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Functional Construction of the Anti-Mucin Core Protein (MUC1) Antibody MUSE11 Variable Regions in a Bacterial Expression System

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A bacterial expression system for the variable region fragments (Fvs) of the anti-MUC1 tumor antigen antibody MUSE11 has been constructed. The Fv fragment showed binding specificity toward TFK-1 cells, with slightly reduced affinity compared to its parent IgG. The single-chain Fv fragment was arranged in two orders, VH-linker-VL and VL-linker-VH. However, linking the regions with a flexible peptide linker $(GGGGS)_3$ or with a shorter linker (GGGGS) led to a dramatic decrease in the biological activity toward the target antigen in both arrangements, suggesting that the MUSE11 antibody loses its activity when the domains are linked with polypeptide linkers. These results indicate that the variable region domains of the anti-MUC1 antibody MUSE11 have specificity only in the Fv form, and that linking the domains strongly reduces the association with its target antigen. Gel filtration analysis indicates that the scFv has a dimeric structure, suggesting that the inactivation of MUSE11 scFv is due to unfavorable intermolecular associations of the scFv chains. To our knowledge, this is the first report of a significant reduction in affinity caused by linking the variable domains in both arrangements, *i.e.*, VH-VL and VL-VH.

Key words: flow cytometry, Fv, MUC1, refolding, scFv.

Recent developments in bacterial expression systems for antibody fragments have made antibodies more feasible targets for engineering [*e.g.* construction of bispecific and/or bivalent antibody fragments (*1–4*), improvement of antibody specificity and affinity (*5, 6*), and enzyme or functional protein-linked antibody fragments (*7–10*)]. The application of antibody engineering to therapy or diagnosis has been investigated extensively (*2, 4, 11, 12*).

Because of their smaller molecular size, the utilization of variable antibody fragments (*i.e.* Fv) is attractive in terms of rapid pharmacokinetics, low immunogenicity, and high tumor penetration (*2, 11–13*). The non-covalent association of Fv fragments, however, has often been relatively weak, and, thus, linking the variable domains with flexible or designed linkers has been attempted to produce physically stabilized Fv fragments (often called single-chain Fv, scFv) (*13–15*).

One of the mucin core proteins, Mucin1 (MUC1), has attracted interest as a potential target for immunotherapy. The cloning of MUC1 cDNA has reported, revealing that it is a type I transmembrane protein with an extracellular domain consisting of a large number of 20-amino acid tandem repeats (*16, 17*). It has become evident that the continuous amino acid sequence PDTRP represents the core epitope of various MAbs against MUC1, almost all of which were prepared against breast cancer-associated antigens (*18*). MAb MUSE11 against adenocarcinoma also has shown to react with the tandem repeat of MUC1, and especially to recognize PDTRPAGP as an epitope (*19, 20*). This PDTRPAGP sequence has been shown to be highly immunogenic, and, therefore, the interaction between MUSE11 and this peptide is intriguing, not only from the viewpoint of immunotherapy, but also in terms of antigen–antibody interaction. A bacterial expression system would provide a powerful method for these concerns. In addition, the engineering of MUSE11 Fv for diagnosis could then be conveniently performed.

Here we report the construction of an expression system for mouse MAbs MUSE11 variable region fragments in *Escherichia coli*. The prepared Fv was found to have biological function almost identical to the IgG. Linking the domains with a flexible polypeptide linker, however, leads to a drastic reduction in its activity.

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Abbreviations: VH, variable region of immunoglobulin heavy chain; VL, variable region of immunoglobulin light chain; Fv, fragment of immunoglobulin variable regions; scFv, single-chain Fv fragment; IPTG, isopropyl β-D-thiogalactopyranoside; PAGE, polyacrylamide-gel electrophoresis; SDS, sodium lauryl sulfate; GuHCl, guanidine hydrochloride; FITC, fluorescein isothiocyanate; IMAC, immobilized metal affinity chromatography.

MATERIALS AND METHODS

Oligodeoxyribonucleotide Primer—Primers for cDNA synthesis, mouse V-gene amplification by PCR, and the preparation of single chain Fv gene were designed according to the partial sequence data of Hinoda *et al.* (*21*). The oligodeoxyribonucleotides were obtained from Nippon Gene Laboratory (Sendai) and purified by polyacrylamide gel electrophoresis.

cDNA Synthesis, PCR Amplifications of Variable Region Genes, and Construction of a Single Chain Fv Gene—The hybridoma cell line MUSE11, producing a mouse monoclonal antibody (IgG1) raised against adenocarcinoma, was used as the source of variable region genes. Total RNA was isolated from hybridoma MUSE11 cells using RNAzol B (Biotecx Laboratories) and the first strand cDNA syntheses were primed with either an IgG constant region primer for heavy chain or a mixture of κ constant region primers for light chains.

The PCR amplifications of each family of VH and Vκ were performed separately using Ig prime (Novagen). The amplified V-genes were cloned into pGEM-T easy vector (Promega), and the DNA was sequenced using an Auto Read Sequencing Kit (Amersham-Pharmacia) according to the recommendations of the manufacturer.

