Intradiabodies, Bispecific, Tetravalent Antibodies for the Simultaneous Functional Knockout of Two Cell Surface Receptors

Nina Jendreyko‡, Mikhail Popkov‡, Roger R. Beerli‡, Junho Chung‡, Dorian B. McGavern§, Christoph Rader‡, and Carlos F. Barbas III‡

From the ¶Department of Molecular Biology and the Skaggs Institute for Chemical Biology and the §Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California 92037

Received for publication, July 1, 2003, and in revised form, August 5, 2003
Published, JBC Papers in Press, August 28, 2003, DOI 10.1074/jbc.M307002200

The specific and high affinity binding properties of intracellular antibodies (intrabodies), combined with their ability to be stably expressed in defined organelles, provides powerful tools with a wide range of applications in the field of functional genomics and gene therapy. Intrabodies have been used to specifically target intracellular proteins, manipulate biological processes, and contribute to the understanding of their functions as well as for the generation of phenotypic knockouts in vivo by surface depletion of extracellular or transmembrane proteins. In order to study the biological consequences of knocking down two receptor-tyrosine kinases, we developed a novel intrabody-based strategy. Here we describe the design, engineering, and characterization of a bispecific, tetravalent endoplasmic reticulum (ER)-targeted intrabody for simultaneous surface depletion of two endothelial transmembrane receptors, Tie-2 and vascular endothelial growth factor receptor 2 (VEGF-R2). Comparison of the ER-targeted intrabody with the corresponding conventional ER-targeted single-chain antibody fragment (scFv) intrabodies demonstrated that the intrabody is significantly more effective with respect to efficiency and duration of surface depletion of Tie-2 and VEGF-R2. In vitro endothelial cell tube formation assays suggest that the bispecific intrabody exhibits strong antiangiogenic activity, whereas the effect of the monospecific scFv intrabodies was weaker. These findings suggest that simultaneous interference with the VEGF and the Tie-2 receptor pathways results in at least additive antiangiogenic effects, which may have implications for future drug developments. In conclusion, we have identified a highly effective ER-targeted intrabody format for the simultaneous functional knockout of two cell surface receptors.

Antibodies can bind almost any molecule with high specificity and affinity, providing powerful biotechnological tools for diagnostic and therapeutic applications. Advances in recombinant DNA technology have facilitated the manipulation of the antigen genes, so that design, cloning, expression, and use of single-chain antibodies have become routine procedures in protein engineering. The potential of single-chain antibody fragments (scFv) for intracellular applications, termed “intrabodies,” has been exploited in a number of laboratories (1–8). To date, intrabodies have been utilized for targeting proteins in a singular fashion. Bispecific and tetravalent antibody fragments could improve and expand the inhibitory potential of intrabodies by exhibiting increased apparent affinity for their antigens and by being more efficient at inhibiting protein function or intracellular trafficking (9). Intrabodies present a potent alternative to methods of gene inactivation that target at the level of DNA or mRNA, such as antisense (10), zinc finger proteins (11), targeted gene disruption, or the relatively new RNA interference (12). Operating at the posttranslational level, intrabodies can be directed to relevant subcellular compartments and precise epitopes on target proteins (2, 13, 14), potentially blocking only one out of several functions of an expressed protein. Numerous studies have reported the development of engineering antibodies that are both multispecific and multivalent (15, 16). Applying this knowledge, the goal of our study was to develop an ER-targeted intrabody format for the simultaneous down-regulation of two independent cell surface receptors, in order to investigate the biological consequences of knocking down two receptor-tyrosine kinases. To accomplish this, an ER-targeted tetravalent antibody construct, with dual specificity, was generated. Using a recombinant adenovirus as a gene delivery system, we could show that this intrabody construct, termed here an “intradiabody,” was expressed in the ER and able to trap both targeted proteins in the same compartment. The intradiabody targets the endothelial transmembrane receptors Tie-2 and VEGF-R2, which are essential for angiogenesis. The interplay of VEGF, VEGF-R2, Tie-2, and Ang-1 and -2 has been suggested as a key modulator in the onset of tumor angiogenesis (17). According to this model, Tie-2 is constitutively engaged with Ang-1 in quiescent blood vessels. The Tie-2/Ang-1 complex stabilizes quiescent blood vessels by promoting their interaction with surrounding perivascular cells, smooth muscle cells, and the extracellular matrix. The constitutive Tie-2/
Ang-1 complex is antagonized by Ang-2, which is up-regulated in endothelial cells that are proximal to the tumor. By competing with Ang-1 for Tie-2 binding, Ang-2 destabilizes the interaction of endothelial cells and their microenvironment. This is thought to sensitize the endothelial cells to VEGF signaling. Thus, Ang-2 produced by endothelial cells promotes tumor angiogenesis in concert with VEGF produced by the tumor. Comparison of the effect of the intradiabody (targeting Tie-2 and VEGF-R2) with the corresponding conventional scFv intrabodies (targeting Tie-2 or VEGF-R2 alone) revealed a remarkable superiority of the intradiabody, as represented by a complete and extended surface depletion of both Tie-2 and VEGF-R2. This finding can be attributed to the extended half-life of our intradiabody, as determined by pulse-chase studies. In addition, we show that the intradiabody strongly inhibits endothelial tube formation beyond that seen with inhibitors of either receptor-tyrosine kinase alone, thus confirming its antiangiogenic properties. Our results confirm that the inhibition of the VEGF receptor pathway cannot be compensated by the Tie-2 pathway, nor vice versa (18), but also demonstrates that targeting both pathways simultaneously results in additive antiangiogenic effects in vitro. Combining the specific and high affinity binding properties of our intradiabody with its ability to be stably expressed in the ER, we identified a very effective intrabody format for the simultaneous functional knockout of two cell surface receptors.

