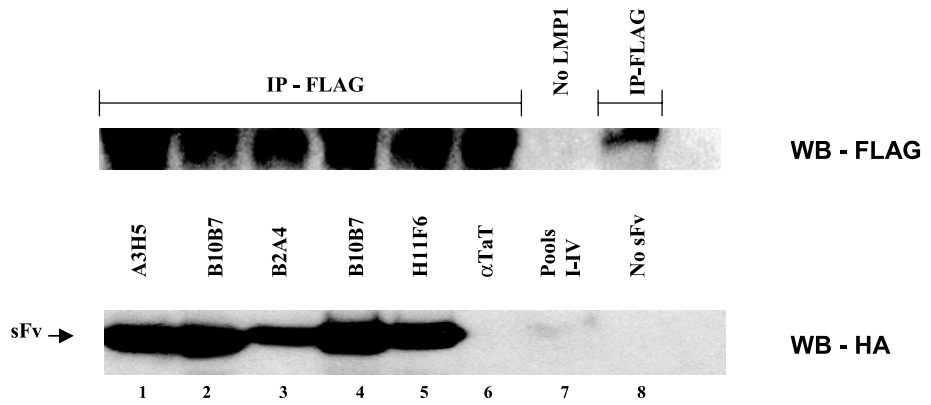

Figure 5. Co-immunoprecipitation of F-LMP1 and anti-LMP1-TES1 intrabodies. The 293 cells were transfected with Flag-LMP1 alone (lane 8) or with the individual anti-TES1 (lanes 1–5) or anti-tat control intrabody (lane 6) or the combined anti-TES1 intrabody pools I–IV alone (lane 7). Cells were disrupted in 1% Brij 58 buffer and the cell lysates were incubated with anti-Flag affinity matrix (SIGMA). Upper panel, Western blot for F-LMP1 expression with anti-Flag. Lower panel, anti-TES1 sFvs co-precipitating with Flag-LMP1 were analyzed by Western blot with anti-HA polyclonal antibody (BabCO).

interpretation of these results that still requires formal testing is that the pooling approach adds a synergistic effect to the phenotypic changes caused by the individual intrabodies. Thus, the DPIS approach may not only add rapidity but increased efficacy in identifying functional of intrabodies from phage libraries.

Over the past several years, considerable effort in the field has focused on the development of more highly efficient screening systems to identify functional intrabodies from sFv libraries. These have included a protein fragment complementation assay for selection of antibody–antigen interactions in the cytoplasm of *E. coli*[40] and a yeast two-hybrid system as a method to select antibodies for intracellular function in eukaryotic cells.[36,39] This latter assay has been used to further characterize structural variants of known intrabodies for improvements in stability, solubility and *in vivo* performance[39,55,56] and in one study for the *de novo* isolation of a single antigen-specific intrabody.[38] In a modification of this technique known as intracellular antibody capture (IAC), *in vitro* phage display is used as a pre-selection enrichment step to adjust for the difference between the complexity of antibody phage libraries and the transformation efficiency in yeast.[57–59] In two published studies, the use of phage display was reported to be a necessary *in vitro* pre-selection step because randomly selected antigen-specific sFvs from this step alone failed to bind the bait in the yeast screens.[36,58] Our results differ in this regard, since all of the anti-TES1 sFvs that were selected by *in vitro* antigen panning functioned well as intrabodies in mammalian cells without the need for a pre-selection step (Figures 4 and 5) or other *in vitro* biochemical studies. Although the reason for the variance in our results compared to the secondary yeast screens mentioned above remains uncertain, they could, in part, reflect unknown differences in the intracellular milieu in yeast compared to mammalian cells. Therefore, this DPIS strategy, which can also be applied to sFvs isolated from ribosomal or other display technologies, represents an important advancement in the field, since it allows for the direct testing of pools of target specific sFvs for the desired intrabody function in mammalian cells without the additional need to sub-clone enriched sFvs pools into a second screening system to identify candidate sFv intrabodies. This DPIS technique also eliminates the *a priori* need to examine sFv expression levels and solubility in *E. coli*, an often used criteria for selecting a stable sFv framework for cytoplasmic expression, since these biochemical parameters are not always predictive of intrabody function.[24,55]

Intra-domain disulfide bonds have been reported to not form or form poorly in sFvs expressed in the cytoplasm of eukaryotic cells;[11,55] however, some sFvs have been shown to tolerate the absence of this bond *in vitro*.[12–15] These observations have been the driving force behind the interest in engineering sFv frameworks for stable cytoplasmic expression and several approaches have been adapted to solve this problem. These include sequence modifications of the VH and VL domains by utilizing random mutations to replace the need for disulfide bonds to obtain stablized sFvs with high intrinsic stability,[13,60] the use of frameworks that prove to be effective *in vivo*[56,61–64] or the transplantation of CDR residues into stable sFv frameworks.[24,56] A semi-synthetic sFv phage library based on a sFv framework that functioned well in both bacterial and plant cytoplasm has been used to select new antigen-specific sFvs that maintained stability and solubility properties of the parent sFv.[64] Thus, although these rational mutation strategies and molecular evolution approaches have yielded more stable sFv frameworks on a case-by-case basis, the results of our studies demonstrate that engineering stable sFv frameworks for intracellular expression is not a

prerequisite for obtaining sFvs with good *in vivo* function.

