Construction and Characterization of Bispecific Costimulatory Molecules Containing a Minimized CD86 (B7-2) Domain and Single-Chain Antibody Fragments for Tumor Targeting

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ABSTRACT

Efficient T-cell activation requires two signals. The first signal, which confers specificity, is provided by interaction of the T-cell receptor with peptides presented by MHC molecules. One of the second costimulatory signals is induced by binding of B7 proteins on the surface of antigen-presenting cells to CD28 on the T-cell surface. Expression of B7 molecules on tumor cells can result in the activation of tumor specific T lymphocytes and induce protective antitumor immunity. However, at present such gene-therapeutic approaches are limited by the inability to selectively target B7 gene expression to cancer cells. As an alternative approach we exploited recombinant antibody fragments to localize a costimulatory B7 molecule to the surface of tumor cells. We constructed chimeric proteins that contain in a single polypeptide chain a portion of human B7-2 (CD86) genetically fused to single-chain (sc) Fv antibody domains specific for the tumor-associated antigens epidermal growth factor receptor and the closely related ErbB2 receptor tyrosine kinase. A small recombinant fragment of human CD86 was characterized that corresponds to amino acid residues 1–111 (CD86\textsubscript{111}) of the mature protein. CD86\textsubscript{111} produced in the yeast Pichia pastoris and CD86\textsubscript{111} expressed in bacteria was functionally active and displayed specific binding to B7 counter receptors. Bacterially expressed CD86\textsubscript{111}-scFv fusion proteins specifically localized to the respective target antigens on the surface of tumor cells and markedly enhanced the proliferation of primary T cells when bound to immobilized tumor antigen.

INTRODUCTION

Human tumors of epithelial origin often overexpress members of the ErbB/EGFR\textsuperscript{2} related family of receptor tyrosine kinases. This receptor family comprises ErbB/EGFR, ErbB2/HER-2/neu, ErbB3, and ErbB4 (1). In particular, overexpression of EGFR and ErbB2 has been shown to directly contribute to malignancy (2, 3). Because of their aberrant expression on tumor cells and their accessibility from the extracellular space, these receptors are suitable targets for directed tumor therapy. Mabs specific for the extracellular domains of such receptors have been shown to interfere with signal transduction and are under evaluation for their therapeutic application in clinical trials (4). To enhance their growth inhibitory potential, recombinant single-chain derivatives of such antibodies have been constructed that target a potent cytotoxin to tumor cells expressing the appropriate receptor (5). In experimental models, this strategy has been applied successfully to reduce tumor load and eliminate metastatic cells (6). However, treatment with such antibodies and cytotoxic antibody fusion proteins does generally not result in the induction of specific antitumor immunity and cannot prevent possible tumor recurrence if disseminated tumor cells escape cytotoxic therapy. To achieve long-lasting antitumor effects, immunotherapeutic strategies have been developed that combine antibodies for specific targeting to tumor cells with cytokines or other immunomodulatory activities for the induction of tumor-specific T lymphocytes (7–9).

For optimal induction and clonal expansion, T cells require at least two activating signals (10). The first signal is antigen-specific and is generated by interaction of the T-cell receptor with peptide-bearing MHC molecules. A second, costimulatory signal is provided by binding of CD28, the major costimulatory signal receptor on CD4 T cells, to its cognate ligands B7-1 (CD80) and B7-2 (CD86), which are expressed on antigen-presenting cells (11, 12). T-cell receptor stimulation without costimulation can result in T-cell anergy or apoptosis. Although many tumor cells express MHC class I molecules and are able to present antigens, most do not provide costimulation, a possible mechanism for tumor cells to evade immune surveillance. Strategies to provide tumor cells with members of the B7 family of costimulatory molecules have led to promising results. In experimental models, rejection of antigenic tumor cells transfected with B7-1 or B7-2 genes has been demonstrated, which also resulted in protection of mice.