MATERIALS AND METHODS

Cell Culture—Human umbilical vein endothelial cells (HUVEC) (BioWhittaker, Walkersville, MD) were cultured at 37 °C in 5% CO2 in EGM medium (BioWhittaker) supplemented with 2% bovine brain extract (BioWhittaker). 293 cells human embryonic kidney (ATCC) cells were cultured at 37 °C in 5% CO2, in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% antibiotics.

Library Generation and Selection—The generation and selection of rabbit/human chimeric Fab libraries, as well as the characterization of 1% antibiotics. supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and anti-human F(ab’2) fragment-depleted phagemid vector pComb3X and purified using goat antibody-coupled protein-G sepharose as described (20). Scramble Fab were expressed from gene III as C-terminal moiety. 

| Fab | $k_{on}/10^{7}$ | $k_{off}/10^{-4}$ | $K_{d}$ |
|-----|----------------|-----------------|--------|
| 1S05 | 9.8            | 13.5            | 15     |
| VC06 | 4.6            | 0.53            | 12.8   |

 Angiopoietins—Angiopoietins are a family of ligands that stimulate the Tie receptor family. Ang-1 is antagonized by Ang-2, which is up-regulated in endothelial cells that are proximal to the tumor. By competing with Ang-1 for Tie-2 binding, Ang-2 destabilizes the interaction of endothelial cells and their microenvironment. This is thought to sensitize the endothelial cells to VEGF signaling. Thus, Ang-2 produced by endothelial cells promotes tumor angiogenesis in concert with VEGF produced by the tumor. Comparison of the effect of the intradiabody (targeting Tie-2 and VEGF-R2) with the corresponding conventional scFv intrabodies (targeting Tie-2 or VEGF-R2 alone) revealed a remarkable superiority of the intradiabody, as represented by a complete and extended surface depletion of both Tie-2 and VEGF-R2. This finding can be attributed to the extended half-life of our intradiabody, as determined by pulse-chase studies. In addition, we show that the intradiabody strongly inhibits endothelial tube formation beyond that seen with inhibitors of either receptor-tyrosine kinase alone, thus confirming its antiangiogenic properties. Our results confirm that the inhibition of the VEGF receptor pathway cannot be compensated by the Tie-2 pathway, nor vice versa (18), but also demonstrates that targeting both pathways simultaneously results in additive antiangiogenic effects in vitro. Combining the specific and high affinity binding properties of our intradiabody with its ability to be stably expressed in the ER, we identified a very effective intrabody format for the simultaneous functional knockout of two cell surface receptors.

Materials and Methods

Cell Culture—Human umbilical vein endothelial cells (HUVEC) (BioWhittaker, Walkersville, MD) were cultured at 37 °C in 5% CO2 in EGM medium (BioWhittaker) supplemented with 2% bovine brain extract (BioWhittaker). 293 cells human embryonic kidney (ATCC) cells were cultured at 37 °C in 5% CO2, in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% antibiotics.

Library Generation and Selection—The generation and selection of rabbit/human chimeric Fab libraries, as well as the characterization of 1% antibiotics. supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and anti-human F(ab’2) fragment-depleted phagemid vector pComb3X and purified using goat antibody-coupled protein-G sepharose as described (20). Scramble Fab were expressed from gene III as C-terminal moiety.

| Fab | $k_{on}/10^{7}$ | $k_{off}/10^{-4}$ | $K_{d}$ |
|-----|----------------|-----------------|--------|
| 1S05 | 9.8            | 13.5            | 15     |
| VC06 | 4.6            | 0.53            | 12.8   |
was SacI/SpeI-cloned into phagemid vector pComb3X. Finally, both constructs were combined by SacI cloning and confirmed by DNA sequence analysis. The T2 scFv was amplified using primers extompseq and TP2-B (5′-TCT AGT GGT CCT GGC TCG GCA CAG GCC GTC GTG GGT CAT CAC GAG CTC GGC CGC CTG TGC CGA GCC ACC CCC AGA ACC-3′), and the scFv for V2 was amplified using dpseq and primer V2-F (5′-TGG GGT GCC TCG GCA CAG GCG GCC GTC GTG ATG ACC CAG ACT-3′). scFv T2 and CH2-CH3 were combined by overlap extension PCR using ext and CH3/V2-B. ScFv V2 was SacI/SpeI cloned into phagemid vector pComb3X. Both constructs were then combined in the same manner as the Tie-2/VEGF-R2 intrabody.