For the assembly of the VH and VL domains of MUSE11, the genes encoding HyHEL-10 VH and the domain of the vector pNSX-2, which has been constructed for the assembly of anti-hen egg-white lysozyme antibody, HyHEL-10 variable domains, were replaced by MUSE11 VH and VL, respectively (Fig. 1). The gene encoding scFv in the order VL-linker-VH was constructed in the same way (*22*).

Expression and Preparation of Soluble ScFv—A plasmid for the overproduction of the MUSE11 scFv fragment was constructed as follows; the *Nco*I–*Sac*II fragment of a selected plasmid containing a complete scFv gene (pMUSE-1), was inserted into the *Nco*I–*Sac*II digested pSNE-4 (*23*), which was constructed for the high expression of the single-chain antibody of HyHEL-10 in *E. coli* (designated as pST-1). For rapid purification of the overexpressed products and determination of specificity, the gene encoding c-myc peptide and 6xHis was added into the *Xba*I–*Bam*HI site of pST-1 (designated as pST-2) (Fig. 1). Two expression vectors for the secretory expression of MUSE11 VH or VL were constructed by deleting of VL or VH fragment from the plasmid, pST-2, respectively. The resulting plasmid for the expression of VH or VL was designated as pST-2-VH and pST-2-VL, respectively. The expression vector for the co-expression of VH with VL was constructed by replacing HyHEL-10 VH and VL chains in the vector, pKTN-2 (*23*) with MUSE11 VH and VL. The gene was tandemly arranged with SD-pelB-MUSE11VH-SD-pelB-MUSE11VL, designated as pST-3 (Fig. 1).

The transformant *E. coli* strain BL21(DE3) harboring (*24*) each expression vector was grown at 28°C in 2× YT (*25*) supplemented with 200 μg/ml ampicillin to the early stationary phase. To induce the expression of soluble scFv, IPTG was added to 1 mM and culture was grown overnight at 28°C. From 200 ml of culture, four fractions (bacterial supernatant, periplasmic, intracellular soluble, and insoluble fractions) were separated as follows (*23*). After the removal of the culture supernatant by centrifugation, the cell pellet was resuspended in 10 ml of 20 mM Tris-HCl (pH 7.5), 0.5 M sucrose, 0.1 mM EDTA and incubated for 5 min at room temperature; then, 40 ml of water was added to produce osmotic shock and the culture was left on ice for 30 min. The cells were centrifuged and the supernatant was saved as the periplasmic fraction. The remaining cells were sonicated after the addition of 20 ml of water and centrifuged to separate the soluble cytoplasmic fraction. The insoluble fraction obtained in the previous step was solubilized in 10 ml of 0.1 M Tris-HCl (pH 8.0) containing 6 M guanidine hydrochloride (GuHCl) by stirring overnight at 4°C. After centrifugation, the supernatant was used as the protein sample obtained from the "intracellular insoluble fraction." All fractions were stored at 4°C.

SDS-PAGE and Western Blotting—One milliliter of culture was taken for analysis. The total proteins in each fraction were precipitated with 6% trichloroacetic acid and 0.083% deoxycholate, and subjected to protein analysis by SDS-PAGE in the same buffer system described by Laemmli (*26*).

In order to detect scFv or Fv samples of the *E. coli* cultures were subjected to SDS-PAGE (15%) followed by electroblotting onto nitrocellulose (Amersham-Pharmacia). The nitrocellulose filters were incubated in blocking buffer [TBS containing 0.05% Tween-20 (Bio-rad), 4% skim-milk (Difco)] at room temperature for 1 h, then incubated in the same buffer containing mouse monoclonal antibody His-Probe (Invitrogen, Japan) directed against 6xHis peptide tag for another hour. The scFv or Fv was finally detected by incubating the filter in the same buffer containing a 1:5,000 dilution of peroxidase-conjugated mouse antibody against mouse IgG, and an ECL detection system (Amersham-Pharmacia).

Purification of Variable Region Fragments by Immobilized Metal Affinity Chromatography (IMAC) under Denaturing Conditions—The insoluble fraction was solubilized in 10 ml of PBS with 6 M GuHCl by stirring overnight at 4°C. After centrifugation, the supernatant was applied to a column of 2 ml of His-bind Resin (Novagen) pre-equilibrated with the same buffer. The column was washed extensively with phosphate-buffered saline (PBS) containing 6 M GuHCl and 20 mM imidazole, and then eluted step-wise with PBS containing 6 M GuHCl and 100 mM imidazole.

Refolding of Insoluble Variable Region Fragments—Denatured variable region fragments eluted from the metal affinity resin were diluted with 6 M GuHCl/PBS to a concentration of about 0.1 mg/ml (corresponding to OD_{280} = 0.2). In the case of the Fv fragment, whose chains were expressed separately, VH and VL were mixed stoichiometrically at the same concentration. In general, the refolding procedures followed the method of Tsumoto *et al.* (*27*). In brief, the denatured purified and reduced variable region fragments (10 ml) were removed by phased-guanidine removing dialysis with GuHCl/PBS (500 ml). The concentration of GuHCl in the dialysis buffer was lowered sequentially (3, 2, 1, 0.5, and 0 M). An oxidizing reagent (glutathione, oxidized form, Sigma) was introduced in 1 and 0.5 M GuHCl/PBS dialysis buffer, and 0.4 M of L-Arg was added during the final dialysis stage. The refolded solution was centrifuged at 4,500 ×*g* for 20 min. The supernatant was concentrated within the range of 0.1–0.2 mg/ml using ultrafiltration membrane centriprep-10 (Amicon Inc., Bev-

erly, MA) and stored at 4°C until flow cytometric analysis.

