tions. To determine the levels of *p53* and c-*myc* siRNAs, 20 μg of total RNA, which was denatured with formamide, was separated on 15% polyacrylamide gels containing 7 *M* urea and electrotransferred to Hybond-N+ membrane (Amersham Biosciences). Loading was checked by ethidium bromide staining. Hybridization was performed with Rapid-Hyb buffer (Amersham Biosciences). Probes which were antisense oligonucleotide (19 bp) of target sequence, were labeled with a MEGALABEL DNA 5′-end labeling kit (TaKaRa Bio, Shiga, Japan). Signals were read with a BAS-2500 (Fujifilm).

RESULTS AND DISCUSSION

Using a combination of Ad vectors and an siRNA expression system is clearly an advantage in gene transfer experiments and therapeutic applications. An inducible siRNA expression system is more desirable, because the degree of gene silencing can be controlled by adjusting the dose or concentration of the inducer. In this study, we developed Ad vectors containing a Dox-inducible siRNA expression system. For inducible siRNA expression, the *tetO* sequence was placed between the TATA box and transcription start site of the H1 promoter (the sequence is described in Materials and Methods). Various Ad vectors, in which target sequences were inserted to express shRNA under the control of the H1 promoter and a mutant H1 promoter containing the *tetO* sequence, were constructed and are shown in Fig. 1. For proof of concept, the expression of endogenous genes *p53* and c-*myc* was silenced.

First, to examine the feasibility of the Ad vector-mediated siRNA expression system, p53 and c-Myc expression was constitutively knocked down by infection with Ad vectors containing the normal H1 promoter or a mutant H1 promoter containing the *tetO* sequence. A549 and HepG2 cells were infected with various concentrations of Ad vector (Ad-H1-p53, Ad-H1-Myc, Ad-H1tetO-p53, Ad-H1tetO-Myc, Ad-H1, Ad-H1tetO, or Ad-null), and cultured without Dox for 3 days. Levels of p53 and c-Myc protein expression were examined by Western blotting (Fig. 2). Expression of actin was also measured as an internal control. Expression of p53 and c-Myc in

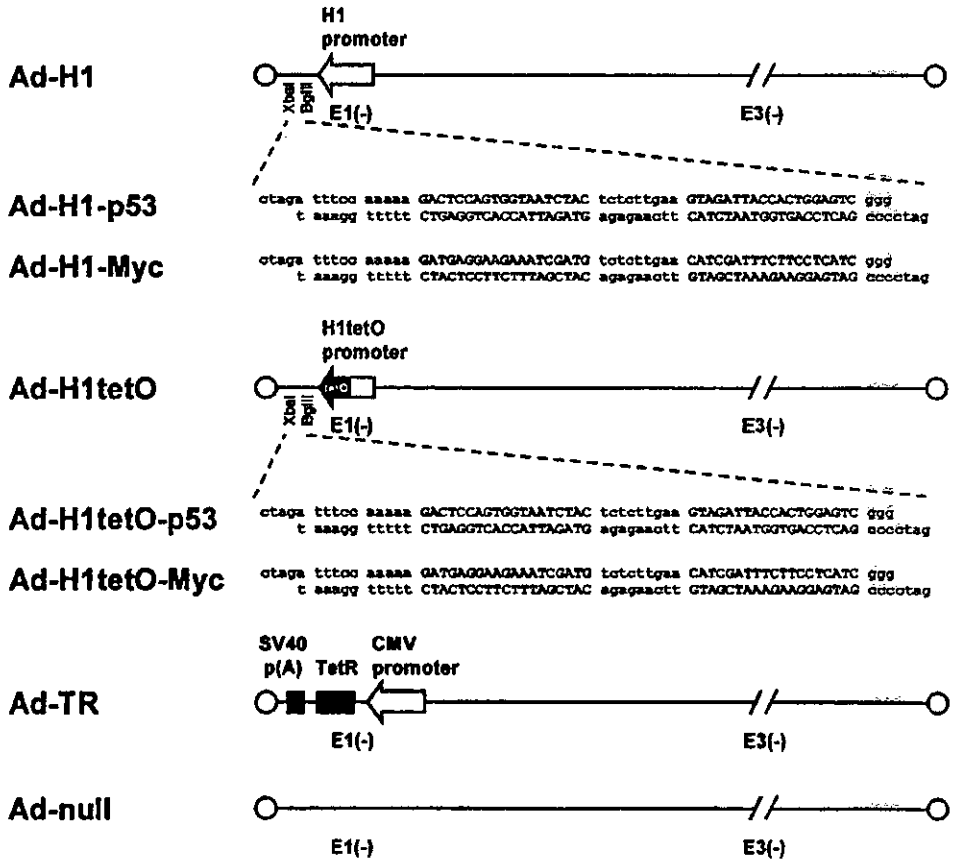

FIG. 1. Structure of Ad vectors used in the present study. The H1 promoter-based siRNA expression cassette was inserted into the E1 deletion region of the Ad genome. For inducible siRNA expression, a tetracycline operator (*tetO*) sequence was introduced downstream of the TATA box in the H1 promoter, as described in Materials and Methods. Target sequences against *p53* and c-*myc* genes are shown in upper case letters. Ad-TR is Ad vector containing a tetracycline repressor sequence under the control of the CMV promoter-enhancer. Ad-null is Ad vector without foreign genes in the E1 deletion region.

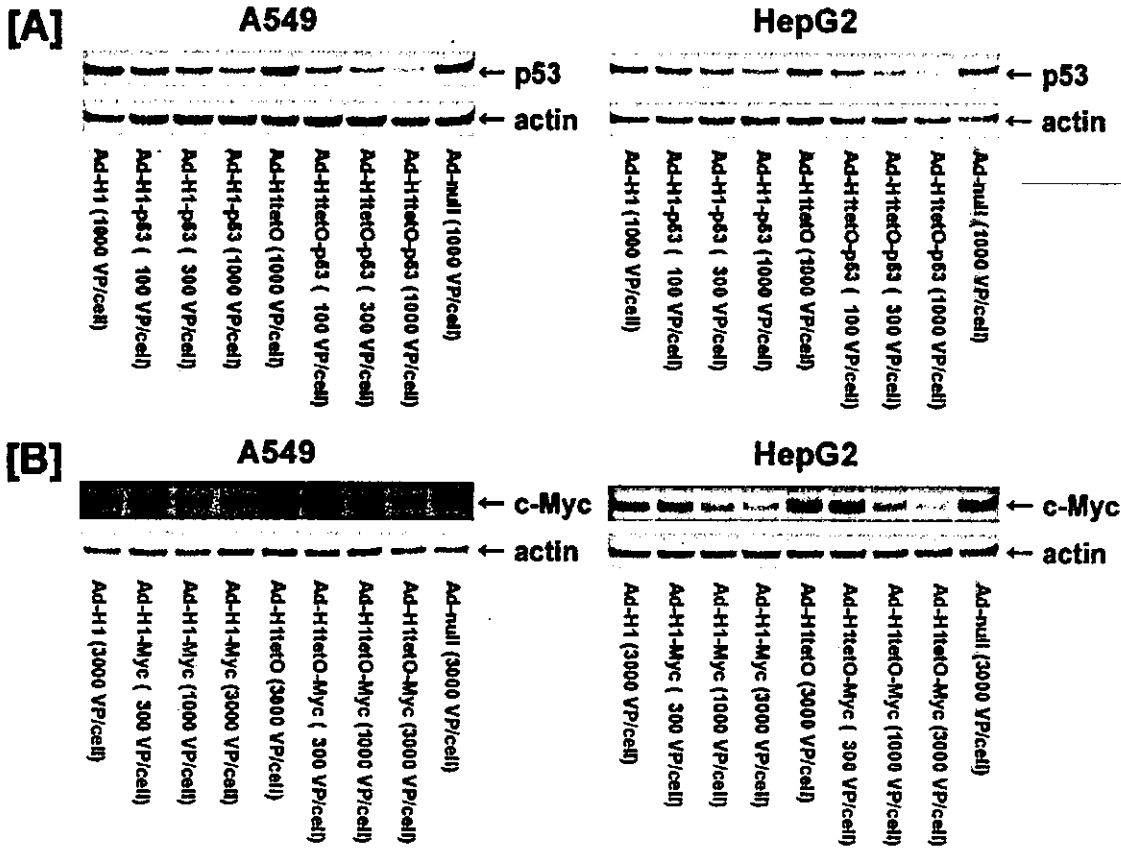

FIG. 2. Dose-dependent suppression of p53 and c-Myc protein expression by Ad vector-delivered siRNA. A549 and HepG2 cells were infected with each Ad vector for 1.5 hr, and then cultured for 3 days. Proteins were then extracted from the cells, and levels of p53 (A) and c-Myc (B) expression were examined by Western blotting. Actin bands served as an internal control for equal total protein loading.

A549 and HepG2 cells decreased in a dose-dependent manner with Ad vector carrying the siRNA expression cassette for p53 or c-Myc (Ad-H1-p53, Ad-H1-Myc, Ad-H1tetO-p53, or Ad-H1tetO-Myc). In the case of p53, a viral concentration of 1000 virus particles (VP)/cell seemed to be enough to knock down expression. Levels of p53 expression in cells treated with Ad-H1-p53 (1000 VP/cell) or Ad-H1tetO-p53 (1000 VP/cell) were decreased to 35–37 or 14–23%, respectively (Fig. 2A), relative to cells treated with Ad-null (1000 VP/cell), according to Image Gauge software (Fujifilm). In the case of c-Myc, a viral concentration of 3000 VP/cell was required to completely knock down expression, although a viral concentration of 1000 VP/cell enabled a moderate knockdown of expression. c-Myc protein expression in cells treated with Ad-H1-Myc (3000 VP/cell) or Ad-H1tetO-Myc (3000 VP/cell) was decreased to 14–44 or 16–35%, respectively (Fig. 2B), relative to cells treated with Ad-null and Ad-H1 (3000 VP/cell). The difference in degree of gene silencing may reflect the effectiveness of the siRNA sequence against each target gene. Compared with Ad-null, Ad-H1 and Ad-H1tetO did not show any effect on gene expression. These results indicate that the *tetO* sequence, placed between the TATA box and the transcription start site of the H1 promoter, does not interfere with promoter activity, and that Ad vectors containing the mutant H1 promoter, as well as the normal H1 promoter-mediated siRNA expression cassette, efficiently silence target gene expression.

Next, we examined whether regulated gene silencing is obtained by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR, the Ad vector expressing TetR, into A549 cells cultured with or without Dox (10 μg/ml). As shown in Fig. 3, in the presence of Dox the silencing effect on p53 expression decreased in proportion to the dose of Ad-TR. Efficient release of gene silencing was obtained with a 1:6 molar ratio of Ad-H1tetO-p53 to Ad-TR, although more Ad-TR might be required to completely release gene silencing. These results suggest that increased amounts of TetR are required to block transcription from the mutant H1 promoter, which contains the *tetO* sequence, in the presence of Dox (Fig. 3A). In the absence of Dox, p53 expression in cells was silenced by coinfection with Ad-H1tetO-p53 and Ad-TR. Therefore, for the regulated silencing of p53 expression, increased amounts of Ad-TR, compared with Ad-H1tetO-p53, were required. A similar result was observed in experiments on c-Myc expression (Fig. 3B), and also in HepG2 cells (both *p53* and c-*myc* genes; data not shown).

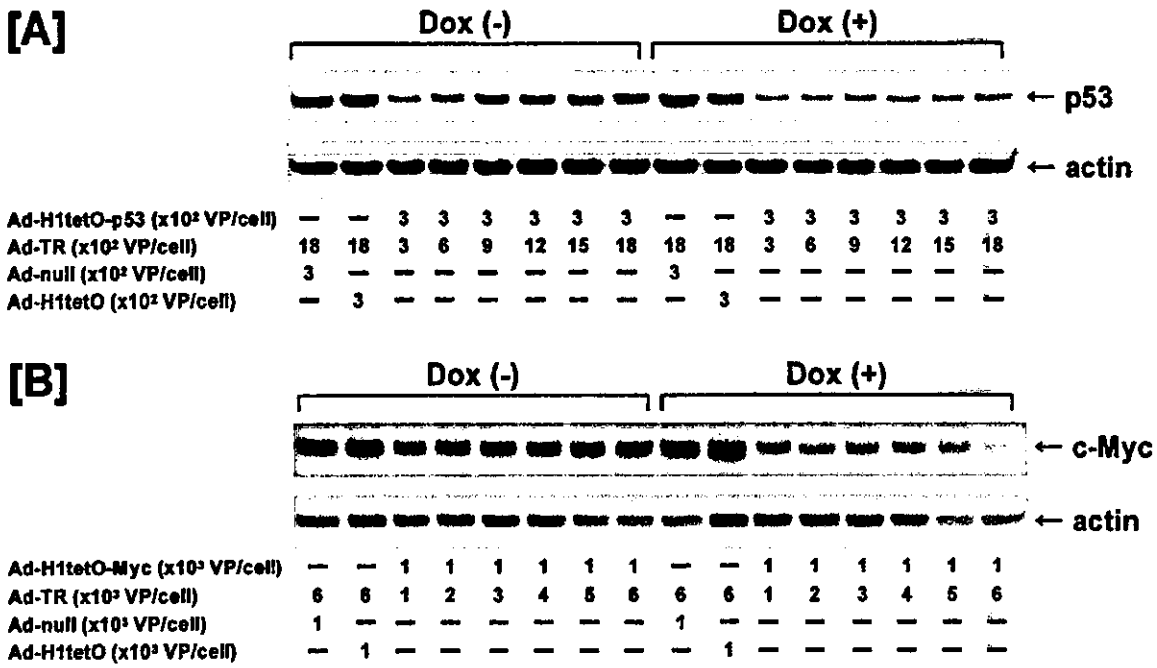

FIG. 3. Regulated suppression of p53 and c-Myc expression by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR. A549 cells were infected with the indicated amounts of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR for 1.5 hr, and then cultured with or without Dox (10 μg/ml) for 3 days. The cells were also infected with Ad-null or Ad-H1tetO plus Ad-TR. Proteins were then extracted from the cells, and levels of p53 (A) and c-Myc (B) expression were examined by Western blotting. Actin bands served as an internal control for equal total protein loading.

Ad-TR expresses TetR from the conventional cytomegalovirus (CMV) promoter-enhancer (Xu *et al.*, 2003a). Addition of the intron A sequence to the CMV promoter-enhancer, or the use of a stronger promoter such as the hybrid promoter of the β-actin promoter and CMV enhancer (Niwa *et al.*, 1991; Xu *et al.*, 2001, 2003b), would result in higher expression of TetR, thus decreasing the concentration of Ad vector expressing TetR needed to obtain inducible gene silencing efficiently. These modifications for TetR expression would make the system more effective, and would therefore enable more widespread use of this system.

We then examined the Dox concentration responsiveness of Ad vector-mediated RNAi (Fig. 4). A549 cells were coinfected with Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR at a molar ratio of 1:6, and were cultured with medium containing various concentrations of Dox. A Dox concentration of 10^{-1} μg/ml was enough to completely suppress the expression of p53 and c-Myc. At a Dox concentration of 10^{-2} μg/ml, intermediate levels of knockdown of p53 and c-Myc expression were obtained. Ad-H1tetO-p53 plus Ad-TR and Ad-H1tetO-Myc plus Ad-TR in the presence of Dox did not interfere with c-Myc and p53 expression, respectively, suggesting that the suppressive effect was target gene specific. These results suggest that the degree of knockdown of target gene expression can be modulated by Dox concentration.

