Construction of single-chain antibodies that bind an overlapping epitope of HIV-1 Nef

Alex H. Chang[a,b,c], James A. Hoxie[d], Sharon Cassol[e], Michael O'Shaughnessy[a], Frank Jirik[c,*]

[a] *B.C. Center for Excellence in HIV/AIDS, 613–1081 Burrard St., St. Paul's Hospital, Vancouver, B.C. V6Z 1Y6, Canada*
[b] *Pathology and Laboratory Medicine, University of British Columbia, Vancouver, B.C. V5Z 4H4, Canada*
[c] *Centre for Molecular Medicine and Therapeutics, 950 West 28th Avenue, University of British Columbia, Vancouver, B.C. V5Z 4H4, Canada*
[d] *Hematology Oncology Section, Rm 664 Clinical Research Building, University of Pennsylvania, 415 Curie Blvd, Philadelphia, PA 19104, USA*
[e] *Division of Infectious Diseases, Department of Medicine, Ottawa General Hospital Research Institute, Box 411, 501 Smyth Rd, Ottawa, Ont. K1H 8L6, Canada*

Received 5 October 1998

Abstract The light and heavy chain variable regions of three mouse hybridoma cell lines (AG11, AE6 and EH1) that produce monoclonal antibodies against an overlapping epitope at the C-terminus of Nef were cloned. Sequence analysis of the light and heavy chain variable regions indicated that clones AG11 and AE6, but not EH1, were highly related. Single-chain antibodies were constructed from the cDNA clones of AG11 and EH1, and subcloned into an eukaryotic expressing vector with the green fluorescent protein as marker for expression. Such intracellular antibodies may provide a way in which to inhibit the function of Nef during HIV-1 infection of cells.

Key words: Single-chain antibody; Monoclonal antibody; Nef; Green fluorescent protein; Human immunodeficiency virus-1

1. Introduction

Nef is an accessory protein produced at all stages of human immunodeficiency virus-1 (HIV-1) gene expression. Although initially Nef was described as being able to repress viral gene transcription [1], these results were not confirmed by subsequent investigations [2,3]. Studies in peripheral blood mononuclear cells (PBMC) [4–7], in specific T-cell lines [8], in rhesus monkeys infected with *nef*-deleted strains of simian immunodeficiency virus [9], and in some of the long-term non-progressors of HIV-1 infection [10,11], demonstrated that Nef is not only necessary for rapid HIV-1 replication in vitro, but that it is also required for efficient viral growth in vivo. However, the precise role of Nef in these processes has not been fully elucidated.

One approach to study the function of cytosolic proteins involves the use of intracellular single-chain antibodies (ScFv) that either block function or sequester the protein of interest. ScFv, which have been shown to have specific binding affinities equivalent to those of the parent monoclonal antibodies [12,13], can be stably expressed intracellularly where they are capable of inactivating specific cellular gene products [14,15]. Intracellular ScFv proteins with specificity for virally encoded proteins thus provide a unique way of studying the role of these viral proteins in HIV-1 infection [16], as well as offering a potential gene therapy strategy for inhibiting the development of AIDS.

We report the cloning and sequencing of the antibody variable regions of three hybridoma cell lines, as well as subsequent ScFv construction and expression. All cell lines studied produced monoclonal antibodies specific to an overlapping epitope at the C-terminus of Nef protein. Single-chain antibodies were then constructed by tethering the V_κ and V_H genes to a DNA sequence encoding a 15 amino acid peptide $(GGGGS)_3$. A green fluorescent protein (GFP) fusion was used as a reporter for monitoring intracellular expression of the single-chain antibodies. Immunoprecipitation studies demonstrated that the ScFv retained the binding activity of their corresponding parent monoclonal antibodies.

2. Materials and methods

2.1. Nef-specific monoclonal antibodies

Anti-Nef hybridoma clones AG11 and AE6 were raised against the recombinant Nef protein of HIV-1_{LAI} strain and were both derived from the same fusion. They both produce IgG1 monoclonal antibodies that recognize Nef of the HIV-1_{LAI}, but not SF2 strain, and are specific for the C-terminus of Nef (epitope: VARELHPEYFKNC) (unpublished data). Clone EH1, raised against the Nef protein of the HIV-1_{SF2} strain, is an IgG1 monoclonal antibody that reacts with Nef from both HIV-1 LAI and SF2 strains. It was also mapped to the C-terminus of Nef protein (epitope: MARELHPEYYKDC) (unpublished data).