The abbreviations used are: EGFR, epidermal growth factor receptor; Mab, monoclonal antibody; sc, single chain; FBS, fetal bovine serum; AOX1, alcohol oxidase 1; IPTG, isopropyl β-thiogalactopyranoside; FACS, fluorescence-activated cell sorting; GST, glutathione S-transferase; PBMC, peripheral blood mononuclear cell.
against subsequent challenge with B7-negative wild-type tumors (13–16).

We recently developed an alternative approach to specifically localize costimulatory activity to the surface of tumor cells that does not require transduction or transfection of the cells with B7 gene constructs (Fig. 1). A soluble fusion protein was constructed that consisted of the extracellular domain of the human CD86 molecule (CD86111), for CD28 activation, genetically fused to an ErbB2-specific scFv antibody fragment for tumor targeting (9). Here we have refined this strategy and have characterized a minimized recombinant CD86 domain (CD86111) that, when expressed in yeast and bacteria, retains binding to B7 counter receptors. Fusion proteins containing this CD86 domain and scFv antibody fragments specific for the human EGFR and ErbB2 molecules specifically localize to the surface of tumor cells expressing the respective target receptors and, upon binding to antigen, stimulate proliferation of primary T lymphocytes.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions. CHO and CHO-CTLA-4 cells expressing glycosylphosphatidyl inositol-anchored human CTLA-4 on the cell surface (17) were maintained in MEMx with deoxyribonucleosides (Life Technologies, Inc., Karlsruhe, Germany), containing 2 mM glutamine, 50 μM β-mercaptoethanol, 10% heat-inactivated FBS, and 1 mg/ml G418 (CHO-CTLA-4). Murine renal carcinoma (Renca) cells stably expressing *Escherichia coli* β-galactosidase and either human ErbB2 (18) or EGFR (6) were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 mg/ml Zeocin, and 0.48 mg/ml G418. *Pichia pastoris* GS 115 yeast cells (Invitrogen, Groningen, the Netherlands) were propagated in buffered glycerol-complex medium and expression of recombinant proteins was induced in buffered methanol-complex medium according to the distributor’s recommendations.

Construction of CD86111 and CD86111-scFv Expression Plasmids. A cDNA fragment encoding the IgV domain of human CD86 (amino acid residues 1–111 of the mature protein; referred to as CD86111) was derived by PCR using CD86 cDNA as a template (17) and the oligonucleotides CD86-sense (5′-aatgtgagctctgctgctcctc-3′) and CD86-IgV-antisense (5′-aatactagcagctgaagcactgacagc-3′; CD86 sequence in upper case), which introduce SalI and NheI restriction sites at the 5′ end and Clal and XbaI restriction sites at the 3′ end of the PCR product. The amplified CD86111 cDNA fragment was digested with SalI and XbaI and ligated in frame to a synthetic sequence that encodes the Myc tag and a cluster of six histidine residues (His tag) in a modified pBluescript KS vector (Stratagene, Heidelberg, Germany; Ref. 17). For expression in the yeast *P. pastoris*, an NheI/NcoI CD86111 fragment including Myc and His tags was isolated from the cloning vector and inserted into the AvrII/NcoI-digested yeast expression vector pPIC9 (Invitrogen), resulting in the plasmid pPIC9-CD86111 (Fig. 2A). CD86111-scFv fusion genes were derived by isolating the ErbB2-specific scFv(FRP5) and the EGFR-specific scFv(14E1) single-chain antibody domains as Clal/XbaI fragments from plasmids pWW152-5 (5) and pWW152-14E1 (19), and inserting them between CD86 and Myc/His tag sequences in the pBluescript cloning vectors. For bacterial expression of recombinant CD86 proteins, plasmid pSW5 was constructed by replacing the *E. coli* ompA signal peptide and synthetic FLAG sequences of the vector pFLAG-1 (IBI Biochemicals, New Haven, CT) with a modified multiple cloning site. Vector backbone, Myc/His tag, and CD86111 and CD86111-scFv fragments were assembled stepwise, resulting in plasmids pSW5-CD86111 (Fig. 2A), pSW5-CD86111-5 and pSW5-CD86111-14E1 (Fig. 3A). In the yeast and *E. coli* expression plasmids, start codons for translation of the gene products are provided by the vector backbones.

