Anti-glycophorin single-chain Fv fusion to low-affinity mutant erythropoietin improves red blood cell-lineage specificity

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The presence of erythropoietin (Epo) receptors on cells besides red blood cell precursors, such as cancer cells or megakaryocyte precursors, can lead to side effects during Epo therapy including enhanced tumor growth and platelet production. It would be ideal if the action of Epo could be limited to erythroid precursors. To address this issue, we constructed single-chain variable fragment (scFv)-Epo fusion proteins that used the anti-glycophorin 10F7 scFv amino-terminal to Epo analogues that would have minimal activity alone. We introduced the Epo mutations N147A, R150A and R150E, which progressively weakened receptor affinity in the context of Epo alone, as defined by cell proliferation assays using TF-1 or UT-7 cells. Fusion of these mutant proteins to the 10F7 scFv significantly rescued the activity of the mutant proteins, but had a relatively small effect on wild-type Epo. For example, fusion to the 10F7 scFv enhanced the activity of Epo(R150A) by 10- to 27-fold, while a corresponding fusion to wild-type Epo enhanced its activity only up to 2.7-fold. When glycophorin was blocked by antibody competition or reduced by siRNA-mediated inhibition of expression, the activity of 10F7 scFv-Epo(R150A) was correspondingly reduced, while such inhibition had essentially no effect on the activity of 10F7 scFv-Epo(wild-type). In addition, potent stimulation of Epo receptors by 10F7 scFv-Epo(R150A) was observed in long-term proliferation and viability assays. Taken together, these results indicate that a combination of targeting and affinity modulation can be used to engineer forms of Epo with enhanced cell-type specificity.

Keywords: antibody fusion/chimeric activator/erythropoietin/glycophorin/protein therapeutics

Introduction

Erythropoietin (Epo) is the major cytokine-regulating red blood cell production, and is used clinically to treat anemia resulting from cancer chemotherapy and from kidney failure. Although Epo is successful in increasing hemoglobin levels in anemic patients, a growing body of clinical trials shows significantly increased risks of tumor progression and death in populations receiving Epo treatment compared with placebo (Henke et al., 2003; Leyland-Jones et al., 2005; Khuri, 2007; Wright et al., 2007). In patients with head and neck carcinomas, disease progression was worse with Epo treatment compared with placebo only for patients whose tumors expressed the Epo receptor, highlighting the role of Epo as a growth factor in adverse reactions (Henke et al., 2006). Epo has also been shown to induce increased motility and proliferation in cultured tumor cells from tissues as diverse as breast, kidney and cervix (Westenfelder and Baranowski, 2000; Lester et al., 2005; Hamadmad and Hohl, 2008).

Additionally, Epo has been implicated in the regulation of platelet production (Beguin, 1999; Stohlwet et al., 2000). Several clinical trials have shown that the use of Epo to raise hemoglobin to normal levels in kidney failure patients carries higher risks of coronary disease, heart attack and stroke (Druce et al., 2006; Singh et al., 2006). This may result from a misregulation of platelet production due to Epo action on erythrocyte precursors that also give rise to megakaryocytes and thus platelets (Stohlwet et al., 2000).

Consequently, the FDA has placed a black-box warning on Epo and other erythropoiesis-stimulating agents (ESAs), recommending their use only in dire cases to reduce transfusions and their discontinuation for quality-of-life improvement in cases of simple anemia (US Food and Drug Administration, 2006; Khuri, 2007). The FDA warning prompted Amgen, the manufacturer of the ESAs in question, to amend medication guides and package inserts to include warnings about associated risks of more rapid tumor progression, thrombotic events and death (US Food and Drug Administration, 2009).

The risks associated with off-target effects underscore the need for engineering Epo to be specifically targeted to erythroid precursor cells. Approaches to engineering Epo for enhanced pharmaceutical utility have involved increasing serum half-life and stability by addition of extra carbohydrate chains, disulfide rearrangement or increasing molecular weight to reduce renal clearance by fusion to Fc antibody fragment or albumin or by PEcylation (Egrie et al., 2003; Bitonti et al., 2004; Way et al., 2005; Long et al., 2006). Epo mimetic peptides have been developed and have analogously been fused to antibody fragments, and a dimeric form of Epo has been shown to have increased activity (Dalle et al., 2001; Bouman-Thio et al., 2008). However, these modifications are not expected to affect specific targeting of Epo to red blood cells or to reduce side effects relative to Epo itself.

Engineering to improve cell-type specificity of other cytokines and cytotoxic proteins has involved fusion to antibodies, antibody single-chain variable fragment (scFv) or other domains with cell-specific targets, but the application
of these approaches to Epo has not been published (Bremer et al., 2004; Schrama et al., 2006). Cironi et al. (2008) reported the development of ‘chimeric activators’, a new approach to improve cell-type specificity of cytokine ligands through mutagenesis to reduce affinity, combined with fusion to a targeting domain. The current work details the approach of this innovation to the creation of a red blood cell lineage-specific Epo fusion, and broadens the chimeric activator concept through the use of an scFv antibody fragment as the targeting element.

