Preferential recognition of the very low-density lipoprotein receptor ligand binding site by antibodies from phage display libraries

Doris M. Pfistermueller, Dieter Blaas*, Regina A. Hodits[1]

Institute of Biochemistry, Medical Faculty, University of Vienna, Dr. Bohr Gasse 9/3, A-1030 Vienna, Austria

Received 8 July 1996; revised version received 12 August 1996

Abstract Screening of a phage library displaying single chain fragments of the variable regions of human immunoglobulins (scFv) for binding to the ovarian chicken very low-density lipoprotein/vitellogenin receptor (OVR) led to the isolation of several antibody fragments with high affinity. As for the natural ligands of OVR, receptor binding of all antibody fragments is strictly Ca^{2+}-dependent and is prevented by receptor-associated protein (RAP). Moreover, attachment of human rhinovirus serotype 2 (HRV2) to this receptor is inhibited by all scFvs. In contrast to conventional immunization, the in vitro selection method thus exclusively led to antibodies that attach to or close to the ligand binding site and thereby block the receptor-ligand interaction.

Key words: Single chain antibody; Phage display; Very low-density lipoprotein receptor; Rhinovirus

1. Introduction

Polypeptides of the low-density lipoprotein receptor (LDLR) family are remarkably related; their common structural motives are clusters of variable numbers of cysteine rich 'binding repeats' of about 40 amino acids in length. Whereas LDLR contains 7 such repeats, the very low-density lipoprotein receptor (VLDLR) contains 8 repeats, and the LDLR-related protein (LRP) carries clusters of 2, 8, 10, and 11 repeats. Megalin or gp330 is the largest member of this family and contains 36 repeats in total. These proteins interact with a large number of unrelated ligands such as lipoproteins and proteinase-inhibitor complexes among several others (for recent reviews see [1,2]). Homologues have been found throughout all species so far examined. In the laying hen, two sets of VLDLR and LRP-related proteins were identified which are specifically expressed in the developing oocyte and in somatic cells, respectively. The ovarian VLDLR (also termed OVR) is of particular interest as it can be rather easily prepared and is available in comparatively large quantities [3–7]. A 39 kDa intracellular protein (receptor-associated protein or RAP) binds to most of the receptors of this protein family, although with different affinities, and thereby blocks the interaction with the ligands [8–17]. Recently, we have shown that a minor receptor group of human rhinoviruses (HRVs), the main causative agents of the common cold [18], bind to several members of the LDLR family including OVR [19,20]; however, the viruses are not replicated in cells other than human or primate [21].

Although negative charges on the surface of the receptor proteins are clearly involved in ligand recognition, the principles underlying ligand discrimination and the exact site of attachment on the receptors have not been fully revealed. Moreover, knowledge of structures involved in virus-receptor interaction is highly desirable in view of blocking viral attachment to the host cell. For example, particular antibodies against intercellular adhesion molecule 1 (ICAM-1), the receptor of the major group of human rhinoviruses, appear promising in antiviral therapy [22,23]. Similar studies with minor group viruses have mostly been hampered by the difficulty in raising antibodies blocking viral attachment by conventional immunization; only in some instances antibodies have been shown to interfere with ligand binding. However, the inhibition is usually weak since in general the antibodies bind to a site remote from the ligand binding site. For example, the monoclonal antibody IgG-C7 [24] was shown to be directed against the N-terminal repeat of LDLR which is not involved in ligand interaction [25]. We therefore investigated whether the screening of a library of phage-displayed antibody fragments would lead to immunological reagents directed towards the ligand binding site and thus prevent ligand attachment to the receptor.

The display of repertoires of antibody fragments on the surface of filamentous bacteriophage, and the selection of antigen-binding phage [26,27] has provided means of obtaining antibodies without immunisation [28]. Thereby antibody repertoires are either derived from the rearranged V-genes of naive lymphocytes [29,30] or from V-gene segments rearranged in vitro [31–34]. We have used a large phage antibody repertoire derived from naive human lymphocytes to screen for antibody fragments against OVR, the oocyte-specific chicken very low-density lipoprotein receptor (VLDLR) [35].

In this communication we show that all single chain Fv antibodies selected at random from those isolated upon selection for attachment to OVR bind to (or close to) the ligand binding site. As for all known ligands, the scFv receptor recognition is strictly Ca^{2+}-dependent and is prevented by the receptor-associated protein (RAP). This indicates that these scFvs bind to a structural epitope which is also used by the various ligands. In contrast to a rabbit hyperimmune serum, they compete for binding of the minor group human rhinovirus HRV2 to OVR. These results indicate that the ligand binding site is a structure particularly prone to immune recognition; however, in vivo the high evolutionary conserva-

*Corresponding author. Fax: (43) (1) 79515-3114.
E-mail: Dieter.Blaas@univie.ac.at

[1] *Present address:* Centre for Protein Engineering, MRC Centre, Hills Road, Cambridge CB2 2QH, UK.