Assembly of Intrabody Constructs in pAdTrackCMV and Generation of Adenoviral Plasmids by Homologous Recombination—Intrabody coding regions were initially assembled in pBabePuro essentially as described (23). In these constructs, the scFv coding regions are flanked by a human κ light chain leader sequence at the 5′-end and a sequence encoding the HA tag (YPYDVPDYA) and the ER retention signal (KDEL) at the 3′-end. The intrabody coding regions were then excised by digestion with BamH1 and Sall and ligated into pAdTrackCMV (24) digested with BglII and SalI and ligated into pAdTrackCMV (24) (KDEL) at the 3′-end. The intrabody coding regions were then excised by digestion with BamH1 and Sall and ligated into pAdTrackCMV (24) digested with BglII and SalI and ligated into pAdTrackCMV (24).

Injection of HUVEC with Intrabodies Using Recombinant Adeno-

was SacI/SpeI-cloned into phagemid vector pComb3X. Finally, both constructs were combined by SacI cloning and confirmed by DNA sequence analysis. The T2 scFv was amplified using primers extompseq and TP2-B (5′-TCT AGT GGT CCT GGC TCG GCA CAG GCC GTC GTG GGT CAT CAC GAG CTC GGC CGC CTG TGC CGA GCC ACC CCC AGA ACC-3′), and the scFv for V2 was amplified using dpseq and primer V2-F (5′-TGG GGT GCC TCG GCA CAG GCG GCC GTC GTG ATG ACC CAG ACT-3′). scFv T2 and CH2-CH3 were combined by overlap extension PCR using ext and CH3/V2-B. ScFv V2 was SacI/SpeI cloned into phagemid vector pComb3X. Both constructs were then combined in the same manner as the Tie-2/VEGF-R2 intrabody.

Assembly of Intrabody Constructs in pAdTrackCMV and Generation of Adenoviral Plasmids by Homologous Recombination—Intrabody coding regions were initially assembled in pBabePuro essentially as described (23). In these constructs, the scFv coding regions are flanked by a human κ light chain leader sequence at the 5′-end and a sequence encoding the HA tag (YPYDVPDYA) and the ER retention signal (KDEL) at the 3′-end. The intrabody coding regions were then excised by digestion with BamH1 and Sall and ligated into pAdTrackCMV (24) digested with BglII and SalI and ligated into pAdTrackCMV (24). High titer viral stocks were produced and purified by CsCl banding. All virus preparations were GFP-corrected (25).

Infection of HUVEC with Intrabodies Using Recombinant Adeno-
donkey anti-rat IgG polyclonal antibodies and streptavidin/rhodamine red-X (both from Jackson Immunoresearch, West Grove, PA) diluted to 1:100 in FACS buffer, 0.1% saponin. Finally, the cells were covered with SlowFade Antifade reagent. Three-color (GFP, rhodamine red-X, and Cy5) three-dimensional data sets were collected with a DeltaVision system (Applied Precision, Issaquah, WA); this consisted of an Olympus IX-70 fluorescence microscope, a motorized high precision xyz stage, a 100-watt mercury lamp, and a KAF1400 chip-based cooled charge-coupled device camera. Exposure times were 0.2–0.5 s (2-binning), and images were obtained with a ×60 oil objective. Three-dimensional reconstructions were generated by capturing 150-nm serial sections along the z axis. Images were deconvolved (based on the Agard-Sadat inverse matrix algorithm) and analyzed with softWorX version 2.5.

Pulse-chase for Determination of Half-life—HUVEC cells were seeded at a density of 1 × 10⁶ cells in T175 flasks and infected 24 h later with recombinant adenoviruses using an MOI of 10. After infection for 24 h, cells were washed with Hepes-buffered saline solution and trypsinized. Cells were starved for 2 h in 10 ml of serum-free, methionine-free, cysteine-free minimal essential medium at 37 °C and swirled periodically. Samples were then labeled with Tran35S-label medium (50 μCi/ml; ICN, Aurora, OH) for 2 h at 37 °C and subsequently chased with EGM medium, containing a 40-fold excess of methionine and 20-fold excess of cysteine for various time points (0, 4, 8, 16, 24, and 48 h). At each time point, cells were washed once with ice-cold PBS containing 1 mM phenylmethylsulfonyl fluoride and lysed (Promega lysis buffer, containing complete protease inhibitor mixture). Supernatants were collected and stored at −80 °C. Samples were precipitated using protein G and monoclonal anti-HA antibody (Covance) for scFv or protein G alone for the diabody. Immunocomplexes were washed twice with lysis buffer and twice with TBS, boiled in 1.5× SDS loading buffer, and separated by a 4–20% gradient SDS-PAGE gel under reducing conditions. The gels were stained, dried, and exposed to autoradiography as well as quantitatively analyzed with a PhosphorImager.