Gel Filtration—Gel-filtration of MUSE11 Fv and scFv was performed using a Superdex 75 column (Amersham-Pharmacia) (1.0 × 100 cm) connected to an FPLC system pre-equilibrated with PBS. Molecular masses were calibrated with calibration proteins for gel chromatography combithek (Boehringer Mannheim, Germany). One milliliter of refolded variable region fragments adjusted to 0.2 mg/ml was subjected to gel filtration at a flow rate of 0.5 ml/min.

Cell Lines—The human bile duct carcinoma (BDC) cell line (TFK-1), reactive with MUSE11 mAb, was used as a target and a human hepatocellular carcinoma cell line (HT-17) as a control (*28*). A MUC1-overexpressing CHO cell line (CHO-MUC1) was obtained by transfecting cells with APR MUC1 cDNA (*29*).

Flow Cytometry—In order to determine the binding ability of scFv or Fv to cells, flow cytometry was carried out. TFK-1 cells (5×10^5) were incubated first with 50 μl of scFv (10 μg/ml), and then with mouse anti-c-myc 9E10 (Santa Cruz Biotech, CA). Finally, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (BioSource International, Camarillo, CA) was used. In the case of MUSE11 IgG binding, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was added after the incubation of 50 ml of IgG (10 μg/ml) with TFK-1 cells (5×10^5). The stained cells were analyzed by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA).

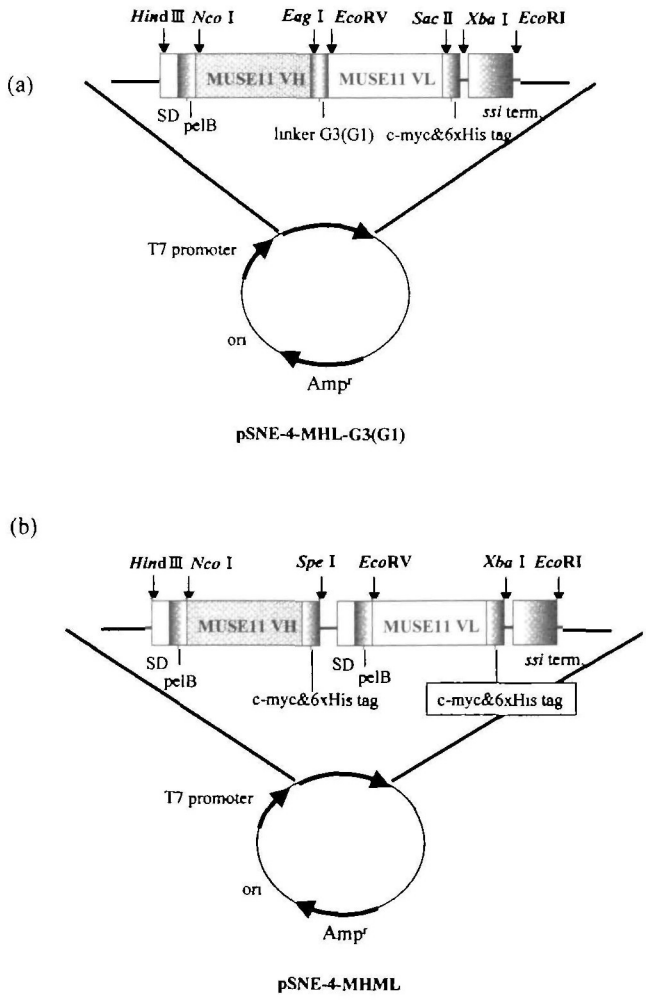

Fig. 1. **Expression vectors of MUSE11 variable domains in *E. coli*.** Schematic representation of the plasmid DNA encoding the MUSE11 variable domains. The genes are under the control of the T7 promoter. Each gene has a *pelB* signal sequence for secretory expression in *E. coli*, c-myc peptide for detection of affinity by monoclonal antibody 9E10, and a 6xHis tag for rapid purification of the gene products. (a) scFv. (b) Fv.

RESULTS

Vector Construction—The antibody variable regions in the cDNA constructed from the mRNA of MUSE11 hybridoma cells were amplified using commercially available primers (Ig primer kit, Novagen, Madison, WI). PCR products of both chains were sequenced, and the results were identical to the sequence described previously (*20*).