Abbreviations: HRV, human rhinovirus; OVR, ovarian very low-density lipoprotein receptor; LDLR, low-density lipoprotein receptor; VLDLR, very low-density lipoprotein receptor; LRP, low-density lipoprotein receptor-related protein; scFv, single chain antibody; RAP, receptor-associated protein

PII S0014-5793(96)00964-7

```
HEAVY CHAIN REGIONS
                                                     CDR1                           CDR2
         1       10        20        30                   40        50                             70
scFv7   QVQLLQSAAEVKKPGASVKVSCKASGYSFTS | .YYMH | WVRQAPGQGLEWMG | IIKPSTGSASYAQKFQG | RVT
scFv6
scFv8       V   GGG VQ   R LRL   A    FT S. | S G   |      K      VS | G SGTS   TY   DSVK  |   F
scFv19      V   G                    T S. | S AI  |                | W NAGN NTK  S        |
scFv21      V   G                    T S. | S AI  |                | W NAGN NTK  S        |
scFv27      V   G                    T S. | S AI  |                | W NAGN NTK  S        |

                                               CDR3
              80         90        100                       120
scFv7   MTRDTSTSTVYMDLSSLRSEDTAVYYCAR | SPIGGGGSDY | WGKGPWSPSP
scFv6                                                       TLVTVS
scFv8   IS   N KN  L  LQMN    A        | VRVYSSAW   |      TLVTVS
scFv19  I      A   A   E          E    | N L  ATGAG | GTTT  TWT GA
scFv21  I      A   .   E          D    | N P RATGAG | GTTT  TWT GA
scFv27  I      A   A   E          D    | N P RATGAG | GTTT  TWT GA

LIGHT CHAIN REGIONS
                                       CDR1                           CDR2
        1        10        20                      40                             60
scFv   DIQMTQSPSTLSASIGDRITITC | RASEGIYHWLA | WYQQKPGKAPKLLIY | KASSLASGA | PSRFSGSGSG

                                    CDR3
        70        80                        100
scFv   TDFTLTISSLQPDDFATYYC | QQYSNYP | LTFGGGTKLEIKR
```

Fig. 1. Amino acid sequences of the selected scFvs as deduced from the cDNA sequence. DNA fragments encoding the heavy and light chains of the 6 scFvs were amplified from bacterial colonies by polymerase chain reaction. Sequences were determined on an automatic DNA sequencer using fluorescent dideoxy-NTPs. CDR regions are shown in bold and are boxed. Only amino acids different from scFv7 are shown for the other scFvs. Since all light chains were identical, the five scFvs were divided into three groups based on sequence similarity of their heavy chains. The sequence of scFv7 is from [35].

tion of this site and/or the presence of various ligands in the blood prevent an efficient immune response to this epitope.

2. Materials and methods

2.1. Materials

Crude membrane extracts and purified OVR were prepared essentially as described in [35]. Purified human placental LRP was generously provided by Dr. J. Gliemann, University of Aarhus, Denmark. HRV2 was propagated and metabolically labelled with [^{35}S]methionine in HeLa cells as described [36]. Rabbit hyperimmune IgGs, obtained from antiserum raised by injection of a rabbit with purified OVR [37], were kindly provided by J. Nimpf, University of Vienna, Austria. GST-RAP was expressed and purified as described [17].

2.2. Selection of antigen binding phage by library panning

The phage antibody repertoire described previously and kindly provided by Cambridge Antibody Technology (CAT), Cambridge, UK, was used for OVR-specific phage selection [35,38]. Briefly, repertoires of human heavy and light chain V-genes amplified from human lymphocytes from tonsils were assembled to encode single chain Fv fragments. The assembled V-genes were cloned into the ampicillin-resistant phagemid pCANTAB6 (CAT, Cambridge, UK) to append a C-terminal hexahistidine (His) tag to allow for purification, and a *myc* tag to facilitate detection of the fragments. In the first round of selection 10^{13} phage were panned for 2 h at room temperature using immunotubes (Maxisorb, Nunc) coated with 10 μg/ml purified OVR in 50 mM $NaHCO_3$ (pH 8.6). Four additional rounds were carried out in immunotubes coated with 5 μg/ml antigen as described [35].

2.3. Screening and sequencing of clones

Phage were isolated from single ampicillin-resistant colonies of infected (suppressor) *E. coli* TG-1 using helper phage *VCS-M13* (Stratagene), and the phage used to infect the (non-suppressor) *E. coli* HB2151. Single ampicillin-resistant colonies were used to inoculate 200 μl of culture broth in microtiter plates, and the expression of soluble scFv fragments induced by addition of 1 mM IPTG to the cultures [35]. Bacteria were pelleted, and the supernatants containing scFv fragments were screened for binding to the antigen by ELISA using the anti-*myc* tag antibody 9E10 (Stratagene) and anti-mouse IgG conjugated with AP. Binding specificity was tested by comparing the signals obtained from plates coated with 100 μl of either 10 μg/ml OVR or of 100 μg/ml BSA. Soluble fragments resulting in an ELISA signal of at least 7 times higher for OVR than BSA were scored positive. Using specific primers [34] the encoded variable heavy and light chain regions were amplified via PCR from single colonies. The amplified DNA was subjected to automatic sequencing by the dideoxy method using the TAQ DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems Inc.) and the PCRHLINK primer [38] for the heavy chain region and FDSEQ1 [32] for the variable light chains.