Materials and methods

Cell culture

TF-1 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI-1640 medium (GIBCO, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Hyclone, Logan UT), 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO) and 2 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF, Invitrogen, Carlsbad, CA) unless otherwise specified. UT-7 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen [German Collection of Microorganisms and Cell Cultures], Braunschweig, Germany) were cultured in MEM Alpha (GIBCO) with 20% FBS (Hyclone), 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO) and maintained with 5 ng/ml GM-CSF (Invitrogen), unless otherwise specified. FreeStyle™ 293F cells (Invitrogen) were cultured in FreeStyle™ 293 medium (Invitrogen) according to the manufacturer’s specifications. All cell lines were cultured at 37°C in a 5% CO2 atmosphere except 293F, which was cultured in 8% CO2 with shaking at 130 RPM. Cells were counted with a hemocytometer (Reichert, Buffalo, NY) and viability was assayed by Trypan blue dye exclusion.

Plasmid constructs

The coding sequence for the wild-type 10F7 scFv-Epo chimera was ordered from Top Gene Technologies (Quebec, Canada) with the following composition: the 246 amino acids of 10F7 scFv specific for human glycoporin A (GI:15149451) with two additional serine residues within the antibody linker, a 35 amino acid Gly/Ser linker and the 166 amino acids of human Epo (GI:5822016). This construct was subcloned using SfiI and ApaI restriction sites into pSecTag2 A vector (Invitrogen), which contains CMV promoter, murine Ig κ-chain leader sequence for protein secretion, and C-terminal c-myec epitope and His6 tag for protein purification. Thus, the encoded protein had this predicted sequence: DAAQPAQVLQKIQSGAEVLPAGASOKLSCA SGYTFSNYFMHWMKQRVPVLQGELWIMIRPNOTTIDY NEKFKNKATLTVKSSNTAYMLQLSTSGDSAVYCA RWEGRSYALDYWGGTITVVSSGGGGGSSSGGGGGSS SSDDIELTQPSAISATLGEKTVMTCRASSVNYMYW YQOKGSGPKLWIYYTSNLSAGPVFGFSGGSGTGYSL TSISVEAESATYCCQFSTSPYTFGGTTLKIEKAREA GGGGSSGSSGSSGSSGSSGSSGSSGSSGSSGSSGAP PRLCDRSVRYHELAEKEAKITTGCAEHSLNKEITV PTDKVNFWKRMREVGGQAVEVQGLALLEAVLRG QALLVKSQPGLQHVDKAVGSLRLTTLRAGL QAIEAISPDDAASAAPLRITTTTADTFKLFRVYNSNLIRGKLYTGEACRTGDGRPEQKLISEEDLNSAVDHHTHHH.

Additionally, four mutant chimeras, with Epo mutations N147A, N147K, R150A or R150E, were constructed using the Quick-Change™ site-directed mutagenesis kit (Stratagene, Cedar Creek, TX). Finally, the sequences encoding wild-type human Epo, each Epo mutant and the 10F7 scFv alone were subcloned using SfiI and ApaI restriction sites into pSecTag2 A vector. All constructs were verified by DNA sequencing. The 10F7 scFv coding sequence was submitted directly by P.D. Kassner to GenBank (AAK85 297.1), but its use has not been published previously.

Protein expression and purification

Plasmids encoding the wild-type and mutant chimeras, wild-type and mutant Epo proteins, and 10F7 scFv were prepared using the Plasmid MAXI Kit (Qiagen, Valencia, CA) and transiently transfected into FreeStyle™ 293F cells using 293fectin reagent (Invitrogen) according to the manufacturer’s protocol. After 5–6 days of culture, cells were pelleted by centrifugation at 500 g and supernatants were visualized for protein expression by Coomassie brilliant blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Coomassie-SDS-PAGE). Empty pSecTag2 A and pSecTag 2 encoding prostate-specific antigen (PSA; Invitrogen) were similarly transfected as negative and positive controls, respectively. To purify expressed protein by His6 tag affinity, 250 ml of culture supernatant was concentrated to 5 ml using Centricon Plus-70 Centrifugal Filter Device (10 000 MWCO; Millipore, Billerica, MA), bound to 0.5 ml of ProBond™ Nickel Chelating Resin (Invitrogen) for 1 h at 4°C and then applied to a Poly-Prep® Chromatography Column (Bio-Rad, Hercules, CA), washed thrice with native purification buffer (50 mM NaH2PO4, 0.5 M NaCl, pH 8.0) plus 20 mM imidazole, and eluted with native purification buffer plus 200 mM imidazole. Eluted proteins were desalted into 1 x phosphate-buffered saline (PBS) using Econo-Pac 10 DG column (Bio-Rad), concentrated to 0.5 ml by an iCon Concentrator (Pierce, Thermo Scientific, Rockford, IL) and contaminating proteins were removed by size-exclusion chromatography on a Sephacore-12 column (GE Healthcare, Little Chalfont, UK). Fractions containing the desired protein were pooled, concentrated to <0.5 ml and quantified on a ND-1000 spectrophotometer (Thermo Scientific, Rockford IL). Protein concentration was verified by Coomassie-SDS-PAGE, and presence of N-linked glycosylation on proteins was verified by digestion with PNGase F (New England Biolabs, Ipswich, MA) according to the supplier’s protocol.