Expression of Truncated CD86111 in the Yeast *P. pastoris*. The pPIC9-derived expression plasmid encodes, under the control of the methanol-inducible AOX1 promoter, the CD86111 fragment fused to an N-terminal α-factor secretion signal. In addition, the plasmid contains a functional histidinol dehydrogenase (*HIS4*) gene for positive selection in the *P. pastoris* HIS4 mutant strain GS115. pPIC9-CD86111 DNA was linearized by SalI digestion and used for transformation of *P. pastoris* GS115 cells by electroporation. His4+/methanol-utilization* (mut−) yeast colonies were isolated on selection media following established protocols (20), and the phenotype of the resulting clones was confirmed by PCR using AOX1 5′ and 3′ primers (Invitrogen).

For expression of recombinant CD86111, a single yeast colony was grown to an absorbance at 600 nm of 3 in buffered glycerol-complex medium (pH 8). The medium was then exchanged with methanol-containing buffered glycerol-complex medium of pH 8, and protein expression was induced for 72 h at 30°C. Yeast cells were removed by centrifugation at 20,000 × g. Supernatant containing soluble CD86111 protein was passed through a 45 μm filter and applied onto a Ni2+-saturated Chelexing Sepharose column (Amersham Pharmacia Biotech, Freiburg, Germany); recombinant protein specifically bound to the column via the COOH-terminal polyhistidine tag was then eluted with PBS containing 250 mM imidazole. Fractions containing the fusion protein were identified by SDS-PAGE and immunoblotting with Mab 9E10 specific for the Myc tag (21), pooled, concentrated, and dialyzed against PBS. A CD86 protein fragment comprising the complete CD86 extracellular domain (CD86122) was expressed in the yeast *P. pastoris* as...
described previously (17). N-Linked glycosylation of purified CD86$_{111}$ protein was analyzed in a deglycosylation reaction. In brief, 0.2 mg of the protein were heated to 100°C for 10 min in PBS containing 0.1% SDS. Triton X-100 at a final concentration of 1% and 1 unit of N-Glycosidase F (Boehringer Mannheim, Mannheim, Germany) were added. After incubation for 16 h at 37°C in a total reaction volume of 100 µl, samples were analyzed by SDS-PAGE and immunoblotting with Mab 9E10.

**Bacterial Expression of CD86$_{111}$ and CD86$_{111}$-scFv Proteins.** Plasmids pSW5-CD86$_{111}$, pSW5-CD86$_{111}$-5 and pSW5-CD86$_{111}$-14E1 were transformed into E. coli BL21 (DE3) trxB$^-$ (22). Single colonies were grown at 37°C to an absorbance at 550 nm of 0.8–1.0 in Luria-Bertani medium containing 0.6% glucose and 100 µg/ml ampicillin. Expression of recombinant proteins was induced with 0.2 mM IPTG for 1 h at room temperature. Cells were harvested by centrifugation at 4°C.
5000 × g for 10 min at 4°C, and a cell pellet from 1 liter of culture was resuspended in 35 ml of PBS containing 8 mM urea. Cells were disrupted in a French press, and the lysates were incubated for 45 min at 6°C. Cells were washed, resuspended in 1 ml of PBS containing 0.5% FBS, and applied on a LS- column (Miltenyi Biotech, Bergisch Gladbach, Germany) for 15 min at 6°C. Cells were incubated with 0.5 µg of CD86111-scFv(14E1) in TTBS [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20] supplemented with normal goat serum (1:100) for 1 h at 37°C. Bound CD86 fusion protein was detected by incubation with 0.5 µg of Mab 9E10 followed by 0.5 µg of FITC-conjugated goat antimouse IgG (PharMingen). Each incubation was carried out in TTBS for 45 min at 37°C and followed by washing steps. Cell nuclei were stained with 50 µg/ml propidium iodide (Roth, Karlsruhe, Germany). Coverslips were mounted on glass slides and analyzed under a fluorescence microscope (Zeiss Axioskop; Carl Zeiss, Jena, Germany).

