We have constructed a novel fusion protein “Scavidin” consisting of the macrophage scavenger receptor class A and avidin. The Scavidin fusion protein is transported to plasma membranes where the avidin portion of the fusion protein binds biotin with high affinity and forms the basis for the targeted delivery of biotinylated molecules. Subcellular fractionation analysis, immunostaining, and electron microscopy demonstrated endosomal localization of the fusion protein. According to pulse-labeling and cross-linking studies Scavidin is found as monomers (55 kDa), dimers, and multimers, of which the 220-kDa form was the most abundant. The biotin binding capacity and active endocytosis of the biotinylated ligands were demonstrated in rat malignant glioma. Local Scavidin gene transfer to target tissues could have general utility as a universal tool to deliver biotinylated molecules at systemic low concentrations for therapeutic and imaging purposes, whereby high local concentration is achieved.

The goal of targeted therapies is to elicit a selective biological effect on specific cells or tissues while minimizing side effects in other organs. One possibility for targeting is to use the very high binding affinity of avidin for biotin. The binding is almost irreversible ($K_d$ $10^{-15}$m). Here we report the construction of a novel fusion protein (Scavidin) for targeting biotinylated molecules to specific tissues. Biotin can be combined to almost any type of molecule through its valeric acid side chain without affecting the biological properties of the molecules (1, 2). This established chemistry allows covalent biotinylation of drugs, liposomes, radiopharmaceuticals, and other ligands.

The fusion protein was generated from the macrophage scavenger receptor class A (MSR-A) (3) and avidin (4). MSR-A belongs to a large family of scavenger receptors, which participate in endocytosis of various ligands, cell adhesion, and defense against microorganisms (5). The C-terminal domain and collagen-like domain containing the ligand-binding site (6) have been removed from the fusion protein. The cytoplasmic domain containing signals for endocytosis, transmembrane domain, and α-helical coiled coil domain have been retained. These domains are sufficient to transport the receptor to the cell membrane and mediate endocytosis of ligands. Avidin, which permits targeted binding of biotinylated ligands, is located extracellularly at the C terminus. In the present study, we demonstrate the functionality of Scavidin fusion protein in vitro and in vivo in rat glioma cells. The Scavidin provides a promising new tool for targeted delivery of biotinylated ligands for local gene and drug therapy and diagnostic applications.

**Experimental Procedures**

Cloning and Vector Construction—The avidin cDNA was generated by 25 cycles of PCR amplification using the following primers: forward, 5′-CGGCCAAGTTGCAGAAAATGCTGCTGCTGG3′; reverse, 5′-TGCTTGGTCATCTACAACCTCCTGTTGCTGGCG3′. Amplified avidin cDNA was joined into the Styl site in MSR-A cDNA after the α-helical coiled coil structure-coding domain and before the collagenous domain in a retrovirus plasmid, pLSaARNL (7). The fusion protein was named Scavidin® (registered UK trademark of Ark Therapeutics, Ltd.). Plasmid sequences were verified by sequencing (A. L. F. DNA sequenator, Amersham Biosciences, Inc.). The same vector backbone containing LacZ cDNA (Bag) (8) was used as a control (Fig. 1).

Production of Retroviruses and Stable Cell Lines—Packaging cell lines were transfected with retroviral plasmids (pLSaARNL or pBag containing LacZ) using the standard calcium phosphate precipitation method as described (7, 9) and were used to transduce BT4C glioma cells (10). After 10–12 days of selection with G418 (400 μg/ml, Sigma) resistant BT4C cells were expanded and selected by the biotin binding capacity with fluorescence-activated cell sorting.

Northern Blot Analysis—Poly(A)+ mRNA (1 μg) was isolated using the SDS/proteinase K method, electrophoresed in a denaturing gel, transferred to nylon membrane (Amersham Biosciences, Inc.), and hybridized with random-primed 32P-labeled probes. Autoradiography was used for signal detection.

Western Blot Analysis—200 μg of protein from Scavidin cells was run in reducing 4–7.5% SDS-PAGE and blotted onto nitrocellulose membranes. The nitrocellulose filter was reacted with anti-avidin antibody (Sigma, 1:4000) and a secondary antibody conjugated to horseradish peroxidase (HRP, 1:3000). Chemiluminescence was detected using 3,3′-diaminobenzidine; (bis)sulfosuccinimidyl suberate, Pierce) for 1 h at room temperature and quenched for 30 min with 50 mM Tris buffer on ice.

