Research Report

Generation and Characterization of Recombinant Vascular Targeting Agents from Hybridoma Cell Lines

BioTechniques 30:190-200 (January 2001)

Claudia Gottstein, Winfried Wels¹, Bertram Ober, and Philip E. Thorpe

University of Texas Southwestern Medical Center, Dallas, TX, USA, and ¹Georg Speyer-Haus, Institute for Biomedical Research, Frankfurt, Germany

ABSTRACT

Vascular targeting agents (VTAs) can be produced by linking antibodies or antibody fragments directed against endothelial cell markers to effector moieties. So far, it has been necessary to produce the components of VTAs (antibody, antibody fragment, linker, and effector) separately and, subsequently, to conjugate them by biochemical reactions. We devised a cloning and expression system to allow rapid generation of recombinant VTAs from hybridoma cell lines. The VTAs consist of a single chain Fv antibody fragment as a targeting moiety and either truncated Pseudomonas exotoxin (resulting in immunotoxins) or truncated human tissue factor (resulting in coaguligands) as effectors. The system was applied to generate recombinant immunotoxins and coaguligands directed against endoglin, vascular endothelial growth factor (VEGF):VEGF receptor (VEGFR) complex and vascular cell adhesion molecule 1 (VCAM-1). The fusion proteins exhibited similar functional activity to analogous biochemical constructs. This is the first report to describe the generation and characteri-

zation of recombinant coaguligands.

INTRODUCTION

Vascular targeting of malignant tumors has been shown to be a new potential approach for the treatment of cancer (12). The general concept of vascular targeting is to impair the ability of the tumor vasculature to supply tumor cells with nutrients and oxygen. This can be achieved by damaging the tumor endothelial cells with immunotoxins (i.e., conjugates of a truncated toxin and a targeting moiety) (4), which then results in activation of the physiological coagulation cascade at the site of injury, or, alternatively, by directly inducing thrombosis in tumor vessels with coaguligands [i.e., conjugates consisting of a coagulation inducing substance like truncated tissue factor (tTF) (10,21) and a targeting moiety]. It has been shown both in an artificial model (12) and in a mouse model for human Hodgkin's lymphoma (18) that this is a valid approach.

Advantages of vascular targeting over conventional antitumor cell therapies are: (i) the target antigen is directly accessible, and penetration of drugs through the tumor tissue, which has been defined as a major problem in the treatment of solid tumors (1,9,13), is not necessary; (ii) there is a potentiation effect because the killing of one endothelial cell results in the death of thousands of tumor cells (7) and the coagulation process is a cascade in which

one molecule of initiating coagulation factor results in the generation of millions of molecules fibrin per minute; (iii) the target cells are unlikely to mutate and to develop a drug resistance (2); and (iv) the same targeted drug can be used for a variety of solid tumors because the target antigen should be present in many different tumors.

So far, it has been necessary to produce the components of vascular targeting agents (VTAs) (antibody, antibody fragment, linker, and effector) separately and, subsequently, to conjugate them by biochemical reactions. The aim of this study was to devise a cloning and expression system for the generation of recombinant VTAs. General advantages of recombinant fusion proteins over biochemical conjugates are: (i) easy production of defined homogeneous proteins; (ii) protein engineering can be performed on the DNA level; and (iii) the constructs can be produced as entirely human proteins, which addresses the previously described problem of immunogenicity (11,16). We describe the development of a cloning and expression system to produce recombinant VTAs in the format of immunotoxins or coaguligands. Using this system, we have functionally expressed several fusion proteins directed against the tumor endothelial markers endoglin, vascular endothelial growth factor (VEGF):VEGF receptor (VEGFR) complex and vascular cell adhesion molecule 1 (VCAM-1).