We next performed a Northern blot analysis of shRNA (siRNA) expression in the presence and absence of Dox (Fig. 5). Levels of shRNA and siRNA expression for *p53* in both A549 and HepG2 cells transduced with Ad-H1tetO-p53 plus Ad-TR in the absence of Dox were significantly reduced compared with those in transduced cells in the presence of Dox (Fig. 5A). The signal of shRNA and siRNA in the absence of Dox was faint. These observations were marked in the case of *c-myc* (Fig. 5B). These results suggested that shRNA expression was tightly regulated in the Ad vector-mediated Dox-inducible RNAi system.

While this work was in progress, a plasmid vector-mediated inducible siRNA expression system using TetR was reported by van de Wetering *et al.* (2003). The mutant H1 promoter in their system contains the *tetO* sequence at a different position (by 1 bp) compared with the position of the *tetO* sequence in the present study. Both positions for insertion of the *tetO* sequence in the H1 promoter seem to be functional for regulated transcription. A similar system using the mutant U6 promoter containing the *tetO* sequence and TetR for inducible RNAi has also been reported (Matsukura *et al.*, 2003). Furthermore, a tetracycline repressor-based system was reported by two groups. In the study by Wiznerowicz and Trono, the *tetO* sequence was placed upstream of the U6 promoter (Chen *et al.*, 2003; Wiznerowicz and Trono, 2003), whereas in the study by Chen *et al.*, the *tetO* sequence was placed in these three regions: (1) upstream of the U6 promoter, (2) between the distal promoter element and the core promoter (PSE) of the U6 promoter, and (3) between the PSE and TATA box of the U6 promoter (Chen *et al.*, 2003; Wiznerowicz and Trono, 2003).

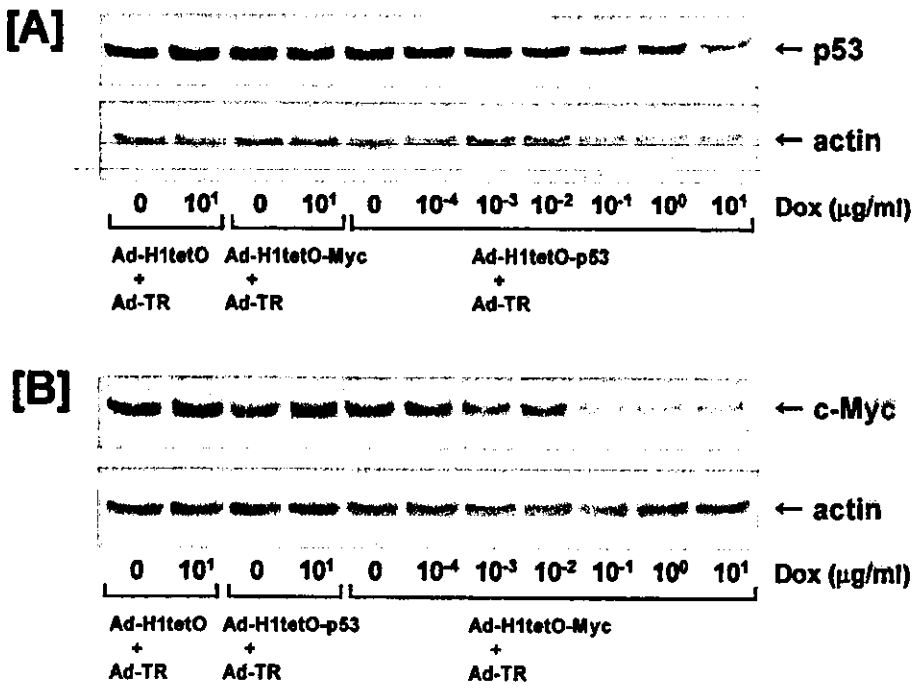

FIG. 4. Dox dose-dependent suppression of p53 and c-Myc expression by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR. A549 cells were infected with Ad-H1tetO-p53 (300 VP/cell) plus Ad-TR (1800 VP/cell) or with Ad-H1tetO-Myc (1000 VP/cell) plus Ad-TR (6000 VP/cell) for 1.5 hr, and then cultured with various concentrations of Dox for 3 days. The cells were also infected with Ad-H1tetO plus Ad-TR. Proteins were then extracted from the cells, and levels of p53 (A) and c-Myc (B) expression were examined by Western blotting. Actin bands served as an internal control for equal total protein loading.

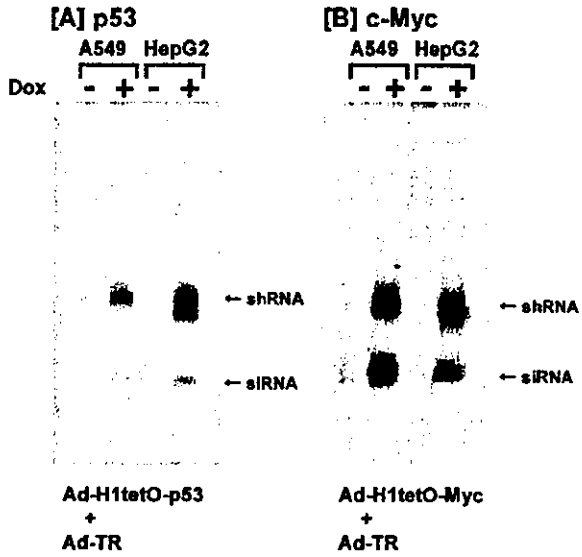

FIG. 5. Dox-inducible *p53* or c-*myc* siRNA expression by coinfection of Ad-H1tetO-p53 or Ad-H1tetO-Myc plus Ad-TR. A549 and HepG2 cells were infected with Ad-H1tetO-p53 (300 VP/cell) plus Ad-TR (1800 VP/cell) or with Ad-H1tetO-Myc (1000 VP/cell) plus Ad-TR (6000 VP/cell) for 1.5 hr, and then cultured with or without Dox (1 μg/ml) for 3 days. Total RNAs were then extracted from the cells, and levels of *p53* and c-*myc* siRNA expression were examined by Northern blotting.

Ad vector-mediated RNAi represents a new strategy for the study of gene function and therapeutic applications. Ad vector-mediated delivery of siRNA allows efficient transduction into a variety of cell types *in vitro* and *in vivo*. Several studies have reported Ad vector-mediated gene silencing using both the Pol II promoter, in which a mutant CMV promoter was used, and the Pol III (H1) promoter (Xia *et al.*, 2002; Zhao *et al.*, 2003). The combination of Ad vectors and an inducible siRNA expression system offers a superior strategy to researchers. To our knowledge, this study is the first to report on the development of Ad vector-mediated inducible RNAi. Various inducible siRNA expression systems, including a tetracycline repressor-based system, and a capsid-modified Ad vector to change viral tropism, can be easily combined. The system described here has great potential for therapeutic use as well as for a variety of applications, including the study of gene function.

ACKNOWLEDGMENTS

We thank Takashi Fukushima and Tomomi Sasaki for technical assistance. T.H. is the recipient of a fellowship from the Japan Society for the Promotion of Science. Z.L.X. is the recipient of a postdoctoral fellowship from the Japan Health Sciences Foundation. This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan.

REFERENCES

BRUMMELKAMP, T.R., BERNARDS, R., and AGAMI, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science **296,** 550–553.

CHEN, Y., STAMATOYANNOPOULOS, G., and SONG, C.Z. (2003). Down-regulation of CXCR4 by inducible small interfering RNA inhibits breast cancer cell invasion *in vitro*. Cancer Res. **63,** 4801–4804.

CURIEL, D.T. (1999). Strategies to adapt adenoviral vectors for targeted delivery. Ann. N.Y. Acad. Sci. **886,** 158–171.

ELBASHIR, S.M., HARBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K., and TUSCHL, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411,** 494–498.

KANEGAE, Y., MAKIMURA, M., and SAITO, I. (1994). A simple and efficient method for purification of infectious recombinant adenovirus. Jpn. J. Med. Sci. Biol. **47,** 157–166.

KOIZUMI, N., MIZUGUCHI, H., SAKURAI, F., YAMAGUCHI, T., WATANABE, Y., and HAYAKAWA, T. (2003a). Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and α_v integrin-binding ablation. J. Virol. **77,** 13062–13072.

KOIZUMI, N., MIZUGUCHI, H., UTOGUCHI, N., WATANABE, Y., and HAYAKAWA, T. (2003b). Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. J. Gene Med. **5,** 267–276.

LEE, N.S., DOHJIMA, T., BAUER, G., LI, H., LI, M.J., EHSANI, A., SALVATERRA, P., and ROSSI, J. (2002). Expression of small interfering RNAs targeted against HIV-1 *rev* transcripts in human cells. Nat. Biotechnol. **20,** 500–505.

MAIZEL, J.V., Jr., WHITE, D.O., and SCHARFF, M.D. (1968). The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. Virology **36,** 115–125.

MATSUKURA, S., JONES, P.A., and TAKAI, D. (2003). Establishment of conditional vectors for hairpin siRNA knockdowns. Nucleic Acids Res. **31,** e77.

MCMANUS, M.T., and SHARP, P.A. (2002). Gene silencing in mammals by small interfering RNAs. Nat. Rev. Genet. **3,** 737–747.

MIYAGISHI, M., and TAIRA, K. (2002). U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. Nat. Biotechnol. **20,** 497–500.

MIZUGUCHI, H., and KAY, M.A. (1998). Efficient construction of a recombinant adenovirus vector by an improved *in vitro* ligation method. Hum. Gene Ther. **9,** 2577–2583.

MIZUGUCHI, H., and KAY, M.A. (1999). A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. Hum. Gene Ther. **10,** 2013–2017.

MIZUGUCHI, H., KOIZUMI, N., HOSONO, T., UTOGUCHI, N., WATANABE, Y., KAY, M.A., and HAYAKAWA, T. (2001). A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. Gene Ther. **8,** 730–735.

NIWA, H., YAMAMURA, K., and MIYAZAKI, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108,** 193–199.

PADDISON, P.J., CAUDY, A.A., BERNSTEIN, E., HANNON, G.J., and CONKLIN, D.S. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. **16,** 948–958.

PAUL, C.P., GOOD, P.D., WINER, I., and ENGELKE, D.R. (2002). Effective expression of small interfering RNA in human cells. Nat. Biotechnol. **20,** 505–508.

SUI, G., SOOHOO, C., AFFAR EL, B., GAY, F., SHI, Y., and FORRESTER, W.C. (2002). A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **99,** 5515–5520.

VAN DE WETERING, M., OVING, I., MUNCAN, V., PON FONG, M.T., BRANTJES, H., VAN LEENEN, D., HOLSTEGE, F.C., BRUMMELKAMP, T.R., AGAMI, R., and CLEVERS, H. (2003). Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO Rep. **4,** 609–615.

WICKHAM, T.J. (2000). Targeting adenovirus. Gene Ther. **7,** 110–114.

WIZNEROWICZ, M., and TRONO, D. (2003). Conditional suppression of cellular genes: Lentivirus vector-mediated drug-inducible RNA interference. J. Virol. **77,** 8957–8961.

XIA, H., MAO, Q., PAULSON, H.L., and DAVIDSON, B.L. (2002). siRNA-mediated gene silencing *in vitro* and *in vivo*. Nat. Biotechnol. **20,** 1006–1010.

XU, Z.L., MIZUGUCHI, H., ISHII-WATABE, A., UCHIDA, E., MAYUMI, T., and HAYAKAWA, T. (2001). Optimization of transcriptional regulatory elements for constructing plasmid vectors. Gene **272,** 149–156.

XU, Z.L., MIZUGUCHI, H., MAYUMI, T., and HAYAKAWA, T. (2003a). Regulated gene expression from adenovirus vectors: A systematic comparison of various inducible systems. Gene **309,** 145–151.

XU, Z.L., MIZUGUCHI, H., MAYUMI, T., and HAYAKAWA, T. (2003b). Woodchuck hepatitis virus post-transcriptional regulation element enhances transgene expression from adenovirus vectors. Biochim. Biophys. Acta **1621,** 266–271.

YAO, F., SVENSJO, T., WINKLER, T., LU, M., ERIKSSON, C., and ERIKSSON, E. (1998). Tetracycline repressor, TetR, rather than the TetR–mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. Hum. Gene Ther. **9,** 1939–1950.

YU, J.Y., DERUITER, S.L., and TURNER, D.L. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **99,** 6047–6052.

ZHAO, L.J., JIAN, H., and ZHU, H. (2003). Specific gene inhibition by adenovirus-mediated expression of small interfering RNA. Gene **316,** 137–141.

Address reprint requests to:
Dr. Hiroyuki Mizuguchi
Division of Cellular and Gene Therapy Products
National Institute of Health Sciences
1-18-1 Kamiyoga, Setagaya-ku
Tokyo 158-8501, Japan

E-mail: mizuguch@nihs.go.jp

Received for publication February 5, 2004; accepted after revision June 16, 2004.

Published online: July 16, 2004.

HUMAN GENE THERAPY 15:1034–1044 (November 2004)

Review

Targeted Adenovirus Vectors

HIROYUKI MIZUGUCHI[1] and TAKAO HAYAKAWA[2]

ABSTRACT

Recombinant adenovirus (Ad) vectors continue to be the preferred vectors for gene therapy and the study of gene function because they are relatively easy to construct, can be produced at high titer, and have high transduction efficiency. However, in some applications gene transfer with Ad vectors is less efficient because the target cells lack expression of the primary receptor, coxsackievirus and adenovirus receptor (CAR). Another problem is the wide biodistribution of vector in tissue following *in vivo* gene transfer because of the relatively broad tissue expression of CAR. To overcome these limitations, various approaches have been developed to modify Ad tropism. In one approach, the capsid proteins of Ad are modified, such as with the addition of foreign ligands or the substitution of the fiber with other types of Ad fiber, in combination with the ablation of native tropism. In other approaches, Ad vectors are conjugated with adaptor molecules, such as antibody and fusion protein containing an anti-Ad single-chain antibody (scFv) or the extracellular domain of CAR with the targeting ligands, or chemically modified with polymers containing the targeting ligands. In this paper, we review advances in the development of targeted Ad vectors.

INTRODUCTION

ADENOVIRUS VECTORS have been expected to play a prominent role in gene therapy because of their extremely high transduction efficiency. However, one of the hurdles confronting gene transfer by adenovirus (Ad) vectors is their inefficient transduction to target cells lacking sufficient expression of the coxsackievirus and adenovirus receptor (CAR), the primary receptor, such cells include many advanced tumor cells, skeletal muscle cells, smooth muscle cells, peripheral blood cells, hematopoietic stem cells, dendritic cells, and so on. A high dose of vector is required to achieve efficient gene transfer to these cell types. This in turn increases unwanted side effects, such as vector-associated immunogenic toxicities.

Another hurdle confronting Ad vector-mediated gene transfer is their nonspecific distribution in tissue after *in vivo* gene transfer because of the relatively broad expression of CAR, α_v integrin (the secondary receptor), and heparan sulfate (the third receptor). This property imposes an increased risk of toxicity due to vector dissemination to nontargeted cells, such as antigen-presenting cells (e.g., macrophages and dendritic cells). This occurs even when Ad vectors are locally administered to the tissue of interest. Vector targeting to a specific tissue or cell type would enhance gene therapy efficacy and permit the delivery of lower doses, which should result in reduced toxicity.