2.4. Preparation of monoclonal reagents

For further characterization of the scFv fragments, the infected HB2151 bacteria were grown in 1 litre of 2×TY medium supplemented with 0.1% glucose and 100 μg/ml ampicillin and were induced overnight with 1 mM IPTG at 28°C. Bacteria were removed using a tangential filtration unit with 45 μm filters (Filtron) and the supernatant was precipitated with 50% $(NH_4)_2SO_4$ overnight. The precipitate was collected by centrifugation and redissolved in 1/10 of the initial volume of PBS. Samples were affinity purified by immobilized metal chelate affinity chromatography (IMAC) using Ni-NTA (Diagen) as described [32]. The fractions eluted with 100 mM imidazole were analyzed by SDS-PAGE on 12.5% polyacrylamide gels under reducing conditions and were estimated to be greater than 90% pure. The concentration was determined spectrophotometrically assuming A_{280} of 1.0=0.7 mg/ml [32]. Labelling of scFv7 with ^{125}I was carried out using the chloramine T method with 0.5 mCi ^{125}I (Amersham) per 70 μg of protein yielding a specific activity of 1.5×10^7 cpm/μg. The determination of binding kinetics by surface plasmon resonance (SPR) was carried out essentially as described [35].

2.5. Competition with GST-RAP

1 μg/ml OVR (100 μl/well) was coated onto ELISA plates in 50 mM $NaHCO_3$, pH 8.6 at 4°C. Plates were blocked with 2% milk powder in PBS for 1 h, washed with PBS and incubated for 1 h at room temperature with the different scFvs in PBS together with the amount of GST-RAP [17] indicated in the figures. Plates were washed 3 times with PBS, 0.2% Tween 20 and 3 times with PBS. ScFvs bound were detected using 9E10 at a dilution of 1:1000 followed by AP-conjugated goat anti-mouse IgG; *p*-nitrophenyl-phosphate at 1 mg/ml in 10 mM ethanolamine, 0.5 mM $MgCl_2$ (pH 9.5) was used as a substrate. Color development was measured at 405 nm using a BioRad ELISA reader after 1 h incubation at room temperature.

2.6. Ca^{2+} dependence of binding

Approximately 1 μg OVR/5 mm gel width was run on a 12.5% SDS-polyacrylamide gel, transferred onto an Immobilon membrane and blocked in PBS containing 2% Tween 20. Binding of rabbit anti-OVR IgG [37] at 85 μg/ml and of scFvs at 1 μg/ml was assayed in the presence of either 2 mM $CaCl_2$ or 20 mM EDTA in PBS on individual strips cut from the membrane. Rabbit antibodies were detected with AP-conjugated anti-rabbit IgG (Promega), scFvs with 9E10 followed by AP-conjugated goat anti-mouse IgG employing BCIP and NBT as substrates.

2.7. Competition assays

Competition assays were carried out using ELISA plates coated with 1 μg/ml OVR or human LRP at 100 μl/well, in 50 mM $NaHCO_3$, pH 8.6 at 4°C overnight. Plates were blocked with 2% milk powder in PBS for 1 h, washed with PBS and incubated with 13 000 cpm/well (about 1 ng) [^{125}I]scFv7 together with the various unlabelled scFvs in PBS for 1 h at room temperature at the concentrations indicated in the figures. The plates were washed 3 times with PBS, 0.2% Tween 20 and 3 times with PBS. Wells were cut, and bound radioactivity was determined using an auto-gamma-counter Cobra II (Packard). In order to test the competition of scFvs for [^{32}S]HRV2 binding to OVR, the antibodies were present at 200 μg/ml, and the virus at 30 000 cpm/well. The assay was essentially done as described above.

3. Results

3.1. Binding characteristics of the selected scFvs

After selection of the repertoire of phage-displayed scFv fragments for binding to OVR, 36 individual clones were chosen at random for further analysis. From these, 27 scored positive in an ELISA-based assay [35]. In order to determine the redundancy of these clones, the regions encoding the heavy and light chain CDRs were amplified by PCR and the cDNA sequences were determined; from these 27 clones, 6 (including the previously described scFv7 [35]) were found to have different sequences in their heavy chains, whereas all had the same light chain sequence. Based on comparisons with the repertoire of human variable genes these scFvs were classified as belonging to three antibody germline families: DP-7 (VH1) for scFv6 and scFv7 [35], DP-49 (VH3) for scFv8, and DP-25 (VH1) for scFvs 19, 21, and 27 [39] (Fig. 1). Note that those clones with identical CDR sequences are slightly different in their framework regions.

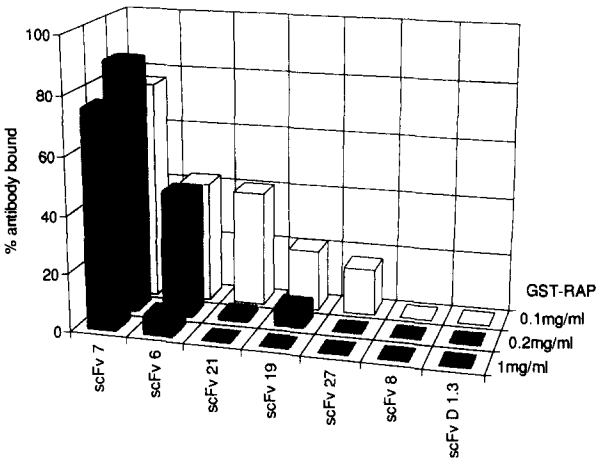

Fig. 2. Competition of GST-RAP for binding of scFvs to OVR. 1 μg/ml (100 μl/well) OVR was coated onto microtiter plates. After blocking, the wells were incubated with the various scFvs at 200 μg/ml in the presence of GST-RAP at the concentrations indicated. After washing, bound antibody fragments were detected with 9E10 and AP-conjugated antiserum and color intensity was measured with a microplate reader. Maximum binding (100%) was determined for each scFv in the absence of competitor and was between 0.35 A_{405} (scFv7) and 0.15 A_{405} (scFv8). Background was 0.1 A_{405} using the irrelevant scFv D1.3; it was subtracted from all values. The mean of two independent experiments is shown. Differences between the duplicates were less than 10%.