Table 1. Sequences of Primers Used for V Gene Amplification of Murine or Rat Heavy and Light Chains

Heavy Chain:	
MVH-Back:	<i>HindIII</i> ATCGATAAGCTTCAG GTSMAACTGCAGSAGTCWGG
MVH-For:	<i>BstEII</i> TGAGGAGACGGTGACCGTGGTCCCTTGGCC CC
Light Chain:	
MVL-Back (ϣ):	1 GAYATTGTGMTGACMCAGWCTCCA 2 GATGTTTTGATGACCCAACTCCA 3 GACATTGTGCTRACCCAGTCTCCA 4 GACATCCAGATGACNCAGTCTCCA 5 CAAATTGTTCTCACCCAGTCTCCA 6 GAAAATGTGCTCACCCAGTCTCCA
MVL-For (ϣ):	<i>XbaI</i> ACTAGTCTAGACGTTTSAKYTCCAGCTT
MVL-Back (λ):	<i>BglII</i> ACTCAGAGATCTGCACTCACCACATCA
MVL-For (λ):	<i>XbaI</i> TACCTCTAGACCTAGGACAGTCAGYTTGGTTCC
<p>The primer sequences have been determined by comparison with known V gene sequences from Kabat or modified from published sequences (17,24). Back stands for toward the 3' end of the antibody gene, and For stands for toward the 5' end of the antibody gene; sequences are given according to the International Union of Pure and Applied Chemistry (IUPAC) nomenclature of mixed bases (R, A or G; Y, C or T; M, A or C; K, G or T; S, C or G; W, A or T; N, A or T or C or G). Note that the MVL-Back (ϣ) primers do not contain a restriction site and therefore have to be reamplified with the analogous primer containing a <i>PvuII</i> site in codons 3 and 4.</p>	

MATERIALS AND METHODS

Antibodies, Cell Lines, and Reagents

Mab Tec11 recognizes human endoglin, which is upregulated on human tumor vessels (23). Mabs GV39 and GV-59, directed against human VEGF, bind preferentially the VEGF:VEGFR complex (3). Anti-murine VCAM-1-mab MK2.7.1 and anti-human tissue factor-mab 10H10 were obtained from ATCC (Manassas, VA, USA). Anti-CD31 antibody 9G11 was from R&D Systems (Minneapolis, MN, USA). M2 antibody directed against the FLAG[®] epitope was purchased from Eastman Kodak (Rochester, NY, USA). Secondary antibodies were from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA).

Human umbilical vein endothelial cells (HUVEC) were from Clonetics (Walkersville, MD, USA). J82 cells expressing tissue factor were from ATCC. BEnd3 cells (mouse endothelial cells expressing VCAM-1 upon stimulation) were kindly provided by Dr. B. Engelhardt (MPI, Bad Nauheim, Germany). 2F2B cells, a murine endothelial cell line constitutively expressing VCAM-1, were purchased from ATCC.

Restriction enzymes were from New England Biolabs (Beverly, MA, USA), and other molecular biology reagents were from Roche Molecular Biochemicals (Indianapolis, IN, USA) unless otherwise specified. Primer oligonucleotides were prepared in the University of Texas Southwestern Medical Center (UTSW) primer synthesis facili-

ties (Dr. Johnston's laboratory).

Tissue culture reagents were from Life Technologies (Rockville, MD, USA). Chemicals, growth factors, and coagulation factors were from Sigma (St. Louis, MO, USA) unless otherwise specified.

Cloning of Vectors

The cloning vectors pww152 and pwwc1 were originally derived from a Bluescript[®] II SK+ plasmid (Stratagene, La Jolla, CA, USA). The construction of pww152 has been described previously (25). The expression vectors are derivatives of the pFLAG-1 expression vector (Eastman Kodak). The construction of psw50 and psw202 has been described (25). Cloning of pwwc1 and the new expression vectors was performed using standard cloning methods (19). To clone the gene for human tTF, RNA was prepared from J82 cells, and DNA encoding for the N-terminal 219 amino acids (extracellular domain) was amplified in a PCR using the primers 5'-CCGGGGTACCTCAGGCA-CTACAAATACT-3' and 5'-GCCAGCGAGCTCTTATTCTCTGAATTCCTTT-3', which add restriction sites *KpnI* and *SacI* at the 5' end and 3' end, respectively. Nucleotide sequences were confirmed by ssDNA sequencing.

Cloning of Fusion Protein Genes

Total RNA was prepared from hybridoma cells in exponential growth following a modified protocol described by Chomczynski and Sage (5). Details for RNA preparation and cDNA synthesis are given in Reference 8.

DNA encoding heavy and light chain variable regions was then generated from the cDNA in a PCR using primers that anneal to framework 1 and framework 4 regions (Table 1) and Expand-Polymerase (Roche Molecular Biochemicals). PCR conditions are described in Reference 8. *BstNI* fingerprinting to rule out amplification of myeloma light chains was performed as described by Clackson et al. (6).