Endothelial Cell Tube Formation Assay—6 × 10⁴ HUVEC cells were infected with recombinant adenovirus using an MOI of 20 in an Eppendorf tube for 45 min and were then transferred to 6-well plates. Three days after infection, cells were washed with Hepes-buffered saline solution, trypsinized, and counted. 2 × 10⁴ cells/well (volume 50 μl) in complete EGM medium were seeded in triplicates for each virus in a 96-well plate coated with Matrigel Basement Membrane Matrix (BD Bioscience, Bedford, MA) and incubated for 15.5 h. Cells were then stained and fixed with the Diff-Quik® staining set (DADE BEHRING Inc., Newark, DE). For this, cells were fixed with Diff-Quik Fixative, followed by Diff-Quik Solution I and II, each with 100 μl for 2–3 min. Cells were then washed four times with distilled H₂O, and pictures were taken under an inverted light microscope at ×2 magnification. The number of tube branches for each virus was counted in triplicates to calculate the average ± S.D.

Cell Proliferation Assay—HUVEC cells were seeded (5 × 10⁴) in 96-well plates in complete EGM medium and infected with different recombinant adenoviral constructs with an MOI of 10 and 50. Three days later, the cells were trypsinized, stained with trypan blue, and counted in triplicate by a hemacytometer. Cell proliferation was also measured by adding [3H]thymidine (ICN Radiochemicals) in a concentration of 0.5 μCi/well (1 Ci = 37 GBq) during the last 24 h of incubation. The cells were then frozen at −80 °C overnight and subsequently processed on a multichannel automated cell harvester (Cambridge Technology, Cambridge, MA) and counted in a liquid scintillation...
Intradiabodies for Simultaneous Knockout of VEGFR2 and Tie2

RESULTS

Selection of Rabbit Fabs Binding to VEGF-R2—Using phage display, several New Zealand White rabbit Fab against VEGF-R2 were selected by panning (19) chimeric rabbit/human antibody libraries (20, 21) on a human VEGF-R2-VEGF complex. For further analysis, chimeric rabbit/human Fab VC06 was selected and produced as soluble Fab in Escherichia coli and purified by affinity chromatography using goat anti-human F(ab’)2 N-hydroxysuccinimide resin columns. Fab VC06 demonstrated a strong binding to human VEGF-R2 in ELISA and to HUVEC in flow cytometry (not shown). Thus, the VEGF-R2 epitope recognized by VC06 is displayed by native VEGF-R2 expressed on the cell surface and is an accessible target for antiangiogenic therapy. Surface plasmon resonance studies of VC06 revealed that it possessed a monovalent dissociation constant of \( \sim 1 \) nM to human VEGF-R2, whereas Fab 1S05, which we described earlier (20), bound with a dissociation constant of 14 nM to human Tie-2 (Table I).

Generation of an Intradiabody against Tie-2 and VEGF-R2 and scFv Intrabodies against Tie-2 or VEGF-R2 Alone—Fabs were converted into scFv, in which the VL and VH fragments were covalently linked with a peptide linker consisting of 18 amino acids. Preserved binding to their respective antigens was confirmed for both scFv VC06 (VEGF-R2) and scFv 1S05 (Tie-2) by ELISA (not shown). Next, scFv 1S05 and scFv VC06 were linked through the second and third heavy chain constant domains of human IgG1, resulting in a scFv-CH2-CH3-scFv expression cassette. As a key feature, the scFv-CH2-CH3-scFv expression cassette provides for the production of a bifunctional tetravalent antibody construct from a single polypeptide.

Through the homophilic interaction of CH3, two scFv-CH2-CH3-scFv molecules associate to form a 150-kDa dimer, which displays both the N-terminal and C-terminal scFv module bivalently as the intradiabody (Fig. 1). The scFv against Tie-2 or VEGF-R2 and the scFv-CH2-CH3-scFv genes were cloned bivalently as the intradiabody (Fig. 1). The scFv against Tie-2 or VEGF-R2 and the scFv-CH2-CH3-scFv genes were cloned into a modified adenovirus shuttle vector pAdTrackCMV (24) using two asymmetric SfiI sites. In these constructs, the scFv coding regions are flanked by a human \( \kappa \) light chain leader sequence at the 5′-end, and a sequence encoding the HA tag (YPYDVPDYA) and the ER retention signal (KDEL) at the 3′-end. The generation of replication-deficient recombinant adenoviruses was done essentially as described (24). The resulting recombinant adenoviruses were purified by CsCl banding, and final yields were between \( 4.3 \times 10^{11} \) particles/ml and \( 1.2 \times 10^{12} \) particles/ml.