The identification of a consensus sequence in the frameworks of sFvs that function as good cytoplasmic intrabodies has been the subject of several investigations. Our results also add to the growing list of sFv frameworks that can function well in the reducing environment of the cytoplasm. Three investigations which used the Sheets sFv library reported the isolation of only VH III heavy chain frameworks that, in part, reflected the initial bias in the phage library.[24,29,57,58] In one of these studies, the combination of VH III and VK (I and IV) frameworks were found for intrabodies that bound TAU antigen and a number of conserved residues that could define an intracellular consensus sequence were identified. In another study that selected intrabodies against BCR and ABL, the majority of light chain genes matched the VK I consensus sequence whereas 6/18 light chains were derived from λ genes.[57] In a third study, only VK1 frameworks were found with anti-Ras intrabodies.[24] In the Mehta II library that was used in this study there is also a predominance of VH III heavy chains and only λ light chains were used in its construction (unpublished results). A comparison of the VH III gene consensus sequence for the four anti-TES-1 sFvs with the VH III genes reported in these three studies and with the VH III consensus sequence from random clones from the Mehta II library (data not shown) reveals three amino acid differences at framework positions (FR1#23A, FR2#47W and FR3#93A) where we observe A → S, W → Y and A → V changes, respectively. The VH III genes found in our study are also rearranged with a different JH segment (JH4b) than with the JH5 segment that has been reported above.[24,57,58] Interestingly, these VH III genes were paired with Vλ6 light chains that differed in multiple FR and CDR amino acid residues. However, our study also demonstrates that for B2A4, a VH I framework when joined to a JH3a gene segment is active as an intrabody framework when paired with a Vλ2 light chain, albeit this sFv had slightly lower binding affinity *in vitro* and inhibitory activity *in vivo* compared to the other four anti-TES1 intrabodies that were recovered (Figures 4 and 5). In addition, in a recent study we demonstrated that five anti-hCyclinT1 intrabodies isolated from the Mehta II library were formed by the pairing of VH III–Vλ1, VH III–Vλ6 and VHI–Vλ1 genes.[65] Thus, it is likely that multiple VH and VL frameworks will serve as good scaffolds for functional intrabodies and that only by screening very large natural or synthetic sFv libraries that contain a broad array of canonical structures will the diversity of structures that meet these requirements be revealed.[66–70] For the cytosolic intrabodies, these requirements will be multifactorial and will depend on proper folding, solubility, steady-state levels of expression, intracellular half-life and on properties of the target antigen itself. These studies also reinforce the finding that repertoires of sFvs that are capable of efficiently binding and inhibiting cytosolic target protein function are present in natural libraries of the size reported here and elsewhere.[24,29,36,57,58] Finally, it will be important to compare the *in vivo* performance of intrabodies that are derived from these naturally occurring canonical structures to those that are isolated from libraries that are built upon intracellular antibody consensus frameworks to determine if any differences in functional performance in mammalian cells will be revealed.[64,71]

The studies presented here also address another important aspect of intrabody screening, namely the nature of the antigen or bait that is used for the sFv or intrabody screening. The production of recombinant proteins for phage panning and *in vitro* biochemical analysis of intrabody function requires additional time and technical support and is subject to the idiosyncratic production and purification properties of each protein. This could be avoided if synthetic peptides from defined hydrophilic segments of proteins could be used to initially isolate sFvs that could then be tested as intrabodies. In the current study, a synthetic polypeptide that encodes a specific effector region (TES1 site) of the LMP1 oncoprotein which is involved in NFκB signal transduction was used to pan the sFv phage library to enrich for target-specific sFvs. This simple procedure eliminated the need to produce recombinant LMP1 protein for the *in vitro* panning procedure and allowed us to isolate anti-TES1 intrabodies that can specifically block NFκB signal transduction through this region. The ability to use synthetic polypeptides as an effective screening reagent is a property that is not unique to the TES1 epitope, as a similar approach was used to isolate intrabodies against the Tat-TAR recognition motif (TRM) of hCyclinT1.[65] In many cases, specific polypeptides can be synthesized with or without post-translation modifications that mimic the (active or inactive) motifs that are involved in signal transductions.