2.2. Inhibition ELISA assay

Recombinant Nef-GST (a gift from Dr. Mark Harris) [17] used as the coating antigen was derived from HIV-1_{BH10}. Nef from HIV-1_{BH10} shares 96% identity with the amino acid sequence of HIV-1_{LAI}. Microtiter wells were coated with 50 μl of Nef-GST per well (12.5 μg/ml in PBS buffer) overnight at 4°C. The wells were blocked with 1% BSA/PBS for 1 h at 37°C. MAb from clone EH1 was biotinylated using the ImmunoPure Sulfo NHS-LC-Biotinylation Kit (Pierce). Serially diluted antibodies from clones AG11 and AE6, as well as a mAb (F14.11) generated against a different epitope of Nef ($Nef_{83–88}$: AAVDLS) [18], were mixed with 5 μg/ml biotinylated EH1 mAb in the wells and incubated for 1 h at 37°C. After three washes with 0.05% Tween 20/PBS, avidin-alkaline phosphatase (Pierce) was added at a dilution of 1:1000 and incubated at 37°C for 1 h and washed as above. Immune complexes were detected by the enzyme-substrate reaction with *p*-nitrophenyl phosphate (Sigma), with the reactions being read at 405 nm after 30 min.

*Corresponding author. Fax: (1) (604) 875-3840.
E-mail: jirik@cmmt.ubc.ca

Abbreviations: ScFv, single-chain Fv (variable fragment) antibody; V_κ, variable region of the kappa light chain; V_H, variable region of the heavy chain; RT, reverse transcriptase; PCR, polymerase chain reaction; GFP, green fluorescent protein; HIV-1, human immunodeficiency virus-1; PBS, phosphate buffered saline; GST, glutathione *S*-transferase; CDR, complementarity determining region

PII: S0014-5793(98)01569-5

2.3. RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was isolated from 10^7 hybridoma cells, using the guanidinium thiocyanate method [19]. The variable regions of the light chain and heavy chain were amplified by RT-PCR, using the following primers: forward primers: V_κForward (5′ AAGCTTCCATGGA[CT][AG]T[TC][TG][TA]GATGAC[CA]CA[GA][TA]CTCC 3′), V_HForward (5′ GGATCCGGTGGTGGTGGTTCTGGTGGTGGTGGTG[AG]GGT[CG]CA[AG]CT[GT][GC][TA]G[GC]AGTC[AT]GG 3′); and reverse primers: V_κReverse (5′ GGATCCACCACCACCATTGATTTCCAGCTTGGTGCCAGCACCGAACG 3′), V_HReverse (5′ AAGCTTCTATGAGGAGACGGTGACCGTGGTCCCGGGGCCCCAG 3′). Primers V_κForward and V_HForward are degenerate primers with alternative bases indicated in the brackets. The synthesis of cDNA was performed using Superscript Moloney leukemia virus (M-MLV) reverse transcriptase (Gibco BRL/Life Technologies) according to the manufacturer's instructions. The reverse primers were used to prime the reverse transcription reactions. Polymerase chain reactions (PCR) were then performed using Taq DNA polymerase (Perkin-Elmer Cetus) in a GeneAmp PCR System 9600 (Perkin Elmer Cetus), for 35 cycles under the following conditions: denaturation at 96°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min; finishing with 72°C for 10 min.

2.4. Cloning and sequencing of amplified products

Amplified DNA fragments were digested with *Bam*HI and *Hin*dIII and gel purified by electrophoresis on 1.5% agarose gels, using the QIAquick gel extraction kit (Qiagen). The purified products were ligated into the *Bam*HI/*Hin*dIII restriction sites of a cloning vector, pGEM-4Z (Promega), and transformed into DH10B competent bacteria (Gibco BRL). Several recombinant clones were selected and sequenced in both directions using dye terminator cycle sequencing kit (Applied Biosystems), and T7/SP6 promoter primers (University Core DNA Services, University of Calgary, Canada) on a 373 automated sequencer (Applied Biosystems). Alignment of the antibody sequences was performed using Clustal V [20]. Sequences of the recombinant clones were also compared with the non-redundant database at National Center for Biotechnology Information (NCBI) using the Blast program [21].