Cell proliferation assays

Induction of cell TF-1 and UT-7 cell proliferation by chimeric proteins and corresponding Epo proteins was tested as follows. TF-1 and UT-7 cells were seeded in RPMI-1640 or MEM Alpha, respectively, with serum and antibiotics but without GM-CSF, into 96-well microtiter plates at a density of 1 x 104 cells in 100 µl. Cells were treated with 20 serial dilutions of engineered proteins, and commercial recombinant human Epo (rhEpo; AbD Serotec, Raleigh, NC), 10F7 scFv and vehicle (PBS) as controls. Cells were grown for 72 h at 37°C in a 5% CO2 atmosphere and cell proliferation was determined by addition of 5 µl of WST-1 Cell Proliferation Assay Reagent (Roche, Penzberg, Germany). After 2 h for UT-7 and 4 h for TF-1, the cleavage of this tetrazolium salt to formazan dye by metabolically active cells was

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measured by reading absorbance at 450 nm with background subtraction at 650 nm on a Victor^3^ V 1420 Multilabel Reader (Perkin Elmer). Resulting data were plotted and fit with logistic regression using Origin8 software (OriginLab, Northampton, MA), from which half maximal effective concentration (EC_{50}) values were obtained. Data represent the average ± SE of three to six replicates from one or two independent experiments.

**Small-interfering RNA knockdown**

Glycophorin A knockdown was performed using the human glycophorin A ON-TARGETplus siRNA SMARTpool and the ON-TARGETplus Non-targeting Pool (Dharmacon, Lafayette, CO) as negative control. Five microgram of specific or non-targeting siRNAs were transfected into 2 × 10^6 TF-1 cells using program T-001 and Nucleofector Solution T in a Nucleofector device (Lonza, Cologne, Germany) according to the manufacturer protocol. Twenty-four hours following Nucleofection, cells were treated with 0.25% Trypsin EDTA (Mediatech, Inc., Manassas, VA) for 10 min at 37°C and allowed to recover overnight before they were assayed for proliferation as described. Glycophorin A levels in treated cells were analyzed by flow cytometry.

**Antibody blocking of glycophorin A**

To measure cell proliferation during antibody blocking of glycophorin A, TF-1 cells were first incubated for 2 h in the presence of 50 nM 10F7 scFv antibody fragment in RPMI-1640 medium lacking growth factors. Cells were then assayed for proliferation as described above.

**Long-term growth assay**

To assay cell proliferation during prolonged exposure to Epo derivatives, TF-1 cells were cultured in RPMI-1640 medium, with serum and antibiotics but without GM-CSF, in the presence of 2 nM Epo derivatives. Cells were initially seeded at a density of 3 × 10^3 cells in 1 ml medium in 12-well plates, and at intervals of 2–3 days, cells were counted, assayed for viability by Trypan blue exclusion and diluted back to approximately 3 × 10^4 cells/ml in fresh medium containing the appropriate growth factor. Cell numbers and viability reported represent the average of two independent counts.

**In vitro kinase assays and western blotting**

For in vitro STAT5 phosphorylation assays, 1 × 10^6 UT-7 cells were washed in dPBS (Gibco) and resuspended in serum-free MEM Alpha (Gibco) for 4 h to induce quiescence. For competitive antibody blocking, 5 μM 10F7 scFv was added during the final 1 h. Cell aliquots were stimulated with assay proteins or controls for 20 min at 37°C in 5% CO_2_ washed in cold dPBS and lysed in 50 μl RIPA buffer (150 mM NaCl, 1% deoxycholate, 10 mM Tris–Cl, 0.1% SDS, 1% Triton X-100, 5 mM EDTA) supplemented with fresh phosphatase inhibitors (25 mM NaF, 10 mM β-glycerophosphate, 1 mM Na_2_VO_3_) and complete protease inhibitor cocktail (Roche). Total cell lysate protein was quantified using Bradford protein assay (Bio-Rad) and normalized, and 60 μg of lysate was separated on 12% SDS-PAGE, transferred to nitrocellulose and subjected to western blot analysis with phospho-STAT5 rabbit polyclonal antibody (Tyr694; Cell Signaling Technology) and actin mouse monoclonal antibody (Chemicon, Temecula, California) as an internal loading control.

**Flow cytometry**

Flow cytometric analyses were performed on a FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ). All steps were performed at 4°C and all washes and incubations were performed in FACS buffer (1 × dPBS, Gibco) supplemented with 0.5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO). To analyze the binding of 10F7 scFv on UT-7 cells, 10^5 cells incubated 45 min with 1 μM scFv, washed, incubated 30 min with 1 μg mouse anti-c-myc mAb (NeoMarkers, Fremont, CA), washed, incubated 30 min with 1:250 dilution of donkey anti-mouse IgG-fluorescein conjugate (Jackson Immunonochemicals, West Grove, PA), and washed a final time. Control populations of cells were stained using a similar protocol, but either omitting the scFv incubation or substituting 1 μg mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for mouse anti-c-myc mAb. Detection was through fluorescein isothiocyanate (FITC) channel, and populations were compared using Super-enhanced Dmax Subtraction (SED) algorithm from FlowJo flow cytometry analysis software (Tree Star Inc., Ashland, OR). Quantification of Epo receptor and glycophorin A expression was performed using the Quantibrite Phycocerythrin Fluorescence Quantitation kit (BD Biosciences, San Jose, CA). Cell labeling was performed using phycocerythrin (PE) conjugated primary antibodies (R&D Systems, Minneapolis, MN) specific for human glycophorin A or human Epo receptor, as well as PE conjugated IgG isotype controls to adjust for non-specific binding. As per supplier instructions, 10^5 washed cells were labeled with 250 ng of antibody or isotype for 30 min at 4°C and washed twice before flow cytometric analysis. Quantifications were performed in three independent experiments.