T-Cell Proliferation Assays. Spleen cells from BALB/c mice were suspended in 900 µl/spleen of PBS containing 0.5% FBS, and incubated with 100 µl of MACS rat antimouse CD90 (Thy 1.2) antibody-conjugated colloidal super-paramagnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 15 min at 6°C. Cells were washed, resuspended in 1 ml of PBS containing 0.5% FBS, and applied on a LS-column (Miltenyi Biotech) placed in a magnetic field. CD90+ cells were eliminated from the column by rinsing with PBS containing 0.5% FBS. Subsequently, the column was removed from the magnetic field, and CD90+ T cells were eluted in 5 ml of the same buffer. The isolated T cells were kept in DMEM medium supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µM β-mercaptoethanol, 10 mM HEPES. Flat-bottomed 96-well plates were coated overnight with the mitogenic antimouse CD3 Mab 2C11 (23) alone or in combination with purified recombinant GST-ErbB2 fusion protein (9). The plates were washed with PBS, and 1 × 10^5 splenic T cells were added to each well with or without the addition of varying concentrations of soluble CD86111-scFv(FRP5). After incubation at 37°C for 60 h, the cells were pulsed with 0.25 Ci/well [3H]thymidine (NEN DuPont, Zaventem, Belgium) for 12 h. The cultures were then harvested, and the incorporation of [3H]thymidine was measured with a liquid scintillation counter (Beckman Instruments, Munich, Germany).

Human PBMCs were isolated by Ficoll gradient centrifuga-
tigation and depleted of monocytic cells by adherence to a plastic surface, and \(2 \times 10^6\) of monocytes were added to each well of flat-bottomed 96-well plates precoated with 1.2 \(\mu\)g/well of a recombinant protein comprising the extracellular domain of human EGFR (kindly provided by D. W. Schneider, Berlex Biosciences, Richmond, CA). After incubation for 3, 6, or 8 days in the presence or absence of CD86111-scFv(14E1) fusion protein, the cells were pulsed with 0.5 \(\mu\)Ci/well of \[^{3}H\]thymidine (Amersham Pharmacia Biotech) overnight. The cultures were then harvested, and the incorporation of \[^{3}H\]thymidine was measured as described above.

**RESULTS**

**Construction and Expression of Recombinant CD86111 Proteins.** We constructed yeast and bacterial expression vectors for the production of a soluble, recombinant CD86 fragment comprising 111 N-terminal amino acids of human CD86 (referred to as CD86111). This fragment includes the IgV-like but not the IgC-like domain of the protein (24–27). A cDNA fragment encoding CD86111 was derived by PCR and inserted into the yeast vector pPIC9 for expression of CD86111 as a secreted protein. The resulting plasmid pPIC9-CD86111 is shown schematically in Fig. 2A and encodes under the control of the methanol-inducible AOX1 promoter the CD86 fragment fused to an N-terminal \(\alpha\)-factor secretion signal from yeast, a COOH-terminal Myc epitope recognized by Mab 9E10, and a polyhistidine cluster, which facilitates protein purification (17).

*P. pastoris* GS115 cells were transformed with the expression construct, recombinant clones were isolated on selection media, and the phenotype of the clones was confirmed by PCR following established protocols (17). Expression of CD86111 was induced by the addition of methanol, and recombinant protein was purified from culture supernatants via Ni\(^{2+}\) affinity chromatography. In SDS-PAGE analysis, the purified material migrates as a smear of bands ranging from 30 to 50 kDa (Fig. 2B, Lane 3). Mab 9E10 immunoblot analysis confirmed that the smear of bands represented recombinant CD86111 (Fig. 2B, Lane 2; Fig. 2C, Lanes 1 and 3). Bands of higher apparent molecular mass represented differentially glycosylated forms of yeast-expressed CD86111, as indicated by the increased electrophoretic mobility of the protein upon N-glycosidase F treatment (Fig. 2C, Lane 2).