Clinical studies have demonstrated that LMP-1 is expressed on the surface of the majority of EBV-associated neoplasms, including post-transplant lymphoproliferative disease (PTLD), AIDS-related lymphomas and nasopharyngeal carcinomas and Hodgkin's disease. In addition to the TES1 site, a second transformation (CTAR2/TES2) site has been identified that comprises the extreme C-terminal 54 residues of the protein (amino acid residues 332–386) and recruits TNFR-associated death domain protein (TRADD) and receptor-interacting protein (RIP).[49,72] These motifs enable LMP-1 to mimic a constitutive active tumor necrosis factor (TNF) family receptor and recruit TRAFs and TRADD in a ligand-independent manner. As a result, LMP-1 constitutively engages signaling pathways, such as the JNK and p38 mitogen-activated protein kinases (MAPK), the transcription factor NF-κB, and the JAK/STAT cascade.[53] LMP-1 also inhibits cell death through the up-regulation of various anti-apoptotic proteins[73–75]

and is responsible for mediating resistance to the anti-proliferative effects of TGF-β on EBV-transformed B cells.[76,77] Because LMP-1 is essential for immortalization by EBV and plays a critical role in preventing apoptosis, it represents a potential therapeutic target for suppression. Interfering with LMP-1 expression using intrabody-mediated phenotypic knockout[78] or antisense[79] strategies have been shown to sensitize EBV-positive lymphoblastoid cell lines (LCLs) to chemotherapeutic drugs and to partially restore sensitivity to the anti-proliferative and apoptotic effects of TGF-β, through reductions of cyclin D2 and Bcl-2 levels, respectively. Thus, this oncogenic protein provides a model system to evaluate the biochemical and therapeutic effects of intrabodies on the selective functional knockout of different well defined functional domains of LMP-1 and the biological consequences of this selective blockade on LMP-1 signaling.

In summary, intrabodies remain a unique and attractive tool for the manipulation of target gene expression that has yet to be fully exploited in the growing field of functional proteogenomics. The DPIS strategy presented here should alleviate some of the barriers to their more widespread use. This approach, combined with our ability to shuttle the sFv genes into different intrabody targeting vectors should provide a more rapid and highly refined approach to isolate and directly test highly specific intrabodies to delineate the precise regions of target proteins that are involved in protein–protein, protein–DNA and protein–RNA interactions. In addition, by further exploiting their potential to be expressed in a spatially and temporally controlled manner through their inducible expression from lentiviral vectors,[80] new avenues of investigation in development biology through their use in transgenic animal and nuclear transfer research will be opened and their potential to be used as gene medicines in the future may be realized.

Methods and Materials

Cells

Human embryonal kidney 293 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 10% (v/v) heat-inactivated fetal bovine serum (FBS).

Plasmids

The LMP-1 expressing plasmids used in this study were pSG5-Flag-LMP1 (F-LMP1), pSG5-Flag-LMP1(1–231) (F-LMP1-TES1) and pSG5-Flag-LMP1(Δ187–351) (F-LMP1-TES2), which are pSG5-based vectors expressing wild-type LMP1, a truncated form containing only the carboxy-terminal TES1 domain and a truncated form containing only the carboxy-terminal TES2 domain, respectively.[46,49] A pcDNA3.1 plasmid (Invitrogen) was used for the construction of a cytoplasmic intrabody expression vector to accommodate *Sfi*I/*Not*I sFv fragments before a HA tag sequence (pcDNA3.1-stuffer-HA).

Phage panning

A 15 billion member non-immune human sFv phage display library was used for these studies (unpublished results). The sFv genes were cloned in the pFARBER phagemid plasmid as sFv fusion proteins with a truncated (Δ1–249 amino acid residues) gene III protein. A biotinylated 44-mer peptide (H-G-Q-R-H-S-D-E-H-H-H-D-D-S-L-P-H-P-Q-Q-A-T-D-D-S-G-H-E-S-D-S-N-S-N-E-G-R-H-H-L-L-V-S-G-K) (TES1 polypeptide) corresponding to the transformation effector site 1/C-terminal activator site region 1 (TES1/CTAR1) of LMP1 encompassing amino acid residues 187–230, was synthesized (Macromolecular Resources, Fort Collins, CO) with biotinylation of the side-chain of the additional carboxy-terminal lysine. For panning, the biotinylated polypeptide was added to the phage at decreasing concentrations starting at 500 nM (rounds 1 and 2), 250 nM (round 3) and 100 nM (rounds 4 and 5) and the bound phage were captured using streptavidin- (M-280, Dynal) paramagnetic beads alternating with avidin (MPG-avidin beads; Millipore, NJ) coated magnetic porous glass beads that were washed with and resuspended in 4% (v/v) MPBST.

For the phage ELISA assays, 96 well plates were coated overnight with streptavidin (100 ng/well) in bicarbonate buffer at 4 °C. The plates were washed five times with Tween-PBS (TPBS) and blocked with 3% (w/v) dried milk in TPBS for two hours at 37 °C. The plates were washed five times with TPBS and then biotinylated TES1 was added to a final concentration of 0.2 μg/ml in a total volume of 100 μl of 1% MPBST. Specificity of binding was analyzed using uncoated plates and plates coated directly with biotinylated TES1, streptavidin or BSA alone. Phage binding was detected with HRP-labeled anti-M13 Mab (Pharmacia).