**Statistical analysis**

Where appropriate, statistical significance was determined using the pair sample t-test algorithm in Origin8 software (OriginLab) by comparing test and control sample means of replicates from proliferation experiments. The program performed a two-tailed student’s t-test given a null hypothesis that the two means were equal.

**Results**

**Molecular design of an Epo targeted to red blood cell precursors**

Cironi et al. (2008) described a method for targeting the activity of cytokine-type molecules to a subset of receptor-expressing cells. This method involved construction of a fusion protein between the cytokine portion (the ‘activity element’) and a targeting element that would bind to a distinct receptor specifically expressed on the target subset of cells to which the given activity element can bind. The method of Cironi et al. (2008) uses two key elements: first, the activity and targeting elements are connected by a linker that is long enough to allow simultaneous binding of both elements to their respective receptors on the surface of a cell; and second, the activity element contains a mutation that significantly reduces its receptor binding and activity. In general, the action of the activity element on non-target cells...
will be reduced in proportion to the strength of the mutation. However, binding to target cells will be driven by the target- ing element, and once bound, the activity element will be driven to bind to its receptor by its high local concentration in spite of the mutation that reduces binding. The current work uses a single-chain antibody as the ‘targeting element’, which greatly generalizes the applicability of the approach.

Following the system developed by Cironi et al. (2008), we sought to develop a targeted form of Epo that would stimulate red blood cell development without also acting on, for example, cancer cells that express the Epo receptor. In the present case, glycoporphin A was chosen as the erythroid-specific target because of its expression on erythroid precursors, lack of expression on other blood stem cells and probable lack of expression on all non-erythroid cancer cells (Sief et al., 1982; Okumura et al., 1992; Southcott et al., 1999).

In the red blood cell lineage, the CFU-E (colony-forming unit-erythroid) cells are generated from a precursor that divides five times, with generation times of 6–7 h in mice, to produce 32 RBCs (Landschulz et al., 1992). Epo is a survival factor during this stage—in the absence of Epo, these cells degenerate (Zamai et al., 2004). After this stereotyped cell lineage, the terminal cells go through several stages of differentiation to produce reticulocytes and mature red blood cells. This process takes about 4 days in culture and presumably in vivo (Okumura et al., 1992). When mice are treated with Epo or darbepoetin, an increase in hematocrit is seen after only 3 days, suggesting that Epo-dependent processes are occurring as late as the final CFU-E divisions and possibly early in terminal differentiation (Egrie and Browne, 2001). Based on this information, we inferred that an Epo derivative whose action is limited to later stages of erythroid development and differentiation is expected to have an effect on hematocrit.

Glycoporphin A is normally present on mature red blood cells at about $6 \times 10^5$ molecules per cell (Merry et al., 1986). Loken et al. (1987) analyzed the expression of glycoporphin on precursors to mature human red blood cells, and found that populations of cells expressing either 10-fold or 100-fold less glycoporphin than mature red blood cells included cells that divided in culture to produce red blood cells, i.e. CFU-E cells. Other studies based on immunostaining of cells have suggested that CFU-E cells do not express glycoporphin, but it is not clear whether lower levels of expression have been scored as positive in these assays (Nijhof and Wierenga, 1983; Landschulz et al., 1992; Okumura et al., 1992). Based on these observations, we infer that glycoporphin is expressed during some Epo-dependent stages of cell division and survival prior to the terminally differentiated state, and that targeting Epo activity to cells expressing glycoporphin could promote red blood cell production.

Antibody 10F7 was chosen as the targeting domain because (1) it binds to both types M and N of glycoporphin A, (2) the V region sequences are publicly available (GenBank AAK85 297.1) and (3) while the structure of glycoporphin is unknown, the binding epitope for 10F7 is within 56 amino acids from the transmembrane anchor sequence (Bigbee et al., 1983). As a point of comparison, structural study of the interaction of Epo and its receptor places the N- and C-termini of Epo about 50 A from the cell surface (Cheetham et al., 1998). The $K_a$ of the 10F7 Fab/glycophorin interaction is $9.5 \times 10^{-8}$ M (Catimel et al., 1993).

Epo mutants N147A, N147K, R150A and R150E were selected because they have been shown to exhibit reduced binding affinity and activity compared with wild-type Epo while still allowing correct protein folding (Elliott et al., 1997). In the final design, the Epo chimeric activators contain an N-terminal 10F7 scFv fused by a 35-amino acid flexible glycine–serine linker to human wild-type or mutant Epo, followed by a c-myc epitope tag and His$_6$ tag (Fig. 1).

One aspect of the design is the consideration that, in a therapeutic context, glycophorin on mature red blood cells is expected to act as a significant ‘sink’ for a 10F7 scFv-Epo fusion protein. The concentration of glycophorin in blood is effectively 5 μM and the $K_a$ of 10F7/glycophorin interaction is roughly 100 nM, so an estimated 98% of a 10F7 scFv-Epo fusion protein is expected to be bound to mature red blood cells in a reversibly inactive state (Catimel et al., 1993). A potential consequence of glycophorin binding might be to significantly extend the serum half-life of a 10F7 scFv-Epo fusion protein, analogously to engineered binding to albumin (Holt et al., 2008). This would have the advantage of reducing the frequency of injections, but could raise a safety issue with respect to overdose. This issue is best addressed with surrogate molecules in animal experiments, and is beyond the scope of the present study.