2.5. Construction and expression of ScFv tagged with GFP

The ScFv were constructed by ligating the V_κ and V_H DNA fragments via the *Bam*HI site at the 3′ end of V_κ and 5′ end of V_H. PCR was used to add the Kozak sequence, GCCACC [22], the start codon, and the restriction sites, *Eco*RI and *Nhe*I, to the 5′ and 3′ ends of the ScFv constructs, respectively. The primers used are: forward primer, GGAATTCCTGCCACCATGGACATTTTGATGACCCAGTCT; reverse primer, CGCCTAGCTAGCTGAGGAGACGGTGACCG. PCR products were subsequently cloned into a mammalian expression vector, pcDEF-GFP, constructed from pcDEF3 [23] and pQBI25 (Quantum). The resulting pDEF-ScFv-GFP vector has a EF-1α promoter and GFP reporter gene at the C-terminus of the expression cassette (Fig. 4). HEK 293 cells were transfected with pcDEF-ScFv-GFP using Superfect (Qiagen).

2.6. Fluorescent microscopy

HEK 293 cells were transfected and cultured overnight in Chamber Slides (Lab-Tek, Nunc). The slides were then washed three times with PBS and fixed for 10 min in 4% paraformaldehyde at room temperature. After three PBS washes, the slides were mounted with Gel/Tol Aqueous Mounting Medium (Immunon, Fisher), and sealed with nail polish. A fluorescent microscope (Zeiss) and CCD camera were used to monitor the GFP fusion protein expression.

2.7. Characterization of expressed ScFv by immunoprecipitation

HEK 293 cells were transfected and cultured overnight in 6-well tissue culture plates (Nunclon Surface, Nunc), and then washed once with cold PBS and lysed in 1 ml of lysis buffer (0.5% NP-40, 100 mM NaCl, 25 mM Tris, pH 7.5, 2 mM EDTA, 10% glycerol, 50 mM NaF, and 10 μg/ml of each of the protease inhibitors: leupeptin, aprotinin, soybean trypsin inhibitor). The supernatant was first cleared of cell debris by centrifugation and subsequently pre-cleared by GST (a gift from Dr. Mark Harris) cross-linked to Sepharose beads (CNBr-activated Sepharose 4B, Pharmacia). The recombinant Nef-GST fusion protein cross-linked to Sepharose beads was used to immunoprecipitate ScFv by incubating for 1 h with rotation at 4°C. The precipitated products were resolved by SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% skim milk in TBS-T buffer (10 mM Tris, 50 mM NaCl, and 0.5% Tween 20) and probed with anti-GFP antibody (1:4000 dilution, Boehringer Mannheim). Immunodetection was accomplished using goat anti-mouse antibody conjugated to horseradish peroxidase, washed with TBS-T and followed by ECL detection (Amersham). The membrane was subsequently exposed to X-ray film (X-OMAT, Kodak).

3. Results

3.1. AG11 and AE6 mAbs bind to an overlapping epitope with mAbs of clone EH1

Using the binding inhibition ELISA assay, it was shown that mAbs from clones AG11 and AE6 inhibited the binding of the biotinylated EH1 mAb to immobilized Nef protein (Fig. 1). This binding inhibition was specific, since another mAb, F14.11, that was mapped to a different epitope failed to inhibit the binding of biotinylated EH1 mAb to immobilized Nef.

3.2. Cloning and sequencing of mouse IgG variable regions

The variable regions of light and heavy chains were cloned from the mouse anti-Nef monoclonal antibody producing hybridoma clones, AG11, AE6 and EH1. At least three clones from two independent RT-PCR reactions were sequenced to minimize the possibility of errors introduced during the amplification step.

A second aberrant V_κ transcript was identified in all three hybridoma cell lines as described [24,25]. Using primers specific for the CDR-1 and CDR-3 regions of the Sp2/0 endogenous κ chain variable region, we were able to eliminate plasmids containing the aberrant κ chain cDNA using the colony PCR method [24]. An internal *Hin*dIII site was found in the PCR amplified κ chain (between CDR-2 and CDR-3, at position 206, Fig. 2A) from clone EH1. Therefore, the TA cloning vector (Invitrogen), instead of pGEM-4Z, was used to subclone this cDNA.