Soluble fragments of each clone were then prepared and affinity purified on Ni-NTA columns using the His tag sequence located at the C-terminus of the protein. These fragments were tested for affinity toward OVR and LRP in Western blots. Receptors in crude membrane extracts from hen ovaries were separated on 12.5% SDS-PAGE and transferred onto Immobilon membranes. Incubation with the respective scFvs followed by immunodetection with AP-conjugated antisera resulted in a single band corresponding to OVR; in a similar experiment purified LRP was run on a 7.5% polyacrylamide gel and transferred to Immobilon. Western blotting revealed that only scFv6 and scFv7 cross-reacted with human LRP whereas the remaining 4 antibody fragments did not bind to LRP (data not shown). The irrelevant scFv D1.3 (which recognizes hen egg lysozyme [26]) was used as a control and showed no reaction in either blot.

3.2. Competition with RAP

RAP, a 39 kDa intracellular protein that copurifies with LRP, is known to compete for binding of all ligands to LRP and VLDLR [13,17,19,40,41]. To determine whether RAP would also interfere with attachment of the scFvs to OVR, the receptor was coated onto ELISA plates and binding of the antibody fragments was assayed in the presence of various concentrations of recombinant GST-RAP. The presence of GST-RAP in the incubation resulted in a concentration dependent decrease of binding of the scFvs to OVR. However, as seen in Fig. 2, the concentration of GST-RAP required to inhibit binding of the individual scFvs to OVR

was different: whereas the binding of scFv8 was completely abolished at 100 μg/ml GST-RAP, the presence of GST-RAP at 1 mg/ml reduced binding of scFv7 to about only 75%. Inhibition of the other scFvs by GST-RAP was found to be intermediate. Closer inspection of the data revealed that those scFvs grouped together by sequence similarity were also similar in their binding properties. ScFv6 and scFv7 were both only inhibited at high concentrations of RAP, while scFv19, 21, and 27, belonging to another group, required moderate amounts of GST-RAP for competition. Finally, scFv8 was most easily replaced by GST-RAP.

3. Ca^{2+} dependence of binding

Members of the LDL receptor family require Ca^{2+} ions for the stabilization of the ligand binding site [42–44]. Therefore, the influence of Ca^{2+} ions on the binding of scFvs to OVR was tested on a Western blot obtained from an oocyte membrane extract. As has been shown for scFv7 [35], binding of all other scFvs to OVR was also strictly dependent on the presence of Ca^{2+} ions and was abolished by addition of excess EDTA. This is in sharp contrast to the IgG fractions of the rabbit hyperimmune serum raised against OVR which bound to the receptor regardless of the absence or presence of EDTA during incubation (Fig. 3).

4. Competition with HRV2 for binding to OVR and to LRP

Competition of RAP for binding of all scFvs to OVR strongly suggests that these ligands recognise the same, or at least an overlapping epitope on the receptor surface. The scFvs were thus assayed for their ability to compete for binding of another ligand of the LDL receptor family, human rhinovirus serotype 2 (HRV2). This picornavirus belongs to the minor rhinovirus receptor group and thus binds to the LDL receptor and to LRP [19]. Although HRVs are not replicated in chicken cells, they also bind to OVR [20]. These viruses can thus be considered universal ligands of the LDLR family since other ligands are less promiscuous as they bind with rather different affinities to different receptors [5,45,46]. As shown in Fig. 4A, the scFvs decreased binding of ^{35}S-labeled HRV2 to OVR immobilized in microtiter plates to

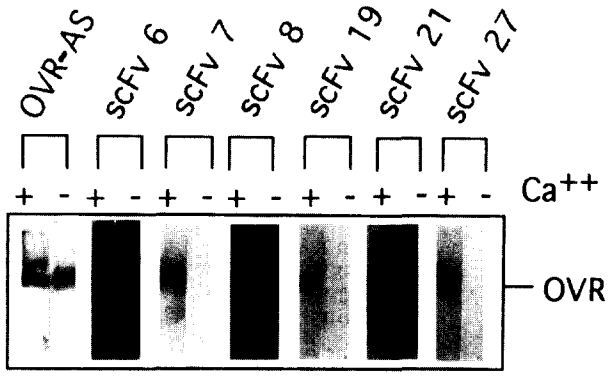

Fig. 3. Binding of all scFvs to OVR is Ca^{2+}-dependent. Purified OVR was run on a 12.5% SDS-polyacrylamide gel and electrophoretically transferred onto an Immobilon membrane. Individual strips from the membrane were incubated either with rabbit anti-OVR IgG (OVR-AS) or with the scFvs as indicated in the presence of 2 mM $CaCl_2$ or of 20 mM EDTA. The blot was developed with 9E10 and AP-conjugated goat anti-mouse IgG, or AP-conjugated anti-rabbit IgG, for scFvs and the rabbit hyperimmune serum, respectively, using BCIP and NBT as substrates.