For the generation of unglycosylated CD86111 protein, a bacterial expression system was used. The CD86111 cDNA fragment and Myc and polyhistidine sequences were assembled in a pFLAG-1-derived *E. coli* expression vector, resulting in the plasmid pSW5-CD86111 (shown schematically in Fig. 2A). After induction of cytoplasmic expression in *E. coli* cells harboring the plasmid, the cells were lysed under denaturing conditions, and recombinant CD86111 protein was purified by Ni\(^{2+}\) affinity chromatography and refolded by dialysis. In SDS-PAGE and immunoblot analysis, the bacterially expressed, purified CD86111 protein was detected as a single band of the expected molecular mass (Fig. 2C, Lane 4).

**Functional Characterization of Recombinant CD86111 Proteins.** Full-length CD86 is expressed as a transmembrane protein and binds to the B7 counter receptors CD28 and CTLA-4 on the surface of T cells (24, 25, 28). To examine the functionality of truncated, soluble CD86111 protein, the interaction of recombinant protein with B7 counter receptors was investigated by FACs analysis using CHO-CTLA-4 cells. These cells stably express the extracellular domain of CTLA-4 attached to the cell membrane via a glycosylphosphatidylinositol anchor (17). CHO-CTLA-4 cells were incubated with CD86111 proteins purified from yeast culture supernatants or bacterial lysates. Yeast-expressed CD86111 protein, which represents a soluble fragment of the complete extracellular domain of CD86 (17), was included as a control. Specifically bound protein was detected with Mab 9E10 and FITC-labeled goat antimouse IgG. Binding of CD86111 as well as truncated CD86111 expressed in yeast and *E. coli* to CHO-CTLA-4 cells but not to CHO control cells could be detected (Fig. 2D). These results demonstrated that the IgV-like domain of CD86 is sufficient to facilitate binding to B7 counter receptors similar to a CD86 protein containing IgV-like and IgC-like domains. Thus, glycosylation of the protein is not required because the bacterially expressed CD86111 protein bound to CTLA-4 with comparable efficiency.

**Construction and Bacterial Expression of Chimeric CD86111-Antibody Fusion Proteins.** To localize the CD86 domain selectively to the surface of tumor cells, chimeric fusion proteins were constructed that contained an N-terminal CD86111 fragment fused to recombinant single-chain antibody (scFv) fragments with specificity for EGFR and ErbB2 (Fig. 1). cDNA fragments encoding the EGFR-specific scFv(14E1) and the ErbB2-specific scFv(FRP5) antibody domains were derived from previously described constructs (5, 19) and assembled with CD86111, Myc, and polyhistidine sequences in the bacterial expression vector pSW5. The resulting constructs pSW5-CD86111-5 and pSW5-CD86111-14E1 are shown schematically in Fig. 3A. Cytoplasmic expression of CD86111-scFv proteins was induced in *E. coli* cells harboring the plasmids by the addition of IPTG; the cells were then lysed under denaturing conditions, and recombinant proteins were purified by Ni\(^{2+}\) affinity chromatography followed by refolding. After a single round of purification, the typical yield of proteins was 1 mg for CD86111-scFv(FRP5) and 3 mg for CD86111-scFv(14E1) per liter of original bacterial culture with a purity of \(70–90\%\), as determined by SDS-PAGE and Coomassie brilliant blue staining (Fig. 3B). The identity of chimeric CD86111-scFv proteins was confirmed by Mab 9E10 immunoblot analysis (Fig. 3C).