Sequences from the three hybridoma cell lines were then

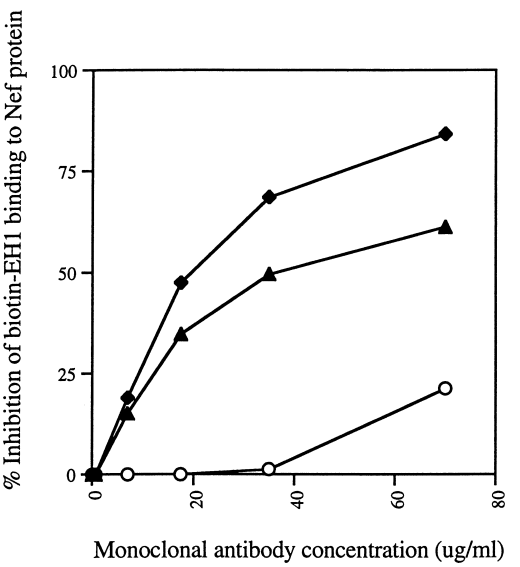

Fig. 1. Binding inhibition of biotinylated EH1 mAb by unlabeled AG11 (♦) and AE6 (▲) mAbs. F14.11 (○) was a control mAb.

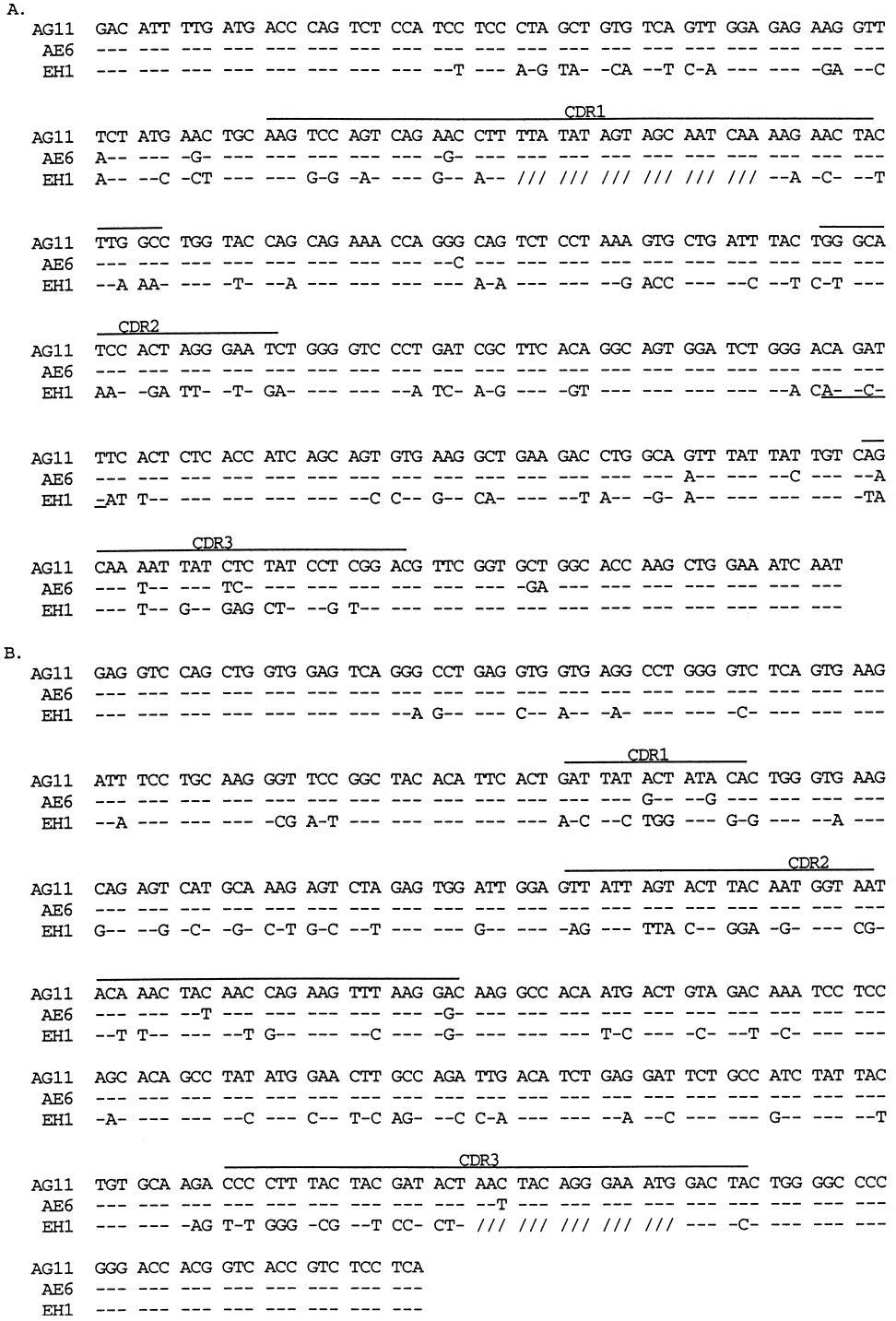

Fig. 2. Alignment of the cDNA sequence of variable regions derived from clones AG11, AE6 and EH1. A: Light chain variable regions. B: Heavy chain variable regions. '-' denotes identical residues; '/' denotes gaps. On the light chain variable region of clone EH1, there is an internal *Hin*dIII restriction site (position 206) (underlined). The complementarity determining regions are indicated.

analyzed. The aligned DNA and predicted amino acid sequence of the light chain and heavy chain variable regions are shown in Figs. 2 and 3. The complementarity determining regions (CDRs) were defined according to the Kabat-Wu numbering scheme [26].