Heavy and light chain DNAs were cloned into the plasmids pww152 or

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pwwc1 by standard techniques.

Western Blot

Supernatants of recombinant *E. coli* were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with M2 or 10H10 as detecting antibody. Western blots were developed with Supersignal chemoluminescence solution (Pierce Chemical, Rockford, IL, USA) and exposed to autoradiography film (Eastman Kodak).

Expression of Recombinant Proteins

Recombinant *E. coli* from freshly transformed plates were cultured in shaker flasks in standard growth media: LB, 2TY, 4TY, and TB, prepared according to Reference 19 in the presence of 1% (w/v) glucose. After overnight culture at 30°C, cells were pelleted and resuspended in fresh medium. Protein expression was induced with isopropyl- β -D-thiogalactoside (IPTG) for 1–2 h at 24°C in the presence of 0.5 μ g/mL leupeptin (Roche Molecular Biochemicals). Bacteria were pelleted, and periplasma preparations were obtained by exposing the bacteria to osmotic shock (modified from Reference 20). Expression conditions were optimized for each protein, comparing yield and function of small-scale samples cultured under various conditions by western blot or functional assays (described in the respective sections).

Purification of Recombinant Proteins

Affinity chromatography was performed on a Ni^{2+} -NTA column (resin from Qiagen, Valencia, CA, USA), eluting with 50–200 mM imidazole. The eluate was dialyzed against phosphate-buffered saline (PBS). When necessary, an FPLC chromatography using a Superdex™ size exclusion column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was performed as a second purification step.

Biochemical Analysis

The purified proteins were analyzed

by SDS-PAGE on 4%–15% or 8%–20% gradient gels using the Phast System® (Amersham Pharmacia Biotech) combined with Coomassie® blue staining. UV spectrograms were obtained on a scanning spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA). FPLC analysis was performed on an FPLC chromatography system (Amersham Pharmacia Biotech) using an analytical Superdex column. Protein concentrations were

calculated from the absorbance at 280 nm, including the information about the purity from Coomassie blue-stained polyacrylamide gels.

Functional Analysis

The functionality of targeting and effector moieties of the fusion proteins was tested in separate assays. To test the targeting moiety (i.e., binding to the respective antigen), we used either

Figure 1. Cloning and expression system for recombinant VTAs. See text for details.

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ELISA assays, flow cytometry, or immunohistochemical analysis, depending on the availability of appropriate cell lines, tissues, or antigens. To test the effector moieties of immunotoxins, we performed a standard protein synthesis inhibition assay. To test the effector moieties of coaguligands, coagulation assays were performed that measure the activation of factor X to factor Xa as a sign of tissue factor activity.

ELISA. Proteins directed against VEGF:VEGFR complex were tested for binding in ELISA assays as described (3) using an anti-human-endoglin scFv as a negative control. M2,

recognizing the FLAG epitope, was used as detecting antibody and goat-anti-mouse-horseradish peroxidase (HRP) conjugate as tertiary antibody.

Flow cytometry. Binding of fusion proteins directed against murine VCAM-1 were tested by flow cytometry. Murine endothelial cells of the cell lines bEnd3 (activated for 4 h with 5 ng/mL interleukin 1 α or 2F2B) were incubated with coaguligands or control antibodies. An indirect immunofluorescent staining was performed using 10H10 as secondary antibody and fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG as tertiary anti-

body. Binding was assessed on a FACS cytofluorometer (Becton Dickinson, Franklin Lakes, NJ, USA) and plotted as histograms demonstrating fluorescence intensity versus counts of cells. L540 human Hodgkin's lymphoma cells were used as an antigen negative control cell line.

Immunohistology. Proteins directed against human endoglin were tested for binding by immunohistology on human primary tumor sections (endometrium carcinoma and Hodgkin's lymphoma) using two irrelevant scFvs as a negative control. As an additional negative control, staining was also performed on guinea pig tumors induced by a guinea pig tumor cell line (Line 10). To confirm identity of blood vessels, sections were also stained with anti-VEGF:VEGFR antibody GV39 or anti-CD31 antibody 9G11. Proteins directed against the VEGF:VEGFR complex were tested for binding by immunohistology on human tumors grown in immuno-deficient mice (HT29 colon carcinoma cells in nude mice). After fixation in 100% acetone, sections were incubated with the recombinant proteins. M2 or 10H10 were used as detecting antibodies. The tertiary antibody was a goat-anti-mouse-HRP conjugate. Sections were developed with carbazole in acetate buffer (pH 5.2) plus H₂O₂ and analyzed microscopically for blood vessel staining.