DNA fingerprinting of sFv genes

PCR amplification of the sFv insert was carried out for 25 cycles at 94 °C for one minute, 52 °C one minute, 72 °C one minute. The restriction analysis on the 800 bp fragment was performed using *Bst*NI.[81]

Standardization of NFκB activity assay

The day before transfection, 293 cells were plated in six well plates (2×10^5 cells/well). The following day, the 60–70% confluent cells were transfected with the calcium phosphate method using 10 μg of total DNA. Co-transfection included 2 μg of the luciferase reporter plasmid 3 × κB-L,[47] 2 μg of pGkβgal (β-galactosidase reporter plasmid to normalize for transfection efficiency), varying concentrations of F-LMP1, F-LMP1-TES1 or F-LMP1-TES2 plasmid DNA and pSG5 to maintain constant 10 μg of total DNA. At 24 hours post-infection cells were lysed in luciferase lysis buffer. Luciferase and β-galactosidase assays were performed on cleared lysates and quantified using the manufacturer's recommended procedures (Promega).

To examine the effects of the anti-LMP1-TES1 intrabody pools or individual intrabodies, the same transfection procedures were used except that 2 μg of 3 × κB-L, 2 μg pGkβgal, 3 μg of (F-LMP1, F-LMP1-TES1

or F-LMP1-TES2) and 3 μg of anti-TES1 pools or individual anti-TES1 or control intrabody were used.

Immunoprecipitation and immunoblotting

The 293 cells were plated in 100 mm dishes and transfected with F-LMP1, and anti-TES1 intrabody expression plasmids using Gene Porter Reagent (Gene Therapy System); 20 μg of total DNA was used that included 10 μg of F-LMP1 plus 10 μg of anti-TES1 (individual or total DNA for pools) or pcDNA3.1. At 48 hours after the transfection cells were harvested in PBS with 5 mM EDTA and lysed in 1 ml of solubilization buffer (0.5% Brij 58 (Sigma, St. Louis, MO), 100 mM NaCl, 10 mM Hepes (pH 7.6) and Protease Inhibitor Mixture (Roche)) for 30 minutes at 4 °C. In some experiments, 293 cells were plated in 150 mm dishes and transfected with F-LMP1 and the pools or individual anti-LMP1-TES1 intrabody plasmids using the calcium phosphate method, using 70 μg of total DNA in the same ratios as described above. Cells were harvested with 5 mM EDTA in PBS and lysed in 1 ml of solubilization buffer (1% Brij 58, 100 mM NaCl, 10 mM Hepes (pH 7.6) and Protease Inhibitor Mixture) for 30 minutes at 4 °C. Cell debris was removed by centrifugation for 15 minutes at 14,000*g*, and cleared cell lysates were incubated with anti-Flag M2 affinity gel (Sigma) or anti-HA monoclonal antibody HA.11 affinity matrix (BabCO, Richmond, California) for four hours at 4 °C on a rocking platform. Beads were then washed six times in solubilization buffer and bound proteins were recovered by incubation in 2 × SDS buffer for one hour at 55 °C. Eluted proteins were separated on an SDS-12% (w/v) polyacrylamide gel. For LMP1 and anti-TES1 immunoblotting the proteins were transferred onto a nitrocellulose filter and detected by anti-Flag-M2 Mab or anti-HA polyclonal antibody followed by HRP-labeled goat anti-mouse IgG or HRP-labeled goat anti-rabbit IgG, respectively (Pierce, Rockford, IL).

DNA sequence analysis

The phage display library samples were purified using a Qiagen miniprep kit. The purified DNA (200–400 μg) was PCR amplified using Applied Biosystems, Big Dye Terminator Chemistry, Version 2. Two sets of primers that flanked the sFv genes in pFARBER were used for each sample to achieve sequence overlap within each sFv gene. The forward primer is 5′-CATAATGAAA TACCTATTGCCTA-3′ and the reverse primer is 5′-CTT ATTAGCGTTTGCCATT-3′. The PCR reactions were cleaned by Biomax Spin-50 column (Odenton, MD). The purified PCR samples were run on an ABI Prism, 310 Genetic Analyzer equipped with a 47 cm × 50 μm capillary and Pop6 gel. The samples were analyzed and assembled with the program Sequencher (Gene Codes Corporation). The family assignments were analyzed by the program DnaPlot†.

Acknowledgements

We thank Dr Quan Zhu for critical review and comments on the manuscript. We also thank Drs Elliott Kieff, Harvard Medical School and Kenneth Izumi, University of Texas Health Science Center for the generous gift of the LMP-1 expression plasmids. This work was supported by the National Institutes of Health grants AI28785 (to W.A.M.) and by a joint Dana-Farber Cancer Institute-Beth Israel Deaconess Medical Center and Children's Hospital Center for AIDS Research (CFAR) grant. F.G. and G.P. were supported by AIDS-ISS grants.