The amplified genes were used to construct expression vectors for Fv and scFv fragments. As shown in Fig. 1, three plasmids were constructed for the expression of the variable region fragments as follows: (i) Fv, (ii) scFv (VH-

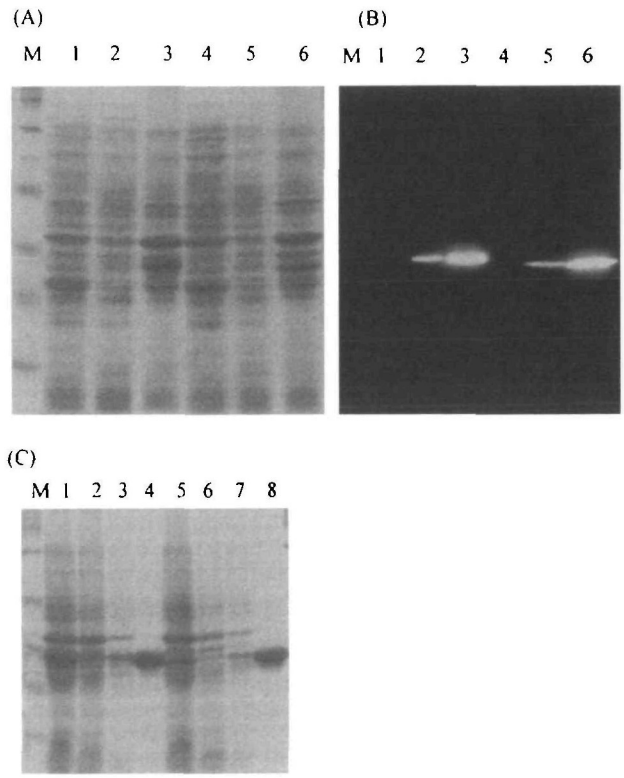

Fig. 2. **Expression and purification of MUSE11 single chain antibody (scFv).** (A) SDS-polyacrylamide gel electrophoresis (PAGE) and (B) Western blotting of proteins from different fractions of *E. coli* BL21 (DE3) cells are shown. Cells from 20 μl of overnight culture were subjected to 12.5% PAGE, and the gel was stained with CBB-R250. Lanes: 1 and 4, culture supernatant of scFv; 2 and 5, periplasmic fraction of scFv; 3 and 6, insoluble fraction of scFv. 1, 2, and 3 are scFv (G3), while 4, 5, and 6 are scFv (G1). G1 and G3 are the linkers (GGGGS) and $(GGGGS)_3$, respectively. (C) SDS-PAGE of immobilized metal-affinity chromatography (IMAC)–purified scFvs. Lanes; 1 and 5, total proteins in the insoluble fraction; 2 and 6, flow through fraction; 3 and 7, wash fraction; 4 and 8, eluted fraction. 1–4, scFv(G3); 5–8, scFv (G1).

VL) for secretory expression, and (iii) scFv (VL-VH) for secretory expression. The secretory expression vector of the Fv fragment was constructed by di-cistronic arrangement of both chains. The gene encoding single-chain Fv fragments were constructed by linking the domains with polypeptide linkers $(GGGGS)_3$ or (GGGGS) as described in "MATERIALS AND METHODS."

Expression and Purification of Variable Region Fragments—The overexpression of MUSE11 scFv or Fv was induced with 1 mM IPTG as described in "MATERIALS AND METHODS." Bacterial supernatant, periplasmic, and intracellular soluble and insoluble fractions were separated to examine the cellular localization of scFv. Almost all of the scFv fragments with the usual linker (designated scFv-G3) and with the short linker (designated scFv-G1) were detected in insoluble fractions, although a small amount of gene product was expressed in the periplasmic fraction (Fig. 2).

In the case of the Fv fragment, the VH fragment was overexpressed in insoluble fractions from SDS-PAGE (Fig. 3), while only a small amount of VL fragment could be observed. However, almost all of the VH fragments flowed unretained through the immobilized metal-affinity column (IMAC) under denaturing conditions, with only the Fv fragment eluting almost stoichiometrically. The VH fragment and VL fragment are expressed separately using two vectors, and almost none of the VH fragment expressed could be detected using anti-6xHis antibody and flowed through the IMAC despite its high expression, while the VL fragment could be detected and purified using IMAC (data not shown). This strongly suggests that the C-terminal region of the VH fragment is subjected to proteolysis *in vivo*. N-terminal sequence analysis of the purified VH and VL chains showed them to be processed *in vivo* correctly, *i.e.* processed at the C-terminal of the *pelB* signal sequence, and retained in the inner membrane of *E. coli*. They could be purified to homogeneity with IMAC followed by gel filtration on Sephacryl S-200 under denaturing conditions. The final yields of scFv and Fv were estimated to be about 5 mg and 1 mg, respectively, per liter of culture.

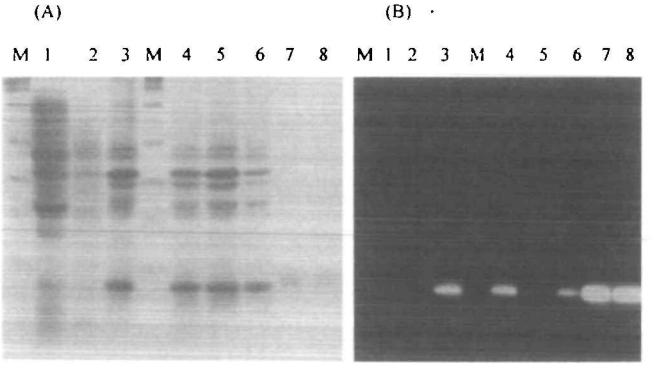

Fig. 3. **Expression and purification of MUSE11 Fv fragment.** (A) SDS–polyacrylamide gel electrophoresis and (B) Western blot of each fraction of *E. coli* BL21 (DE3) cells expressing MUSE11 Fv fragments. Lanes: 1, culture supernatant of Fv; 2, periplasmic fraction of Fv; 3, insoluble fraction of Fv; 4, samples subjected to IMAC; 5, flow through fraction; 6, wash fraction; 7, eluted fraction; 8; refolded Fv. It is noteworthy that most of the expressed VH fragments flowed unretained through the resin, while Fv could be stoichiometrically eluted, *i.e.* the molar ratio of VH is equal to VL.