Proliferation assay. ³H-Leucin incorporation assays were performed as described previously (22). Briefly, HUVEC cells (passages 5–7) were seeded in microplates at 3000 cells per well and incubated in triplicate with different concentrations of immunotoxins and controls. After 24 h, they were labeled with 1 μ Ci ³H-leucin per well for 24 h and then harvested on a glass fiber filter. Radioactivity was measured in a liquid scintillation counter. Inhibition of protein synthesis is given as percent cpm of untreated control cells. The antigen negative cell line IMR5 (human neuroblastoma) was used as a negative control line.

Coagulation assay. A two-stage coagulation assay was performed to test for activity of the effector moiety tissue factor. Tissue factor-containing samples and controls at various concentra-

Figure 2. Determination of expression conditions by western blot screening. Shown here is a western blot for the protein GV59-tTF, expressed with varying concentrations of IPTG, using two different growth media. M, molecular weight marker; lane 1, positive control (whole cell lysate); lane 2, LB medium/0.2 mM IPTG; lane 3, LB medium/0.1 mM IPTG; lane 4, LB medium/0.05 mM IPTG; lane 5, LB medium/0.05 mM IPTG; lane 6, LB medium/0.025 mM IPTG; lane 7, LB medium/0.0125 mM IPTG; lane 8, 4TY medium/0.05 mM IPTG; and lane 9, 4TY medium/0.1 mM IPTG.

Figure 3. Immunostaining of tumor vessels in human endometrium carcinoma. Staining of tumor vasculature (red color) with anti-endoglin antibody Tec11. (A) Negative control, (B) parental Tec11 antibody, and (C) recombinant Tec11-scFv.

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tions were incubated with phospholipids (either in soluble form or membrane bound). A mixture of coagulation factors containing factors VIIa, X (Sigma), and calcium ions was added. The reaction was stopped with 100 mM EDTA, and a substrate specific for factor Xa, S2675 (Chromogenix, Milano, Italy) at 0.2 mM final concentration was added. Factor Xa generation was measured at various time points as an increase in the absorbance at 405 nm on an ELISA reader.

RESULTS AND DISCUSSION

Cloning and Expression System

A modular system was devised for the cloning and expression of murine antibody V genes and fusion proteins from hybridoma cell lines. The respective plasmids are illustrated in Figure 1.

Cloning vectors. Pww152 was designed for cloning murine scFvs with κ light chains, containing the first two amino acids of κ light chains and a naturally occurring *Pvu*II site at codons 3 and 4 (according to Reference 14) for cloning $\nu\kappa$ For the cloning of murine scFvs with λ light chains, pwwc1 was constructed. The nucleotides encoding for the first two amino acids of κ light chains and the *Pvu*II site in pww152 were replaced by the first six amino acids of λ light chains and a *Bgl*III site. *Bgl*III is a naturally occurring restriction site at codon position 7 and 8 (according to Reference 14) in murine λ light chains.

Expression vectors. Pswc5 for expression of scFvs is a derivative of psw50, lacking 51 irrelevant bp between the *Xba*I site and the stop codon, which are present in psw50. Pswc4, for expression of coaguligands, was constructed in two steps. Restriction sites *Kpn*I and *Sac*I were inserted into psw50 downstream of the *Xba*I site, resulting in the plasmid pswc4'. DNA encoding for the first 219 amino acids of TF, lacking the transmembrane and intracellular domains, was then inserted into pswc4' using *Kpn*I and *Sac*I. Using the primer duplex insertion method, we constructed pswc7 for the expression of untargeted tTF from pswc4 by deletion of the

cloning site for insertion of scFvs.

The system was designed for minimization of PCR errors. Integrating the linker sequence into the cloning plasmid reduces the number of PCR steps because no overlap extension PCR step is needed and the amplified fragments are all smaller than 400 bp.