**Characterization of CD86111-scFv Binding Specificity.** In the chimeric CD86111-scFv(FRP5) and CD86111-scFv(14E1) proteins, antibody domains were included as a means for anchoring and presentation of the CD86 domain on the surface of cells via binding to the respective target antigen. The functionality of the scFv domains was examined by FACs analysis using murine Renca-lacZ/ErbB2 and Renca-lacZ/EGFR cells stably expressing human ErbB2 and EGFR, respectively (6, 18). The cells were incubated with purified CD86111-scFv(FRP5) and CD86111-scFv(14E1) proteins. Cells treated with CD86111 protein lacking a scFv domain served as a control. Specifically bound proteins were detected with Mab 9E10 and FITC-labeled goat antimouse IgG. The results are shown in Fig. 4.
cells was also analyzed (A and goat antimouse IgG. As a control, the binding of CD86 scFv to CTLA-4 was investigated by FACS analysis with Mab 9E10 and FITC-labeled goat antimouse IgG. Control cells were incubated with secondary antibodies in the absence of CD86 scFv proteins (A and B, open peaks).

Fig. 4  Binding of CD86 scFv fusion proteins to the surface of tumor cells. The binding of purified CD86(scFv(FRP5) to ErbB2-expressing Renca-lacZ/ErbB2 cells (A, filled peak) and of purified CD86(scFv(14E1) to EGFR-expressing Renca-lacZ/EGFR cells (B, filled peak) was detected by FACS analysis with Mab 9E10 and FITC-labeled goat antimouse IgG. As a control, the binding of CD86 control protein to the cells was also analyzed (A and B, open peaks).

C, immunofluorescence microscopy of Renca-lacZ/EGFR cells incubated with CD86(scFv(14E1) followed by Mab 9E10 and FITC-labeled goat antimouse IgG. D, Renca-lacZ/ErbB2 control cells incubated with EGFR-specific CD86(scFv(14E1) and secondary antibodies.

4. Specific binding of CD86(scFv(FRP5) to ErbB2-expressing tumor cells (Fig. 4A) and CD86(scFv(14E1) binding to EGFR-expressing tumor cells (Fig. 4B) was observed. The CD86 control protein did not bind to either tumor cell line. Upon treatment of adherent Renca-lacZ/EGFR cells with CD86(scFv(14E1) and subsequent immunofluorescence microscopy, strong membrane staining was found (Fig. 4C), whereas Renca-lacZ/ErbB2 control cells incubated with the EGFR-specific CD86(scFv(14E1) and secondary antibodies displayed only weak nuclear staining (Fig. 4D), most likely because of cross-reactivity of Mab 9E10 with endogenous murine c-Myc (29). Binding of the CD86(scFv proteins to their target receptors was also confirmed by ELISA (19). The apparent binding affinities of CD86(scFv(14E1) to EGFR and CD86(scFv(FRP5) to ErbB2, calculated as the half-maximal saturation value, were 32 and 150 nM, respectively (data not shown).

To confirm that the chimeric CD86(scFv fusion proteins are bispecific and in addition to tumor cell recognition facilitate interaction with B7 counter receptors, the binding of CD86(scFv(FRP5) and CD86(scFv(14E1)) to CTLA-4 was investigated by FACS analysis. CHO-CTLA-4 cells were incubated with purified CD86(scFv(FRP5) and CD86(scFv(14E1)) proteins. Specifically bound protein was detected with Mab 9E10 and FITC-labeled goat antimouse IgG. As shown in Fig. 5, binding of both CD86(scFv proteins to CTLA-4-expressing CHO cells could be demonstrated. These results illustrate that both the B7 domain and the tumor cell recognition domain of the fusion proteins are functional.