All of the sequences of the variable regions from the three hybridomas contained open reading frames. At the DNA level, the total CDRs of clone AG11 was 95.1% similar to that of clone AE6. Both antibodies recognize the C-terminus of Nef from LAI strain (Table 1). The total CDRs of clone AG11, in contrast, was only 57.9% similar to clone EH1, which recognizes an overlapping epitope at the C-terminus of Nef (Table 1). A low percentage of sequence similarity was also found at the amino acid level when clones AG11 and EH1 were compared (Table 1). Thus, while there was 91.2% similarity when the amino acids of the total CDRs of clones AG11 and AE6 were compared, there was only 36.8% identity when clone AG11 was compared with EH1.

```
A.
                                        CDR1
AG11  DILMTQSPSSLAVSVGEKVSMNCKSSQNLLYSSNQKNYLAWYQQKPGQSP
 AE6  --------------------T-S-----S----------------------
 EH1  ----------MYA-L--R-TIT--AN-//////DI-T--N-F-----K--

          CDR2                                        CDR3
AG11  KVLIYWASTRESGVPDRFTGSGSGTDFTLTISSVKAEDLAVYYCQQNYLY
 AE6  ----------------------------------------I-----Y-S-
 EH1  -T---R-NRLVD---S--S-----QAYS-----LEH--MGI---L-YDEL

      __
AG11  PRTFGAGTKLEIN
 AE6  -----G-------
 EH1  -W-----------

B.
                                      CDR1                __
AG11  EVQLVESGPEVVRPGVSVKISCKGSGYTFTDYTIHWVKQSHAKSLEWIGV
 AE6  --------------------------------AM----------------
 EH1  --------A-LMK--A-------AT-----N-W-E---ERPGHG---V-E

      __CDR2_________                                    __
AG11  ISTYNGNTNYNQKFKDKATMTVDKSSSTAYMELARLTSEDSAIYYCARPL
 AE6  ---------------G----------------------------------
 EH1  -LPGS-R-Y--E---G---F-A-T--N----QFSS-------V----KSG

      __CDR3____
AG11  YYDTNYREMDYWGPGTTVTVSS
 AE6  ----------------------
 EH1  SYP/////L-S-----------
```

Fig. 3. Deduced amino acid sequence alignment of the variable regions of clones AG11, AE6 and EH1. A: Light chain variable regions. B: Heavy chain variable regions. '-' denotes identical residues; '/' denotes gaps.

3.3. Construction and expression of intracellular ScFv tagged with a GFP reporter

Single-chain antibodies (ScFv) were constructed for clones AG11 and EH1 as described in Section 2. The cDNA of the light chain variable region was tethered to the heavy chain variable region through a linker DNA encoding $(GGGGS)_3$. The ScFv cDNA constructs were ligated to the *Eco*RI and *Nhe*I sites of the pDEF-GFP expression vector (Fig. 4).

To assess the intracellularly expressed single-chain antibodies, HEK 293 cells were transfected and cultured overnight. The transfected cells were then examined using a fluorescent microscope and attached CCD camera (Fig. 5). Comparable level of expression was achieved with using all ScFv-GFP constructs, as well as the control vector expressing only GFP.

The ability of the intracellularly expressed ScFv to bind with Nef protein was assessed by immunoprecipitation of ScFv-GFP, performed using recombinant Nef protein-immobilized on Sepharose beads. The results demonstrated that the immobilized Nef was able to precipitate intracellularly expressed ScFv-GFP (Fig. 6A), but not GFP alone (Fig. 6B,C).