References

1. Van Dijk, M. A. & van de Winkle, J. G. J. (2001). Human antibodies as next generation therapeutics. *Curr. Opin. Chem. Biol.* **5**, 368–374.
2. Zhu, Q. & Marasco, W. A. (2003). Intracellular targeting of antibodies in mammalian cells. In *New Comprehensive Biochemistry: Gene Transfer and Expression in Mammalian Cells* (Makrides, Savvas C., ed.), Elsevier, London, in the press.
3. Biocca, S. & Cattaneo, A. (1995). Intracellular immunization: antibody targeting to subcellular compartments. *Trends Cell Biol.* **5**, 248–252.
4. Richardson, J. H. & Marasco, W. A. (1995). Intracellular antibodies: development and therapeutic potential. *Trends Biotechnol.* **13**, 306–310.
5. Richardson, J. H., Sodroski, J. G., Waldman, T. A. & Marasco, W. A. (1995). Phenotypic knockout of the high-affinity human interleukin 2 receptor by intracellular single-chain antibodies against the α subunit of the receptor. *Proc. Natl Acad. Sci. USA*, **92**, 3137–3141.
6. Marasco, W. A. (1995). Intracellular antibodies (intrabodies) as research reagents and therapeutic molecules for gene therapy. *Immunotechnology*, **1**, 1–19.
7. Marasco, W. A. (1997). Designing intrabodies: structural features and the use of intracellular trafficking signals. In *Intrabodies: Basic Research and Clinical Gene Therapy Applications* (Marasco, W. A., ed.), pp. 1–22, LANDES Publishers, London.
8. Mhashilkar, A. M., Doebis, C., Seifert, M., Busch, A., Zani, C., Soo Hoo, J. *et al.* (2002). Intrabody-mediated phenotypic knockout of major histocompatibility complex class 1 expression in human and monkey cell lines and in primary human keratinocytes. *Gene Ther.* **9**, 307–319.
9. Thompson, J. D. (2002). Applications of antisense and siRNAs during preclinical drug development. *Drug Discov. Today*, **7**, 912–917.
10. Scherr, M., Morgan, M. A. & Eder, M. (2003). Gene silencing mediated by small interfering RNAs in mammalian cells. *Curr. Med. Chem.* **10**, 245–256.
11. Biocca, S., Ruberti, F., Tafani, M., Pierandrei-Amaldi, P. & Cattaneo, A. (1995). Redox state of single chain Fv fragments targeted to endoplasmic reticulum, cytosol and mitochondria. *Biotechnology*, **13**, 1110–1115.
12. Rudikoff, S. & Pumphrey, J. G. (1986). Functional antibody lacking a variable-region disulfide bridge. *Proc. Natl Acad. Sci. USA*, **83**, 7875–7878.
13. Proba, K., Worn, A., Honegger, A. & Pluckthun, A. (1998). Antibody scFv fragments without disulfide bonds made by molecular evolution. *J. Mol. Biol.* **275**, 245–253.
14. Martineau, P., Jones, P. & Winter, G. (1998).