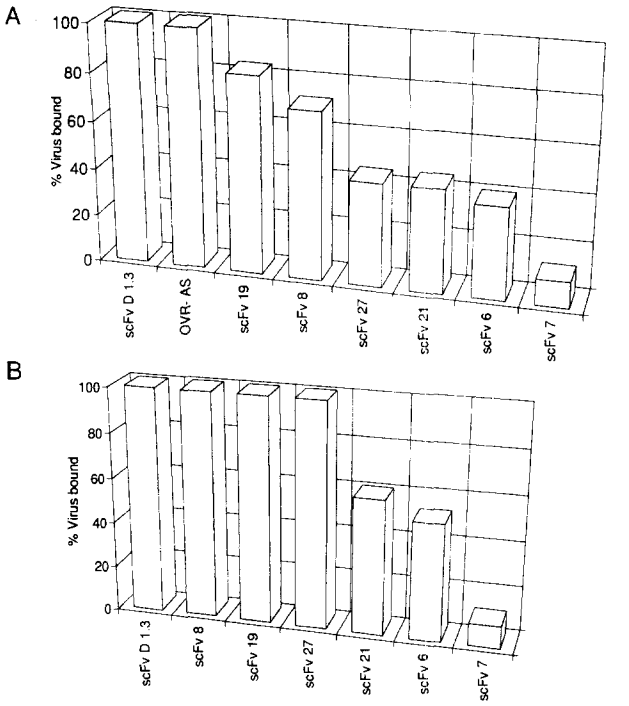

Fig. 4. Competition of HRV binding to OVR (A) and LRP (B) by scFvs. OVR and LRP were immobilized to microtiter wells as in Fig. 2 and incubated with 30000 cpm/well [^{35}S]HRV2 in the presence of 200 μg/ml of the scFvs or of 5 mg/ml of the IgG fraction of the hyperimmune serum against OVR (OVR-AS). After washing, the wells were cut and bound radioactivity was determined by liquid scintillation counting. Maximal binding (100%) was between 1000 and 2000 cpm to OVR and between 700 and 1000 cpm to LRP. Background binding to wells coated with BSA was 30 cpm. No reduction in binding was seen with the control scFv D1.3. The mean of at least two independent experiments is shown.

various degrees. At 200 μg/ml scFv6, 7, 21, and 27 reduced HRV2 binding to between 40% and 10%, while the other three scFvs decreased HRV2 binding to between 70% and 85%.

As expected from earlier experiments which had demonstrated binding of scFv7 to human LRP and inhibition of virus infection of human fibroblasts deficient in LDLR but expressing LRP [35], scFv7, scFv6, and scFv21 also decreased HRV2 binding to immobilized LRP to 10, 50, and 60%, respectively when added at 200 μg/ml. As anticipated from the lack of cross-reactivity with LRP in Western blots (see above), scFv 8, 19, 21, and 27 had no detectable influence on HRV2 binding to this receptor (Fig. 4B). Note, however, that binding of these scFvs to LRP could be demonstrated in surface plasmon resonance experiments (see below). The control scFv D1.3 used at the same concentration did not interfere with HRV2 binding.

In order to examine whether the differences in specificity for OVR or LRP in ligand competition were only a matter of different affinities of the individual scFvs for the respective receptor proteins, the affinity constants were determined by plasmon surface resonance technology. Purified OVR and LRP were immobilized on CM5 sensor chips as described previously [35]. Purified scFvs were applied at three different concentrations with each measurement being carried out three times. Affinity constants were calculated from the on- and off-rates and are summarized in Table 1A for OVR and in Table 1B for LRP. ScFv6 showed a dissociation constant in the nanomolar range for OVR and LRP and is thus very similar

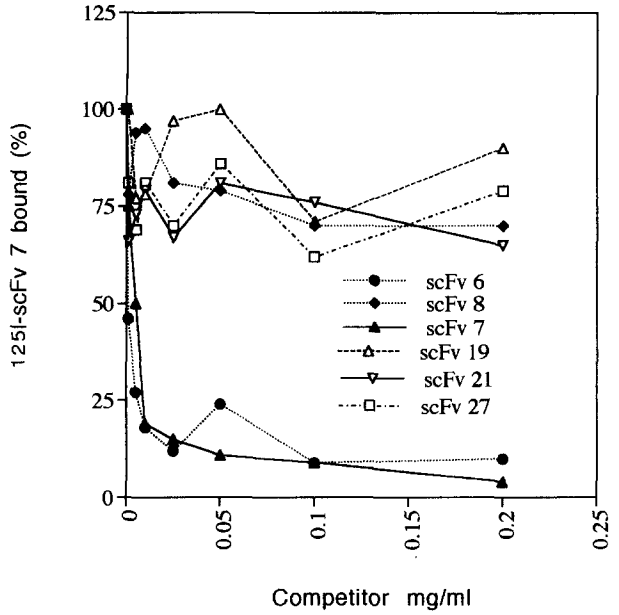

Fig. 5. Cross-competition of scFv binding to OVR. OVR was immobilized to microtiter wells as in Fig. 2 and incubated with 13000 cpm/well (1 ng/100 μl) of ^{125}I-labelled scFv7 in the presence of increasing concentrations of unlabelled scFvs. Bound radioactivity was determined from wells cut out with a scalpel in a Packard Cobra II γ-counter.

to scFv7 [35]; scFv19, 21 and 27 showed slightly lower affinity to both receptors. As expected from the binding data on Western blots and from competition with GST-RAP, the affinity of scFv8 for OVR as well as for LRP was found to be about three times less than that of scFv6 and scFv7.