Refolding of MUSE11 Variable Region Fragments—Renaturing of the purified variable domain fragments (Fv and scFv) was performed according to the methods of Tsumoto *et al.* (27). The denatured purified and reduced variable region fragments (5 μM) were removed by dialysis with GuHCl at sequentially decreased concentrations (3, 2, 1, 0.5, and 0 M). An oxidizing reagent (glutathione, oxidized form, Sigma) was introduced into 1 and 0.5 M GuHCl/PBS dialysis buffer, and 0.4 M L-Arg was added during the final dialysis stage. Four micromoles of fragments were retained in the solution, indicating about 75% recovery of the soluble proteins. Gel filtration analyses of refolded MUSE11 Fv fragments showed that all of the refolded proteins had a monomeric structure (*ca.* 25 kDa), indicating the stoichiometric association of VH with VL in Fv. On the other hand, scFv-G3 and scFv-G1 were found to have dimeric structures (*ca.* 50 kDa) (Fig. 4) by analytical gel filtration.

Flow Cytometry Analysis of Fv- and scFv-Binding to TFK-1 Cells and CHO-MUC1 Cells—The reactivities of the variable region fragments to the test cells are summarized in Fig. 5. The co-expressed and refolded Fv fragment showed high reactivity toward MUC1-positive TFK1 cells. The Fv fragment did not bind MUC-1–negative HT-17 cells, suggesting the specific binding of the Fv fragment to MUC-1 (data not shown). The slight decrease in the reactivity of Fv observed (Fig. 5a) might originate from the number of antigen-binding sites in the antibody, *i.e.* Fv is monovalent, while the parent IgG is bivalent. However, regardless of the length of the linker, *i.e.*, $(GGGGS)_3$ or GGGGS, the refolded scFv fragments with VH-VL and VL-VH arrangements show a greatly reduced affinity (Fig. 5), suggesting that the linking of the domains strongly affects the association of the scFv fragments with their target antigens. As shown in

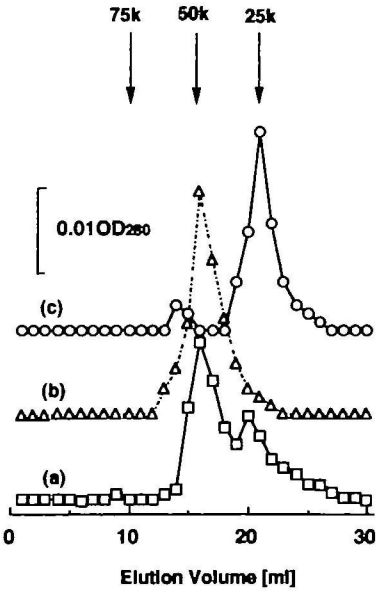

Fig. 4. **Gel filtration of purified and refolded MUSE11 variable domain fragments.** Purified and refolded MUSE11 variable domain fragments were subjected to gel filtration on Superdex-75 (Pharmacia) (1.0 × 100 cm) connected to an FPLC system, pre-equilibrated with PBS. 1 ml of refolded variable region fragments adjusted to 0.2 mg/ml was subjected to gel filtration analysis at a flow rate of 0.5 ml/min. (a) scFv (G1); (b) scFv (G3); (c) Fv.