† http://www.dnaplot.de/

Expression of an antibody fragment at high levels in the bacterial cytoplasm. *J. Mol. Biol.* **280**, 117–127.
15. Worn, A. & Pluckthun, A. (1998). Mutual stabilization of VL and VH in single-chain antibody fragments, investigated with mutants engineered for stability. *Biochemistry*, **37**, 13120–13127.
16. Levy, R., Weiss, R., Chen, G., Iverson, B. L. & Georgiou, G. (2001). Production of correctly folded Fab antibody fragment in the cytoplasm of *Escherichia coli* trxB gor mutants *via* the coexpression of molecular chaperones. *Protein Expr. Purif.* **23**, 338–347.
17. Jurado, P., Ritz, D., Beckwith, J., de Lorenzo, V. & Fernandez, L. A. (2002). Production of functional single-chain Fv antibodies in the cytoplasm of *Escherichia coli*. *J. Mol. Biol.* **320**, 1–10.
18. Grim, J. E., Siegal, G. P., Alvarez, R. D. & Curiel, D. T. (1998). Intracellular expression of the anti-erbB-2 sFv N29 fails to accomplish efficient target modulation. *Biochem. Biophys. Res. Commun.* **250**, 699–703.
19. Beerli, R. R., Graus-Porta, D. & Hynes, N. E. (1997). Intracellular antibodies as tools to study ErbB receptor tyrosine kinases. In *Intrabodies: Basic Research and Clinical Gene Therapy Applications* (Marasco, W. A., ed.), LANDES Publishers, London.
20. Zhu, Q., Zeng, C., Huhalov, A., Yao, J., Turi, T. G., Danley, D. *et al.* (1999). Extended half-life and elevated steady-state level of a single-chain Fv intrabody are critical for specific intracellular retargeting of its antigen, Capase-7. *J. Immunol. Methods*, **231**, 207–222.
21. Rajpal, A. & Turi, T. G. (2001). Intracellular stability of anti-caspase-3 intrabodies determines efficacy in retargeting the antigen. *J. Biol. Chem.* **276**, 33139–33146.
22. Mhashilkar, A. M., Bagley, J., Chen, S.-Y., Szilvay, A. M., Helland, D. G. & Marasco, W. A. (1995). Inhibition of HIV-1 tat-mediated LTR transduction and HIV-1 infection by anti-tat single chain intrabodies. *EMBO J.* **14**, 1542–1551.
23. Arafat, W., Gomez-Navarro, J., Xiang, J., Siegal, G. P., Alvarez, R. D. & Curiel, D. T. (2000). Antineoplastic effect of anti-erbB-2 intrabody is not correlated with scFv affintiiy for its target. *Cancer Gene Ther.* **7**, 1250–1256.
24. Tanaka, T. & Rabbitts, T. H. (2003). Intrabodies based on intracellular capture frameworks that bind the RAS protein with high affinity and impair oncogenic transformation. *EMBO J.* **22**, 1025–1035.
25. Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L. *et al.* (1994). Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J.* **13**, 3245–3260.
26. Nissim, A., Hooggenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D. & Winter, G. (1994). Antibody fragments from a 'single pot' phage display library as immunochemical reagents. *EMBO J.* **13**, 692–698.
27. Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C. *et al.* (1996). Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nature Biotechnol.* **14**, 309–314.
28. De Kruif, J., Boel, E. & Logtenberg, T. (1995). Selection and application of human single chain Fv antibody fragments from a semi-synthetic phage antibody display library with designed CDR3 regions. *J. Mol. Biol.* **248**, 97–105.
29. Sheets, M. D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R. *et al.* (1998). Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. *Proc. Natl Acad. Sci. USA*, **95**, 6157–6162.
30. De Haard, H. J., van Neer, N., Reurs, A., Hufton, S. E., Roovers, R. C., Henderikx, P. *et al.* (1999). A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. *J. Biol. Chem.* **274**, 18218–18230.
31. Hanes, J. & Pluckthun, A. (1997). *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc. Natl Acad. Sci. USA*, **94**, 4937–4942.
32. Feldhaus, M. J., Siegel, R. W., Opresko, L. K., Coleman, J. R., Weaver Feldhaus, J. M. *et al.* (2003). Flow-cytometric isolation of human antibodies from a nonimmune *Saccharomyces cerevisiae* surface display library. *Nature Biotechnol.* **21**, 163–170.
33. Daugherty, P. S., Olsen, J. J., Iverson, B. L. & Georgiou, G. (1999). Development of an optimized expression system for the screening of antibody libraries displayed on the *Escherichia coli* surface. *Protein Eng.* **12**, 613–621.
34. Chen, G., Hayhurst, A., Thomas, J. G., Harvey, B. R., Iverson, B. L. & Georgiou, G. (2001). Isolation of high-affinity ligand-binding proteins by periplasmic expression with cytometric screening (PECS). *Nature Biotechnol.* **19**, 537–542.
35. Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D. & Winter, G. (1991). By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* **222**, 581–597.
36. Visintin, M., Tse, E., Axelson, H., Rabbitts, T. H. & Cattaneo, A. (1999). Selection of antibodies for intracellular function using a two-hybrid *in vivo* system. *Proc. Natl Acad. Sci. USA*, **96**, 11723–11728.
37. Cattaneo, A. & Biocca, S. (1999). The selection of intracellular antibodies. *Trends Biotechnol.* **17**, 115–121.
38. Portner-Taliana, A., Russell, M., Froning, K. J., Budworth, P. R., Comiskey, J. D. & Hoeffler, J. P. (2000). *In vivo* selection of single-chain antibodies using a yeast two-hybride system. *J. Immunol. Methods*, **238**, 161–172.
39. Auf der Maur, A., Escher, D. & Barberis, A. (2001). Antigen-independent selection of stable intracellular single-chain antibodies. *FEBS Letters*, **508**, 407–412.
40. Mossner, E., Koch, H. & Pluckthun, A. (2001). Fast selection of antibodies without antigen purification: adaptation of the protein fragment complementation assay to select antigen-antibody pairs. *J. Mol. Biol.* **308**, 115–122.
41. Lebowitz, D. (1998). Epstein-Barr virus and a cellular signaling pathway in lymphomas from immunosuppressed patients. *N. Engl. J. Med.* **338**, 1413–1421.
42. Cohen, J. I. (2000). Epstein-Barr virus infection. *N. Engl. J. Med.* **338**, 481–492.
43. Knecht, H., Berger, C., Rothenberger, S., Odermatt, B. F. & Brousset, P. (2001). The role of Epstein-Barr virus in neoplastic transformation. *Oncology*, **60**, 289–302.
44. Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C. & Kieff, E. (1995). The Epstein-Barr virus transforming protein LMP1