Colocalization of Intradiabody and Targeted Proteins in the ER—To investigate whether our intrabody constructs were expressed in the ER and able to trap the targeted proteins in the same compartment, we infected HUVEC with an MOI of 50 and verified the endoplasmatic reticulum localization of intrabodies and proteins by deconvolution microscopy on day 3 after infection (Fig. 2). As a control, HUVEC were infected with an intrabody against integrin \( \alpha_\beta_3 \) (scFv JC7U). To visualize the intrabodies in the ER, intracellular staining was carried out with saponin and rat anti-HA monoclonal antibody, followed by donkey anti-rat Cy5 polyclonal antibodies. Both intrabody constructs were found to be expressed in the ER, as indicated by a staining of the characteristic tubular network. To confirm that the targeted proteins were trapped in the ER, another staining protocol using biotinylated Tie-2 and VEGF-R2 polyclonal antibodies, followed by streptavidin-rhodamine red-X polyclonal antibodies was performed. As shown in Fig. 2 and in three-dimensional movies (see Supplemental Material), the intradia-
body against Tie-2/VEGF-R2 was found to be co-localized with both targeted proteins in the ER, indicated by the \textit{purple color} in the merged picture. On the other hand, the control intrabody (JC7U), although expressed in the ER, did not retain either targeted proteins or the control diabody construct T2V2 (c) formed capillary tubes. By contrast, cells infected with the intradiabody (f) were strongly inhibited in their capability to form capillary tubes. B: the intradiabody revealed an inhibitory effect of 90 ± 4%, whereas scFv intrabodies showed inhibitory effects to 44 ± 4.3% (A, d, VC06 against VEGF-R2) and 12 ± 5.8% (A, e, 1S05 against Tie-2). The number of tube branches in three independent wells per sample were counted and averaged with S.D. values.

Extended Surface Depletion of Tie-2 and VEGF-R2 with Intradiabody—To compare the effect of the intradiabody with that of the scFv intrabodies on the surface expression of Tie-2 and VEGF-R2, HUVEC were infected with an MOI of 10 and analyzed by flow cytometry over a period of 15 days. The surface expression of human Tie-2 and VEGF-R2 on HUVEC infected with the intradiabody, was specifically blocked up to 57% (Tie-2) and 78% (VEGF-R2), respectively, on day one. On day three after infection, blockade of Tie-2 and VEGF-R2 was 98% complete. Fifteen days after infection, surface expression remained efficiently blocked, 97.5 and 96% for Tie-2 and VEGF-R2, respectively. In comparison, the surface expression of HUVEC infected with the scFv intrabodies, was blocked with 80% (Tie-2) and 83% (VEGF-R2) efficiency on day one and 84% (Tie-2) and 90% (VEGF-R2) on day three postinfection. However, on day 15, surface depletion was only 68 and 5% for Tie-2 and VEGF-R2, respectively (Fig. 3, a and b). No down-regulation of integrins $\alpha_\beta_3$ and $\alpha_\beta_5$ was observed during 15 days of the experiment (data not shown).

Extended Half-life of the Intradiabody—To determine whether the remarkable superiority of the intradiabody with respect to effectiveness and duration of surface depletion of the targeted proteins can be attributed to an extended half-life, pulse-chase studies were performed. In these experiments, we compared the intradiabody with the 1S05 scFv intrabody, which had been found to be more efficient than the VC06 scFv intrabody. In brief, 10^6 HUVEC cells were infected with an MOI of 10 with each virus for 24 h. Proteins were labeled with $[^{35}\text{S}]$methionine/cysteine, and HUVEC were lysed following different time points of chase. Following immunoprecipitation, proteins were separated on a 4–20% gradient SDS-PAGE gel under reducing conditions, visualized by autoradiography, and quantified using a PhosphorImager. Pulse-chase experiments revealed a half-life of the intradiabody beyond 48 h ($t = 230$ h), whereas the half-life of the scFv intrabody 1S05 was $\sim 22$ h (Fig. 4, a and b). The half-life of the scFv intrabody VC06 was even shorter than 22 h (data not shown).