**Protein expression, purification and characterization**

Chimeric proteins and unfused Epo controls were produced in human 293F cells. After purification by nickel resin affinity to His$_6$ tag and size exclusion chromatography, between 3.3 and 8 mg/l of protein (>95% pure) was obtained for each construct (Fig. 2A). Western blot analysis verified the presence of correct protein domains (data not shown) and treatment of Epo and chimeric proteins with PNGase F showed the presence of extensive N-linked glycosylation (Fig. 2B), which has been implicated in Epo activity and stability (Dube et al., 1988; Delorme et al., 1992). Functionality of scFv domain was shown by specific binding to UT-7 cells as determined by flow cytometry (Fig. 3). The test UT-7 population scored positive for scFv binding at 88.6 and 77.8% when compared with negative controls omitting scFv or substituting isotype control for anti-myc, respectively.

**Characterization of Epo receptor and glycophorin A expression in TF-1 and UT-7**

To develop a test system for targeted activity of anti-glycophorin-Epo fusion proteins, we examined cell lines for expression of glycophorin and Epo receptor. The TF-1 and UT-7 cell lines both express Epo receptor and UT-7 expresses glycophorin A while TF-1 reportedly does not (Kitamura et al., 1989; Komatsu et al., 1991). When the presence of surface molecules was quantified via FACS, as expected, Epo receptor expression was found in both cell lines (1620 ± 140 molecules/cell for TF-1 and 7880 ± 1830 molecules/cell for UT-7), and UT-7 expressed glycophorin A (28 ± 200 molecules/cell); however, substantial glycoporphin A expression was also observed in TF-1 cells (3860 ± 780 molecules/cell). Expression of glycophorin A by each cell line was also verified by western blot (data not shown). These levels of glycophorin are much lower than the level of $6 \times 10^5$/cell on a mature red blood cell but are consistent with the idea that dividing red blood cell precursors express glycophorin at low levels (Merry et al., 1986).
Epo mutations reduce cell proliferation activity. For targeting to function efficiently, chimeric activators generally should have an activity element that binds weakly to its receptor, so that binding to a target cell is initially driven by the targeting element and subsequent binding of the activity element overcomes its receptor-binding deficit and facilitates ligand-receptor interaction. On non-target cells lacking antigen for the targeting element, the activity element has little effect due to poor affinity for its receptor.

**Fig. 1.** (A) Schematic diagram of a chimeric activator, showing the activity element with a mutation that reduces receptor binding, a flexible peptide linker, and the targeting element, depicted here as a scFv antibody fragment. (B) Molecular model/schematic of the 10F7 scFv-Epo construct used in this study, showing Epo bound to its receptor (structure 1EER), with the N-terminus of Epo connected to a 35-amino acid linker (with alpha-carbons shown as spheres) to the C-terminus of the scFv light chain. The linker between heavy and light chain V regions is omitted for clarity. The antibody-binding epitope is shown as a red segment. (C–E) Illustration of the chimeric activator principle. (C) A chimeric activator initially binds to a target cell via the targeting element. (D) Increased local concentration of the mutated activity element overcomes its receptor-binding deficit and facilitates ligand-receptor interaction. (E) On non-target cells lacking antigen for the targeting element, the activity element has little effect due to poor affinity for its receptor.

**Fig. 2.** Characterization of expressed proteins. (A) One μg of each protein (assayed by OD) was run on SDS-PAGE to verify purity and equivalence of measured concentration. Shown are 10F7 scFv-Epo fusion proteins (lanes 1–5) and corresponding non-fused Epo derivatives (lanes 6–10) with molecular weight marker (lane 11). (B) Release of N-linked carbohydrate chains by enzyme PNGase F. Shown are untreated 10F7 scFv-Epo (lane 1), treated 10F7 scFv-Epo (wild-type and mutants N147A, R150A and R150E) (lanes 2–5), untreated Epo and (lane 6) and treated Epo and mutants N147A, R150A and R150E (lanes 7–10), and molecular weight marker (lane 11). PNGase enzyme seen at 32 kDa in all treated lanes.

**Fig. 3.** Flow cytometric analysis of scFv binding to UT-7 cells. Flow cytometry histogram of FITC fluorescence intensity for binding of: 10F7 scFv (including a c-myc epitope tag), mouse c-myc primary and anti-mouse FITC conjugated secondary (thick black line); 10F7 scFv, non-immunized mouse IgG and anti-mouse FITC conjugate (thick grey line); or mouse c-myc and anti-mouse FITC conjugate (thin grey line). Histograms are scaled for comparison.