Several approaches have been developed to overcome these hurdles, including genetic modification of Ad capsid proteins, such as fiber, penton base, hexon, and protein IX (pIX), and conjugation-based modification of virus such as antibody or bispecific fusion protein, and chemical modification by polymers containing the targeting ligands (Fig. 1). To improve gene transfer efficiency, modification of tropism is required. To target gene transfer, both the ablation of natural tropism and introduction of cell-specific tropism are required. In this paper we review approaches to developing targeted Ad vectors.

[1]Project III, National Institute of Health Sciences, Osaka Branch, Fundamental Research Laboratories for Development of Medicine, Osaka 567-0085, Japan.
[2]National Institute of Health Sciences, Tokyo 158-8501, Japan.

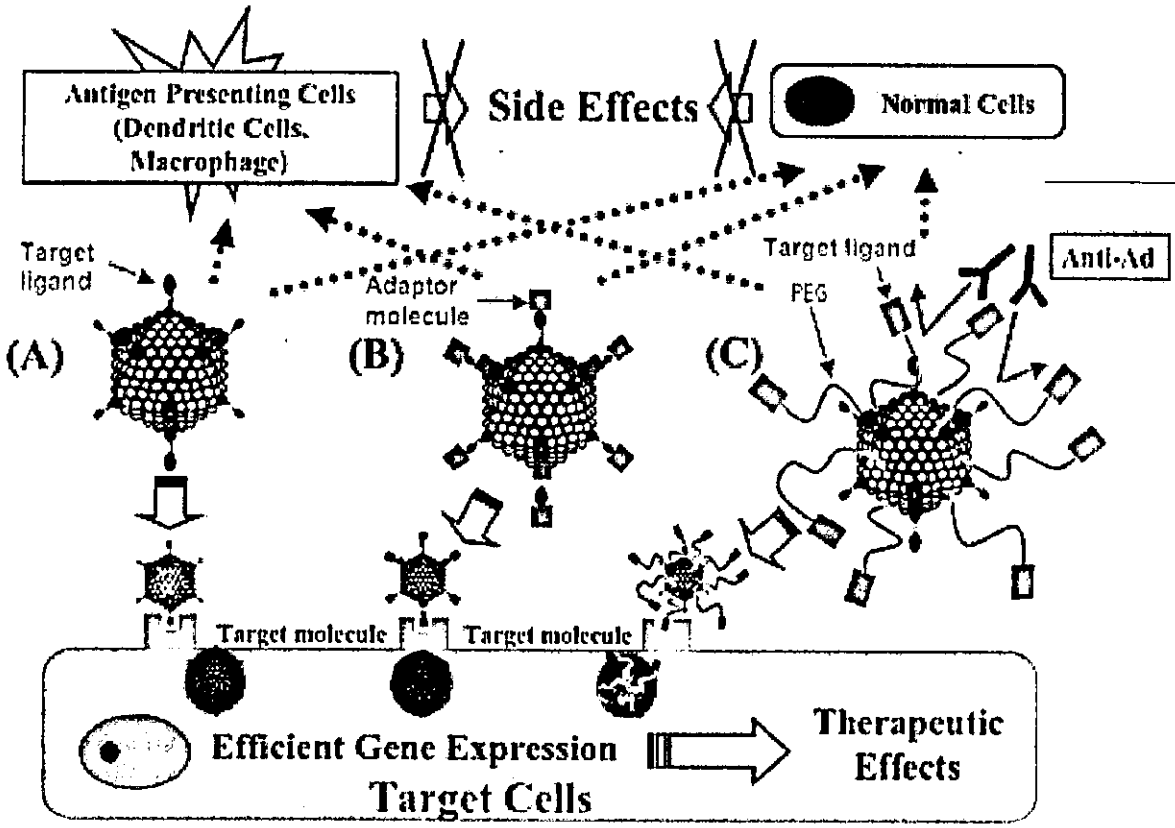

FIG. 1. Three approaches to developing targeted Ad vectors. (A) Genetic modification of virus capsid. (B) Modification by the use of adaptor molecules. (C) Chemical modification by polymers with ligands.

KINETICS OF ADENOVIRUS VECTOR-MEDIATED GENE TRANSFER *IN VIVO*

Important determinants of virus clearance from the bloodstream include interactions between viral components and cellular receptors, virion size, net charge of the viral particle, and anatomical barriers, such as tightness of the basal membrane of endothelial cells. Understanding factors that impact on the kinetics of blood clearance and the biodistribution of Ad vectors would be beneficial to advancing their application as therapeutic agents.

Systemically administered Ad vectors are rapidly cleared from the blood of mice, with a half-life of less than 3 min (Alemany *et al.*, 2000; Koizumi *et al.*, 2003a; Sakurai *et al.*, 2003b). Liver Kupffer cells play a central role in clearing Ad genomes from the bloodstream (Lieber *et al.*, 1997; Wolff *et al.*, 1997; Worgall *et al.*, 1997). Activated Kupffer cells (and monocytes and resident macrophages) release proinflammatory cytokines/chemokines such as interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), interferon γ-inducible protein 10 (IP-10), and RANTES (regulated on activation, normal T cell expressed and secreted), causing the activation of an innate immune response (Liu and Muruve, 2003). It has been proposed that a low dose of Ad vectors ($\sim 10^{10}$ vector particles) is rapidly sequestered by Kupffer cells (nonparenchymal cells), whereas higher doses of Ad vectors are delivered into both Kupffer cells and parenchymal cells, leading to a nonlinear dose response in hepatic transgene expression (Tao *et al.*, 2001). At a dose of 3.0×10^{10} vector particles, Ad vectors are likely to be equally distributed to Kupffer and parenchymal cells (Koizumi *et al.*, 2003a).

The liver directivity of the systematically administered Ad vectors can also be applied when local administration of the vectors is performed. Even if the Ad vector is injected into local tissues such as tumors, large amounts of vector are distributed into the bloodstream and targeted into the liver, causing unwanted side effects (Mizuguchi and Hayakawa, 2002b; Okada *et al.*, 2003). The process of Ad vector-mediated liver transduction is influenced by interactions between viral components and cellular receptors (discussed in Truly Targeted Adenovirus Vectors, below), the size of the sinusoidal fenestrae (Fechner *et al.*, 1999; Lievens *et al.*, 2004), and the complement system (Zinn *et al.*, 2004). Lievens *et al.* showed that Ad vector-mediated liver transduction in Dutch Belt rabbits, with 124-nm sinusoidal fenestrae, is significantly higher than that in New Zealand White rabbits, which have 108-nm sinusoidal fenestrae, and Fauve de Bourgogne rabbits with 105-nm sinusoidal fenestrae (Lievens *et al.*, 2004). The increase in sinusoidal fenestrae to 123 nm in New Zealand White rabbits by the intraportal injection of sodium decanoate enhances Ad vector-mediated liver transduction, confirming that the size of the sinusoidal

fenestrae is an important determinant for liver transduction (Lievens *et al.*, 2004). For targeting Ad vector to extrahepatic tissues, it is important to avoid distribution into parenchymal and nonparenchymal (Kupffer) cells of the liver as well as other tissues, such as spleen.

APPROACHES TO DEVELOPING TARGETED ADENOVIRUS VECTORS

Genetic modification of the virus capsid

Modification of virus tropism. Modification of the fiber proteins has been used to successfully overcome barriers to transduction due to a paucity of CAR. Two approaches have been used for this purpose. One is the addition of foreign peptides to the HI loop or C terminus of the fiber knob (Wickham *et al.*, 1997; Dmitriev *et al.*, 1998; Krasnykh *et al.*, 1998; Mizuguchi *et al.*, 2001; Koizumi *et al.*, 2003b). Another is the substitution of fibers derived from other Ad serotypes, which bind to receptor molecules other than CAR (Gall *et al.*, 1996; Stevenson *et al.*, 1997; Chillon *et al.*, 1999; Shayakhmetov *et al.*, 2000; Mizuguchi and Hayakawa, 2002a). Both approaches allow Ad tropism to be expanded (or changed) via binding of the modified fiber protein with a different cellular receptor.

Expanded and higher rates of gene transfer have been reported on the basis of the use of mutant fiber proteins containing an Arg-Gly-Asp (RGD) peptide (Wickham *et al.*, 1997; Dmitriev *et al.*, 1998; Hidaka *et al.*, 1999; Mizuguchi *et al.*, 2001) or a stretch of lysine residues (KKKKKKK [K_7] peptide) (Wickham *et al.*, 1997; Hidaka *et al.*, 1999), which target α_v integrins or heparan sulfates to the cellular surface, respectively. The RGD peptide has been displayed in the HI loop or C terminus of the fiber knob, whereas the K_7 peptide has been displayed at the C terminus of the fiber knob. There have also been reports of inserting the peptides into the HI loop of the fiber knob, including those discovered by phage display library to show high affinity for vascular endothelial cells (Nicklin *et al.*, 2000), cancer cells (Nicklin *et al.*, 2003), transferrin receptor (Xia *et al.*, 2000), and vascular smooth muscle cells (Work *et al.*, 2004).

Altered vector tropism was reported by substitution of the Ad type 5 (Ad5) fiber protein into that of Ad3, Ad7, Ad11, Ad16, Ad17, Ad35, and others (Gall *et al.*, 1996; Stevenson *et al.*, 1997; Chillon *et al.*, 1999; Shayakhmetov *et al.*, 2000; Goossens *et al.*, 2001; Havenga *et al.*, 2001; Rea *et al.*, 2001; Stecher *et al.*, 2001; Mizuguchi and Hayakawa, 2002a). Most Ad serotypes belonging to the subgroups A, C, D, E, and F use CAR as the initial receptor for the virion (Roelvink *et al.*, 1998), whereas Ad serotype B uses other molecules for infection (Roelvink *et al.*, 1998; Arnberg *et al.*, 2000a,b; Law and Davidson, 2002; Burmeister *et al.*, 2004). Ad8, Ad19, and Ad37, which belong to serotype D, use sialic acids as the primary receptor (Arnberg *et al.*, 2000a,b; Burmeister *et al.*, 2004). CD46, CD80, and CD86 were identified as cellular receptor(s) of Ad belonging to subgroup B, including Ad3, Ad11, Ad14, Ad16, Ad21, Ad35, and Ad50 (Gaggar *et al.*, 2003; Segerman *et al.*, 2003; Short *et al.*, 2004). Human CD34-positive cells, dendritic cells, synoviocytes, vascular endothelial cells (ECs), and smooth muscle cells (SMCs), which were poorly transfectable by conventional Ad vectors, were efficiently transfected by fiber-substituted Ad vectors (Shayakhmetov *et al.*, 2000; Goossens *et al.*, 2001; Havenga *et al.*, 2001; Okada *et al.*, 2001; Rea *et al.*, 2001). Mercier *et al.* described the creation of a chimeric Ad vector encoding the reovirus attachment protein σ1, which targets cells expressing the junctional adhesion molecule 1 (JAM1) (Mercier *et al.*, 2004).

When modified Ad vectors are injected locally into target tissue expressing corresponding receptors, the affinity of the vector for the cells increases, thereby resulting not only in higher transduction efficiency, but also in decreased vector dissemination. We reported that the intratumoral administration of luciferase-expressing Ad vectors containing the RGD peptide in the HI loop of the fiber knob resulted in nearly 40 times more transgene production in tumor, but 8 times less transgene expression in liver in the B16 mouse melanoma model as compared with conventional Ad vectors (Mizuguchi and Hayakawa, 2002b).

Other candidate locations for insertion of foreign ligands into the Ad capsid are the pIX, the penton base, and the hypervariable region (HVR) 5 of hexon loop L1 (Wickham *et al.*, 1995; Vigne *et al.*, 1999; Dmitriev *et al.*, 2002; Vellinga *et al.*, 2004). Among them, pIX seems to be the most promising. pIX is a minor structural protein that is contained in the Ad virion, and enhances the structural integrity of the particles by stabilizing hexon–hexon interaction (Ghosh-Choudhury *et al.*, 1987; Furcinitti *et al.*, 1989). It also plays a role in transcriptional activity and nuclear reorganization (Rosa-Calatrava *et al.*, 2001). Foreign ligands are displayed at the C terminus of the pIX of Ad (Dmitriev *et al.*, 2002). The attractive characteristics of ligand insertion into the pIX region is that the C terminus of pIX tolerates the insertion of large peptides. By incorporation of the pIX–green fluorescent protein (GFP) fusion protein, a fluorescent Ad was generated (Le *et al.*, 2004; Meulenbroek *et al.*, 2004). The insertion of higher affinity ligands such as single-chain antibodies (scFv) would be ideal, although generating such Ad vectors might be difficult because of impaired assembly of complex scFv–pIX fusion proteins in the nucleus. One problem with pIX fusions is that Ad pIX resides below the top of the hexon capsomer, within the core of the virus. This problem was circumvented by incorporating an α-helical spacer into the ligand–pIX fusion protein so as to lift the ligand and expose it to the surface of the capsid (Vellinga *et al.*, 2004). However, Ad vectors containing the RGD motif in the C terminus of pIX with α-helical spacers are likely to be less efficient than Ad vectors containing the RGD motif in the HI loop of the fiber knob (Vellinga *et al.*, 2004). Additional modification may be required for improved efficacy and specificity of retargeting.

Several groups have developed an Ad vector from an entire Ad35, and have demonstrated higher transduction efficiency for the Ad35 vector into human CD34-positive cells and dendritic cells compared with the conventional Ad5 vector (Gao *et al.*, 2003; Sakurai *et al.*, 2003a,b; Seshidhar Reddy *et al.*, 2003; Vogels *et al.*, 2003). In addition, Ad35 vectors have the advantage of evading humoral immune responses against Ad5. However, fiber-substituted Ad5 vectors containing fiber proteins of another serotype do not circumvent the immune response against Ad5 (Gall *et al.*, 1996; Ophorst *et al.*, 2004), because hexon is the major target of host-neutralizing antibodies in Ad5 infec-

tion (Gall *et al.*, 1996, 1998; Roy *et al.*, 1998). The Ad35 vector would be an effective alternative for use in persons with neutralizing antibodies in Ad5, and in the second injection when the Ad5 vector is used in the first injection of *in vivo* gene therapy.

Truly targeted adenovirus vectors. Although modifications described above yield Ad vectors with greatly improved transduction to many cells lacking in CAR expression, when systemically administered, vector dissemination, resulting in accumulation in liver, is unavoidable. To create a strictly targeted Ad vector, two basic requirements must be met. The first is construction of vectors that abolish natural viral tropism. The second is identification and incorporation of a foreign ligand with high affinity for a specific cellular receptor into the capsid of Ad vectors.