engages signaling proteins for the tumor necrosis factor receptor family. *Cell*, **80**, 389–399.
45. Mitchell, T. & Sugden, B. (1995). Stimulation of NF-κB-mediated transcription by mutant derivatives of the latent membrane protein of Epstein-Barr virus. *J. Virol.* **69**, 2968–2976.
46. Izumi, K. M., Kaye, K. M. & Kieff, E. D. (1997). The Epstein-Barr virus LMP1 amino acid sequence that engages tumor necrosis factor receptor associated factors is critical for primary B lymphocyte growth transformation. *Proc. Natl Acad. Sci. USA*, **94**, 1447–1452.
47. Izumi, K. M., Kaye, K. M. & Kieff, E. D. (1994). Epstein-Barr virus recombinant molecular genetic analysis of the LMP1 amino-terminal cytoplasmic domain reveals a probable structural role, with no component essential for primary B-lymphocyte growth transformation. *J. Virol.* **68**, 4369–4376.
48. Huen, D. S., Henderson, S. A., Croom-Carter, D. & Rowe, M. (1995). The Epstein-Barr virus latent membrane protein 1 (LMP1) mediates activation of NF-κB and cell surface phenotype *via* two effector regions in its carboxy-terminal cytoplasmic domain. *Oncogene*, **10**, 549–560.
49. Izumi, K. M. & Kieff, E. D. (1997). The Epstein-Barr virus oncogene product latent membrane protein 1 engages the tumor necrosis factor receptor-associated death domain protein to mediate B lymphocyte growth transformation and activate NF-κB. *Proc. Natl Acad. Sci. USA*, **94**, 12592–12597.
50. Brodeur, S. R., Cheng, G., Baltomore, D. & Thorley-Lawson, D. A. (1997). Localization of the major NF-$_{\kappa}$B-activating site and the sole TRAF3 binding site of LMP1 defines two distinct signaling motifs. *J. Biol. Chem.* **272**, 19777–19784.
51. Devergne, O., Hatzivassiliou, E., Izumi, K. M., Kaye, K. M., Kleijner, M., Kieff, E. & Mosialos, G. (1996). TRAF1, TRAF2 and TRAF3 effect NF-κB activation by an Epstein-Barr virus LMP1 domain important for B lymphocyte transformation. *Mol. Cell. Biol.* **16**, 7098–7107.
52. Devergne, O., McFarland, E. C., Mosialos, G., Izumi, K., Ware, C. F. & Kieff, E. (1998). Role of the TRAF binding site and NF-κB activation in Epstein-Barr virus latent membrane protein 1-induced cell ggene expression. *J. Virol.* **72**, 7900–7908.
53. Eliopoulos, A. G., Waites, E. R., Blake, S. M. S., Davies, C., Murray, P. & Young, L. S. (2003). TRAF1 is a critical regulator of NJK signaling by the TRAF-binding domain of the Epstein-Barr virus-encoded latent infection membrane protein 1 but not CD40. *J. Virol.* **77**, 1316–1328.
54. Chothia, C. & Lesk, A. M. (1987). Canonical structures for the hypervariable regions of immunoglobulins. *J. Mol. Biol.* **196**, 901–917.
55. Worn, A., Auf der Maur, A., Escher, D., Honegger, A., Barberis, A. & Pluckthun, A. (2000). Correlation between *in vitro* stability and *in vivo* performance of anti-GCN4 intrabodies as cytoplasmic inhibitors. *J. Biol. Chem.* **275**, 2795–2803.
56. Auf der Maur, A., Zahnd, C., Fischer, F., Spinelli, S., Honegger, A., Cambillau, C. *et al.* (2002). Direct *in vivo* screening of intrabody libraries constructed on a highly stable single-chain framework. *J. Biol. Chem.* **277**, 45075–45085.
57. Tse, E., Natividad Lobato, M., Forster, A., Tanaka, T., Chung, G. T. Y. & Rabbitts, T. H. (2002). Intracellular antibody capture technology: application to selection of intracellular antibodies recognizing the BCR-ABL oncogenic protein. *J. Mol. Biol.* **317**, 85–94.
58. Visintin, M., Settanni, G., Maritan, A., Graziosi, S., Marks, J. D. & Cattaneo, A. (2002). The intracellular antibody capture technology (IACT): towards a consensus sequence for intracellular antibodies. *J. Mol. Biol.* **317**, 73–83.
59. Hyland, S., Beerli, R. R., Barbas, C. F., III, Hynes, N. E. & Wels, W. (2003). Generation of functional characterization of intracellular antibodies interacting with the kinase domain of human EGF receptor. *Oncogene*, **22**, 1557–1567.
60. Worn, A. & Pluckthun, A. (1998). An intrinsically stable antibody scFv fragment can tolerate the loss of both disulfide bonds and fold correctly. *FEBS Letters*, **427**, 357–361.
61. Ohage, E. & Steipe, B. (1999). Intrabody construction and expression. I. The critical role of V_L domain stability. *J. Mol. Biol.* **291**, 1119–1128.
62. Ohage, E. C., Wirtz, P., Barnikow, J. & Steipe, B. (1999). Intrabody construction and expression. II. A synthetic catalytic Fv fragment. *J. Mol. Biol.* **291**, 1129–1134.
63. Wirtz, P. & Steipe, B. (1999). Intrabody construction and expression III: engineering hyperstable V_H domains. *Protein Sci.* **8**, 2245–2250.
64. Desiderio, A., Franconi, R., Lopez, M., Villani, M. E., Viti, F., Chiaraluce, R. *et al.* (2001). A semi-synthetic repertoire of intrinsically stable antibody fragments derived from a single-framework scaffold. *J. Mol. Biol.* **310**, 603–615.
65. Bai, J., Sui, J., Zhu, R.-Y., Tallarico, A. St. C., Gennari, F., Zhang, D. & Marasco, W. A. (2003). Inhibition of tat-mediated transactivation and HIV-1 replication by human anti-hCyclin T1 intrabodies. *J. Biol. Chem.* **278**, 1433–1442.
66. Martin, A. C. R. & Thornton, J. M. (1996). Structural families in loops of homologous proteins: automatic classification, modelling and application to antibodies. *J. Mol. Biol.* **263**, 800–815.
67. Al-Lazikani, B., Lesk, A. M. & Chothia, C. (1997). Standard conformations for the canonical structures of immunoglobulins. *J. Mol. Biol.* **273**, 927–948.
68. Morea, V., Tramontano, A., Rustici, M., Chothia, C. & Lesk, A. M. (1998). Conformations of the third hypervariable region in the VH domain of immunoglobulins. *J. Mol. Biol.* **275**, 269–294.
69. Chothia, C., Gelfand, I. & Kister, A. (1998). Structural determinants in the sequences of immunoglobulin variable domain. *J. Mol. Biol.* **278**, 457–479.
70. Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G. *et al.* (2000). Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CFRs randomized with trinucleotides. *J. Mol. Biol.* **296**, 57–86.
71. Tanaka, T., Chung, G. T. Y., Forster, A., Natividad Lobato, M. & Rabbitts, T. H. (2003). *De novo* production of diverse intracellular antibody libraries. *Nucl. Acids Res.* **31**, 20036.
72. Izumi, K. M., McFarland, E. C., Riley, E. A., Rizzo, D., Chen, Y. & Kieff, E. (1999). The residues between the two transformation effector sites of Epstein-Barr virus latent membrane protein 1 are not critical for B-lymphocyte growth transformation. *J. Virol.* **73**, 9908–9916.
73. Henderson, S., Rowe, M., Gregory, C., Croom-Carter, D., Wang, F., Longnecker, R. *et al.* (1991). Induction of bcl-2 expression by Epstein-Barr virus latent