3.5. *Cross-competition of scFv*

Since all scFvs had been found to compete for HRV2 binding to OVR, although to various degrees, we wanted to determine whether this competition was the result of binding to the same epitope or to epitopes in the close vicinity. As scFv7 competes most strongly with HRV2, this antibody fragment was labelled with ^{125}I and its binding to OVR was monitored in the presence of the other scFvs at various concentrations (Fig. 5). Unlabelled scFv7 and scFv6 reduced binding of ^{125}I-labelled scFv7 in a concentration-dependent manner with reduction to background values at 50 μg/ml. ScFv8, 19, 21, and 27 showed no significant inhibition of the interaction between scFv7 and OVR, even at 200 μg/ml (Fig. 5).

4. Discussion

Antibodies that block the interaction of multiple ligands to the LDL receptor family should help to narrow down sites involved in ligand binding and to understand the principles underlying differences in affinity of various ligands for each particular receptor. Moreover, as has been demonstrated for major receptor group rhinoviruses, antibodies against the receptor can be used to block infection [22]. In this case the only portal of entry is the intercellular adhesion molecule 1 (ICAM-1) whereas minor group viruses bind to several although closely related receptors. Therefore, antibodies with wide cross-reactivity towards members of the LDLR family could possibly be used to block the attachment of minor group human rhinoviruses to their natural host cells and thus inhibit infection.

Although antisera and monoclonal antibodies have been obtained against several members of the LDLR family, none have unequivocally been demonstrated to bind to the ligand binding site, although inhibition of ligand uptake through down-regulation of the receptors was frequently observed. For instance, the monoclonal antibody IgG-C7 interferes with the interaction of LDL and apo-E-rich lipoproteins, with the LDL receptor, but only to a moderate extent [24]. Since this antibody binds to the amino-terminal cysteine-rich repeat – which is not required for ligand binding [25] – it is not particularly surprising that it inhibits viral attachment only moderately [19]. The difficulty in obtaining antibodies with the desired properties by conventional means may be due to the conserved nature of the binding epitopes on the receptors between different species, or the presence of ligands in the serum of the animal to be immunised rendering the binding site inaccessible. We therefore employed the technique of antibody display phage libraries for the production of scFvs with affinity for the chicken OVR. This receptor was chosen because of the ease of purification and the comparatively large amount being expressed in the growing oocyte. HRVs strongly bind to this protein although virus is not replicated in chicken cells [20]. A repertoire consisting of 10^9 different clones was screened for binding to OVR. We succeeded in the isolation of six different scFvs which were all found to bind strongly to OVR. All of these antibody fragments competed for HRV binding to this receptor, but to different extents. As expected from results with antisera or monoclonal antibodies raised against LDLR or LRP [24,47,48], a hyperimmune serum obtained by immunization with OVR strongly bound to its antigen as seen by Western blotting but failed to show any influence on HRV2 binding to OVR (Fig. 4).

Cross-competition experiments showed that at least scFv6

Table 1
Affinity constants for scFv binding to OVR (A) and LRP (B)

	K_{on} $(M^{-1}\ s^{-1})\times10^4$	K_{off} $(s^{-1})\times10^{-3}$	K_{aff} $(M^{-1})\times10^7$	Conc $(M)\times10^{-6}$
A				
scFv6	10	0.5	20	0.45–1
scFv7	8	0.3	26.6	0.1 –2
scFv8	6	1.2	5	0.33–0.5
scFv19	15	1.1	13.6	0.09–0.13
scFv21	7	0.5	14	0.13–0.4
scFv27	15	1	15	0.09–0.27
B				
scFv6	2.5	0.27	9	0.5 –1.5
scFv7	3	0.3	10	0.4 –2
scFv8	9	2.5	3.6	0.3 –1
scFv19	5	0.75	6.7	0.2 –0.4
scFv21	4	0.6	6.7	0.1 –0.5
scFv27	8	1	8	0.2 –0.4

35 μl of pure scFv preparations at the concentrations indicated were passed over a sensorchip surface to which 1200 resonance units (RU) of OVR (A) and of LRP (B) had been coupled. On/off rates, affinity constants and the range of concentrations used for the determinations are shown. Constants are the mean values of determinations at three different concentrations. All measurements were carried out in triplicate. Values for scFv7 were taken from [35]. The irrelevant scFv D1.3 passed over both surfaces showed neither binding to OVR nor to LRP.

and scFv7 bind to the same epitope on the receptor surface. However, the remaining scFvs failed to compete for the site recognized by scFv6 and scFv7 (Fig. 5). Since all of the scFvs inhibit binding of HRV2 to OVR they all must bind to (or close to) the viral attachment site. The viral binding site is highly conserved within the LDLR family since minor group HRVs bind to LDLR, to LRP, to OVR, and to megalin (unpublished). Upon Western blotting only scFv6 and 7 recognized LRP whereas scFv8, 19, 21, and 27 did not, again indicating that the former antibodies attach to the most conserved structure within these receptors. The site in LRP corresponding to that site in OVR which is recognized by scFv19, 21, and 27 might be in a somewhat altered conformation after SDS gel electrophoresis since determination of the affinity constants by surface plasmon resonance revealed that these antibodies had only a slightly lower affinity than scFv6 and 7; scFv8 binds with about four-fold lower affinity. These data, together with the differences in ligand competition and the data from the cross-competition, suggest that scFv19, 21, and 27 bind to an epitope different from that recognized by scFv6 and 7. Nevertheless both epitopes must be close to (or overlapping with) each other since all antibodies are competed by RAP and all antibodies prevent HRV2 binding to OVR.