Antiangiogenic Effect of Intradiabody—To test whether our intradiabody can inhibit angiogenesis \textit{in vitro}, we used a three-dimensional capillary tube formation assay. This \textit{in vitro} assay has been used as a model of the early organization of new blood vessels and is consistently found to recapitulate to a large extent the process of angiogenesis \textit{in vivo} (26, 27). HUVEC were infected with an MOI of 20 of each virus. Cells were transferred to the Matrigel-coated 96-well plate 3 days after infection to achieve maximum surface depletion of the targeted proteins. All cells were incubated in the presence of exogenous growth factors and serum (complete EGM medium). Controls consisted of mock-infected cells (empty vector) and a control intradiabody without specificity for any known target (T2V2). These controls were used to determine possible toxic or growth inhibition (antiproliferative) effects of either the vector or the intradiabody format. As shown in Fig. 5, formation of capillaries in Matrigel was inhibited 90 ± 3% with the intradiabody (VEG-R2/Tie-2), 44 ± 4.3% with the scFv intrabody VC06 (VEGF-R2), and 11 ± 5.8% with the scFv intrabody 1S05 (Tie-2). Significantly, cells infected with the empty vector and the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5}
\caption{Inhibition of capillary tube formation \textit{in vitro}. A, HUVEC cells were infected with different recombinant adenoviruses for 5 days and then seeded in 96-well plates coated with Matrigel. After incubation for 15.5 h, cells were stained and fixed, and pictures were taken. Uninfected cells (a), mock-infected cells (b), and cells infected with the control diabody construct T2V2 (c) formed capillary tubes. By contrast, cells infected with the intradiabody (f) were strongly inhibited in their capability to form capillary tubes. B: the intradiabody revealed an inhibitory effect of 90 ± 4%, whereas scFv intrabodies showed inhibitory effects to 44 ± 4.3% (A, d, VC06 against VEGF-R2) and 12 ± 5.8% (A, e, 1S05 against Tie-2). The number of tube branches in three independent wells per sample were counted and averaged with S.D. values.}
\end{figure}
control intradiabody (T2V2) were able to form capillaries to the same extent as uninfected cells.

Intradiabody Does Not Affect Cell Viability—In order to determine that the antiangiogenic effects of the intradiabody are not related to cytotoxic effects on HUVEC, cell proliferation and survival were assessed via $[^{3}H]$thymidine incorporation assay and trypan blue staining. HUVEC were infected with an MOI of 10 and 50 of each virus. Cell viability was assessed 3 days after infection. All cells were incubated in complete EGM medium. As shown in Fig. 6, cell survival and proliferation of cells infected with the intradiabody are similar as for the scFv intrabodies (1S05 and VC06) and the control intradiabody (T2V2) were able to form capillaries to the same extent as uninfected cells.

Discussion

The use of scFv fragments as intrabodies has received considerable attention over the past 10 years. Intrabodies have been utilized to neutralize the function of endogenous target proteins using several different strategies (13, 28, 29). Among these, the misdirected localization of the target to another subcellular region features as the most popular methodology employed. We hypothesized that an ER-targeted intrabody with dual specificity and valency could be generated by fusing two scFv modules with unique specificity to the N terminus and C terminus of an Fc domain, respectively. Although there are reports of presenting functional bispecific antibodies in scFv-Fc formats (30, 31), none of those antibody constructs have been studied as functional intrabodies targeted to the ER. Furthermore, these antibody constructs bind in a monovalent fashion to each of the target proteins. As a prototype and proof of concept, we demonstrate here the development of an ER-targeted intradiabody format for the simultaneous down-regulation of two independent cell surface receptors using an adenovirus-mediated gene delivery system. Our prototypical intradiabody targets the endothelial transmembrane tyrosine kinase receptors Tie-2 and VEGFR-2. Both of these receptors have been shown to be key in angiogenesis.