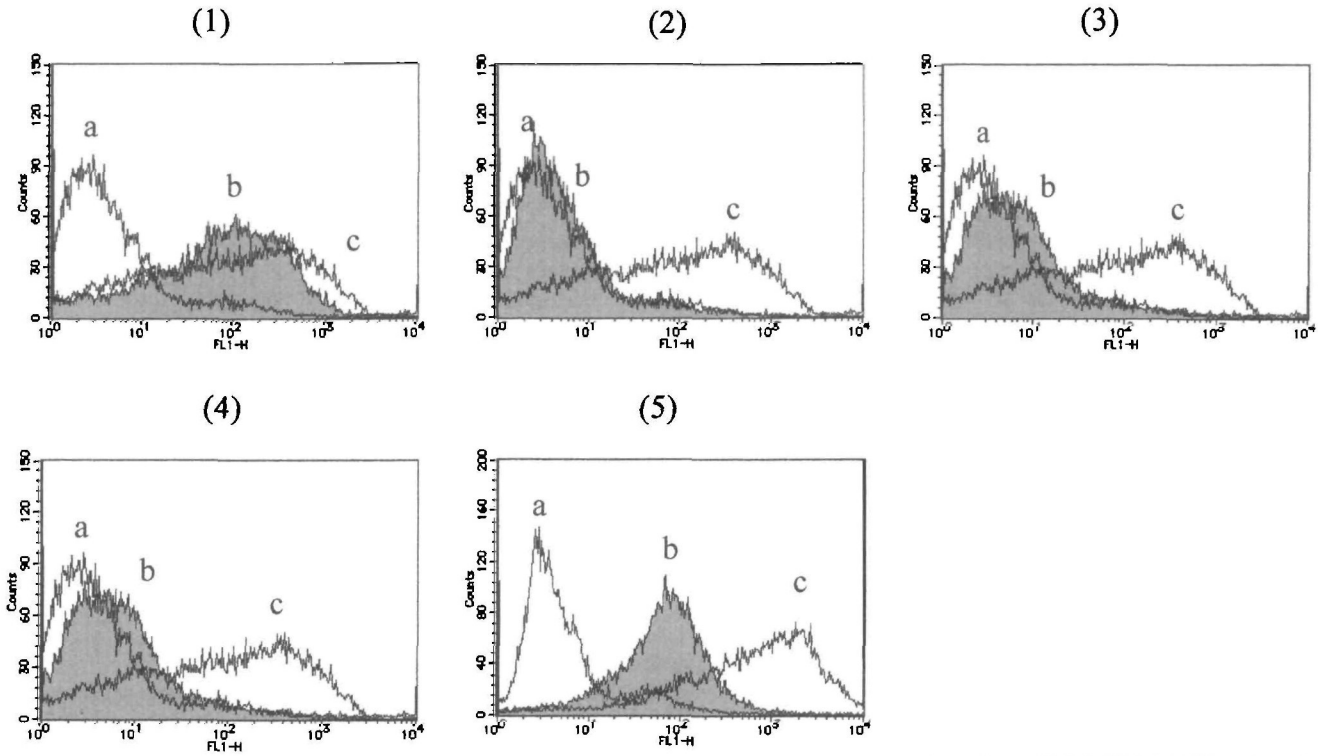

Fig. 5. **Flow cytometry profiles of TFK-1 cells (1–4) and MUC1-overexpressing CHO cells (5).** Open histograms, cells incubated with PBS as a negative control (a) and MUSE11 Ab as a positive control (c); filled histograms, cells incubated with MUSE11 Fv (1), scFvHL (G3) (2), scFvLH (G3) (3) or scFvHL (G1) (4). When these Fv fragments (10 μg/ml) were used as the first Abs, mouse anti–c-myc 9E10 Ab was the second Ab. Staining was done with FITC-conjugated goat anti–mouse IgG.

Fig. 5, the refolded Fv and scFv fragments reacted with anti–c-myc peptide tag mouse IgG, suggesting that these peptide tags (c-myc peptide for detection of binding to MUC1, and 6xHis peptide for the purification of variable domains) were expressed and not degraded under the conditions used. Thus, it is concluded that the reduction in affinity toward the target antigen by linking the domains originates from the significant decrease in the affinity of single-chain Fv.

In order to determine whether the Fv fragments recognize mucin MUC1 on tumor cells as the parent IgG, MUC1 overexpressing CHO cells were subjected to flow cytometric analysis (Fig. 5). The Fv fragment reacted specifically with MUC1(+) expressing cells, indicating that the Fv fragment shows high specificity for MUC1. A competitive blocking assay was also performed by flow cytometry, and the reactivity of Fv the fragment decreased in a dose-dependent fashion with the increase in the number of competitive cells (data not shown).

Isolated VH and VL chains prepared from insoluble inclusion bodies also show activity toward MUC1, despite a lower affinity than Fv (data not shown). The addition of soluble VL fragment to scFv, however, did not result in reactivity toward MUC1, and refolding of the denatured scFv fragment in the presence of VL fragment did not increase the reactivity in comparison with Fv (Asano *et al.*, manuscript in preparation). These results suggest that the linking of the domain in itself results in a significant reduction in the affinity for MUC1.

DISCUSSION

A remarkable feature of the MUSE11 variable domains prepared in our *E. coli* expression system is that the variable domains show specificity and affinity for MUC1 only in the Fv form, and that linking the domains significantly reduces the affinity regardless of the linker length. This suggests that linking the variable domains of MUSE11 strongly reduces the association with MUC1 on tumor cells. To our knowledge, this is the first report of a significant reduction in affinity caused by linking the variable domains in both arrangements, *i.e.*, VH-VL and VL-VH.

Gel filtration analysis indicated that the prepared single-chain Fv fragment was dimeric (Fig. 3). Multimerization of single-chain Fv, such as to dimers and trimers (*30–32*, *4*), has been reported by several groups. For instance, the dimerization of scFv has been applied to the construction of a bi-specific antibody, diabody (*33*, *34*). However, these dimeric scFvs have been reported to dissociate to monomers at low concentrations (*35*). The present scFv, however, has a stable dimeric structure even at concentrations as low as 1 μM. Thus, the MUSE11 scFv forms stable dimers with significantly reduced affinity toward MUC1. Understanding the folding mechanism of the scFv is intriguing and is now under investigation.

Isolated VH and VL chains prepared from insoluble inclusion bodies also have activity toward MUC1, although the affinity is lower than that of Fv (data not shown). This suggests that scFv fragments are active if the antigen bind-

ing sites are correctly folded. However, scFv in the dimeric form shows a significantly reduced affinity toward MUC1, suggesting that the scFv has folded into a conformation different from Fv or the isolated variable domains. The scFv might not be correctly folded due to the linking of the domains with a polypeptide linker, G1 or G3. The addition of VL to scFv did not enhance the affinity of the antibody fragment (data not shown), suggesting that scFv forms a conformation different from Fv, perhaps due to intermolecular interactions.