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element only occurs because of a high local concentration (Cironi et al., 2008). As a point of reference, the $K_d$ of wild-type Epo for its receptor on cells is ~1 nM, while the $K_d$ of a 10F7 scFv fragment is ~95 nM; these values suggest that for the binding of the 10F7 scFv-Epo fusion protein to be driven by the antibody element, the Epo element should have significantly reduced activity (Landschulz et al., 1992; Catimel et al., 1993). Elliott et al. (1997) characterized a series of Epo mutants with reduced activity. From this mutant collection we chose the mutants N147A, N147K, R150A and R150E because they appeared to reduce but not abolish activity, and because they involved surface residues involved in receptor contact and were therefore less likely to affect folding (Elliott et al., 1997; Cheetham et al., 1998).

To verify that Epo mutants exhibit reduced cell proliferation activity, growth of TF-1 and UT-7 cells was assayed in presence of serial dilutions of Epo wt and mutants. Cell growth was plotted against protein concentration and fit with logistic regression to obtain half-maximal effective concentration (EC$_{50}$) values. As expected, mutations N147A, R150A and R150E showed progressively lowered activity, with respective reductions of 7-, 49- and 180-fold and 7-, 23- and 150-fold in TF-1 and UT-7 cell proliferation assays, respectively, when compared with wild-type Epo (Fig. 4). Mutant N147K showed extremely reduced activity and was not used for further experiments (data not shown).

**Targeting effect of 10F7 scFv-Epo chimeric activators depends on reduction of Epo activity**

The targeting effect created by fusion of 10F7 scFv to Epo was evaluated by comparing proliferation of TF-1 and UT-7 cells induced by the 10F7 scFv-Epo chimeric proteins and the corresponding Epo controls. Upon logistic fit of proliferation data for TF-1 cells, each 10F7 scFv-Epo fusion showed higher activity than the corresponding Epo protein, with the minimal increase observed for wild-type Epo and the maximal increase observed for the mutant R150A protein (Fig. 4A–D). Similarly, the 10F7 scFv-Epo(wild-type) fusion and the Epo(wild-type) control induced proliferation of UT-7 cells to about the same extent, while the maximal activity increase due to 10F7 scFv fusion was observed for the Epo(R150A) mutant protein (Fig. 4E–H). To compare EC$_{50}$ values, the targeting effect of a chimera was defined as EC$_{50}$(10F7 scFv-Epo)/EC$_{50}$(Epo); i.e., the targeting effect due to 10F7 scFv corresponds to the fold difference between chimera and Epo activity. To compensate for the baseline targeting effect of 10F7 scFv fusion on Epo(wild-type)-targeting effects for each mutant were normalized accordingly, with the maximal normalized targeting effect of about 10-fold observed for Epo(R150A) in both cell lines (Table I).

**Long-term proliferation of TF-1 stimulated by 10F7 scFv-Epo(R150A)**

To verify that targeting was not a transient effect, long-term Epo treatment was simulated *in vitro* by culturing TF-1 cells for 16 days without GM-CSF, but in the presence of 2 nM 10F7 scFv-Epo(R150A), Epo(R150A), Epo(wild-type) or no growth factors by day 12 (Fig. 5B). This experiment illustrates that the reduction of Epo activity induced by mutation R150A can be rescued over the long term by using 10F7 scFv to target Epo to cells expressing glycophorin A.

**Targeting of mutant Epo by 10F7 scFv requires presence of glycophorin A**

To prove that targeting is a glycophorin-dependent effect, we used two methods to reduce the number of available glycophorin molecules present on the surface of TF-1 cells. First, the 10F7 scFv fragment was prebound to TF-1 cells to block binding to glycophorin A. Subsequent proliferation of these blocked cells was then assayed as described above, using Epo(R150A) and 10F7 scFv-Epo(R150A). In this experiment, activity of 10F7 scFv-Epo(R150A) was reduced by the 10F7 scFv, whereas there was no effect on the activity of Epo(R150A), even at the highest blocking concentration (Fig. 6A). Using 50 nM 10F7 scFv, the EC$_{50}$ for 10F7 scFv-Epo(R150A) increased to 11.4 nM from 4.1 nM, whereas the EC$_{50}$ for Epo(wild-type) was essentially unchanged.

Second, a siRNA pool specific for glycophorin A was used to reduce expression. TF-1 cells transfected with siRNA showed a reduction in glycophorin A expression of approximately 60%, as determined by FACS (data not shown). When proliferation of transfected cells was assayed, the activity of 10F7 scFv-Epo(R150A) was significantly reduced ($P = 0.01$) compared with control cells transfected with a scrambled siRNA pool, but no difference in activity was observed for Epo(R150A) between the two populations (Fig. 6B). For 10F7 scFv-Epo(R150A), EC$_{50}$ increased from 2.5 nM in control cells to 5.4 nM in knockdown cells, whereas EC$_{50}$ for Epo R150A was essentially unchanged.

Additionally, blocking of glycophorin on UT7 cells by 10F7 scFv competition prevented phosphorylation of signal transducer and activator of transcription 5 (STAT5) from being induced by 10F7 scFv-Epo(R150A) (Fig. 7). This mutant fusion protein showed similar potency to Epo(wild-type) or 10F7 scFv-Epo(wild-type) for induction of STAT5 phosphorylation, with signaling saturating around 10 nM (Fig. 7A and B), whereas Epo(R150A) was less potent, showing signaling saturation around 30 nM (Fig. 7A). When UT7 cells were pre-incubated with 5 μM 10F7 scFv, STAT5 phosphorylation was abolished (Fig. 7C). Taken together, these experiments indicate that the enhanced potency of mutant Epo fusions is a specific effect produced by scFv domain binding to cell-surface glycophorin A.