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rabbit anti-avidin and anti-rabbit-HRP conjugate (Bio-Rad). The signal was detected by chemiluminescence (with SuperSignal™ Substrate, Pierce).

**Metabolic Labeling and Immunoprecipitation**—Monolayers of Scavidin cells were washed with medium without methionine, pulse-labeled for 5 and 40 min with [35S]Met-Cys (500 Ci/mmol) medium, and either immediately harvested by lysis on ice or chased for the indicated time periods in a medium supplemented with unlabeled methionine (1.5 mg/ml) before the lysis. Scavidin was immunoprecipitated at 4 °C with anti-avidin antibody in 20% BSA overnight, bound to protein A-Sepharose for 1 h, and collected by centrifugation. SDS-PAGE sample buffer (with 10% [v/v] 2-mercaptoethanol) was added, and protein A-Sepharose beads were dissociated at 100 °C for 5 min. Samples were separated in 5–15% gradient SDS-PAGE gel.

**Percoll Fractionation**—Fractionation of the cells was performed as described earlier (11). Briefly, stably BT4C/Scavidin cells were pelleted for 5 min at 37 °C in the 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium with 2 mg/ml HRP in the 10% Dulbecco’s modified Eagle’s medium and washed with ice-cold BSA (5 mg/ml) in PBS on ice. Cells were resuspended in Percoll buffer (3% Percoll, 0.85 mM imidazole, 0.25 M sucrose, 1 mM EDTA), and nuclei were pelleted by centrifugation (2 h, 30,000 g, 4 °C). The cells were fixed in methanol for 6 min at -20 °C and blocked for 1 h at 4 °C with 1% (v/v) PBS and embedded in OCT compound (Miles Scientific, Elkhart, IN). The goat anti-avidin antibody (1:250, Vector Laboratories) was used for immunostainings. The sections were counterstained with hematoxylin. An avidin-biotin-HRP system (Vector Elite, Vector Laboratories) was used for signal detection. The bound biotinylated HRP was detected from the sections by direct DAB staining. The LacZ activity was visualized by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Sigma) staining.

**RESULTS**

**The Scavidin Fusion Protein Is Expressed in Cells in Multimeric Forms and Is Stable for Several Hours**—The Scavidin fusion protein was constructed in a retroviral vector as described under “Experimental Procedures” (Fig. 1). A full-length mRNA encoding Scavidin was produced in transfected cells (Fig. 2a). 110-kDa dimers and 55–60-kDa monomers were detected from retrovirus-transfected cells (Fig. 2b). Size was calculated according to the molecular mass of the non-glycosylated monomeric Scavidin, which is 45 kDa. From the previously known quaternary structure of MSR-A it was anticipated that Scavidin fusion protein forms trimers (from non-covalently associated disulfide cross-linked dimers at Cys-83) and monomers (15). Instead, in cross-linking studies, the strongest signal was from a 220-kDa protein, which was denatured to a 110-kDa dimer and a 55-kDa monomer suggesting the formation of tetramers instead of trimers (Fig. 2c). These results indicate that the avidin moiety remains soluble and is capable of forming multimers, such as tetramers, even when attached to MSR-A. However, the results do not rule out the existence of trimers since a band of ~170 kDa was seen in both acetylation and cross-linking studies.

To study the synthesis and degradation of the Scavidin protein, the cells were pulse-labeled with [35S]methionine and then immediately processed or chased for the indicated time periods (Fig. 2, d–f). Scavidin protein remained stable for at least 10 h of chase, and only one-third of the protein was degraded. Monomers were already seen after 5 min of chase. The lower molecular mass monomers disappeared by 3 h. The conversion of monomers into dimers and multimers occurred within 40 min. The intensity of the dimers and multimers increased within 5 h of chase simultaneously with the decrease in the intensity of monomers. Multiple bands are likely to represent different glycosylated forms of Scavidin. All of the dimers and multimers were denatured into monomers in the presence of 2-mercaptoethanol (Fig. 2e).