Costimulatory Activity of Chimeric CD86(scFv) Protein. We have previously shown that recombinant human CD86 comprising the complete extracellular domain of the protein (CD86) provides a costimulatory signal to murine T cells when immobilized on a plastic surface but not in solution (9, 17). Similarly, truncated yeast or bacterially expressed CD86 proteins characterized in this study enhanced the proliferation of murine T cells when coated on a plastic surface together with an anti-CD3 antibody (data not shown). In the case of the chimeric CD86(scFv proteins, binding to their target antigens is intended to immobilize the B7 domain in high density on the surface of tumor cells, thereby facilitating multiple contacts with neighboring T cells and enabling the CD28 clustering required for efficient costimulation (30).

To test the dependence of CD86(scFv-mediated co-stimulation on the binding to the scFv target antigen, we designed a simplified in vitro assay. Mitogenic 2C11 anti-CD3 antibody (23) and bacterially expressed GST-ErbB2 fusion protein representing the epitope recognized by scFv(FRP5) (9) were immobilized on the surface of tissue culture plates. Splenic T cells from BALB/c mice were added and incubated for 60 h in the presence of purified CD86(scFv(FRP5) protein. Control cells were grown in the absence of GST-ErbB2, anti-CD3, and/or CD86(scFv(FRP5). T-cell proliferation was then measured by [3H]thymidine incorporation. The results are shown in Fig. 6A. CD86(scFv(FRP5) alone had no effect on T-cell growth, whereas stimulation of the cells with anti-CD3 antibody alone resulted in a basic level of T-cell proliferation, similar to previous observations (17). In the presence of immobilized anti-CD3 antibody, addition of CD86(scFv(FRP5) resulted in markedly enhanced T-cell proliferation. For this
effect, the presence of immobilized GST-ErbB2 was required, indicating that CD86\(_{111}\) scFv(FRP5) clustered by binding to the target antigen, but not monovalent CD86\(_{111}\) scFv(FRP5) in solution, elicits costimulatory activity and mimics the effect of natural transmembrane B7 on antigen-presenting cells.

The costimulatory activity of the EGFR-specific CD86\(_{111}\) scFv(14E1) protein was tested in a similar assay using human PBMCs. The cells were grown for 3, 6, or 8 days in the presence of purified CD86\(_{111}\) scFv(14E1) on tissue culture plates precoated with a recombinant protein comprising the extracellular domain of human EGFR (19). Control cells were grown in the absence of the CD86 fusion protein. T-cell proliferation was then measured by \[^{3}H\]thymidine incorporation as described in “Materials and Methods.” Each value was determined in triplicates. Bars, SD.

**Fig. 6** Costimulation of primary T cells by CD86\(_{111}\)–scFv fusion proteins. A, costimulatory activity of CD86\(_{111}\)–scFv(FRP5). Mitogenic 2C11 anti-CD3 antibody and recombinant GST-ErbB2 fusion protein were immobilized on 96-well plates as indicated. Splenic T cells from BALB/c mice were added and grown in the presence of purified CD86\(_{111}\)–scFv(FRP5) protein. Control cells were grown in the absence of GST-ErbB2, anti-CD3, and/or CD86\(_{111}\)–scFv(FRP5) as indicated. B, costimulatory activity of CD86\(_{111}\)–scFv(14E1). Recombinant EGFR protein was immobilized on 96-well plates (1.2 \(\mu\)g/well). Human PBMCs were added and grown in the presence or absence of purified CD86\(_{111}\)–scFv(14E1) protein for the indicated time periods. Proliferation of murine and human cells was measured by \[^{3}H\]thymidine incorporation as described in “Materials and Methods.” Each value was determined in triplicates. Bars, SD.

**DISCUSSION**

Efficient activation of T cells requires interaction of the T-cell receptor with peptides presented by MHC molecules and induction of a costimulatory signal by binding of B7 proteins on the surface of antigen-presenting cells to CD28 on the surface of the T cells (11, 12). Direct expression of B7 molecules on tumor cells can result in the activation of tumor-specific T lymphocytes and induce protective antitumor immunity. Strategies for the presentation of B7 proteins on cancer cells include transduction or infection of the cells with viral vectors (13–15) and direct injection of B7 plasmid constructs (31). However, the broad application of such gene therapeutic approaches is limited by the inability of available vectors to selectively target B7 gene expression to the tumor.