The capsid proteins determine the tropism of Ad. Because the fiber knob binds with CAR, this interaction first must be abolished. Mutation of the AB, DE, or FG loop of the fiber knob has been reported to abolish the fiber–CAR interaction (Bewley *et al.*, 1999; Kirby *et al.*, 1999; Roelvink *et al.*, 1999). These mutations of the fiber knob greatly reduce the transduction efficiency of Ad vectors to CAR-positive cells *in vitro*. In another strategy, Nakamura *et al.* replaced the tail, shaft, and knob domains of the Ad5 fiber with those of the Ad40 short fiber, which is hypothesized not to bind to any receptors (Nakamura *et al.*, 2003). In addition, interaction of the RGD motif of the penton base with α_v integrin must be abolished, although this interaction might be minor, at least *in vitro* (Mizuguchi *et al.*, 2002). The ablation of α_v integrin binding was accomplished by deletion of the RGD motif of the penton bases. Several articles reported that a single mutation of either the fiber knob or penton base does not change the biodistribution of Ad vectors in mice after *in vivo* injection (Alemany and Curiel, 2001; Leissner *et al.*, 2001; Mizuguchi *et al.*, 2002), whereas double mutation reduces liver transduction (Einfeld *et al.*, 2001; Koizumi *et al.*, 2003a), although two groups showed that double mutation also does not reduce liver transduction (Martin *et al.*, 2003; Smith *et al.*, 2003b). The reason for this discrepancy is unclear. However, Nicol *et al.* reported that combining fiber knob and penton base mutations reduces liver transduction by 509-fold in rats, an effect not observed in parallel experiments in mice (Nicol *et al.*, 2004). Subtle differences among the vectors, such as differences in mutated amino acids, experimental animal strains used, or injected doses, might have caused these discrepancies. Furthermore, the fiber shaft domain of Ad5 was reported to be involved in accumulation in the mouse liver of systemically administrated Ad vectors (Nakamura *et al.*, 2003; Smith *et al.*, 2003b), possibly because of the interaction of the KKTK (Lys-Lys-Thr-Lys) motif on the fiber shaft of Ad5 with heparan sulfate (Smith *et al.*, 2003b). This effect was also observed in nonhuman primate (cynomolgus monkey) models (Smith *et al.*, 2003a). According to our data, triple mutation of

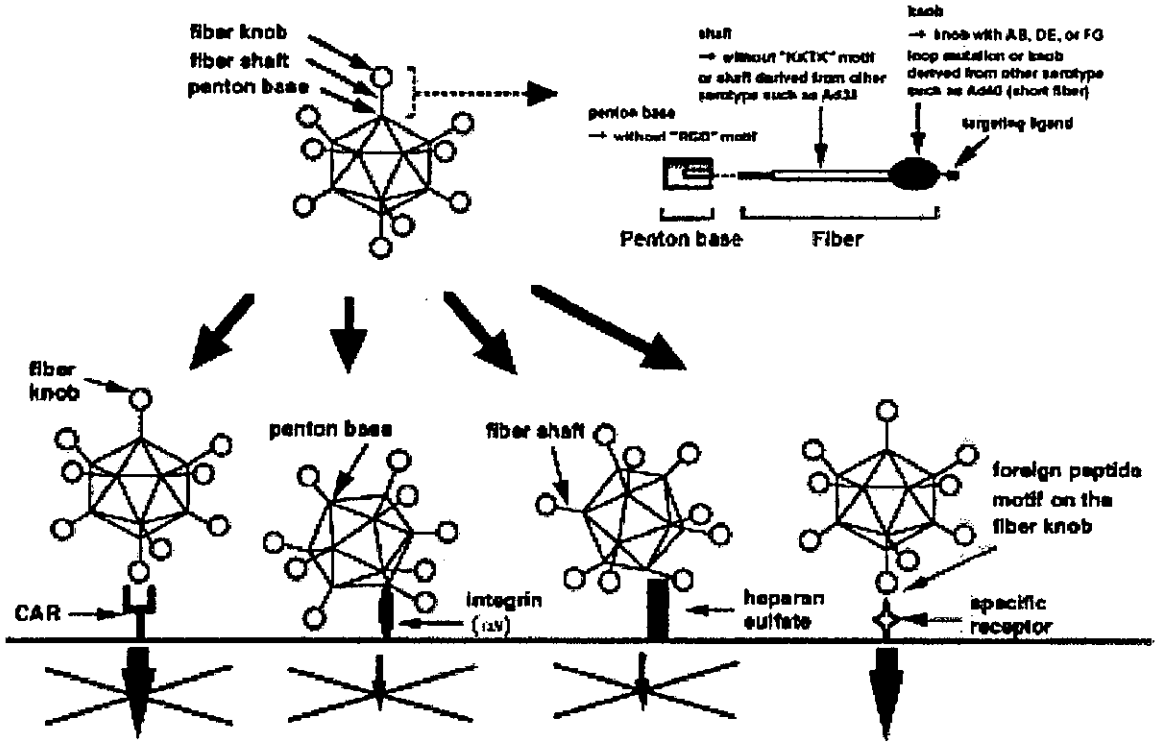

FIG. 2. Schematic diagram of Ad vectors targeted by the genetic approach. The CAR-, α_v integrin-, and heparan sulfate-binding activities of the Ad capsid are completely ablated by mutations in the fiber knob, the fiber shaft, and the penton base, respectively (Koizumi *et al.*, 2003a; Nicol *et al.*, 2004). Targeting ligands should be incorporated in the virus capsid, such as the fiber, the penton base, the hexon, or pIX. The resulting targeted Ad vectors transduce cells via the incorporated foreign ligand-dependent, CAR-, α_v integrin-, and heparan sulfate-independent pathway.

the fiber knob, shaft, and penton base mediated levels of liver transduction more than 30,000-fold lower than that of conventional Ad vectors (Koizumi *et al.*, 2003a). This vector contains a CAR-binding ablated mutant fiber knob derived from Ad5, a fiber shaft derived from Ad35 (the fiber shaft of Ad35 does not have the KKTK motif and is shorter than that of Ad5 [Ad5 fiber shaft, 6 β repeats; Ad5 fiber shaft, 22 β repeats]), a fiber tail derived from Ad5, and a mutant penton base of Ad5 without the RGD motif. Ad vectors, with mutations in two domains of the fiber knob, the fiber shaft, and the penton base, showed a level of liver transduction intermediate between that of conventional Ad vectors and the triple-mutant Ad vectors (Koizumi *et al.*, 2003a). Nicol *et al.* reached a similar conclusion in a rat model (Nicol *et al.*, 2004). Thus, Ad tropism would be determined by at least three factors: the fiber knob, the fiber shaft, and the RGD motif of the penton base (Figs. 1A and 2). Triple mutations, including the fiber knob, the fiber shaft, and the RGD motif of the penton base, should be preferable for the platform of targeted Ad vectors.

A detailed study on vector distribution to the liver, however, suggested that triple-mutant Ad vectors distribute to nonparenchymal cells to a similar extent as conventional vectors, and that both vectors are cleared rapidly from the bloodstream, having a half-life of less than 2 min (Koizumi *et al.*, 2003a). This nonparenchymal cell-mediated clearance might present an obstacle to the development of targeted Ad vectors that incorporate a foreign ligand into the viral capsid. One promising strategy to overcome this problem might be intraperitoneal, not intravenous, injection of the vector. Akiyama *et al.* reported that the intraperitoneal administration of CAR and integrin binding-ablated Ad vectors increases their persistence in the bloodstream, although the mechanism by which this occurs is unknown (Akiyama *et al.*, 2004). Extended release of the vector from the cavity might change its pharmacokinetics. More detailed study is needed to clarify nonparenchymal cell-mediated vector clearance. Lower clearance from the bloodstream may lead to increased delivery of the vector to the tissue of interest, if an appropriate targeting ligand is incorporated into the vector.

The identification of targeting ligands that are displayed in the capsid, such as fiber and pIX, is another challenge. A display library using filamentous phage is widely used for the identification of functional peptides for targeting. Although some success in identifying peptide ligands for the targeted Ad vectors was reported (Nicklin *et al.*, 2000; Xia *et al.*, 2000; Work *et al.*, 2004), most peptides that are identified by phage display libraries are not functional when they are displayed in the fiber knob of Ad vectors. Foreign peptides inserted into the HI loop of the fiber knob are constrained at both the N and C termini, whereas peptides inserted at the C terminus of the fiber knob are constrained only at the N terminus. In contrast, peptides identified by filamentous phage display library are constrained only at the C terminus, when the peptides are displayed as a fusion protein with the product of gene III of the phage. The lack of efficacy of peptides inserted in the fiber knob could be due to this difference when the peptides are identified. Furthermore, the lack of efficacy would be dependent on conformational changes after ligation of the peptide to the fiber knob. To overcome these limitations, Pereboev *et al.* employed a modified filamentous phage-displayed system, pJuFo, which was originally designed to display C-terminal protein fragments (Pereboev *et al.*, 2001). They developed a system for displaying peptides in the context of the fiber knob on the surface of the phage. A display system based on phage λ, which expresses a functional Ad fiber knob on the surface, was also developed (Fontana *et al.*, 2003). By using these systems, Ad vectors containing novel peptide ligands were generated, transducing NIH3T3 and dendritic cells at 100- to 1000-fold higher efficiency than conventional vectors (Fontana *et al.*, 2003). The development and evaluation of the next generation of targeted vectors by incorporating the novel peptides into native tropism-ablated Ad vectors is expected. In the case of the adeno-associated virus (AAV) vector, a method for incorporating random small peptides in the viral capsid has been developed (Muller *et al.*, 2003). This type of screening for ligands might be useful for targeted Ad vector, although the creation of an Ad library with wide diversity is a challenge.

Propagation of modified Ad vectors that no longer bind with cellular receptors (CAR, α_v integrin, and heparan sulfate) requires a special packaging cell line. Two types of packaging cell lines have been reported. One utilizes 293 cells modified to express an artificial receptor molecule (Douglas *et al.*, 1999; Roelvink *et al.*, 1999) that should not have any natural analogs, such as the anti-His single-chain antibody (scFv) and anti-hemagglutinin (HA) scFv. The other approach is to use 293 cells expressing Ad5 fiber protein (Fiber-293 cells) (Von Seggern *et al.*, 1998; Legrand *et al.*, 1999; Koizumi *et al.*, 2003a). In the case of cell lines expressing anti-His scFv, a His tag sequence has been introduced into the C-terminal region of the fiber knob in Ad vectors (Douglas *et al.*, 1999), whereas in the case of cell lines expressing anti-HA scFv, an HA tag sequence has been introduced into the HI loop of the fiber knob or the penton base instead of the RGD motif (Roelvink *et al.*, 1999). Modified Ad vectors are generated by interaction of the tag sequence in the virus with the scFv against the tag sequence on the cells. When the modified Ad vectors are propagated in Fiber-293 cells, wild-type fibers are incorporated in the virus during amplification, resulting in the virus containing both wild-type fibers and mutated fibers. This virus infects 293 (Fiber-293) cells via the wild-type fiber. At the final stage of viral amplification, mutated Ad vectors are allowed to infect normal 293 cells. The recovered viruses should contain only mutant fiber proteins. When Fiber-293 cells have been used as packaging cell lines, either the HI loop or the C-terminal region of the fiber knob as well as the penton base can be used to display a foreign ligand on the vectors. This makes these cells advantageous over cell lines expressing anti-His scFv or anti-HA scFv. In both methods, modified vectors were generated to particle titers similar to that of conventional Ad vectors (Douglas *et al.*, 1999; Roelvink *et al.*, 1999; Koizumi *et al.*, 2003a).

Another strategy to ablate CAR binding by Ad vectors is to proteolytically remove the knob domain of Ad fibers via the insertion of a single factor Xa cleavage site in the fiber shaft, between the cellular ligand and knob domain (Magnusson *et al.*, 2001; Hong *et al.*, 2003; Gaden *et al.*, 2004). As cellular ligands, the RGD peptide and a 58-residue oligopeptide termed the affibody, which binds specifically to the human IgG1 Fc domain, were introduced and ligand-mediated gene transfer was reported (Magnusson *et al.*, 2001; Hong *et al.*, 2003; Gaden *et al.*, 2004).

Ad vectors in which the fiber protein was replaced with phage T4 fibritin were also developed (Krasnykh *et al.*, 2001;

Belousova *et al.*, 2003; Papanikolopoulou *et al.*, 2004). In these vectors, structural similarity between the Ad fiber and bacteriophage T4 fibritin proteins was used, and the fiber shaft and knob domains were replaced with T4 fibritin and a receptor-binding ligand. The human CD40 ligand was functionally displayed in the chimeric fiber of the Ad vectors (Belousova *et al.*, 2003). This approach seems to overcome structural conflicts between the fiber and the targeting ligand.

As described above, several types of vector systems have been developed, using a genetic strategy. These vectors would provide a platform for future targeted Ad vector development. Future efforts should be directed toward novel ligands for specific tissue targeting.

Modification by the use of adaptor molecules

Retargeting of Ad infection can also be achieved through the use of bispecific or bifunctional adaptor molecules composed of an anti-fiber antibody fragment and a cell-binding component. Douglas *et al.* conjugated folate to the neutralizing Fab fragment of an anti-fiber monoclonal antibody (mAb). This Fab–folate conjugate was complexed with an Ad vector and shown to redirect, at high efficiency, the Ad infection of target cells via the folate receptor (Douglas *et al.*, 1999). The Fab fragment of the anti-fiber mAb has been utilized to conjugate with several other ligands including fibroblast growth factor 2 (FGF-2) (Goldman *et al.*, 1997; Sosnowski *et al.*, 1999), epidermal growth factor receptor (EGFR) (Miller *et al.*, 1998), and an anti-CD40 mAb fragment (Tillman *et al.*, 1999). Reynolds *et al.* succeeded in targeting pulmonary endothelial cells *in vivo* by the intravenous injection of Ad vectors complexed with bispecific antibody against the Ad fiber knob and angiotensin-converting enzyme (Reynolds *et al.*, 2000). In a similar strategy, the anti-Ad fiber knob scFv (Watkins *et al.*, 1997; Haisma *et al.*, 2000; Nettelbeck *et al.*, 2001) or the extracellular domain of CAR (Dmitriev *et al.*, 2000; Itoh *et al.*, 2003) was used as the attachment molecule with the virus. Fusion proteins or complexes of ligands with the anti-Ad fiber knob scFv or CAR were used as adaptor molecules (Fig. 1B).

Combination of the adaptor molecule and genetically modified capsids of the Ad vector has also been reported. The Fc-binding domain of staphylococcal protein A was genetically incorporated into the Ad fiber protein (Henning *et al.*, 2002; Korokhov *et al.*, 2003; Volpers *et al.*, 2003). Two studies incorporated the Fc-binding domain into either the HI loop or C terminus of the fiber knob (Korokhov *et al.*, 2003; Volpers *et al.*, 2003), whereas one study incorporated the Fc-binding domain into a knob-deleted fiber containing seven shaft repeats and an external trimerization motif (Henning *et al.*, 2002). Targeting components such as the antibody and fusion protein of the ligand with the Fc domain of immunoglobulin effectively bind to the modified Ad vectors, resulting in specific gene delivery. Because the target-specific ligands such as antibodies are simply changed in this system, these types of Ad vectors should be useful for systematic screening and detection of the target-specific ligands, as well as for therapeutic applications.

Metabolically biotinylated Ad vectors have been developed as another type of vector with adaptor molecule and genetically modified capsid. Barry and colleagues designed a system based on the fusion of a truncated form of the *Propionibacterium shermanii* 1.3S transcarboxylase domain (PSTCD), which functions as a biotin acceptor peptide (BAP) and is efficiently biotinylated by human holocarboxylase synthetase, to the C terminus of the Ad fiber protein (Parrott *et al.*, 2003) or the C terminus of the Ad pIX protein (Campos *et al.*, 2004). In this system, Ad vectors containing BAP are metabolically biotinylated during vector production by the endogenous biotin ligase in 293 cells, resulting in covalently biotinylated virions. Biotinylated Ad vectors are useful as a platform for avidin-based ligand screening and vector targeting by conjugating biotinylated ligands to the virus, using high-affinity tetrameric avidin. Their group performed ligand screening for dendritic cells, using biotinylated Ad vectors (Parrott *et al.*, 2003).