**Discussion**

Undesired effects of Epo during therapeutic use, including increased tumor progression rate and thrombotic events,
present significant safety concerns given the widespread use of Epo (Henke et al., 2003; Leyland-Jones et al., 2005; Drueke et al., 2006; Singh et al., 2006; Khuri, 2007; Wright et al., 2007). Various groups have generated Epo variants with increased bioactivity and serum half-life that reduce the dosing frequency, but development of an erythroid lineage-specific variant has not been described (Dalle et al., 2001; Egrie et al., 2003; Bitonti et al., 2004; Way et al., 2005; Long et al., 2006). The current work demonstrates specific targeting of Epo to cells expressing the erythroid-specific marker glycophorin A by combining antibody coupling and affinity reduction of the erythropoietin moiety.

The goal of the experiments described here was to determine whether the approach described by Cironi et al. (2008) could be applied to Epo, and to expand the field of ‘targeting elements’ to include single-chain antibody fragments. Specifically, is it possible to construct a targeted form of Epo that would only act on red blood cell precursors and not on Epo receptors that might be present on cancer cells or on precursors to platelets/megakaryocytes and other blood cells? The strategy of Cironi et al. (2008) involves constructing a fusion protein that can simultaneously bind to two proteins on the same cell surface. The activity element within the fusion protein is mutated to reduce its binding, so that the initial binding event is driven by the targeting element. While Cironi et al. (2008) provided a proof-of-concept for this idea, the experiments here applied this strategy in the context of a specific therapeutic need, and used a more practical and general targeting element.

To implement this strategy, we fused the 10F7 scFv anti-glycophorin antibody N-terminally to Epo, and also introduced mutations into the Epo moiety to reduce its receptor affinity and biological activity. (The fusion order is in

Fig. 4. Fusion of 10F7 scFv to mutant Epo causes greater enhancement of biological activity than fusion to wild-type Epo. Absorbance from WST-1 proliferation reagent, indicating cell number, was plotted against Epo-based protein concentration. TF1 cells (A–D) or UT7 cells (E–H) were treated with 10F7 scFv-Epo fusion proteins (filled boxes) or corresponding Epo proteins (open boxes). Epo variants used were human wild type (WT), and point mutations N147A, R150A and R150E. Treated cells were allowed to proliferate for 72 h after addition of Epo-based protein before addition of WST-1 reagent. Data represent mean ± SE of three replicates from one experiment and are fit with logistic regression.
principle arbitrary, and can be varied depending on spatial constraints of the simultaneous ligand-receptor interactions on the cell surface.) The scFv and Epo elements were connected via a 35-amino acid linker consisting of glycine and serine. The resulting fusion proteins and the corresponding mutant forms of Epo were tested for activity in Epo-dependent proliferation-based assays using cell lines (UT-7 and TF-1) that express both glycophorin and Epo receptor.

Based on these experiments, we identified a form of targeted Epo whose activity largely depended on binding to glycophorin. For example, the chimeric activator 10F7 scFv-Epo(R150A) stimulated cell proliferation with a potency only slightly less than that of Epo itself. Considered by itself, the R150A mutant of Epo was 20- to 50-fold less active than Epo. In contrast, when 10F7 scFv is fused to wild-type Epo, there is little or no enhancement of activity relative to unfused Epo. In control experiments where available glycophorin was reduced by siRNA inhibition of expression or by competitive antibody binding, the activity of 10F7 scFv-Epo(R150A) was correspondingly reduced, indicating that activity of this fusion is dependent on the presence of glycophorin on target cells.

Table 1. Enhancement of targeting effect by mutations that reduce Epo activity

<table>
<thead>
<tr>
<th>Form of Epo</th>
<th>Cell tested</th>
<th>EC50 (Epo alone)</th>
<th>EC50 (fusion)</th>
<th>Targeting effect due to antibody fusion</th>
<th>Normalized targeting effect due to mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>TF-1</td>
<td>0.78</td>
<td>0.29</td>
<td>2.7</td>
<td>1</td>
</tr>
<tr>
<td>Wild type</td>
<td>UT-7</td>
<td>0.47</td>
<td>0.51</td>
<td>0.92</td>
<td>1</td>
</tr>
<tr>
<td>N147A</td>
<td>TF-1</td>
<td>5.81</td>
<td>0.89</td>
<td>6.5</td>
<td>2.4</td>
</tr>
<tr>
<td>N147A</td>
<td>UT-7</td>
<td>3.23</td>
<td>0.78</td>
<td>4.1</td>
<td>4.5</td>
</tr>
<tr>
<td>R150A</td>
<td>TF-1</td>
<td>38.06</td>
<td>1.37</td>
<td>27</td>
<td>10.3</td>
</tr>
<tr>
<td>R150A</td>
<td>UT-7</td>
<td>10.59</td>
<td>1.10</td>
<td>9.6</td>
<td>10.5</td>
</tr>
<tr>
<td>R150E</td>
<td>TF-1</td>
<td>143.46</td>
<td>22.69</td>
<td>6.3</td>
<td>2.3</td>
</tr>
<tr>
<td>R150E</td>
<td>UT-7</td>
<td>72.18</td>
<td>18.34</td>
<td>3.9</td>
<td>4.3</td>
</tr>
</tbody>
</table>

EC50 values are taken from Fig. 4. The targeting effect due to antibody fusion is the ratio of EC50s for 10F7 scFv-fused vs. unfused Epos. The normalized targeting effect due to mutation is the ratio of the targeting effect seen with a given mutant form of Epo normalized to the targeting effect observed for wild-type Epo.