**Scavidin Was Located in a Vesicular Fraction and Was Able to Bind and Endocytose Biotin**—The intracellular localization of the Scavidin fusion protein was determined using subcellu-
lar fractionation of Scavidin-expressing BT4C cells in a Percoll gradient (Fig. 3a). Cells were also allowed to endocytose HRP for 5 min in order to label early endosomes. Comparison of Scavidin distribution in fractions, analyzed by immunoelectron microscopy (15 min after internalization of antibody/protein Scavidin fusion protein was demonstrated by immunoelectron microscopy) and cell fractionation studies, the vesicular localization of labeled by 10 nm of protein A-gold particles (Fig. 3b). In addition to cell fractionation studies, the vesicular localization of Scavidin fusion protein was demonstrated by immunoelectron microscopy 15 min after internalization of antibody/protein A-gold particles (Fig. 3, d and e).

The Scavidin protein was detected by anti-avidin antibody staining in BT4C cells using fluorescence microscopy. Abundant protein in the cytoplasm was detected as granular structures (Fig. 4a). The protein was not uniformly distributed in the cells, but the perinuclear region was most intensively stained. To study the biotin binding and endocytosis capacity of the Scavidin fusion protein, Scavidin-transduced BT4C cells were incubated with b-IgG as a ligand and analyzed by fluorescence microscopy (Fig. 4, c–h). We found partial colocalization with CI-MPR suggesting that at least a fraction of the Scavidin/b-IgG is translocated to late endosomes. Endocytosis of b-IgG was followed up from 5 to 30 min in the BT4C cells. Before the follow-up, the cells were incubated with the ligand on ice, under conditions were no endocytosis occurs, and washed well to remove unbound ligand. After a 5-min chase without the ligand, immunoreactivity was found in numerous vesicles randomly distributed through the cytoplasm (Fig. 4, c–e). After 15 min, the ligand had accumulated in numerous vesicles randomly distributed through the cytoplasm (Fig. 4, c–e). After 15 min, the ligand had accumulated in numerous vesicles randomly distributed through the cytoplasm (Fig. 4, c–e). After 15 min, the ligand had accumulated in numerous vesicles randomly distributed through the cytoplasm (Fig. 4, c–e). After 15 min, the ligand had accumulated in numerous vesicles randomly distributed through the cytoplasm (Fig. 4). The distribution pattern showed clear colocalization with the CI-MPR antibody, which was detected in the juxtanuclear area (Fig. 4h). No significant binding of b-IgG was seen in the LacZ-transduced BT4C control cells (Fig. 4h).

The Scavidin Fusion Protein Expressed in Rat Malignant Glioma Cells Was Able to Bind Biotinylated Ligands in Vivo—Rat malignant glioma cells were transduced with VSV-G pseudotyped retroviruses containing the Scavidin or LacZ cDNA ex vivo and implanted in the rat brain followed by intratumoral delivery of biotinylated HRP (1 mg/ml) for 10 min. 30 min after the injection of biotinylated HRP rats were sacrificed ex vivo and processed for immunohistochemistry. Scavidin was expressed on an average of 20% of the transduced cells in the rat malignant glioma tumors (Fig. 5a) as detected with anti-avidin immunostaining. No anti-avidin reactivity or specific binding of the biotinylated HRP was found in control animals having LacZ-transduced tumors (Fig. 5, c and d). Instead, binding of the biotinylated HRP was seen in the same tumor area where
Scavidin immunoreactivity was detected (Fig. 5, e and f) confirming the ability of the Scavidin fusion protein to bind biotinylated ligands in vivo.

**DISCUSSION**

Targeting of therapeutic compounds to specific cells or tissues is of great interest for the drug development. Targeting increases the concentration of therapeutic or diagnostic agents as compared with non-targeted tissues and reduces side effects in non-target tissues. Several in vivo studies have shown promising results of avidin and streptavidin-directed biotinylated monoclonal antibodies (16, 17). For instance, the “pretarget” technology based on antibody-avidin/streptavidin conjugates, which target radionuclides to tumors, has been successfully used in man without significant toxicity (18). In this study, we generated a Scavidin fusion protein that is expressed on plasma membrane and is capable of high-affinity binding and endocytosis of biotinylated molecules. Utilization of a transmembrane protein that mediates endocytosis of the ligands...
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shown its promise in improving the efficiency of gene delivery into cells by using transferrin-streptavidin-DNA-conjugated vectors in mice (19). Also, the interaction of avidin with biotin could be utilized in targeting biotin-coated vectors and liposomes to specific sites of tissues after local gene transfer of Scavidin.