Theoretically, in all the approaches discussed above, any conjugates with one component directed against the Ad capsid (or modified capsid) and the second component directed against the cell surface protein can be applied to increase transduction of target cells. The advantage is that the natural tropism of the fiber knob is usually ablated, possibly as a result of steric hindrance by adaptor molecules. One limitation is that complexes of Ad vectors and adaptor molecules are nonuniform, and batch-to-batch difference of the vectors might occur.

Chemical modification by polymers

Chemical modification with polyethylene glycol (PEG; PEGylation) is frequently used in pharmaceutic preparations to provide a hydrophilic coat and to increase the blood persistence of therapeutic peptides and proteins (Harris and Chess, 2003). Modification of Ad vectors with PEG, in which the activated PEG reacts preferentially with the ε-amino terminal of lysine residues on the capsid, including the hexon, fiber, and penton base, prolongs persistence in the blood and circumvents neutralization of the Ad vectors by antibodies (O'Riordan *et al.*, 1999; Romanczuk *et al.*, 1999; Alemany *et al.*, 2000; Croyle *et al.*, 2000, 2001, 2002; Lanciotti *et al.*, 2003; Eto *et al.*, 2004; Ogawara *et al.*, 2004) (Fig. 1C). Furthermore, PEGylated Ad vectors attenuate the ability of the vector to be taken up by antigen-presenting cells, thereby reducing inflammatory responses. Animals administered PEGylated Ad vectors exhibited reduced levels of both cell-mediated and humoral immune responses, resulting in significant gene expression on readministration of unmodified Ad vectors in the lung (O'Riordan *et al.*, 1999; Croyle *et al.*, 2001). However, the PEGylation of Ad vectors leads to loss of infectivity due to steric hindrance by PEG chains (O'Riordan *et al.*, 1999; Alemany *et al.*, 2000; Croyle *et al.*, 2000, 2001, 2002; Lanciotti *et al.*, 2003; Eto *et al.*, 2004; Ogawara *et al.*, 2004). The extent of loss of infectivity and extension of blood retention half-time are dependent on the degree of PEG modification (Eto *et al.*, 2004). The efficiency of transduction of 34% modified PEGylated Ad vectors was approximately 200-fold lower than that of unmodified Ad (Eto *et al.*, 2004).

To overcome the decreased efficiency of infection of PEGylated Ad vectors, vectors containing functional molecules on the tip of PEG have been developed (Lanciotti *et al.*, 2003; Eto *et al.*, 2004; Ogawara *et al.*, 2004). Lanciotti *et al.* reported targeted Ad vectors, using heterofunctional PEG and FGF-2 (Lanciotti *et al.*, 2003). The transduction of Ad/PEG/FGF2 is dependent on the FGF-2 receptor, and is independent of CAR. In

an intraperitoneal model of ovarian cancer, Ad/PEG/FGF2 mediated increased transgene expression in tumor tissue and reduced localization of the vectors to nontarget tissues compared with unmodified Ad vectors (Lanciotti *et al.*, 2003). Ogawara *et al.* reported PEGylated Ad vectors containing E-selectin-specific antibody at the tip of PEG, which target activated endothelial cells (Ogawara *et al.*, 2004). They showed that the systemic administration of PEGylated Ad vectors with anti-E-selectin antibody selectively targeted inflamed skin and mediated local transgene expression in mice with a delayed-type hypersensitivity (DTH) inflammation. As for modification with peptides, α_v integrin-specific RGD peptide-modified PEGylated Ad vectors have been developed (Eto *et al.*, 2004; Ogawara *et al.*, 2004).

Ad vectors coated with polymers other than PEG have also been developed. Seymour's group used a multivalent hydrophilic polymer based on poly[*N*-(2-hydroxypropyl)methacrylamide] to modify Ad vectors (Fisher *et al.*, 2001; Green *et al.*, 2004). Their vector showed an extended plasma circulation time and decreased toxicity, and evaded neutralizing antibodies.

Approaches by chemical modification with polymers are advantageous, in that many ligands, such as peptides, antibodies, and antigens, may be applied to the tips of the polymers. A great deal of knowledge and techniques about chemical modification have been acquired in the study of pharmaceutic preparations for drug delivery systems. Although improved pharmacokinetic properties of polymer (including PEG)-coated Ad vectors without ligands have been reported, those of polymer-coated Ad vectors with ligands have not been reported in detail. The exact nature of those vectors must be characterized further.

CONCLUSIONS

In this review, we have focused on the development of targeted Ad vectors based on specific virus entry mechanisms. These approaches are easily combined with transcriptional targeting, using tissue/cell-specific promoters. Ideally, combining a better targeted vector containing a modified capsid with a fully deleted Ad genome (i.e., helper-dependent Ad vectors) is desirable to reduce the innate and acquired immunogenicity of the vectors. These combined vectors should be carefully evaluated in terms of transgene expression profile, distribution of the vectors, and pharmacokinetics, including circulation half-life, interaction with blood components, and so on. Anatomical barriers, such as the tightness of endothelial cells, should also be taken into account, because the vectors must pass endothelial barriers to reach target tissues. Although progress still needs to be made in perfecting targeted Ad vectors, steady improvements have been achieved through comprehensive approaches. Targeted Ad vectors are a source of great promise for gene therapy in future, because they enhance gene therapy efficacy and permit the delivery of lower doses, which should result in reduced toxicity.

ACKNOWLEDGMENTS

We thank Z.-L. Xu and F. Sakurai for their discussions with us. H.M. is supported by grants from the Ministry of Health, Labor, and Welfare of Japan and by a Grant-in-Aid for Scientific Research on Priority Areas of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

REFERENCES

AKIYAMA, M., THORNE, S., KIRN, D., ROELVINK, P.W., EINFELD, D.A., KING, C.R., and WICKHAM, T.J. (2004). Ablating CAR and integrin binding in adenovirus vectors reduces nontarget organ transduction and permits sustained bloodstream persistence following intraperitoneal administration. Mol. Ther. 9, 218–230.

ALEMANY, R., and CURIEL, D.T. (2001). CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. Gene Ther. 8, 1347–1353.

ALEMANY, R., SUZUKI, K., and CURIEL, D.T. (2000). Blood clearance rates of adenovirus type 5 in mice. J. Gen. Virol. 81, 2605–2609.

ARNBERG, N., EDLUND, K., KIDD, A.H., and WADELL, G. (2000a). Adenovirus type 37 uses sialic acid as a cellular receptor. J. Virol. 74, 42–48.

ARNBERG, N., KIDD, A.H., EDLUND, K., OLFAT, F., and WADELL, G. (2000b). Initial interactions of subgenus D adenoviruses with A549 cellular receptors: Sialic acid versus α_v integrins. J. Virol. 74, 7691–7693.

BELOUSOVA, N., KOROKHOV, N., KRENDELSHCHIKOVA, V., SIMONENKO, V., MIKHEEVA, G., TRIOZZI, P.L., ALDRICH, W.A., BANERJEE, P.T., GILLIES, S.D., CURIEL, D.T., and KRASNYKH, V. (2003). Genetically targeted adenovirus vector directed to CD40-expressing cells. J. Virol. 77, 11367–11377.

BEWLEY, M.C., SPRINGER, K., ZHANG, Y.B., FREIMUTH, P., and FLANAGAN, J.M. (1999). Structural analysis of the mechanism of adenovirus binding to its human cellular receptor, CAR. Science 286, 1579–1583.

BURMEISTER, W.P., GUILLIGAY, D., CUSACK, S., WADELL, G., and ARNBERG, N. (2004). Crystal structure of species D adenovirus fiber knobs and their sialic acid binding sites. J. Virol. 78, 7727–7736.

CAMPOS, S.K., PARROTT, M.B., and BARRY, M.A. (2004). Avidin-based targeting and purification of a protein IX-modified, metabolically biotinylated adenoviral vector. Mol. Ther. 9, 942–954.

CHILLON, M., BOSCH, A., ZABNER, J., LAW, L., ARMENTANO, D., WELSH, M.J., and DAVIDSON, B.L. (1999). Group D adenoviruses infect primary central nervous system cells more efficiently than those from group C. J. Virol. 73, 2537–2540.

CROYLE, M.A., YU, Q.C., and WILSON, J.M. (2000). Development of a rapid method for the PEGylation of adenoviruses with enhanced transduction and improved stability under harsh storage conditions. Hum. Gene Ther. 11, 1713–1722.

CROYLE, M.A., CHIRMULE, N., ZHANG, Y., and WILSON, J.M. (2001). "Stealth" adenoviruses blunt cell-mediated and humoral immune responses against the virus and allow for significant gene expression upon readministration in the lung. J. Virol. 75, 4792–4801.

CROYLE, M.A., CHIRMULE, N., ZHANG, Y., and WILSON, J.M. (2002). PEGylation of E1-deleted adenovirus vectors allows significant gene expression on readministration to liver. Hum. Gene Ther. 13, 1887–1900.

DMITRIEV, I., KRASNYKH, V., MILLER, C.R., WANG, M., KASHENTSEVA, E., MIKHEEVA, G., BELOUSOVA, N., and CURIEL, D.T. (1998). An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus. J. Virol. 72, 9706–9713.

DMITRIEV, I., KASHENTSEVA, E., ROGERS, B.E., KRASNYKH, V., and CURIEL, D.T. (2000). Ectodomain of coxsackievirus and adenovirus receptor genetically fused to epidermal growth factor me-

diates adenovirus targeting to epidermal growth factor receptor-positive cells. J. Virol. 74, 6875–6884.

DMITRIEV, I.P., KASHENTSEVA, E.A., and CURIEL, D.T. (2002). Engineering of adenovirus vectors containing heterologous peptide sequences in the C terminus of capsid protein IX. J. Virol. 76, 6893–6899.

DOUGLAS, J.T., MILLER, C.R., KIM, M., DMITRIEV, I., MIKHEEVA, G., KRASNYKH, V., and CURIEL, D.T. (1999). A system for the propagation of adenoviral vectors with genetically modified receptor specificities. Nat. Biotechnol. 17, 470–475.

EINFELD, D.A., SCHROEDER, R., ROELVINK, P.W., LIZONOVA, A., KING, C.R., KOVESDI, I., and WICKHAM, T.J. (2001). Reducing the native tropism of adenovirus vectors requires removal of both CAR and integrin interactions. J. Virol. 75, 11284–11291.

ETO, Y., GAO, J.-Q., SEKIGUCHI, F., KURACHI, S., KATAYAMA, K., MIZUGUCHI, H., HAYAKAWA, T., MAEDA, M., KAWASAKI, K., TSUTSUMI, Y., MAYUMI, T., and NAKAGAWA, S. (2004). PEGylated adenovirus vectors containing RGD peptides on the tip of PEG show high transduction efficiency and antibody evasion ability. J. Gene Med. (in press).

FECHNER, H., HAACK, A., WANG, H., WANG, X., EIZEMA, K., PAUSCHINGER, M., SCHOEMAKER, R., VEGHEL, R., HOUTSMULLER, A., SCHULTHEISS, H.P., LAMERS, J., and POLLER, W. (1999). Expression of coxsackie adenovirus receptor and α_V-integrin does not correlate with adenovector targeting *in vivo* indicating anatomical vector barriers. Gene Ther. 6, 1520–1535.

FISHER, K.D., STALLWOOD, Y., GREEN, N.K., ULBRICH, K., MAUTNER, V., and SEYMOUR, L.W. (2001). Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. Gene Ther. 8, 341–348.

FONTANA, L., NUZZO, M., URBANELLI, L., and MONACI, P. (2003). General strategy for broadening adenovirus tropism. J. Virol. 77, 11094–11011.

FURCINITTI, P.S., VAN OOSTRUM, J., and BURNETT, R.M. (1989). Adenovirus polypeptide IX revealed as capsid cement by difference images from electron microscopy and crystallography. EMBO J. 8, 3563–3570.

GADEN, F., FRANQUEVILLE, L., MAGNUSSON, M.K., HONG, S., MERTEN, M.D., LINDHOLM, L., and BOULANGER, P. (2004). Gene transduction and cell entry pathway of fiber-modified adenovirus type 5 vectors carrying novel endocytic peptide ligands selected on human tracheal glandular cells. J. Virol. 78, 7227–7247.

GAGGAR, A., SHAYAKHMETOV, D.M., and LIEBER, A. (2003). CD46 is a cellular receptor for group B adenoviruses. Nat. Med. 9, 1408–1412.

GALL, J., KASS-EISLER, A., LEINWAND, L., and FALCK-PEDERSEN, E. (1996). Adenovirus type 5 and 7 capsid chimera: Fiber replacement alters receptor tropism without affecting primary immune neutralization epitopes. J. Virol. 70, 2116–2123.

GALL, J.G., CRYSTAL, R.G., and FALCK-PEDERSEN, E. (1998). Construction and characterization of hexon-chimeric adenoviruses: Specification of adenovirus serotype. J. Virol. 72, 10260–10264.

GAO, W., ROBBINS, P.D., and GAMBOTTO, A. (2003). Human adenovirus type 35: Nucleotide sequence and vector development. Gene Ther. 10, 1941–1949.

GHOSH-CHOUDHURY, G., HAJ-AHMAD, Y., and GRAHAM, F.L. (1987). Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. EMBO J. 6, 1733–1739.

GOLDMAN, C.K., ROGERS, B.E., DOUGLAS, J.T., SOSNOWSKI, B.A., YING, W., SIEGAL, G.P., BAIRD, A., CAMPAIN, J.A., and CURIEL, D.T. (1997). Targeted gene delivery to Kaposi's sarcoma cells via the fibroblast growth factor receptor. Cancer Res. 57, 1447–1451.

GOOSSENS, P.H., HAVENGA, M.J., PIETERMAN, E., LEMCKERT, A.A., BREEDVELD, F.C., BOUT, A., and HUIZINGA, T.W. (2001). Infection efficiency of type 5 adenoviral vectors in synovial tissue can be enhanced with a type 16 fiber. Arthritis Rheum. 44, 570–577.

GREEN, N.K., HERBERT, C.W., HALE, S.J., HALE, A., MAUTNER, V., HARKINS, R., HERMISTON, T., ULBRICH, K., FISHER, K.D., and SEYMOUR, L.W. (2004). Extended plasma circulation time and decreased toxicity of polymer-coated adenovirus. Gene Ther. 11, 1256–1263.

HAISMA, H.J., GRILL, J., CURIEL, D.T., HOOGELAND, S., VAN BEUSECHEM, V.W., PINEDO, H.M., and GERRITSEN, W.R. (2000). Targeting of adenoviral vectors through a bispecific single-chain antibody. Cancer Gene Ther. 7, 901–904.

HARRIS, J.M., and CHESS, R.B. (2003). Effect of pegylation on pharmaceuticals. Nat. Rev. Drug Discov. 2, 214–221.

HAVENGA, M.J., LEMCKERT, A.A., GRIMBERGEN, J.M., VOGELS, R., HUISMAN, L.G., VALERIO, D., BOUT, A., and QUAX, P.H. (2001). Improved adenovirus vectors for infection of cardiovascular tissues. J. Virol. 75, 3335–3342.

HENNING, P., MAGNUSSON, M.K., GUNNERIUSSON, E., HONG, S., BOULANGER, P., NYGREN, P.A., and LINDHOLM, L. (2002). Genetic modification of adenovirus 5 tropism by a novel class of ligands based on a three-helix bundle scaffold derived from staphylococcal protein A. Hum. Gene Ther. 13, 1427–1439.