The endothelial cell receptor-tyrosine kinases, VEGFR-2 and Tie-2, as well as their ligands, VEGF and angiopoietin-1 and -2, respectively, play key roles in tumor angiogenesis. A model for the interplay of VEGF, VEGFR-2, Tie-2, and Ang-1 and -2 in tumor angiogenesis is discussed by Holash et al. (17). From a therapeutic perspective, the Tie-2/Ang-2 complex and the VEGF/VEGFR-2 complex, which are formed on the surface of proliferating endothelial cells, are attractive targets for antiangiogenic agents. Several reports (32, 33) have been published showing the inhibition of tumor growth by interference with the VEGF or Tie-2 receptor pathway by means of antibodies and soluble or dominant-negative receptor domains (34, 35). As of yet, no reports have been published based on the use of an ER-targeted intrabody to inhibit expression of the two endothelial transmembrane receptors Tie-2 and VEGFR-2, either separately or simultaneously. Our results demonstrate the feasibility of expressing functional, bispecific, and tetravalent antibodies intracellularly and the possibility of inhibiting the transit of two integral membrane proteins simultaneously through the ER by means of diabody expression. The potential for inhibiting angiogenesis by this approach should have implications for the treatment of cancer, since VEGFR-2 and Tie-2 are essential for tumor angiogenesis. In vitro angiogenesis assays using Matrigel showed that the intradiabody significantly reduces the number of capillary tubes formed, demonstrating its potential as an antiangiogenic drug. Interestingly, monospecific scFv intrabodies against VEGFR-2 or Tie-2 inhibited capillary tube formation to a significantly lesser extent. Moreover, the toxicity of the bispecific intradiabody and the control intradiabody (T2V2) are comparable. The relative efficiency of the intradiabody over the traditional intrabody-based approach could be explained by several reasons: first, the bispecificity of the intradiabody versus the monospecificity of the scFv intrabodies; second, simply their monovalency versus the bivalency of the intradiabody. A third possibility is that the relative stability of the proteins could also account for these findings. Fourth, the bivalent display of the ER retention signal may lead to better retention of the targeted proteins. We found that the superiority of the intradiabody with respect to effectiveness and duration of surface depletion of the targeted proteins can be attributed to an extended half-life. This was demonstrated in pulse-chase studies, which revealed a half-life of the intradiabody beyond 48 h ($t_{1/2} < 230$ h), whereas the half-life of the scFv intrabody 1S05 was $22$ h and the half-life of scFv intrabody VC06 was even shorter (data not shown). The extended half-life of the intradiabody correlates well with its more efficient and durable surface depletion of the targeted proteins compared with the scFv intrabody. A plausible explanation for the extended half-life of the intradiabody format is the bivalent display of the ER retention signal (KDEL) through the homodimerization of the constant domains (Fig. 1).
may increase the efficiency of recovery of the intrabody from the Golgi by the KDEL receptor (36). In addition, fusion to constant domains per se has been suggested as a means of increasing the stability of intrabodies (37). Although the increased valency of the intrabody, which results in a higher apparent affinity (avidity) for the antigen, is probably an important factor for the efficient surface depletion we observed, other factors might contribute to the superiority of the intrabody. In particular, it is known that intrabodies with extended half-lives achieve higher steady-state expression levels, which increases effectiveness in target molecule inactivation. In fact, intracellular stability can be more important than affinity for the antigen (28, 38, 39).

A major application for intrabodies lies in functional genom-ics (3, 5, 6). Since the number of proteins with unknown function and their interactions with other proteins steadily increases, bispecific and tetravalent intrabodies could provide an attractive proteomic tool to analyze these networks. This novel tool provides for the simultaneous surface depletion of two independent receptors while improving both the efficiency and duration of the resulting phenotypic knockout. Intrabodies may be used to investigate linked regulatory pathways or block redundant pathways. In addition, intrabodies may extend the therapeutic applicability of intrabodies. For example, our prototype developed in this study provides a precise tool for the simultaneous silencing of two independent signaling pathways essential for angiogenesis (18).

Acknowledgment—We thank Roberta Fuller for assistance.