Another reason for the reduction in affinity may originate from the recognition mechanism of MUSE11 for MUC1. Recent crystallographic studies of antigen-antibody complexes have pointed out the significance of the relative orientation of the variable domains (*36–38*). The linker might interfere with the correct orientation of the variable chains.

For the stable utilization of MUSE11 Fv and scFv in immunotherapy, stabilization of the fragments by site-directed and/or random mutation followed by selection (*39*) is required. Fusion of the variable domain fragments with other stable proteins such as thioredoxin (*40*) and superantigen (*41*) might provide one method for stabilization.

REFERENCES

1. Cao, Y. and Suresh, M.R. (1998) Bispecific antibodies as novel bioconjugates. *Bioconjug. Chem.* **9**, 635–644
2. Plückthun, A. and Pack, P. (1997) New protein engineering approaches to multivalent and bispecific antibody fragments. *Immunotechnology* **3**, 83–105
3. King, D.J., Turner, A., Farnsworth, A.P.H., Adair, J.R., Owens, R.J., Pedley, R.B., Baldock, D., Proudfoot, K.A., Lawson, A.D.G., Beeley, N.R.A., Millar, K., Millican, A., Boyce, B.A., Antoniw, P., Mountain, A., Begent, R.H.J., Shochat, D., and Yarranton, G.T. (1994) Improved tumor targeting with chemically cross-linked recombinant antibody fragments. *Cancer Res.* **54**, 6176–6185
4. Hudson, P.J. (1998) Recombinant antibody fragments. *Curr. Opin. Biotechnol.* **9**, 395–402
5. Winter, G., Griffiths, A.D., Hawkins, R.E., and Hoogenboom, H.R. (1994) Making antibodies by phage display technology. *Annu. Rev. Immunol.* **12**, 433–455
6. Griffiths, A.D. and Duncan, A.R. (1998) Strategies for selection of antibodies by phage display. *Curr. Opin. Biotechnol.* **9**, 102–108
7. Pastan, I. (1997) Targeted therapy of cancer with recombinant immunotoxins. *Biochim. Biophys. Acta* **1333**, C1–6
8. Neri, D., de Lalla, C., Petrul, H., Neri, P., and Winter, G. (1995) Calmodulin as a versatile tag for antibody fragments. *Bio/Technology* **13**, 373–377
9. Suzuki, C., Ueda, H., Tsumoto, K., Mahoney, W.C., Kumagai, I., and Nagamune, T. (1999) Open Sandwich ELISA with VH-/VL-alkaline phosphatase fusion proteins. *J. Immunol. Methods* **224**, 171–184
10. Yang, J., Moyana, T., and Xiang, J. (1995) A genetically engineered single-chain Fv/TNF molecule possesses the anti-tumor immunoreactivity of Fv as well as the cytotoxic activity of tumor necrosis factor. *Mol. Immunol.* **32**, 873–881
11. Hudson, P.J. (1999) Recombinant antibody constructs in cancer therapy. *Curr. Opin. Immunol.* **11**, 548–557
12. Carter, P. and Merchant, A.M. (1997) Engineering antibodies for imaging and therapy. *Curr. Opin. Biotechnol.* **8**, 449–454
13. Yokota, T., Milenic, D.E., Whitlow, M., and Schlom, J. (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.* **52**, 3402–3408
14. Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S.M., Lee, T., Pope, S.H., Riordan, G.S., and Whitlow, M. (1988) Single-chain antigen-binding proteins. *Science* **242**, 423–426
15. Huston, J.S., Margolies, M.N., and Haber, E. (1996) Antibody binding sites. *Adv. Protein Chem.* **49**, 329–450
16. Gendler, S., Taylor-Papadimitriou, J., Duhig, T., *et al.* (1988) A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. *J. Biol. Chem.* **263**, 12820–12823
17. Siddiqui, J., Abe, M., Hayes, D., *et al.* (1988) Isolation and sequencing of a cDNA coding for the human DF3 breast carcinoma-associated antigen. *Proc. Natl. Acad. Sci. USA* **85**, 2320–2323
18. Burchell, J., Taylor-Papadomitriou, J., Boshell, M., Gendler, S., and Duhig, T. (1989) A short sequence, within the amino acid tandem repeat of a cancer-associated mucin, contains immunodominant epitopes. *Int. J. Cancer* **44**, 691–696
19. Ban, T., Imai, K., and Yachi, A. (1989) Immunohistological and immunochemical characterization of a novel pancreatic cancer-associated antigen MUSE11. *Cancer Res.* **49**, 7141–7146
20. Hinoda, Y., Nakagawa, N., Ohe, Y., Kakiuchi, H., Tsujisaki, M., Imai, K., and Yachi, A. (1990) Recognition of the polypeptide core of mucin by monoclonal antibody MUSE11 against an adenocarcinoma-associated antigen. *Jpn. J. Cancer Res.* **81**, 1206–1209
21. Hinoda, Y., Arimura, Y., Itoh, F., Adachi, M., Tsujisaki, M., Imai, K., and Yachi, A. (1993) Primary structure of the variable regions of a monoclonal antibody MUSE11 recognizing the tandem repeat domain of a mucin core protein, MUC1. *J. Clin. Lab. Anal.* **7**, 100–104
22. Tsumoto, K., Nakaoki, Y., Ueda, Y., Ogasahara, K., Yutani, K., Watanabe, K., and Kumagai, I. (1994) Effect of the order of antibody variable regions on the expression of the single-chain HyHEL10 Fv fragment in *E. coli* and the thermodynamic analysis of its antigen-binding properties. *Biochem. Biophys. Res. Commun.* **201**, 546–551
23. Tsumoto, K., Ueda, Y., Maenaka, K., Watanabe, K., Ogasahara, K., Yutani, K., and Kumagai, I. (1994) Contribution to antibody-antigen interaction of structurally perturbed antigenic residues upon antibody binding. *J. Biol. Chem.* **269**, 28777–28782
24. Studier, F.W. and Moffatt, B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**, 113–130
25. Sambrook, J., Fritsch, F.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Horber, NY
26. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
27. Tsumoto, K., Shinoki, K., Kondo, H., Uchikawa, M., Juji, T., and Kumagai, I. (1998) Highly efficient recovery of functional single-chain Fv fragments from inclusion bodies overexpressed in *Escherichia coli* by controlled introduction of oxidizing reagent—application to a human single-chain Fv fragment. *J. Immunol. Methods* **219**, 119–129
28. Saijyo, S., Kudo, T., Suzuki, M., Katayose, Y., Shinoda, M., Muto, T., Fukuhara, K., Suzuki, T., and Matsuno, S. (1995) Establishment of a new extrahepatic bile duct carcinoma cell line, TFK-1. *Tohoku J. Exp. Med.* **177**, 61–71
29. Batra, S.K., Kern, H.F., Worlock, A.J., Metzgar, R.S., Hollingsworth, M.A. (1991) Transfection of the human Muc 1 mucin gene into a poorly differentiated human pancreatic tumor cell line, Panc1: integration, expression and ultrastructural changes. *J. Cell Sci.* **100**, 841–849
30. Kortt, A.A., Malby, R.L., Caldwell, J.B., Gruen, L.C., Ivancic, N., Lawrence, M.C., Howlett, G.J., Webster, R.G., Hudson, P.J., and Colman, P.M. (1994) Recombinant anti-sialidase single-chain variable fragment antibody. Characterization, formation of dimer and higher-molecular-mass multimers and the solution of the crystal structure of the single-chain variable fragment/sialidase complex. *Eur. J. Biochem.* **221**, 151–157
31. McGregor, D.P., Molloy, P.E., Cunningham, C., and Harris, W.J. (1994) Spontaneous assembly of bivalent single chain antibody