HIDAKA, C., MILANO, E., LEOPOLD, P.L., BERGELSON, J.M., HACKETT, N.R., FINBERG, R.W., WICKHAM, T.J., KOVESDI, I., ROELVINK, P., and CRYSTAL, R.G. (1999). CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts. J. Clin. Invest. 103, 579–587.

HONG, S.S., MAGNUSSON, M.K., HENNING, P., LINDHOLM, L., and BOULANGER, P.A. (2003). Adenovirus stripping: A versatile method to generate adenovirus vectors with new cell target specificity. Mol. Ther. 7, 692–699.

ITOH, A., OKADA, T., MIZUGUCHI, H., HAYAKAWA, T., MIZUKAMI, H., KUME, A., TAKATOKU, M., KOMATSU, N., HANAZONO, Y., and OZAWA, K. (2003). A soluble CAR–SCF fusion protein improves adenoviral vector-mediated gene transfer to c-Kit-positive hematopoietic cells. J. Gene Med. 5, 929–940.

KIRBY, I., DAVISON, E., BEAVIL, A.J., SOH, C.P.C., WICKHAM, T.J., ROELVINK, P.W., KOVESDI, I., J. SUTTON, B.J., and SANTIS, G. (1999). Mutations in the DG Loop of adenovirus type 5 fiber knob protein abolish high-affinity binding to its cellular receptor CAR. J. Virol. 73, 9508–9514.

KOIZUMI, N., MIZUGUCHI, H., SAKURAI, F., YAMAGUCHI, T., WATANABE, Y., and HAYAKAWA, T. (2003a). Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and α_V integrin-binding ablation. J. Virol. 77, 13062–13072.

KOIZUMI, N., MIZUGUCHI, H., UTOGUCHI, N., WATANABE, Y., and HAYAKAWA, T. (2003b). Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. J. Gene Med. 5, 267–276.

KOROKHOV, N., MIKHEEVA, G., KRENDELSHCHIKOV, A., BELOUSOVA, N., SIMONENKO, V., KRENDELSHCHIKOVA, V., PEREBOEV, A., KOTOV, A., KOTOVA, O., TRIOZZI, P.L., ALDRICH, W.A., DOUGLAS, J.T., LO, K.M., BANERJEE, P.T., GILLIES, S.D., CURIEL, D.T., and KRASNYKH, V. (2003). Targeting of adenovirus via genetic modification of the viral capsid combined with a protein bridge. J. Virol. 77, 12931–12940.

KRASNYKH, V., DMITRIEV, I., MIKHEEVA, G., MILLER, C.R., BELOUSOVA, N., and CURIEL, D.T. (1998). Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. J. Virol. 72, 1844–1852.

KRASNYKH, V., BELOUSOVA, N., KOROKHOV, N., MIKHEEVA, G., and CURIEL, D.T. (2001). Genetic targeting of an adenovirus vector via replacement of the fiber protein with the phage T4 fibritin. J. Virol. 75, 4176–4183.

LANCIOTTI, J., SONG, A., DOUKAS, J., SOSNOWSKI, B., PIERCE, G., GREGORY, R., WADSWORTH, S., and O'RIORDAN, C. (2003). Targeting adenoviral vectors using heterofunctional polyethylene glycol FGF2 conjugates. Mol. Ther. 8, 99–107.

LAW, L.K., and DAVIDSON, B.L. (2002). Adenovirus serotype 30 fiber does not mediate transduction via the coxsackie–adenovirus receptor. J. Virol. 76, 656–661.

LE, L.P., EVERTS, M., DMITRIEV, I.P., DAVYDOVA, J.G., YAMAMOTO, M., and CURIEL, D.T. (2004). Fluorescently labeled adenovirus with pIX–EGFP for vector detection. Mol. Imaging 3, 105–116.

LEGRAND, V., SPEHNER, D., SCHLESINGER, Y., SETTELEN, N., PAVIRANI, A., and MEHTALI, M. (1999). Fiberless recombinant adenoviruses: Virus maturation and infectivity in the absence of fiber. J. Virol. 73, 907–919.

LEISSNER, P., LEGRAND, V., SCHLESINGER, Y., HADJI, D.A., VAN RAAIJ, M., CUSACK, S., PAVIRANI, A., and MEHTALI, M. (2001). Influence of adenoviral fiber mutations on viral encapsidation, infectivity and *in vivo* tropism. Gene Ther. 8, 49–57.

LIEBER, A., HE, C.-Y., MEUSE, L., SCHOWALTER, D., KIRILLOVA, I., WINTHER, B., and KAY, M.A. (1997). The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. J. Virol. 71, 8798–8807.

LIEVENS, J., SNOEYS, J., VEKEMANS, K., VAN LINTHOUT, S., DE ZANGER, R., COLLEN, D., WISSE, E., and DE GEEST, B. (2004). The size of sinusoidal fenestrae is a critical determinant of hepatocyte transduction after adenoviral gene transfer. Gene Ther. 11, 1523–1531.

LIU, Q., and MURUVE, D.A. (2003). Molecular basis of the inflammatory response to adenovirus vectors. Gene Ther. 10, 935–940.

MAGNUSSON, M.K., HONG, S.S., BOULANGER, P., and LINDHOLM, L. (2001). Genetic retargeting of adenovirus: Novel strategy employing "deknobbing" of the fiber. J. Virol. 75, 7280–7289.

MARTIN, K., BRIE, A., SAULNIER, P., PERRICAUDET, M., YEH, P., and VIGNE, E. (2003). Simultaneous CAR- and α_V integrin-binding ablation fails to reduce Ad5 liver tropism. Mol. Ther. 8, 485–494.

MERCIER, G.T., CAMPBELL, J.A., CHAPPELL, J.D., STEHLE, T., DERMODY, T.S., and BARRY, M.A. (2004). A chimeric adenovirus vector encoding reovirus attachment protein sigma1 targets cells expressing junctional adhesion molecule 1. Proc. Natl. Acad. Sci. U.S.A. 101, 6188–6193.

MEULENBROEK, R.A., SARGENT, K.L., LUNDE, J., JASMIN, B.J., and PARKS, R.J. (2004). Use of adenovirus protein IX (pIX) to display large polypeptides on the virion: Generation of fluorescent virus through the incorporation of pIX-GFP. Mol. Ther. 9, 617–624.

MILLER, C.R., BUCHSBAUM, D.J., REYNOLDS, P.N., DOUGLAS, J.T., GILLESPIE, G.Y., MAYO, M.S., RABEN, D., and CURIEL, D.T. (1998). Differential susceptibility of primary and established human glioma cells to adenovirus infection: Targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. Cancer Res. 58, 5738–5748.

MIZUGUCHI, H., and HAYAKAWA, T. (2002a). Adenovirus vectors containing chimeric type 5 and type 35 fiber proteins exhibit altered and expanded tropism and increase the size limit of foreign genes. Gene 285, 69–77.

MIZUGUCHI, H., and HAYAKAWA, T. (2002b). Enhanced anti-tumor effect and reduced vector dissemination with fiber-modified adenovirus vectors expressing herpes simplex virus thymidine kinase. Cancer Gene Ther. 9, 236–242.

MIZUGUCHI, H., KOIZUMI, N., HOSONO, T., UTOGUCHI, N., WATANABE, Y., KAY, M.A., and HAYAKAWA, T. (2001). A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. Gene Ther. 8, 730–735.

MIZUGUCHI, H., KOIZUMI, N., HOSONO, T., ISHII-WATABE, A., UCHIDA, E., UTOGUCHI, N., WATANABE, Y., and HAYAKAWA, T. (2002). CAR- or α_V integrin-binding ablated adenovirus vectors, but not fiber-modified vectors containing RGD peptide, do not change the systemic gene transfer properties in mice. Gene Ther. 9, 769–776.

MULLER, O.J., KAUL, F., WEITZMAN, M.D., PASQUALINI, R., ARAP, W., KLEINSCHMIDT, J.A., and TREPEL, M. (2003). Random peptide libraries displayed on adeno-associated virus to select for targeted gene therapy vectors. Nat. Biotechnol. 21, 1040–1046.

NAKAMURA, T., SATO, K., and HAMADA, H. (2003). Reduction of natural adenovirus tropism to the liver by both ablation of fiber-coxsackievirus and adenovirus receptor interaction and use of replaceable short fiber. J. Virol. 77, 2512–2521.

NETTELBECK, D.M., MILLER, D.W., JEROME, V., ZUZARTE, M., WATKINS, S.J., HAWKINS, R.E., MULLER, R., and KONTERMANN, R.E. (2001). Targeting of adenovirus to endothelial cells by a bispecific single-chain diabody directed against the adenovirus fiber knob domain and human endoglin (CD105). Mol. Ther. 3, 882–891.

NICKLIN, S.A., WHITE, S.J., WATKINS, S.J., HAWKINS, R.E., and BAKER, A.H. (2000). Selective targeting of gene transfer to vascular endothelial cells by use of peptides isolated by phage display. Circulation 102, 231–237.

NICKLIN, S.A., DISHART, K.L., BUENING, H., REYNOLDS, P.N., HALLEK, M., NEMEROW, G.R., VON SEGGERN, D.J., and BAKER, A.H. (2003). Transductional and transcriptional targeting of cancer cells using genetically engineered viral vectors. Cancer Lett. 201, 165–173.

NICOL, C.G., GRAHAM, D., MILLER, W.H., WHITE, S.J., SMITH, T.A., NICKLIN, S.A., STEVENSON, S.C., and BAKER, A.H. (2004). Effect of adenovirus serotype 5 fiber and penton modifications on *in vivo* tropism in rats. Mol. Ther. 10, 344–354.

OGAWARA, K., ROTS, M.G., KOK, R.J., MOORLAG, H.E., VAN LOENEN, A.M., MEIJER, D.K., HAISMA, H.J., and MOLEMA, G. (2004). A novel strategy to modify adenovirus tropism and enhance transgene delivery to activated vascular endothelial cells *in vitro* and *in vivo*. Hum. Gene Ther. 15, 433–443.

OKADA, N., SAITO, T., MASUNAGA, Y., TSUKADA, Y., NAKAGAWA, S., MIZUGUCHI, H., MORI, K., OKADA, Y., FUJITA, T., HAYAKAWA, T., MAYUMI, T., and YAMAMOTO, A. (2001). Efficient antigen gene transduction using Arg-Gly-Asp fiber-mutant adenovirus vectors can potentiate antitumor vaccine efficacy and maturation of murine dendritic cells. Cancer Res. 61, 7913–7919.

OKADA, Y., OKADA, N., MIZUGUCHI, H., HAYAKAWA, T., MAYUMI, T., and MIZUNO, N. (2003). An investigation of adverse effects caused by the injection of high-dose TNFα-expressing adenovirus vector into established murine melanoma. Gene Ther. 10, 700–705.

OPHORST, O.J., KOSTENSE, S., GOUDSMIT, J., DE SWART, R.L., VERHAAGH, S., ZAKHARTCHOUK, A., VAN MEIJER, M., SPRANGERS, M., VAN AMERONGEN, G., YUKSEL, S., OSTERHAUS, A.D., and HAVENGA, M.J. (2004). An adenoviral type 5 vector carrying a type 35 fiber as a vaccine vehicle: DC targeting, cross neutralization, and immunogenicity. Vaccine 22, 3035–3044.

O'RIORDAN, C.R., LACHAPELLE, A., DELGADO, C., PARKES, V., WADSWORTH, S.C., SMITH, A.E., and FRANCIS, G.E. (1999). PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody *in vitro* and *in vivo*. Hum. Gene Ther. 10, 1349–1358.

PAPANIKOLOPOULOU, K., FORGE, V., GOELTZ, P., and MITRAKI, A. (2004). Formation of highly stable chimeric trimers by fusion of an adenovirus fiber shaft fragment with the foldon domain of bacteriophage T4 fibritin. J. Biol. Chem. 279, 8991–8998.

PARROTT, M.B., ADAMS, K.E., MERCIER, G.T., MOK, H., CAMPOS, S.K., and BARRY, M.A. (2003). Metabolically biotinylated

adenovirus for cell targeting, ligand screening, and vector purification. Mol. Ther. 8, 688–700.

PEREBOEV, A., PEREBOEVA, L., and CURIEL, D.T. (2001). Phage display of adenovirus type 5 fiber knob as a tool for specific ligand selection and validation. J. Virol. 75, 7107–7113.

REA, D., HAVENGA, M.J., VAN DEN ASSEM, M., SUTMULLER, R.P., LEMCKERT, A., HOEBEN, R.C., BOUT, A., MELIEF, C.J., and OFFRINGA, R. (2001). Highly efficient transduction of human monocyte-derived dendritic cells with subgroup B fiber-modified adenovirus vectors enhances transgene-encoded antigen presentation to cytotoxic T cells. J. Immunol. 166, 5236–5244.

REYNOLDS, P.N., ZINN, K.R., GAVRILYUK, V.D., BALYASNIKOVA, I.V., ROGERS, B.E., BUCHSBAUM, D.J., WANG, M.H., MILETICH, D.J., GRIZZLE, W.E., DOUGLAS, J.T., DANILOV, S.M., and CURIEL, D.T. (2000). A targetable, injectable adenoviral vector for selective gene delivery to pulmonary endothelium *in vivo*. Mol. Ther. 2, 562–578.

ROELVINK, P.W., LIZONOVA, A., LEE, J.G., LI, Y., BERGELSON, J.M., FINBERG, R.W., BROUGH, D.E., KOVESDI, I., and WICKHAM, T.J. (1998). The coxsackievirus–adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. J. Virol. 72, 7909–7915.

ROELVINK, P.W., MI LEE, G., EINFELD, D.A., KOVESDI, I., and WICKHAM, T.J. (1999). Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing Adenoviridae. Science 286, 1568–1571.

ROMANCZUK, H., GALER, C.E., ZABNER, J., BARSOMIAN, G., WADSWORTH, S.C., and O'RIORDAN, C.R. (1999). Modification of an adenoviral vector with biologically selected peptides: A novel strategy for gene delivery to cells of choice. Hum. Gene Ther. 10, 2615–2626.

ROSA-CALATRAVA, M., GRAVE, L., PUVION-DUTILLEUL, F., CHATTON, B., and KEDINGER, C. (2001). Functional analysis of adenovirus protein IX identifies domains involved in capsid stability, transcriptional activity, and nuclear reorganization. J. Virol. 75, 7131–7141.

ROY, S., SHIRLEY, P.S., MCCLELLAND, A., and KALEKO, M. (1998). Circumvention of immunity to the adenovirus major coat protein hexon. J. Virol. 72, 6875–6879.

SAKURAI, F., MIZUGUCHI, H., and HAYAKAWA, T. (2003a). Efficient gene transfer into human CD34$^+$ cells by an adenovirus type 35 vector. Gene Ther. 10, 1041–1048.

SAKURAI, F., MIZUGUCHI, H., YAMAGUCHI, T., and HAYAKAWA, T. (2003b). Characterization of *in vitro* and *in vivo* gene transfer properties of adenovirus serotype 35 vector. Mol. Ther. 8, 813–821.

SEGERMAN, A., ATKINSON, J.P., MARTTILA, M., DENNERQUIST, V., WADELL, G., and ARNBERG, N. (2003). Adenovirus type 11 uses CD46 as a cellular receptor. J. Virol. 77, 9183–9191.