REFERENCES
1. Marasco, W. A., Haseltine, W. A., and Chen, S. Y. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7889–7893
2. Beers, R. R., Wiel, W., and Hynes, N. E. (1994) J. Biol. Chem. 269, 23931–23936
3. Deshane, J., Siegal, G. P., Alvarez, R. D., Wang, M. H., Feng, M., Cabrera, G., Liu, T., Kay, M., and Curiel, D. T. (1995) J. Clin. Invest. 96, 2880–2889
4. Deshane, J., Siegal, G. P., Wang, M., Wright, M., Buyc, R. P., Alvarez, R. D., and Curiel, D. T. (1997) Gynecol. Oncol. 64, 378–385
5. Marasco, W. A., Chen, S., Richardson, J. H., Ramstedt, U., and Jones, S. D. (1998) Hum. Gene Ther. 9, 1627–1642
6. Visintin, M., Tse, E., Axelson, H., Rabbitts, T. H., and Cattaneo, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11725–11728
7. Ruberti, F., Capsoni, S., Comparini, A., Di Daniel, E., Franziot, J., Gonzoni, S., Rossi, G., Berardi, N., and Cattaneo, A. (2000) J. Neurosci. 20, 2589–2601
8. Auf Der Maur, A., Zahnd, C., Fischer, F., Spinelli, S., Honegger, A., Cambillau, C., Escher, D., Püschlik, A., and Barberis, A. (2002) J. Biol. Chem. 277, 45075–45085
9. Fitzgerald, K., Hooliger, P., and Winter G. (1997) Protein Eng. 10, 1221–1225
10. Wagner, R. W., and Flanagan, W. M. (1997) Mol. Med. Today 3, 31–38
11. Beers, R. R., and Marbas, C. F., III (2002) Nat. Biotechnol. 20, 135–141
12. Hannon, G. J. (2002) Nature 418, 244–251
13. Marasco, W. A. (1997) Gene Ther. 4, 11–15
14. Bai, J., Sui, J., Zhu, R. Y., St. Clair Tallarico, A., Gennari, F., Zhang, D., and Marasco, W. A. (2003) J. Biol. Chem. 278, 1433–1442
15. Todorovska, A., Roovers, R. C., Dolezal, O., Kortt, A. A., Hoogenboom, H. R., and Hudson, P. (2001) J. Immunol. Methods 248, 47–66
16. Hudson, P., and Soureian, C. (2003) Nat. Med. 9, 129–134
17. Holash, J., Maisonnierre, P. C., Compton, D., Boland, P., Alexander, C. R., Zagag, D., Yancopoulos, G. D., and Wiegand, S. J. (1999) Science 284, 1994–1998
18. Siemeister, G., Schirner, M., Weindel, K., Reusch, P., Menrad, A., Marone, D., and Martinay-Baron, G. (1999) Cancer Res. 59, 3185–3191
19. Barbas, C. F., III, Burton, D. R., Scott, J. K., and Silverman, G. J. (2001) Phage Display: A Laboratory Manual, pp. 9.1–9.113, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Popkov, M., Mage, R. G., Alexander, C. B., Thumvalappilu, S., Barbas, C. F., III, and Rader, C. (2003) J. Mol. Biol. 325, 325–335
21. Rader, C., Ritter, G., Nathan, S., Ehia, M., Gout, I., Jungbluth, A. A., Cohen, L. S., Welt, S., Old, L. J., and Barbas, C. F., III (2000) J. Biol. Chem. 275, 13668–13676
22. Rader, C., Popkov, M., Neves, J. A., and Barbas, C. F., III (2002) FASEB J. 16, 2060–2062
23. Steinberger, P., Andris-Wadhuf, J., Buehler, B., Torbett, B. E., and Barbas, C. F., III (1997) Proc. Natl. Acad. Sci. U. S. A. 97, 805–810
24. Hoe, T., Zhou, S., Da Costa, L. T., Yu, J., Kinzer, K. W., and Vogelstein, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3529–3534
25. Hitt, D. C., Booth, J. L., Dandpani, V., Pennington, L. R., Gimble, J. M., and Metcalfe, J. (2000) Mol. Biotechnol. 14, 197–203
26. Dallabrida, S. M., DeSouza, M. A., and Farell, D. H. (2000) J. Biol. Chem. 275, 32281–32288
27. Henderson, A. M., Wang, S.-J., Taylor, A. C., Attkenhead, M., and Hughes, C. C. W. (1991) J. Biol. Chem. 266, 6169–6176
28. Zhu, Q., Zeng, C., Huhakey, V., Yao, J., Turi, T. G., Danley, D., Hynes, T., Cong, Y., Dimattia, D., Kennedy, S., Dauny, G., Schaeffer, E., Marasco, W. A., and Huston, J. S. (1999) J. Immunol. Methods 231, 207–222
29. Liu, M., Horn, I. R., Cardinale, A., Messina, S., Nielsen, U. B., Rybak, S. M., Hoogenboom, H. R., Cattaneo, A., and Biseca, S. (2000) Eur. J. Biochem. 267, 1196–1205
30. Coloma, M. J., and Morrison, S. L. (1997) Nat. Biotechnol. 15, 159–163
31. Muller, K. M., Arndt, K. M., and Plu, C. C. W. (2001) J. Biol. Chem. 276, 6176–6179
32. Millauer, B., Longhi, M. P., Plate, K. H., Shawer, L. K., Risau, W., Ullrich, A., and Strawn, L. M. (1996) Cancer Res. 56, 1615–1620
33. Goldman, C. K., Kendall, R. L., Cabrera, G., Soroceanu, L., Heike, Y., Gillespie, G. Y., Siegal, G. P., Mao, X., Bett, A. J., Huckle, W. R., Thomas, K. A., and Curiel, D. T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8795–8800
34. Lin, P., Pulverini, P., Dewhirst, M., Shan, S., Rao, P. S., and Peters, K. (1997) J. Clin. Invest. 100, 2672–2678
35. Lin, P., Buxton, J. A., Acheson, A., Radziejewski, C., Maisonnierre, P. C., Yancopoulos, G. D., Channon, K. M., Hale, L. P., Dewhirst, M. W., George, S. E., and Peters, K. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8829–8834
36. Munro, S., and Pelham, H. R. (1987) Cell 48, 899–907
37. Masih, M., Bagley, J., Chen, S. Y., Szilavy, A. M., Helland, D. G., and Marasco, W. A. (1995) EMBO J. 14, 1542–1551
38. Strube, R. W., and Chen, S. Y. (2002) J. Immunol. Methods 263, 149–167
39. Rajap, A., and Turi, T. G. (2001) J. Biol. Chem. 276, 33139–33146

Intradiabodies for Simultaneous Knockout of VEGFR2 and Tie2 47819