fragments in *Escherichia coli*. *Mol. Immunol.* **31**, 219–226
32. Schier, R., McCall, A., Adams, G.P., Marchall, K.M., Merritt, H., Yim, M., Crawford, R.S., Weiner, L.M., Marks, C., and Marks, J.D. (1996) Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. *J. Mol. Biol.* **263**, 551–567
33. Holliger, P., Prospero, T., and Winter, G. (1993) "Diabodies": small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. USA* **90**, 6444–6448
34. Kipriyanov, S.M., Moldenhauer, G., Strauss, G., and Little, M. (1998) Bispecific CD3 × CD19 diabody for T cell-mediated lysis of malignant human B cells. *Int. J. Cancer* **77**, 763–772
35. Arndt, K.M., Müller, K.M., and Plückthun, A. (1998) Factors influencing the dimer to monomer transition of an antibody single-chain Fv fragment. *Biochemistry* **37**, 12918–12926
36. Stanfield, R.L., Takimoto-Kamimura, M., Rini, J.M., Profy, A.T., and Wilson, I.A. (1993) Major antigen-induced domain rearrangement in an antibody. *Structure* **1**, 83–93
37. Banfield, M.J., King, D.J., Mountain, A., and Brady, R.L. (1997) VL:VH domain rotations in engineered antibodies: crystal structures of the Fab fragments from two murine antitumor antibodies and their engineered human constructs. *PROTEINS: Struct. Funct. Genet.* **29**, 161–171
38. Kondo, H., Shiroishi, M., Matsushima, M., Tsumoto, K., and Kumagai, I. (1999) Crystal structure of anti-hen egg white lysozyme antibody (HyHEL-10) Fv-antigen complex. *J. Biol. Chem.* **274**, 27623–27631
39. Worn, A. and Plückthun, A. (1998) Mutual stabilization of VL and VH in single-chain antibody fragments, investigated with mutants engineered for stability. *Biochemistry* **37**, 13120–13127
40. Schodin, B.A., Schlueter, C.J., and Kranz, D.M. (1996) Binding properties and solubility of single-chain T cell receptors expressed in *E. coli*. *Mol. Immunol.* **33**, 819–829
41. Sakurai, N., Kudo, T., Suzuki, M., Tsumoto, K., Takemura, S., Kodama, H., Ebara, S., Teramae, A., Katayose, Y., Shinoda, M., Kurokawa, T., Hinoda, Y., Imai, K., Matsuno, S., and Kumagai, I. (1999) SEA-scFv as a bifunctional antibody: construction of a bacterial expression system and its functional analysis. *Biochem. Biophys. Res. Commun.* **256**, 223–230