4. Discussion

The technique of constructing artificial antibodies by tethering immunoglobulin variable regions to various linkers, combined with in vitro selection strategies, offers an efficient route to the development of research reagents, as well as diagnostic and therapeutic molecules [27]. In this study, we compared the sequences of the variable regions from three monoclonal antibodies that recognize an overlapping epitope of Nef protein. Amongst these, two of the antibody clones, AG11 and AE6, derived from the same hybridoma fusion, were almost identical in nucleic acid and the deduced amino acid sequences. Although the variable region sequences of EH1 clone were significantly different from those of the other two mAbs, EH1 recognizes an overlapping epitope within the binding site of the AG11 and AE6 mAbs. This is supported by the binding inhibition assay which demonstrated that the antibodies from clones AG11 and AE6 inhibited the binding of the biotin-labeled EH1 mAb to Nef protein.

Table 1
Comparison of the variable region sequences of clones AE6 and EH1 with AG11

Antibody	Similarity[a] relative to clone AG11 (%)					
	Nucleic acid sequence			Amino acid sequence		
	V_κ	V_H	Total CDRs[b]	V_κ	V_H	Total CDRs
AE6	96.5	98.6	95.1	93.8	97.5	91.2
EH1	73.8	76.6	57.9	61.7	63.2	36.8

[a]The similarity study was done according to the overlapping sequences.
[b]Total CDRs: the total complementarity determining regions of both V_κ and V_H.

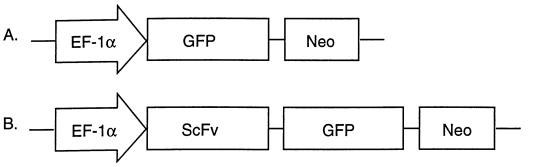

Fig. 4. The single chain antibody assembly in pDEF-GFP expression vector. A: pDEF-GFP vector which express GFP. B: pDEF-ScFv-GFP vector which express single chain antibody-GFP fusion protein.

The fact that EH1 antibody recognizes the recombinant Nef from both LAI and SF2 strains, but that clones AG11 and AE6 could only react to the Nef from LAI strain likely indicated that the fine specificity of the EH1 antibody is different from that of the other two antibodies. More detailed analysis using Ala-scan mutagenesis or comparison of crystal structures of the antibody-antigen complex would be required to elucidate this further.

Intracellular expression of single-chain antibodies is a useful technique for the study of cellular proteins, and may generate potential reagents for gene therapy [28]. We have utilized a strategy which uses green fluorescent protein [29] as a reporter for monitoring ScFv expression in eukaryotic cells. The advantage of GFP is that the ScFv expression can be readily monitored in either live or fixed cells. The binding specificity of intracellularly expressed ScFv was confirmed by immunoprecipitation using immobilized Nef protein.

In summary, we have successfully constructed anti-Nef single chain antibodies which retain the binding activity of their corresponding parental monoclonal antibodies when expressed intracellularly. Using GFP as a reporter, intracellular ScFv expression can be readily evaluated. The anti-Nef ScFv that we have generated can be used to study the intracellular role of Nef in the pathogenesis of HIV-1 infection, and could potentially be used in a gene therapy model for the treatment of HIV-1 infection.

Acknowledgements: The authors wish to thank Drs. Kevin Leslie for providing the primers used to amplify the variable regions of the monoclonal antibodies; Mark Harris for the recombinant Nef-GST fusion protein and GST protein, as well as their corresponding bacterial expression vectors; Rita De Santis for the mAb clone, F14.11; Jerome A. Langer for the pDEF-3 vector; Wayne A. Vogl for his assistance in fluorescent microscopy. This research was supported in part by the National Health Research and Development Program through a National Health Fellowship to A.H.C. and also supported by the province of British Columbia and the B.C. Centre for Excellence in HIV/AIDS.