Recently, Horn and coworkers [49] have selected an antibody fragment blocking the binding of pro-urokinase and urokinase-plasminogen-activator-inhibitor-1 complexes to LRP from a phage-displayed Fab library. However, the phage library was made from mRNA recovered from the lymphocytes from a mouse which had been immunized with the antigen. Moreover, selection was biased towards antibodies binding to a structural epitope by eluting phage during the panning procedure by EDTA. In our case a library obtained from naive human lymphocytes was used and no selection for antibodies binding to a structural epitope was made. Nevertheless, binding of all scFvs isolated was Ca^{2+}-dependent (Fig.), they were displaced from the receptor by RAP (Fig. 2), and they competed with HRV2 for binding to OVR (Fig. 4). In the first two respects, the antibody fragments behave very similarly to all known ligands of the LDL receptor family. They clearly recognize sites which are not immunodominant in vivo indicating that they might be hidden for the immune system or that an immune reaction against these sites is specifically suppressed. In addition to the previous finding that phage display technology yields antibodies which are not obtained by conventional means our results indicate that strongly exposed sites in evolutionarily highly conserved proteins might not be seen by the immune system but are perfectly targeted by antibodies which are not subject to the selection mechanisms operating in vivo. It will be of interest to investigate the basis for the strong (in vitro) immunogenic behavior of these particular sites in OVR.

Acknowledgements: This work was supported by Austrian Science Foundation Grant P-10384-MOB. We want to thank T. Hiesberger for labeling scFv7 with ^{125}I, J. Nimpf for the kind gift of the hyperimmune serum against OVR, J. Gliemann for LRP, Z. Rattler for discussions, and A. Weinberger and S. Krenn for hen ovaries.

References

[1] Strickland, D.K., Kounnas, M.Z. and Argraves, W.S. (1995) FASEB J. 9, 890–898.
[2] Schneider, W.J. and Nimpf, J. (1993) Curr. Opin. Lipidol. 4, 205–209.
[3] Stifani, S., George, R. and Schneider, W.J. (1988) Biochem. J. 250, 467–475.
[4] Stifani, S., Barber, D.L., Aebersold, R., Steyrer, E., Shen, X., Nimpf, J. and Schneider, W.J. (1991) J. Biol. Chem. 266, 19079–19087.
[5] Stifani, S., Barber, D.L., Nimpf, J. and Schneider, W.J. (1990) Proc. Natl. Acad. Sci. USA 87, 1955–1959.
[6] Nimpf, J., Stifani, S., Bilous, P.T. and Schneider, W.J. (1994) J. Biol. Chem. 269, 212–219.
[7] Bujo, H., Hermann, M., Kaderli, M.O., Jacobsen, L., Sugawara, S., Nimpf, J., Yamamoto, T. and Schneider, W.J. (1994) EMBO J. 13, 5165–5175.
[8] Medh, J.D., Fry, G.L., Bowen, S.L., Pladet, M.W., Strickland, D.K. and Chappell, D.A. (1995) J. Biol. Chem. 270, 536–540.
[9] Warshawsky, I., Bu, G. and Schwartz, A.L. (1994) J. Biol. Chem. 269, 3325–3330.
[10] Simonsen, A.C.W., Heegaard, C.W., Rasmussen, L.K., Ellgaard, L., Kjoller, L., Christensen, A., Etzerodt, M. and Andreasen, P.A. (1994) FEBS Lett. 354, 279–283.
[11] Battey, F.D., Gafvels, M.E., Fitzgerald, D.J., Argraves, W.S., Chappell, D.A., Strauss, J.F. and Strickland, D.K. (1994) J. Biol. Chem. 269, 23268–23273.
[12] Willnow, T.E., Sheng, Z.Q., Ishibashi, S. and Herz, J. (1994) Science 264, 1471–1474.
[13] Kounnas, M.Z., Argraves, W.S. and Strickland, D.K. (1992) J. Biol. Chem. 267, 21162–21166.
[14] Warshawsky, I., Bu, G.J. and Schwartz, A.L. (1995) Biochemistry 34, 3404–3415.
[15] Bu, G.J., Geuze, H.J., Strous, G.J. and Schwartz, A.L. (1995) EMBO J. 14, 2269–2280.
[16] Orlando, R.A., Kerjaschki, D., Kurihara, H., Biemesderfer, D. and Farquhar, M.G. (1992) Proc. Natl. Acad. Sci. USA 89, 6698–6702.
[17] Herz, J., Goldstein, J.L., Strickland, D.K., Ho, Y.K. and Brown, M.S. (1991) J. Biol. Chem. 266, 21232–21238.
[18] Stott, E.J. and Killington, R.A. (1972) Annu. Rev. Microbiol. 26, 503–524.
[19] Hofer, F., Gruenberger, M., Kowalski, H., Machat, H., Huettinger, M., Kuechler, E. and Blaas, D. (1994) Proc. Natl. Acad. Sci. USA 91, 1839–1842.
[20] Gruenberger, M., Wandl, R., Nimpf, J., Hiesberger, T., Schneider, W.J., Kuechler, E. and Blaas, D. (1995) J. Virol. 69, 7244–7247.
[21] Yin, F.H. and Lomax, N.B. (1986) J. Gen. Virol. 67, 2335–2340.
[22] Hayden, F.G., Gwaltney, J., Jr. and Colonno, R.J. (1988) Antiviral Res. 9, 233–247.
[23] Sperber, S.J. and Hayden, F.G. (1989) Antiviral Res. 12, 231–238.
[24] Beisiegel, U., Schneider, W.J., Goldstein, J.L., Anderson, R.G. and Brown, M.S. (1981) J. Biol. Chem. 256, 11923–11931.
[25] Esser, V., Limbird, L.E., Brown, M.S., Goldstein, J.L. and Russell, D.W. (1988) J. Biol. Chem. 263, 13282–13290.
[26] McCafferty, J., Griffiths, A.D., Winter, G. and Chiswell, D.J. (1990) Nature 348, 552–554.
[27] Kang, A.S., Barbas, C.F., Janda, K.D., Benkovic, S.J. and Lerner, R.A. (1991) Proc. Natl. Acad. Sci. USA 88, 4363–4366.
[28] Marks, J.D., Griffiths, A.D., Malmqvist, M., Clackson, T.P., Bye, J.M. and Winter, G. (1992) Bio-Technology 10, 779–783.
[29] Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D. and Winter, G. (1991) J. Mol. Biol. 222, 581–597.
[30] Griffiths, A.D. (1993) Curr. Opinion Immunol. 5, 263–267.
[31] Barbas, C.F., Bain, J.D., Hoekstra, D.M. and Lerner, R.A. (1992) Proc. Natl. Acad. Sci. USA 89, 4457–4461.
[32] Griffiths, A.D., Williams, S.C., Hartley, O., Tomlinson, I.M., Waterhouse, P., Crosby, W.L., Kontermann, R.E., Jones, P.T., Low, N.M., Allison, T.J., Prospero, T.D., Hoogenboom, H.R., Nissim, A., Cox, U.P.L., Harrison, J.L., Zaccolo, M., Gherardi, E. and Winter, G. (1994) EMBO J. 13, 3245–3260.
[33] Hoogenboom, H.R., Marks, J.D., Griffiths, A.D. and Winter, G. (1992) Immunol. Rev. 130, 41–68.
[34] Nissim, A., Hoogenboom, H.R., Tomlinson, I.M., Flynn, G., Midgley, C., Lane, D. and Winter, G. (1994) EMBO J. 13, 692–8.