The possibility to generate functional recombinant B7 proteins might provide a basis for novel therapeutic concepts. The transmembrane and intracellular domains of CD80 and CD86 are not required for their costimulatory activity. When expressed in mammalian cells, recombinant proteins comprising the extracellular domain of B7 molecules retain the ability to bind to B7 counter receptors on the T-cell surface (32, 33). We have previously demonstrated that functional soluble forms of human CD80 (CD80\(_{208}\)) and CD86 (CD86\(_{225}\)) can also be produced in recombinant form in the yeast *P. pastoris*. When coated to a plastic surface, such proteins transmit a costimulatory signal and in the presence of immobilized anti-CD3 antibody facilitate the proliferation of primary T cells (17). Here we have characterized a smaller recombinant fragment of human CD86 that corresponds to amino acid residues 1–111 (CD86\(_{111}\)) of the mature protein. The extracellular domains of CD80 and CD86 contain an IgV-like and an IgC-like domain (26). Whereas for CD80 sequences, both domains appear to be critical for CD28 binding (34), in the case of CD86, the IgV-like domain contained within CD86\(_{111}\) is sufficient for functional activity (this report and Ref. 27). Thus, for CD86\(_{225}\) and CD86\(_{111}\) from yeast or CD86\(_{111}\) produced in bacteria, no obvious differences in the binding to CTLA-4-expressing CHO cells were noted. Likewise, the proteins displayed similar costimulatory activity when immobilized on a plastic surface (Ref. 17 and data not shown). This indicates that previous difficulties in isolating functional recombinant CD86 from *E. coli* were not attributable to the absence of
glycosylation in bacterially expressed protein but most likely were attributable to folding problems, which appear to be more severe for bacterially expressed CD86_{111} (17) than for the shorter CD86_{111} molecule analyzed here.

Combining the ability of antibodies to specifically localize to tumor-associated antigens on the surface of cancer cells with the potent costimulatory activity of recombinant B7 molecules might overcome present limitations of B7 gene therapeutic strategies and antibody-based therapies. Here we report the construction of fusion proteins that contain in a single polypeptide chain the costimulatory IgV-like domain of human CD86 and scFv antibody domains for specific tumor targeting. EGF-R and the closely related ErbB2 receptor tyrosine kinases were chosen as suitable targets for such molecules on the tumor cell surface because of their enhanced expression on a wide variety of human tumors and their involvement in malignant transformation (2, 3). We demonstrated that the bacterially expressed CD86-scFv fusion proteins are bifunctional. They localize specifically to the respective target antigens on the surface of tumor cells and interact with natural B7 counter receptors. Importantly, as shown for the ErbB2-specific CD86-scFv(FRP5) protein, the molecule was able to markedly enhance the proliferation of primary T cells when bound to immobilized tumor antigen, but not in solution. Likewise, the EGFR-specific CD86-scFv(14E1) protein strongly enhanced the proliferation of human PBMCs grown in the presence of immobilized EGFR. This suggests that in an in vivo situation, T-cell activation would be restricted to the tumor site because the multiple contacts between B7 molecules and CD28 required for CD28 clustering and activation (30) could be provided by cell-bound, but not circulating, monovalent fusion protein.

Because of their smaller size, recombinant scFv antibody fragments penetrate tumors more easily than whole antibodies (35). This will also be of importance for the in vivo application of reagents that link antibodies with costimulatory activity (9, 36, 37). Here we showed that the CD86_{111} fragment comprising the IgV-like domain of human CD86 is sufficient to provide tumor-specific scFv antibody fragments with costimulatory effect functions, thereby restricting these chimeric molecules to the minimal functional domains required for activity. Such B7-scFv fusion proteins can be expressed in yeast or E. coli, a potential advantage for their production at higher yields and for further evaluation of their potential for specific cancer immunotherapy in suitable in vivo models.

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