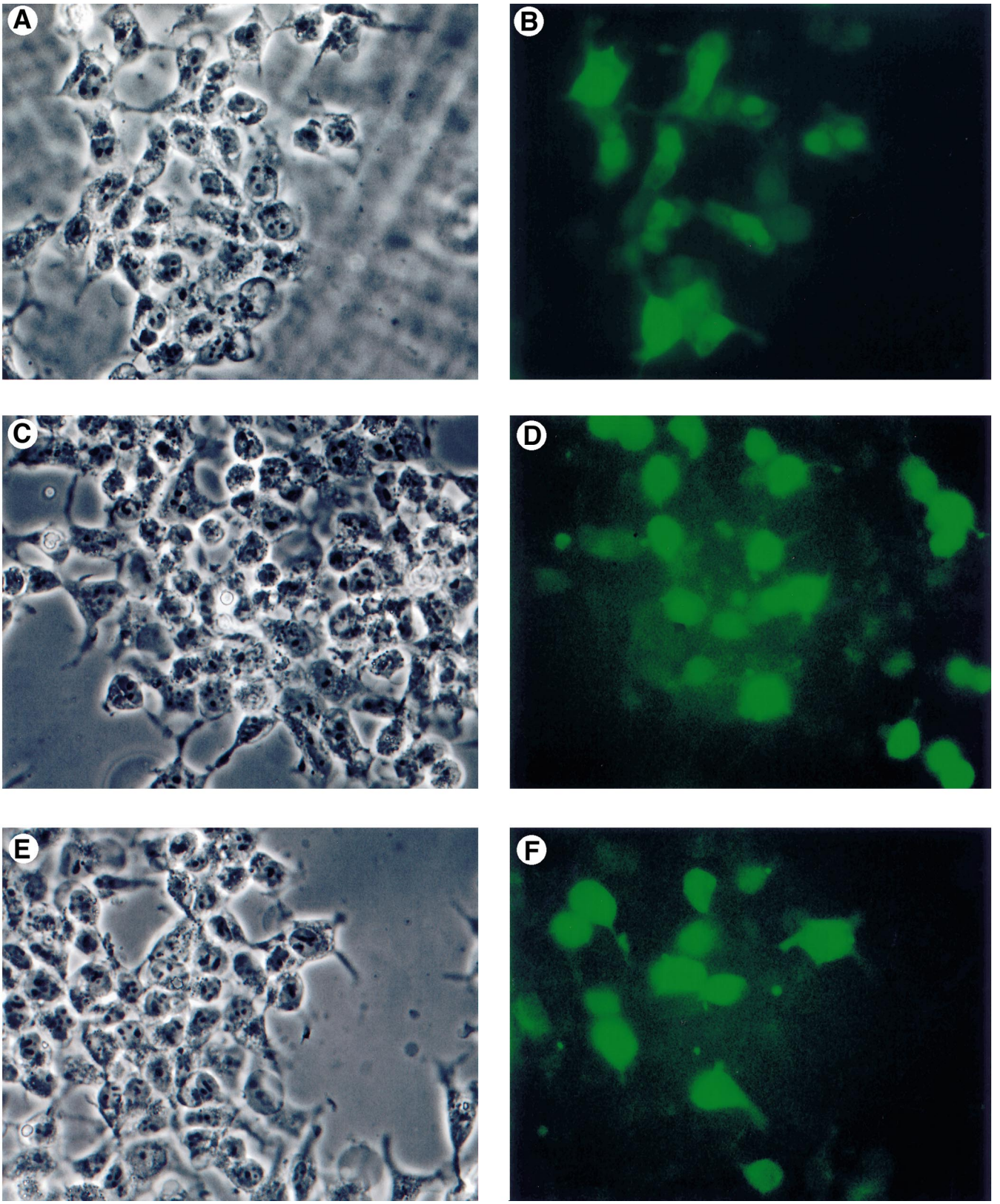

Fig. 5. HEK 293 cells transfected with pDEF-GFP or pDEF-ScFv-GFP, and expressing: A: GFP (phase). B: GFP (fluorescence). C: AG11 ScFv tagged with GFP (phase). D: AG11 ScFv tagged with GFP (fluorescence). E: EH1 ScFv tagged with GFP (phase). F: EH1 ScFv tagged with GFP (fluorescence). Magnification, ×36.

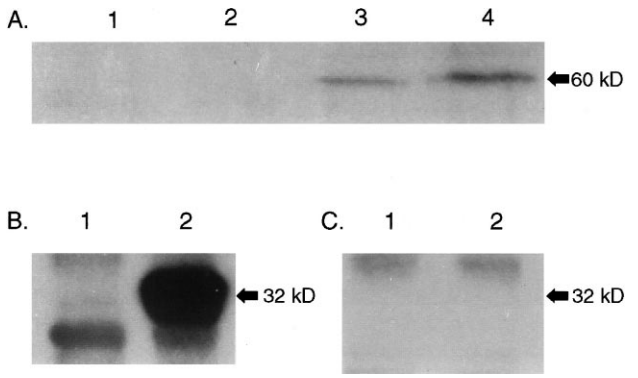

Fig. 6. Intracellularly expressed ScFv is immunoprecipitated by immobilized recombinant Nef-GST protein. A: Immunoprecipitation of ScFv with Nef-GST cross-linked to Sepharose beads from cells transfected with the control vector (1), pDEF-GFP (2), pDEF-(AG11)ScFv-GFP (3), and pDEF-(EH1)ScFv-GFP (4). B: Immunoblot of the total cell lysate of the cells transfected with control vector (1) and pDEF-GFP (2). C: Immunoprecipitation of cells expressing the control vector (1) and pDEF-GFP (2) corresponding to B, by Nef-GST cross-linked with Sepharose beads.