SESHIDHAR REDDY, P., GANESH, S., LIMBACH, M.P., BRANN, T., PINKSTAFF, A., KALOSS, M., KALEKO, M., and CONNELLY, S. (2003). Development of adenovirus serotype 35 as a gene transfer vector. Virology 311, 384–393.

SHAYAKHMETOV, D.M., PAPAYANNOPOULOU, T., STAMATOYANNOPOULOS, G., and LIEBER, A. (2000). Efficient gene transfer into human CD34$^+$ cells by a retargeted adenovirus vector. J. Virol. 74, 2567–2583.

SHORT, J.J., PEREBOEV, A.V., KAWAKAMI, Y., VASU, C., HOLTERMAN, M.J., and CURIEL, D.T. (2004). Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. Virology 322, 349–359.

SMITH, T.A., IDAMAKANTI, N., MARSHALL-NEFF, J., ROLLENCE, M.L., WRIGHT, P., KALOSS, M., KING, L., MECH, C., DINGES, L., IVERSON, W.O., SHERER, A.D., MARKOVITS, J.E., LYONS, R.M., KALEKO, M., and STEVENSON, S.C. (2003a). Receptor interactions involved in adenoviral-mediated gene delivery after systemic administration in non-human primates. Hum. Gene Ther. 14, 1595–1604.

SMITH, T.A., IDAMAKANTI, N., ROLLENCE, M.L., MARSHALL-NEFF, J., KIM, J., MULGREW, K., NEMEROW, G.R., KALEKO, M., and STEVENSON, S.C. (2003b). Adenovirus serotype 5 fiber shaft influences *in vivo* gene transfer in mice. Hum. Gene Ther. 14, 777–787.

SOSNOWSKI, B.A., GU, D.L., D'ANDREA, M., DOUKAS, J., and PIERCE, G.F. (1999). FGF2-targeted adenoviral vectors for systemic and local disease. Curr. Opin. Mol. Ther. 1, 573–579.

STECHER, H., SHAYAKHMETOV, D.M., STAMATOYANNOPOULOS, G., and LIEBER, A. (2001). A capsid-modified adenovirus vector devoid of all viral genes: Assessment of transduction and toxicity in human hematopoietic cells. Mol. Ther. 4, 36–44.

STEVENSON, S.C., ROLLENCE, M., MARSHALL-NEFF, J., and MCCLELLAND, A. (1997). Selective targeting of human cells by a chimeric adenovirus vector containing a modified fiber protein. J. Virol. 71, 4782–4790.

TAO, N., GAO, G.P., PARR, M., JOHNSTON, J., BARADET, T., WILSON, J.M., BARSOUM, J., and FAWELL, S.E. (2001). Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. Mol. Ther. 3, 28–35.

TILLMAN, B.W., DE GRUIJL, T.D., LUYKX-DE BAKKER, S.A., SCHEPER, R.J., PINEDO, H.M., CURIEL, T.J., GERRITSEN, W.R., and CURIEL, D.T. (1999). Maturation of dendritic cells accompanies high-efficiency gene transfer by a CD40-targeted adenoviral vector. J. Immunol. 162, 6378–6383.

VELLINGA, J., RABELINK, M.J., CRAMER, S.J., VAN DEN WOLLENBERG, D.J., VAN DER MEULEN, H., LEPPARD, K.N., FALLAUX, F.J., and HOEBEN, R.C. (2004). Spacers increase the accessibility of peptide ligands linked to the carboxyl terminus of adenovirus minor capsid protein IX. J. Virol. 78, 3470–3479.

VIGNE, E., MAHFOUZ, I., DEDIEU, J.F., BRIE, A., PERRICAUDET, M., and YEH, P. (1999). RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection. J. Virol. 73, 5156–5161.

VOGELS, R., ZUIJDGEEST, D., VAN RIJNSOEVER, R., HARTKOORN, E., DAMEN, I., DE BETHUNE, M.P., KOSTENSE, S., PENDERS, G., HELMUS, N., KOUDSTAAL, W., CECCHINI, M., WETTERWALD, A., SPRANGERS, M., LEMCKERT, A., OPHORST, O., KOEL, B., VAN MEERENDONK, M., QUAX, P., PANITTI, L., GRIMBERGEN, J., BOUT, A., GOUDSMIT, J., and HAVENGA, M. (2003). Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: Efficient human cell infection and bypass of preexisting adenovirus immunity. J. Virol. 77, 8263–8271.

VOLPERS, C., THIRION, C., BIERMANN, V., HUSSMANN, S., KEWES, H., DUNANT, P., VON DER MARK, H., HERRMANN, A., KOCHANEK, S., and LOCHMULLER, H. (2003). Antibody-mediated targeting of an adenovirus vector modified to contain a synthetic immunoglobulin G-binding domain in the capsid. J. Virol. 77, 2093–2104.

VON SEGGERN, D.J., KEHLER, J., ENDO, R.I., and NEMEROW, G.R. (1998). Complementation of a fibre mutant adenovirus by packaging cell lines stably expressing the adenovirus type 5 fibre protein. J. Gen. Virol. 79, 1461–1468.

WATKINS, S.J., MESYANZHINOV, V.V., KUROCHKINA, L.P., and HAWKINS, R.E. (1997). The "adenobody" approach to viral targeting: Specific and enhanced adenoviral gene delivery. Gene Ther. 4, 1004–1012.

WICKHAM, T.J., CARRION, M.E., and KOVESDI, I. (1995). Targeting of adenovirus penton base to new receptors through replacement of its RGD motif with other receptor-specific peptide motifs. Gene Ther. 2, 750–756.

WICKHAM, T.J., TZENG, E., SHEARS, L.L.N., ROELVINK, P.W., LI, Y., LEE, G.M., BROUGH, D.E., LIZONOVA, A., and KOVESDI, I. (1997). Increased *in vitro* and *in vivo* gene transfer by adenovirus vectors containing chimeric fiber proteins. J. Virol. 71, 8221–8229.

WOLFF, G., WORGALL, S., VAN-ROOIJEN, N., SONG, W.R., HARVEY, B.G., and CRYSTAL, R.G. (1997). Enhancement of *in vivo* adenovirus-mediated gene transfer and expression by prior depletion of tissue macrophages in the target organ. J. Virol. 71, 624–629.

WORGALL, S., WOLFF, G., FALCK-PEDERSEN, E., and CRYSTAL, R.G. (1997). Innate immune mechanisms dominate elimination of adenoviral vectors following *in vivo* administration. Hum. Gene Ther. 8, 37–44.

WORK, L.M., NICKLIN, S.A., BRAIN, N.J., DISHART, K.L., VON SEGGERN, D.J., HALLEK, M., BUNING, H., and BAKER, A.H. (2004). Development of efficient viral vectors selective for vascular smooth muscle cells. Mol. Ther. 9, 198–208.

XIA, H., ANDERSON, B., MAO, Q., and DAVIDSON, B.L. (2000). Recombinant human adenovirus: Targeting to the human transferrin receptor improves gene transfer to brain microcapillary endothelium. J. Virol. 74, 11359–11366.

ZINN, K.R., SZALAI, A.J., STARGEL, A., KRASNYKH, V., and CHAUDHURI, T.R. (2004). Bioluminescence imaging reveals a significant role for complement in liver transduction following intravenous delivery of adenovirus. Gene Ther. 11, 1482–1486.

Address reprint requests to:
Dr. Hiroyuki Mizuguchi
Project III, National Institute of Health Sciences
Osaka Branch, Fundamental Research Laboratories for Development of Medicine
Asagi 7-6-8, Saito, Ibaraki, Osaka 567-0085, Japan

E-mail: mizuguch@nihs.go.jp

Received for publication September 14, 2004; accepted after revision October 7, 2004.

Published online: October 28, 2004.

RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2004; 18: 1156–1160
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/rcm.1465

RCM

Improved sensitivity for insulin in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry by premixing α-cyano-4-hydroxycinnamic acid matrix with transferrin

Tetsu Kobayashi*, Hiroshi Kawai, Takuo Suzuki, Toru Kawanishi and Takao Hayakawa
Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Received 27 February 2004; Revised 25 March 2004; Accepted 25 March 2004

This report describes an enhancement of the signal intensities of proteins and peptides in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). When α-cyano-4-hydroxycinnamic acid (CHCA) premixed with human transferrin (Tf) was used as a matrix, the signal intensity of insulin was amplified to more than ten times that of the respective control in CHCA without Tf. The detection limit of insulin was 0.39 fmol on-probe in the presence of Tf, while it was 6.3 fmol in the absence of Tf. The signal intensity of insulin was also enhanced when the CHCA matrix was premixed with proteins other than Tf (80 kDa), such as horse ferritin (20 kDa), bovine serum albumin (BSA, 66 kDa), or human immunoglobulin G (150 kDa). The optimum spectrum of insulin was obtained when the added amount of protein was in the range 0.26–0.62 pmol, regardless of the molecular weight of the added protein. Tf and BSA outperformed the other tested proteins, as determined by improvements in the resulting spectra. When the mass spectra of several peptides and proteins were recorded in the presence of Tf or BSA, the signal intensities of large peptides such as glucagon were enhanced, though those of smaller peptides were not enhanced. In addition, the signal enhancement achieved with Tf and BSA was more pronounced for the proteins, including cytochrome C, than for the large peptides. This enhancement effect could be applied to improve the sensitivity of MALDI-TOFMS to large peptides and proteins. Copyright © 2004 John Wiley & Sons, Ltd.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) and electrospray ionization mass spectrometry have been widely used in studies of protein chemistry, including proteomics studies aimed at sequence identification or quantitative analyses following enzymatic digestion by isotope-coded affinity tags and other tagging systems.[1–8] In particular, MALDI-TOFMS has been used for the qualitative and quantitative analysis of intact proteins.[9–11] When the MALDI technique was first introduced as an ionization method for proteins, a mixture of fine metal powder and glycerol, or nicotinic acid, was used as the matrix.[12,13] Progress has been made with other matrix materials such as sinapinic acid, 2,5-dihydroxybenzoic acid (DHB), and α-cyano 4-hydroxycinnamic acid (CHCA), which have some desirable properties such as less intense adduct peaks and a relative insensitivity to contamination.[14–16] With the MALDI approach, analyte proteins are dispersed on a surface in a thin layer of matrix. The energy of an incident pulse of laser photons is absorbed by the matrix to form a jet of matrix vapor that lifts the analyte proteins from the surface and transforms some of them into ions.[13]

However, the mechanisms by which laser light irradiation is able to generate macromolecular ions have not been fully verified to date. It has been reported that the ionization of macromolecules by the MALDI process is affected by several factors. For example, peptide signal intensity was increased by the use of acetone as the solvent for CHCA matrix instead of employing the commonly used solvent, a mixture of acetonitrile and aqueous 0.1% trifluoroacetic acid (TFA).[17] The signal-to-noise (S/N) ratios for macromolecules are low in DHB matrix, but the addition of suitable additives (fructose, glucose, fucose, or 2-hydroxy-5-methoxybenzoic acid) to the DHB matrix improved its performance in the high molecular mass range.[18–21] In the CHCA and sinapinic acid matrices, the detection of higher molecular weight proteins was improved by using polytetrafluoroethylene (Teflon) as sample support.[22,23]

Recently, we investigated a method of identifying and quantifying proteins in blood using mass spectrometry. During the present study, we discovered that the signal intensity of human insulin was augmented more than 10-fold when transferrin (Tf) was mixed with the CHCA matrix

*Correspondence to: T. Kobayashi, Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan.
E-mail: kobayash@nihs.go.jp
Contract/grant sponsor: Ministry of Health, Labor and Welfare, Japan.

solution used for MALDI-TOFMS. This phenomenon was not specific to either insulin or Tf, which suggested that such enhancements could be used more generally to improve the sensitivity of protein analysis with MALDI-TOFMS.

EXPERIMENTAL

Materials

Human atrial natriuretic peptide (hANP), glucagon, insulin, insulin-like growth factor-1 (IGF-1), transferrin (Tf), bovine serum albumin (BSA), horse spleen ferritin (106 mg/mL in 0.15 M NaCl), and ProteoMass Peptide & Protein, were purchased from Sigma (St. Louis, MO, USA). Human immunoglobulin G (IgG, 11.3 mg/mL in 0.01 M sodium phosphate, 0.5 M NaCl, pH 7.6) was obtained from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). Human insulin, IGF-1, glucagon, and hANP stock solutions were prepared at concentrations of 100 pmol/μL by dissolving them in 0.1% TFA. Tf and BSA stock solutions were prepared at concentrations of 10 mg/mL by dissolving the materials in Millipore deionized water. ProteoMass Peptide & Protein stock solutions, which include bradykinin fragment 1-7, human angiotensin II, synthetic peptide $P_{14}R$, human ACTH fragment 18-39, bovine insulin oxidized B chain, bovine insulin, equine cytochrome C, equine apomyoglobin, rabbit aldolase, and BSA, were prepared at concentrations of 100 pmol/μL each, according to the manufacturer's instructions.

Sample application and data acquisition

The Tf-mixed CHCA was a 5:1 mixture of the CHCA solution (10 mg/mL in 50% acetonitrile in 0.1% aqueous TFA) and Tf solution (0.10 μg/μL; the final concentration was approximately 8.3 ng/μL), corresponding to 0.21 pmol Tf on each well of the target plate, if not otherwise noted. The control CHCA was a mixture of the CHCA solution and deionized water (5:1). A portion of each sample solution was immediately mixed with an equal volume of the matrix solution with or without Tf, and an aliquot of 2 μL (corresponding to 1 μL of sample solution) was applied to a stainless steel target plate. Mass spectrometric analyses were performed using an AB4700 proteomics analyzer (Applied Biosystems, Foster, CA, USA). The operating conditions were as follows: Nd:YAG laser (355 nm), linear mode, and detection of positive ions. The spectra were generated by signal averaging 50 laser shots into a single spectrum. The signal intensity was obtained after performing background correction and noise reduction using the Data Processor software (Applied Biosystems). This software was also used to determine the detection limit.

To confirm whether or not the matrix solution was at an optimum composition, serially diluted CHCA, DHB, or sinapinic acid solutions (from 10 to 0.078 mg/mL in 50% acetonitrile, 50% 0.1% TFA) were added to the insulin solution (100 fmol/μL). The most intense signal was obtained when 10 mg/mL CHCA was added to the insulin solution.