Fig. 5. Long-term proliferation of TF-1 cells in response to treatment with 10F7 scFv-Epo(R150A). Cells were grown for 16 days with back-dilution every 2–3 days to avoid reaching confluence. The graphs show TF-1 cells in RPMI-1640 medium with 2 nM wild-type Epo (WT; filled boxes), 10F7 scFv-Epo(R150A) (open boxes), Epo(R150A) (open circles) or no growth factors (filled circles): (A) total number of viable cells accounting for dilution and (B) percent cell viability. Data presented are mean of two counts.

Fig. 6. Targeting of 10F7 scFv-Epo(R150A) depends on glycophorin A. (A) Proliferation of TF-1 cells plotted against Epo-based protein concentration in the presence or absence of competing 10F7 scFv ('GlyA blocking'). Shown is proliferation induced by 10F7 scFv-Epo(R150A) with or without 50 nM scFv (open and filled boxes, respectively) and by Epo R150A with or without 50 nM blocking (open and filled grey circles, respectively). (B) Proliferation of TF-1 cells after siRNA knockdown of glycophorin A. Shown is proliferation induced by 10F7 scFv-Epo(R150A) of TF-1 cells transfected with scrambled siRNA control (filled boxes) or glycophorin A-specific siRNA (open boxes) and by unfused Epo(R150A) of TF-1 cells transfected with siRNA control (filled grey circles) or glycophorin siRNA (open grey circles). Data shown represent mean ± SE for three replicates and are fit by logistic regression.
N147A, R150A and R150E. Of these, R150A had the great-

four forms of Epo, ranked by activity, were tested: wild-type,
given mutation in the activity element and is useful in

targeting element and IFNa as an activity element. These

indicated that this concept may be successful for a wide set of protein
pairs, including single-chain antibodies as targeting

The previous work of Cironi et al. (2008) used EGF as a
targeting element and IFNα as an activity element. These
proteins were chosen because they are particularly well
understood with respect to their on-rates, off-rates and struc-
tures of ligand/receptor complexes, and these characteristics
appeared to be favorable. While the quantitative and struc-
tural characteristics of the Epo/EpoR interaction are also well
understood, the geometry of the glycophorin/10F7 interaction
is not known, and the equilibrium binding is relatively weak
(95 nM; Catimel et al., 1993). Nonetheless, application of the chimeric
activator concept to the 10F7 scFv-Epo fusion protein proceeded in a straightforward manner, suggesting
that this concept may be successful for a wide set of protein
pairs, including single-chain antibodies as targeting

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Fig. 7. Blocking glycophorin by 10F7 scFv reduces 10F7 scFv-Epo
signaling by as measured by STAT5 phosphorylation in UT-7 cells. (A)
Control western blot showing STAT5 phosphorylation induced in quiescent
UT-7 cells by vehicle or recombinant human Epo (lanes 1–2), 10F7
scFv-Epo(R150A) (lanes 3–7) or unfused Epo(R150A) (lanes 8–12). (B) Control
blot showing STAT5 phosphorylation induced in UT7 by unfused
wild-type Epo WT (lanes 1–5) or 10F7 scFv-Epo(wild-type) (lanes 6–10).
(C) UT7 cells or cells pre-incubated with 5 μM anti-glycophorin scFv
induced by chimera R150A when antibody pre-incubation is absent. Samples
were normalized for total protein before western blot; actin is shown as a
loading control for each panel.

To quantitate the value of a given mutation in the activity
element of such fusion proteins, it is useful to define a ‘nor-
malized targeting effect’ for a mutation as follows:

\[
\text{Normalized targeting effect (mutation)} = \frac{EC_{50} \text{ (mutant activator alone)}}{EC_{50} \text{ (wild-type activator targeted fusion)}}
\]

\[
\text{Normalized targeting effect (mutation)} = \frac{EC_{50} \text{ (wild-type activator alone)}}{EC_{50} \text{ (wild-type activator targeted fusion)}}
\]

In the case of the R150A mutation, the normalized targeting
effect was about 10-fold, based on assays in both UT-7 and

TF-1 cells (Table I). This number represents the utility of a
given mutation in the activity element and is useful in

ranked testing mutations. In the experiments described here,
four forms of Epo, ranked by activity, were tested: wild-type,
N147A, R150A and R150E. Of these, R150A had the greatest

normalized targeting effect, indicating that this effect is
not a simple function of the strength of a mutation.

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Improving cell-type specificity of erythropoietin

<table>
<thead>
<tr>
<th>A</th>
<th>Vehicle (PBS)</th>
<th>10F7 scFv-Epo(R150A)</th>
<th>Epo(R150A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Epo(WT)</td>
<td>10F7 scFv-Epo(WT)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Vehicle (PBS)</td>
<td>10F7 scFv-Epo(R150A)</td>
<td>Epo(R150A)</td>
</tr>
</tbody>
</table>

pSTAT5

Actin

Antibody blocking 5 μM 10F7 scFv

Table I