[35] Hodits, R.A., Nimpf, J., Pfistermueller, D.M., Hiesberger, T., Schneider, W.J., Vaughan, T.J., Johnson, K.S., Haumer, M., Kuechler, E., Winter, G. and Blaas, D. (1995) J. Biol. Chem. 270, 24078–24085.
[36] Neubauer, C., Frasel, L., Kuechler, E. and Blaas, D. (1987) Virology 158, 255–258.
[37] Barber, D.L., Sanders, E.J., Aebersold, R. and Schneider, W.J. (1991) J. Biol. Chem. 266, 18761–18770.
[38] Vaughan, T.V., Williams, A.J., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, J.C., McCafferty, J., Hodits, R.A., Wilton, J. and Johnson, K.S. (1996) Nat. Biotechnol. 14, 309–314.
[39] Tomlinson, I.M., Walter, G., Marks, J.D., Llewelyn, M.B. and Winter, G. (1992) J. Mol. Biol. 227, 776–798.
[40] Bu, G.J., Williams, S., Strickland, D.K. and Schwartz, A.L. (1992) Proc. Natl. Acad. Sci. USA 89, 7427–7431.
[41] Argraves, K.M., Battey, F.D., Maccalman, C.D., McCrae, K.R., Gafvels, M., Kozarsky, K.F., Chappell, D.A., Strauss, J.F. and Strickland, D.K. (1995) J. Biol. Chem. 270, 26550–26557.
[42] Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H. and Stanley, K.K. (1988) EMBO J. 7, 4119–27.
[43] George, R., Barber, D.L. and Schneider, W.J. (1987) J. Biol. Chem. 262, 16838–16847.
[44] Moestrup, S.K., Kaltoft, K., Sottrup Jensen, L. and Gliemann, J. (1990) J. Biol. Chem. 265, 12623–12628.
[45] Willnow, T.E., Goldstein, J.L., Orth, K., Brown, M.S. and Herz, J. (1992) J. Biol. Chem. 267, 26172–26180.
[46] Moestrup, S.K. (1994) Biochim. Biophys. Acta 1197, 197–213.
[47] Moestrup, S.K. et al. (1993) J. Biol. Chem. 268, 13691–13696.
[48] Moestrup, S.K., Kaltoft, K., Petersen, C.M., Pedersen, S., Gliemann, J. and Christensen, E.I. (1990) Exp. Cell Res. 190, 195–203.
[49] Horn, I.R., Moestrup, S.K., van den Berg, B.M., Pannekoek, H., Nielsen, M.S. and van Zonneveld, A.J. (1995) J. Biol. Chem. 270, 11770–11775.