RESULTS AND DISCUSSION

Human insulin solution (6.3 fmol/μL) was mixed with an equal volume of Tf-mixed CHCA or control CHCA. When

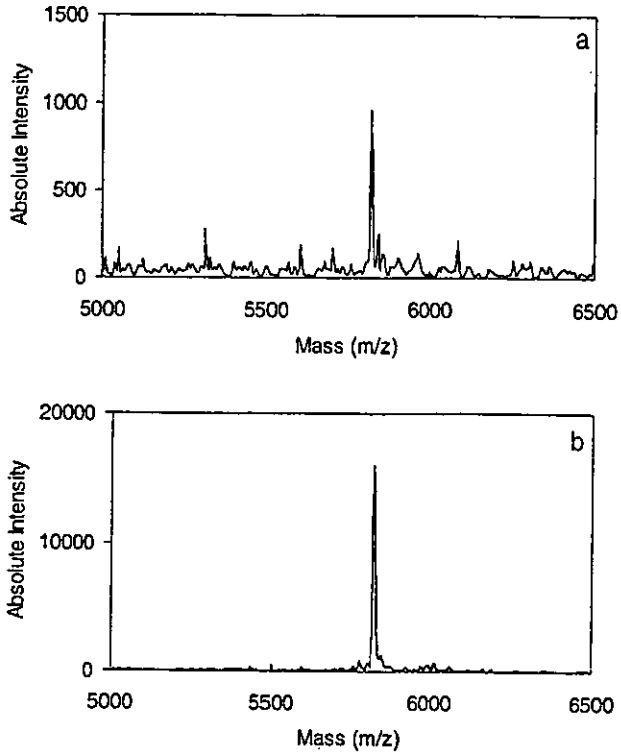

Figure 1. MALDI mass spectra of human insulin. The insulin solution (6.3 fmol/μL) and matrix solution were mixed together in equal volumes; 2 μL of the resulting mixture were applied to a target plate, allowed to dry, and analyzed by MALDI-TOFMS (see Experimental). The matrix solution was a 5:1 mixture of CHCA solution (10 mg/mL in 50% acetonitrile in 0.1% aqueous TFA) with deionized water or Tf solution (0.10 μg/μL). (a) Control CHCA used as matrix. (b) Tf-mixed CHCA used as matrix.

the Tf-mixed CHCA was used as matrix, the signal intensity of insulin in the MALDI-TOFMS detection system was amplified more than 10-fold relative to that achieved with the control CHCA (Fig. 1). To assess the sensitivity of insulin detection, the matrix solution was added to serially diluted insulin solutions (from 100 to 0.20 fmol/μL in deionized water), and samples were then spotted on a target plate. The detection limit of insulin was 0.39 fmol on the target plate in a Tf-mixed CHCA matrix under the present experimental conditions, whereas this limit was 6.3 fmol in the case of CHCA without Tf (Fig. 2).

To obtain the optimum concentration of Tf for the enhancement of insulin measurement sensitivity, the CHCA solution was mixed with serially diluted Tf solutions (from 1.0 μg/μL to 7.8 ng/μL) before addition to the insulin solution (100 fmol/μL). The signal intensity increased in a Tf-concentration-dependent manner (Fig. 3(a)). However, the S/N ratio decreased when the Tf concentration was more than 125 ng/μL (Fig. 3(b)), though it should be noted that the S/N value was still higher than the corresponding control value, i.e., 15 ± 7. A signal for 0.39 fmol/μL insulin was detected in the CHCA solution mixed with 0.1 μg/μL Tf (Fig. 2), whereas the signal for 1.6 fmol/μL insulin was not detected in the CHCA solution mixed with 1.0 μg/μL Tf (data not shown). These results suggest that the detection limit was also decreased in the presence of a high concentration of Tf.

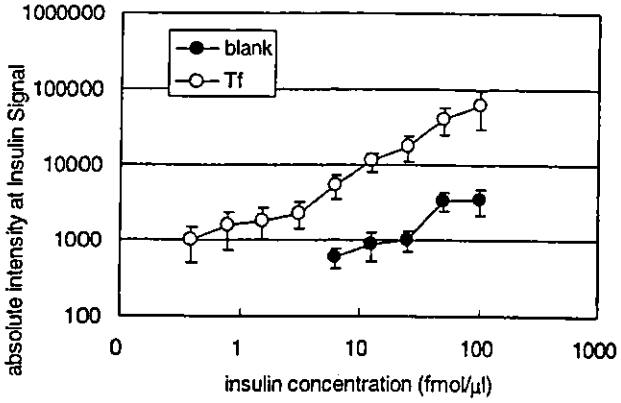

Figure 2. Dependence of insulin signals on insulin concentration. Sequentially diluted human insulin solution (100 to 0.20 fmol/μL in deionized water) and matrix solution were mixed in equal volumes. The matrix solution was a 5:1 mixture of the CHCA solution with either deionized water or Tf solution (0.10 μg/μL). The absolute intensity of the insulin signal obtained from Tf-mixed CHCA (open circles) is compared with that obtained for the control CHCA (closed circles). Each point represents the mean ± S.E. of four tests.

It is known that an excess amount of protein components can strongly influence the behavior of the MALDI process, resulting in partial or complete ion signal suppression.[24] In addition, the optimum mass ratio between the analyte and matrix for MALDI analysis has been demonstrated empirically.[15] When the CHCA was mixed with 1.0 μg/μL Tf, the excess amount of Tf might have suppressed the signal intensity of insulin as well. However, if that amount is appropriate, Tf appears somehow capable of enhancing the signal.

To determine whether or not the enhancement of the insulin MALDI-TOFMS signal intensity was specific to Tf, the CHCA solution was mixed with serially diluted solutions of several peptides and proteins before its addition to the insulin solution. The insulin signal intensity was also enhanced in the presence of ferritin (20 kDa), BSA (66 kDa), or IgG (150 kDa) (Fig. 4(a)). However, this was not found to occur in a simple concentration-dependent manner in the case of either ferritin or IgG; furthermore, when the CHCA solution was mixed with more than 2.0 μg/μL of these protein solutions, no insulin signal was detected. The enhancement of the insulin signal intensity was relatively small in the presence of peptides such as hANP (3.1 kDa) and glucagon (3.4 kDa). In addition, when the CHCA solution was mixed with more than 77 ng/μL of hANP or 87 ng/μL of glucagon, no insulin signal was detected. Among the tested peptides and proteins, the insulin signal intensity was enhanced most effectively in the presence of Tf (80 kDa) or BSA. Therefore, it is probable that this type of enhancement requires an added protein of moderate molecular weight, namely 66–80 kDa.

With regard to the results for the serial dilutions of the added peptides and proteins, the highest S/N values were obtained at 4.8 ng/μL hANP, 5.4 ng/μL glucagon, 66 ng/μL ferritin, 0.13 μg/μL BSA, 0.13 μg/μL Tf, or 0.57 μg/μL

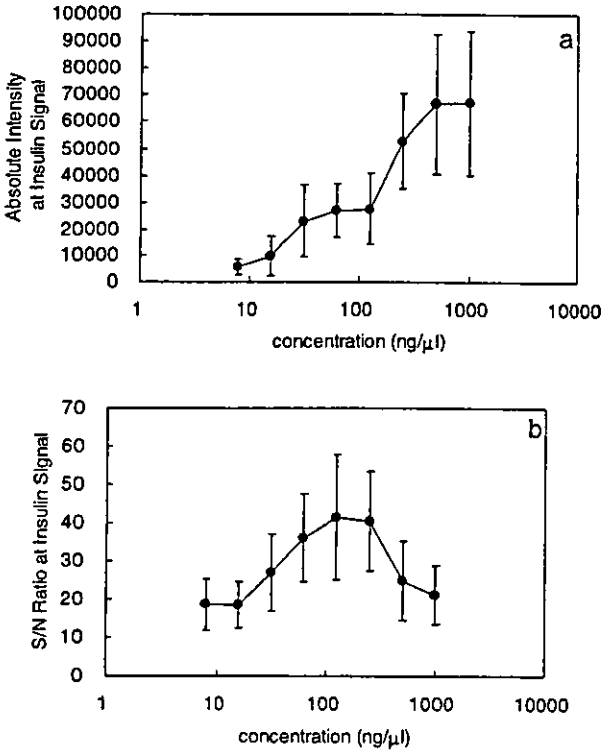

Figure 3. Dependence of insulin signal on Tf concentration. Serially diluted Tf solution was added to five volumes of the CHCA solution before mixing the resulting solution with an equal volume of human insulin (100 fmol/μL): (a) absolute intensity (arbitrary units) and (b) S/N ratio of the insulin signal in the MALDI analysis. Each point represents the mean ± S.E. of four tests.

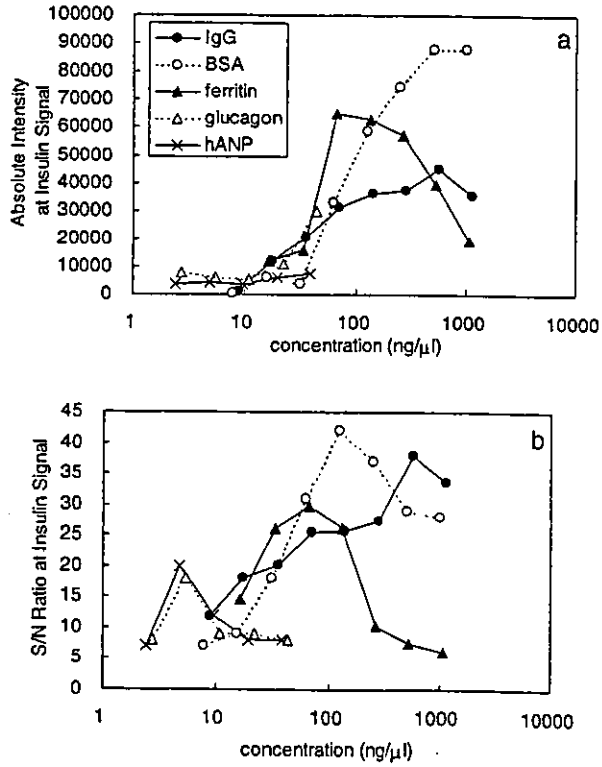

Figure 4. Dependence of insulin signal on concentrations of various added proteins. Serially diluted IgG, BSA, ferritin, glucagon, or hANP solution was added to the CHCA solution before the solution was mixed with the human insulin solution (100 fmol/μL): (a) absolute intensity (units) and (b) S/N ratio of the insulin signal. Each point represents the average of duplicate samples.

IgG (Figs. 3(b) and 4(b)), which correspond to 0.26 pmol, 0.26 pmol, 0.50 pmol, 0.32 pmol, 0.26 pmol, and 0.62 pmol, respectively, in each well. Thus, the optimum molar concentrations occurred in the same scale order, although the optimum mass concentrations of polypeptides required to enhance the signal differed markedly between the proteins and small peptides. In addition, the molar concentrations of excess peptides or proteins required to suppress the insulin signal were also found to exhibit the same scale in the same order. The ionization of insulin appeared to depend on the molar concentration of the peptide or protein which was mixed with the CHCA matrix solution.

To examine whether or not the signal enhancement was specific to human insulin, the CHCA solution premixed with Tf or BSA (0.10 μg/μL) was added to a solution of peptides and proteins, which included hANP, glucagon, human insulin, IGF-I, and ProteoMass Peptide & Protein at concentrations of 50 fmol/μL each. The signal intensities of [angiotensin II]$^+$ (1046 Da), [synthetic peptide $P_{14}R$]$^+$ (1534 Da), and [ACTH fragment]$^+$ (2465 Da) were either not enhanced or were reduced in the matrix premixed with Tf or BSA (Table 1). However, the signal intensities of [hANP]$^+$ (3080 Da), [glucagon]$^+$ (3483 Da), [insulin B chain]$^+$ (3494 Da), and [bovine insulin]$^+$ (5730 Da) were enhanced as well as that of [human insulin]$^+$ (5808 Da) (Table 1, Fig. 5). The signal intensities of [IGF-I]$^+$ (7649 Da), [cytochrome C]$^+$ (12 362 Da), and [cytochrome C]$^{2+}$ were enhanced more than that of human insulin in the presence of Tf or BSA. In addition, the signals of [apomyoglobin]$^+$ (16 952 Da) and [apomyoglobin]$^{2+}$ were clearly observed in the presence of Tf or BSA, although their signals were not detected in the control matrix. In this latter case, the signal of [apomyoglobin]$^+$ overlapped with that of BSA, but not of Tf; therefore, it was more advantageous to use Tf than BSA for detecting this signal. Since BSA was included in the ProteoMass Peptide & Protein solution, the signals of [BSA]$^+$ (66 430 Da), [BSA]$^{2+}$, [BSA]$^{3+}$, and [BSA]$^{4+}$ were also detected in the presence of Tf (Table 1, Fig. 5(b)).

The results reported above demonstrate that the enhancement of the signal intensity achieved with the use of Tf and BSA was observed for both peptides and proteins, and this effect was not specific to human insulin. The degree of enhancement was dependent on the molecular weights of the peptides and proteins, and no such enhancement was observed in the case of small peptides; in this regard a dividing line appeared to exist between [ACTH fragment]$^+$ (2465 Da) and [hANP]$^+$ (3080 Da).

The mechanism by which signal intensity enhancement was achieved with the use of peptides and proteins mixed with the matrix solution remains unclear. However, when super DHB (a co-matrix of DHB and 2-hydroxy-5-methoxybenzoic acid) was used as the matrix, ion yields and S/N ratio improved, especially for the high-mass range.[20] It has been suggested that this signal enhancement was caused by a disorder in the DHB lattice, allowing 'softer' desorption. This type of signal enhancement has also been observed in the case of substance P in CHCA after fast evaporation of an acetone solvent, which resulted in the more homogeneous distribution of matrix and analytes.[18] In addition, better mass resolution has been observed in the spectra of cytochrome C in a CHCA matrix desorbed from polyethylene and polypropylene membranes than has been observed with a CHCA matrix desorbed from stainless steel; it was thus suggested that such improved resolution might be due at least in part to the formation of relatively small matrix crystals within the membrane lattice structure.[25] In the present study, Tf and other proteins might have led to a similar disorganization in the CHCA lattice, resulting in the homogeneous distribution of insulin in the CHCA. However, the mechanism may differ from that suggested here, since the disorder in the CHCA lattice cannot reasonably account for why both Tf and BSA were able to enhance the insulin signal more effectively than either hANP or glucagon. As the next step, we are now planning to compare the crystals of the additive macromolecules plus matrix with those of the control matrix, using microscopic examination, to help elucidate the enhancement mechanism. We also intend to investigate whether the enhancement effect is observed in matrices other than CHCA. If crystallization is important,

Table 1. Signal intensities for proteins and peptides obtained using a matrix premixed with deionized water or with solutions of Tf or BSA

	Water	Tf	BSA
[Angiotensin II]$^+$	27 834 ± 10 757	17 057 ± 5021	19 755 ± 11 237
[P14R]$^+$	41 689 ± 15 289	30 675 ± 8588	29 237 ± 13 330
[ACTH 18–39]$^+$	4371 ± 1586	3801 ± 2246	5458 ± 3826
[hNAP]$^+$	5158 ± 1323	6889 ± 2879	9523 ± 6384
[human glucagon]$^+$	435 ± 183	674 ± 324	978 ± 566
[insulin B chain]$^+$	367 ± 257	997 ± 251	715 ± 479
[bovine insulin]$^+$	639 ± 100	6266 ± 2736	7498 ± 5331
[human insulin]$^+$	1267 ± 130	13 321 ± 5057	12 982 ± 6863
[equine cytochrome C]$^{2+}$	166 ± 83	5668 ± 1975	3460 ± 1442
[human IGF-I]$^+$	459 ± 81	7667 ± 1808	6263 ± 2872
[equine apomyoglobin]$^{2+}$	nd	2249 ± 994	2217 ± 1087
[equine cytochrome C]$^+$	114 ± 43	7629 ± 1804	4006 ± 1981
[BSA]$^{4+}$	nd	52 ± 14	2459 ± 604
[equine apomyoglobin]$^+$	nd	1347 ± 700	2090 ± 1316
[BSA]$^{3+}$	nd	155 ± 13	3721 ± 1426
[BSA]$^{2+}$	nd	114 ± 27	3624 ± 1681
[BSA]$^+$	nd	25 ± 8	634 ± 433

Each entry is the average of the most intense signals from four samples. nd: no signal was detected.