The modular nature of the cloning and expression system bears two advantages. First, easy and reliable cloning is achieved because the cloning vector is a high copy plasmid. VH and VL (κ or λ) can be cloned separately into pww152 or pwwc1. The remaining steps of scFv assembly and fusion to effectors are only subcloning procedures. Second, the system is very flexible. The targeting and effector moieties can be switched with others, allowing evolving targeting or effector moieties to be easily integrated.

Cloning of Recombinant VTAs

Using the system described above, a total of 20 different V genes has been cloned and sequenced.

We were able to amplify heavy chain V genes of all hybridomas tested so far ($n = 10$) with one MVH primer set. Since framework 1 in murine κ light chains is not as well conserved, a panel of primers had to be tested to give a good amplification (Table 1). Amplification of myeloma light chain genes was avoided by *Bst*NI fingerprinting of κ light chain PCR products.

The primers we used seem to be useful not only for hybridoma cell lines of mouse origin but also for those of rat origin because the variable regions of a hybridoma cell line from rat origin could also be successfully cloned.

In 3 out of 20 sequenced V genes, we found two or three different sequences in the same hybridoma line. For the fusion proteins described in this report, only one heavy or light chain V gene was detected from the parental hybridoma. The expression of aberrant unfunctional heavy and light chains by hybridoma cells has been described as a problem in cloning scFvs (15). We did not see expression of aberrant chains frequently; however, our experimental procedure was not designed to specifically detect these. If this prob-

lem occurs, more than one scFv per hybridoma has to be cloned and characterized, which can be performed in parallel, and therefore will not significantly increase the amount of time needed. Alternatively, a phage display-based method has been described that includes a screening step to select the functional chains (15).

Expression and Purification of Recombinant VTAs

Several scFvs and fusion proteins against endoglin, VEGF:VEGFR complex, and VCAM-1 have been expressed. A selection is shown in Table 2. First, optimal expression conditions were determined for each protein. An example is shown in Figure 2. Larger-scale *E. coli* cultures (one to several liters) were then used for the generation of recombinant proteins. Protein preparations were purified using Ni-NTA affinity chromatography. After this chromatography step, the proteins were usually between 50% and 95% pure; in the case of MK2.7-tTF, the purity was only about 10%. This protein was further purified on an FPLC size exclusion column to approximately 80% purity. FPLC analysis showed that dimer and oligomer formation occurred to a variable extent with the monomeric portion being between 30% and 90%. Protein yields ranged between 0.4 and 3.8 mg purified product per liter of *E. coli* culture (Table 2).

In addition to the proteins listed in Table 2, we have functionally expressed untargeted tissue factor and recombinant fusion proteins directed against tumor-associated antigens, which will be described elsewhere.

The method for the expression of proteins is simple and inexpensive. The ompA leader sequence in the expression vectors allows the preparation of native protein from the periplasmic space, which circumvents the process of refolding. We have also used denaturing/renaturing protocols for purification of proteins from whole cell lysates or inclusion bodies. In some cases, we saw higher yields (two- to threefold increase), but in our hands the functional activity was better preserved when native periplasmic proteins were purified (D. Aisner and C. Gottstein, unpub-

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Table 2: Single Chain Fvs and Fusion Proteins Expressed in *E. coli* and Affinity Purified

Target Antigen	Basis of Target Selectivity	Parental Hybridoma (species and subtype)	Expressed Proteins	Yields per Liter <i>E. coli</i> Culture
Endoglin (human)	proliferation marker	Tec 11 (mouse, IgM)	scFv immunotoxin	3.8 mg 0.4 mg
VEGF:VEGF-R	captured growth factor	GV39, GV59 (mouse, IgM)	scFv coaguligand	0.4 mg 0.5 mg
VCAM-1(murine)	inducible endothelial cell marker	MK2.7 (rat, IgG)	scFv coaguligand	0.7 mg 0.5 mg

Yields are given as (1/100) ×(% purity) ×(total protein concentration).

lished results).