References

[1] Trono, D. (1995) Cell 82, 189–192.
[2] Hammes, S.R., Dixon, E.P., Malim, M.H., Cullen, B.R. and Greene, W.C. (1989) Proc. Natl. Acad. Sci. USA 86, 9549–9553.
[3] Kim, S.Y., Byrn, R., Groopman, J. and Baltimore, D. (1989) J. Virol. 63, 3708–3713.
[4] de Ronde, A., Klaver, B., Keulen, W., Smit, L. and Goudsmit, J. (1992) Virology 188, 391–395.
[5] Zazopoulos, E. and Haseltine, W.A. (1993) Virology 194, 20–27.
[6] Miller, M.D., Warmerdam, M.T., Gaston, I., Greene, W.C. and Feinbergh, M.B. (1994) J. Exp. Med. 179, 101–113.
[7] Spina, C.A., Jesse Kwoh, T., Chowers, M.Y., Guatelli, J.C. and Richman, D.D. (1994) J. Exp. Med. 179, 115–123.
[8] Chowers, M.Y., Spina, C.A., Jesse Kwoh, T., Fitch, N.J.S., Richman, D.D. and Guatelli, J.C. (1994) J. Virol. 68, 2906–2914.
[9] Kestler III, H.W., Ringler, D.J., Mori, K., Panicali, D.L., Sehgal, P.K., Daniel, M.D. and Desrosiers, R.C. (1991) Cell 65, 651–662.
[10] Kirchhoff, F., Greenough, T.C., Brettler, D.B., Sullivan, J.L. and Desrosiers, R.C. (1995) New Engl. J. Med. 332, 228–232.
[11] Deacon, N.J., Tsykin, A., Soloman, A., Smith, K., Ludford-Menting, M., Hooker, D.J., McPhee, D.A., Greenway, A.L., Ellet, A., Chatfield, C., Lawson, V.A., Crowe, S., Maerz, A., Sonza, S., Learmont, J., Sullivan, J.S., Cunningham, A., Dwyer, S., Dowton, D. and Mills, J. (1995) Science 270, 988–991.
[12] Whitlow, M. and Filpula, D. (1991) Methods Enzymol. 2, 97–105.
[13] Winter, G. and Milstein, C. (1991) Nature 349, 293–299.
[14] Carlson, J.R. (1988) Mol. Cell. Biol. 8, 2538–2546.
[15] Biocca, S., Nurberger, M.S. and Cattaneo, A. (1990) EMBO J. 9, 101–108.
[16] Marasco, W.A., Haseltine, W.A. and Chen, S.Y. (1993) Proc. Natl. Acad. Sci. USA 90, 7889–7893.
[17] Harris, M., Hislop, S., Patsilinacos, P. and Neil, J.C. (1992) AIDS Res. Hum. Retroviruses 8, 537–543.
[18] De Santis, R., Anastasi, A.M., Marcolini, S., Valesini, G., Pezzella, M., Vonesch, N., Sturchio, E. and Mele, A. (1991) AIDS Res. Hum. Retroviruses 7, 315–321.
[19] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[20] Higgins, D.G., Bleasby, A.J. and Fuchs, R. (1992) Comput. Appl. Biosci. 8, 189–191.
[21] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.
[22] Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8148.
[23] Goldman, L.A., Cutrone, E.C., Kotenko, S.V., Krause, C.D. and Langer, J.A. (1996) BioTechniques 21, 1013–1015.
[24] Duan, L. and Pomerantz, R.J. (1994) Nucleic Acids Res. 22, 5433–5438.
[25] Carroll, W.L., Mendel, E. and Levy, S. (1988) Mol. Immunol. 25, 991–995.
[26] Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S. and Foeller, C. (1991) Sequences of Proteins of Immunological Interest, United States Department of Health and Human Services, Washington, DC.
[27] Johnson, K.S. and Chiswell, D.J. (1993) Curr. Opin. Struct. Biol. 3, 564–571.
[28] Marasco, W.A. (1995) Immunotechnology 1, 1–19.
[29] Cubitt, A.B., Roger, H., Adams, S.R., Boyd, A.E., Gross, L.A. and Tsien, R.Y. (1995) Trends Biochem. Sci. 20, 448–455.