membrane protein 1 protects infected B cells form programmed cell death. *Cell*, **65**, 1107–1115.
74. Wang, S., Rowe, M. & Lundgren, E. (1996). Expression of Epstein-Barr virus transforming protein LMP1 causes a rapid and transient stimulation of the Bcl-2 homolog Mcl-1 levels in B-cell lines. *Cancer Res.* **56**, 4610–4613.
75. Fries, K. L., Miller, W. E. & Raab-Traub, N. (1996). Epstein-Barr virus latent membrane protein 1 blocks p53-mediated apoptosis through induction of the A20 gene. *J. Virol.* **70**, 8653–8659.
76. Chaouchi, N., Arvanitakis, L., Auffredou, M. T., Blanchard, D. A., Vazquez, A. & Sharma, S. (1995). Characterization of transforming growth factor-β1 induced apoptosis in normal human B cells and Lymphoma B cell lines. *Oncogene*, **11**, 1615–1622.
77. Arvantakis, L., Yaseen, N. & Sharma, S. (1995). Latent membrane protein-1 induces cyclin D2 expression, pRb hyperphosphorylation, and loss of TGF-β1-mediated growth inhibition in EBV-positive B cells. *J. Immunol.* **155**, 1047–1056.
78. Piche, A., Kasono, K., Johanning, F., Curiel, T. J. & Curiel, D. T. (1998). Phenotypic knock-out of the latent membrane protein 1 of Epstein-Barr virus by an intracellular single-chain antibody. *Gene Ther.* **5**, 1171–1179.
79. Kenney, J. L., Guinnes, M. E., Reiss, M. & Lacy, J. (2001). Antisense to the Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP-1) sensitizes EBV-immortalized B cells to transforming growth factor-beta and chemotherapeutic agents. *Int. J. Cancer*, **91**, 89–98.
80. Ogueta, S. B., Yao, F. & Marasco, W. A. (2001). Design and *in vitro* characterization of a single regulatory module for efficient control of gene expression in both plasmid DNA and a self-inactivating lentiviral vector. *Mol. Med.* **7**, 569–579.
81. Clackson, T., Hoogenboom, H. R., Griffiths, A. D. & Winter, G. (1991). Making antibody fragments using phage display libraries. *Nature*, **352**, 624–628.

Edited by I. Wilson

(Received 6 May 2003; received in revised form 26 September 2003; accepted 30 September 2003)