Functional Analysis of Recombinant VTAs

Binding analysis. The anti-endoglin constructs were analyzed for binding on human tumor sections. The anti-endoglin scFv stained human tumor vasculature in the same pattern as the parental antibody. Titrating the recombinant fusion protein from 100 to 5 $\mu\text{g/mL}$ showed clear binding to blood vessels down to 5 $\mu\text{g/mL}$. Figure 3 demonstrates the staining with the parental antibody (cell culture supernatant) and the recombinant fusion

protein (here: 50 $\mu\text{g/mL}$). All negative controls (irrelevant IgM, irrelevant recombinant coaguligand, and secondary antibody only control, which is shown in Figure 3) revealed no staining of blood vessels. No specific staining was detected on guinea pig tumor sections.

VEGF:VEGFR fusion protein binding was assayed either in a VEGF-based ELISA or by immunohistology. The coaguligand GV39-tTF showed similar binding as the parental antibody in a VEGF-based ELISA. The fusion protein GV59-tTF specifically stained human tumor xenografts grown in immunodeficient mice.

Binding of the anti-VCAM-1 fusion

protein was verified by flow cytometry on murine endothelial cell lines expressing VCAM-1. The staining of the recombinant protein seemed to be weaker than that of the parental antibody, but this could be due to different secondary antibodies. No binding was seen on an antigen negative control cell line.

Effector function analysis. To test the activity of *Pseudomonas* exotoxin conjugates, which exert their function via inhibition of protein synthesis, ^3H -leucine incorporation assays were performed. These assays test both the binding and the effector activity. The anti-endoglin immunotoxin Tec11scFv-PE inhibited the proliferation of endothelial cells to a similar extent as its biochemical counterpart (Figure 4A). No significant toxicity was seen on endoglin-negative cells at these concentrations.

Effector function of coaguligands was tested in coagulation assays, where tissue factor activity was measured in terms of factor Xa activation. The coagulation activities of the fusion proteins were compared to those of the corresponding biochemical conjugate, where possible. Coagulation activities of the anti-VEGF:VEGFR fusion protein GV39-tTF and the anti-VCAM-1 fusion protein rMKT were comparable to the analogous biochemical construct. A representative experiment is shown in Figure 4B. For GV59-tTF, no biochemical construct was available for comparison.

Figure 4. Function analysis. Two representative experiments of a protein synthesis inhibition assay and a two-stage coagulation assay are shown. (A) Protein synthesis inhibition assay with recombinant and biochemically linked anti-endoglin immunotoxins (see Materials and Methods). Closed circles, Tec11-scFv; open squares, biochemical Tec11-Ricin-A; open triangles, recombinant Tec11-*Pseudomonas* exotoxin. Error bars, standard deviation of triplicate samples. (B) Indirect coagulation assay of GV39-scFv-tTF (see Materials and Methods) measuring Factor Xa activity. Closed circles, tTF (positive control); open squares, biochemical GV39-tTF; open triangles, recombinant GV39-tTF.

CONCLUSION

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In this paper, we report a cloning and expression system for the generation of recombinant VTAs. The system has been employed to functionally express a number of recombinant immunotoxins and coaguligands directed against tumor endothelial cell markers. The immunogenicity of these proteins in humans is expected to be low because the targeting moiety consists only of variable regions of antibodies and the effector moiety is a human protein. Though biochemically constructed VTAs have been reported (12,18), this is the first paper, to our knowledge, to describe the generation and characterization of recombinant coaguligands.

Pilot experiments in tumor-bearing mice have shown specific coagulation of tumor vessels in vivo with recombinant coaguligands generated using the system described here (data not shown). The most promising agents are currently further evaluated in preclinical studies.

ACKNOWLEDGMENTS

C.G. was supported by a research grant from the Deutsche Forschungsgemeinschaft (Az Go 706/1-1). This work was supported in part by National Institutes of Health grant nos. 5 RO1 CA74951-03 and 7 RO1 CA59569-06. Dr. E. Derbyshire and Dr. B. Gao provided control reagents (biochemical constructs and unconjugated tTF). We are grateful to L. Watkins, S. Park, T. Furlough, and M. Unruh for excellent technical assistance. D. Aisner and T. Wise contributed to this work as rotating students. We thank Dr. S. Ward for helpful discussions. FLAG and anti-FLAG are registered trademarks of Immunex Corporation.

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Received 29 November 1999; accepted 11 August 2000.

Address correspondence to:

Dr. Claudia Gottstein
University of Cologne
Laboratory for Tumortherapy
LF1 E4 R707
50924 Cologne, Germany
e-mail: claudia.gottstein@uni-koeln.de