Scavidin fusion protein was translated into a functional protein in vitro and in vivo. According to the protein analysis, the most abundant form of Scavidin was an ~220-kDa multimer. This protein was 2-mercaptoethanol-sensitive, dissociating into 110-kDa dimers. This was anticipated, based on the knowledge of the MSR-A quaternary structure indicating that disulfide (Cys-83) cross-linked dimers and monomers non-covalently associate to form Scavidin trimers (15). However, tetramerization may be favored because of the fact that avidin forms tetramers composed of two structural units (4, 20), whose association is very tight and can force two dimeric Scavidin molecules to tetramerize at their avidin domains. Also, the triple helix-stabilizing sequences, which locate in the collagenous domain of MSR-A, were excluded from the Scavidin fusion protein (21). Recent construction of dimeric and monomeric avidins (22) will further broaden the potential uses of avidin fusion proteins. Targeting to the plasma membrane or inner organelles via transcytosis and endocytosis can be achieved by modulating the $K_m$ value. With the aid of the dimeric avidin moiety, which shows lower and reversible biotin binding, acid-dissociable ligands could be released from endosomes and transported to cellular compartments of interest, such as the nucleus. Utilization of the different oligomerization states of avidin will allow further possibilities for generating a broad spectrum of fusion proteins with different characteristics. It is also noteworthy that Scavidin is relatively stable ($t_{1/2} > 10$ h) in cells. Tetramerization of the fusion protein might be a stabilizing factor, as it is with avidin (22).

The electron microscopy study revealed that Scavidin was expressed on cell membranes and on the newly formed vesicles. We also demonstrated the functionality of Scavidin in mediating endocytosis: biotinylated IgG was detectable in the endocytic compartment of the cells, which serves as a route for introducing therapeutic compounds into the cells. More importantly, the functionality of Scavidin was demonstrated in vivo in the rat glioma model using biotinylated HRP as a ligand. The binding of biotinylated ligand was seen in the areas of Scavidin expression. This indicated that Scavidin could be used as a targeting molecule for various biotinylated ligands.

In cancer therapy, there is an increasing interest in eliminating tumor cells by the use of biotinylated antibodies that are targeted to unique tumor antigens, followed by delivery of biotinylated therapeutic agents via avidin (17, 23–25). These studies have shown that avidin-biotin-based therapies are generally well tolerated and that they facilitate targeting of therapeutic or diagnostic compounds in experimental animals and in man (23–26). However, in most cases the antibodies are also bound to antigens found in normal tissues. Because of the cross-reactivity with epitopes in tissues, the benefit gained from an improvement in the therapeutic window of cytotoxic drugs by conjugation with antibodies has often been compromised by concerns of toxicity. An important limitation of these approaches is that they require engineering of unique chimeric molecules for each specific application or tumor type. Scavidin overcomes these problems by the use of a fusion protein targeting system, which only requires two steps: Scavidin gene transfer into the target tissue and a biotinylated drug. The elimination of the antibody-step and the local gene delivery by using Scavidin gene transfer enhance the possibility of a true targeted therapeutic effect. The development of better vectors,
**Fig. 5.** Binding of biotinylated HRP was seen in the same areas as Scavidin gene expression. Scavidin or LacZ gene ex vivo transduced rat glioma BT4C cells were implanted into corpus callosum in rat brain. a, Scavidin expression in the glioma as detected with an anti-avidin antibody. b, non-immune control without the anti-avidin antibody showed no staining. c, X-gal staining of LacZ transduced glioma cells showing an extensive blue color indicating transgene expression. d, no anti-avidin staining was seen in LacZ-transduced malignant glioma. The arrowhead indicates the biotinylated HRP inoculation site into the tumor. e, binding of biotinylated HRP (1 mg/ml) was seen in the Scavidin-expressing tumor cells. Biotinylated HRP was injected into a depth of 2.5 mm into the tumor. e, anti-avidin antibody detection of Scavidin protein in the same area where the biotinylated HRP binding was observed with direct DAB staining in f. Inserts in e and f show the higher magnifications from the marked areas. Asterisks indicates the same location in serial sections. Scale bars in the images; b, e, f = 50 μm; a = 100 μm; image c, d = 200 μm. All of the tissues were counterstained with hematoxylin.

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tissue-specific promoters and regulated gene expression systems will further increase the potential of Scavidin-mediated therapy.

In summary, we have demonstrated that biotinylated molecules can be targeted using the Scavidin fusion protein and that the targeted ligands are efficiently endocytosed. The Scavidin fusion protein is a promising new tool for in vivo targeting offering the possibility of enhanced local effect and decreased systemic exposure of therapeutic compounds.

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