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C2-Streptavidin Mediates the Delivery of Biotin-Conjugated Tumor Suppressor Protein P53 into Tumor Cells

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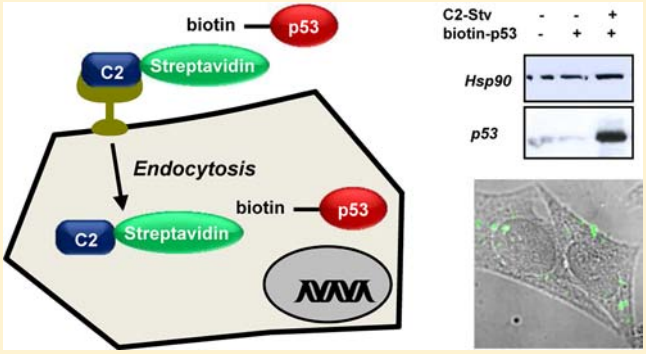
ABSTRACT: We have previously generated a recombinant C2-streptavidin fusion protein for the delivery of biotin-labeled molecules of low molecular weight into the cytosol of mammalian cells. A nontoxic moiety of *Clostridium botulinum* C2 toxin mediates the cellular uptake, whereas the streptavidin unit serves as a binding platform for biotin-labeled cargo molecules. In the present study, we used the C2-streptavidin transporter to introduce biotin-conjugated p53 protein into various mammalian cell lines. The p53 tumor suppressor protein is inactivated in many human cancers by multiple mechanisms and therefore the restoration of its activity in tumor cells is of great therapeutic interest. Recombinant p53 was expressed in insect cells and biotin-labeled. Biotin-p53 retained its specific high-affinity DNA-binding as revealed by gel-shift analysis. Successful conjugation of biotin-p53 to the C2-streptavidin transporter was monitored by an overlay blot technique and confirmed by real-time surface plasmon resonance, providing a K_D-value in the low nM range. C2-streptavidin significantly enhanced the uptake of biotin-p53 into African Green Monkey (Vero) epithelial cells as shown by flow cytometry. Using cell fractionation, the cytosolic translocation of biotin-p53 was detected in Vero cells as well as in HeLa cervix carcinoma cells. In line with this finding, confocal microscopy displayed cytoplasmic staining of biotin-p53 in HeLa and HL60 leukemia cells. Internalized biotin-p53 partially colocalized with early endosomes, as confirmed by confocal microscopy. In conclusion, our results demonstrate the successful conjugation of biotin-p53 to C2-streptavidin and its subsequent receptor-mediated endocytosis into different human tumor cell lines.



INTRODUCTION

The *Clostridium botulinum* C2 toxin is a bacterial AB-type toxin, which consists of two nonlinked proteins, C2I and C2II.[1] The enzyme component C2I catalyzes the mono-ADP-ribosylation of G-actin upon cell entry, which is mediated by the binding and translocation component C2II. To gain biological activity, C2II (80 kDa) requires proteolytic activation to C2IIa (60 kDa).[2] C2IIa binds to a carbohydrate receptor on mammalian cells, followed by the assembly with C2I, and the C2IIa/C2I complex is then internalized by clathrin-dependent endocytosis involving PI3K-Akt signaling.[3−5] The subsequent acidification of the endosomal lumen results in a conformational switch of C2IIa and its insertion into the endosomal membrane, forming a transmembrane pore. This allows for C2I translocation into the cytosol, a process that is facilitated by an array of host cell chaperones including Hsp90, cyclophilin A, and FK506-binding protein 51.[6−8] In the cytosol, C2I ADP-ribosylates G-actin, which in turn causes a breakdown of actin filaments and leads to delayed caspase-dependent apoptosis.[9,10]

The N-terminal domain of C2I (C2IN, amino acid residues 1−225) lacks the enzyme domain that elicits cytotoxicity, but is crucial for C2IIa-dependent internalization into eukaryotic cells.[11] This has previously prompted us to generate a fusion protein consisting of C2IN and streptavidin, which is internalized into mammalian cells in a C2IIa-dependent manner.[12] Streptavidin noncovalently binds to biotin with an exceptionally high binding affinity, which has paved the way for its use in a broad range of applications, such as cellular delivery systems.[13,14] The streptavidin variant used for the C2-based transporter is a dimeric mutant that displays a reduced binding affinity for biotin, thereby serving as a reversible binding platform for biotin-labeled molecules. We demonstrated previously that the C2IN-streptavidin/C2IIa (C2-streptavidin) delivery system directs the specific uptake of low molecular weight compounds into Vero cells and leukocytes.[12,15] Importantly, the transporter did not exert any cytotoxicity in the cell lines tested nor interfered with intrinsic cellular processes such as phagocytosis, as shown in primary human macrophages.[12,15]

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The p53 tumor suppressor is a transcription factor that is involved in the control of cell cycle progression, apoptosis, and DNA repair.[16] In response to diverse stress conditions such as DNA damage and hypoxia, p53 binds to its DNA consensus sequence inducing the transcription of specific target genes such as p21 and Bax.[17] The p53 tumor suppressor protein is inactivated in many human cancers by distinct mechanisms, such as mutations in its DNA binding domain and enhanced proteasomal degradation upon ubiquitination.[18,19] Due to its vital functions, p53 has attracted a great deal of attention, and several approaches have been pursued to deliver p53 into tumor cells. Thus, polyethylenimine-conjugated p53 plasmid DNA was internalized into HepG2 and HeLa cells via transferrin-receptor-mediated endocytosis.[20] A more recent study showed the nanoparticle-dependent uptake of a p53 expression plasmid together with the anticancer drug doxorubicin into HeLa cells.[21] Apart from gene delivery, other studies focused on the internalization of p53 protein fused to cell-penetrating peptides. Takenobu and colleagues generated a fusion protein consisting of p53 and polyarginine capable of mediating its uptake into oral cancer cells.[22] In addition, p53 was expressed as fusion protein linked to gonadotropin releasing hormone, which triggered receptor-mediated endocytosis into GnRH-positive cells.[23]

In this study, we attached biotin groups to recombinant human p53 protein retaining its specific DNA-binding activity, as shown by gel-shift analysis. Subsequently, we conjugated biotin-p53 with the C2-streptavidin transporter in a non-covalent manner building on biotin-streptavidin chemistry, which was monitored by overlay blot technique and real-time surface plasmon resonance (SPR) analysis. Next, we provided evidence that C2-streptavidin efficiently mediated the internalization of biotin-p53 into different tumor cell lines such as HeLa cells and facilitated the translocation of the biotin-p53 cargo into the cytosol of these cells.

EXPERIMENTAL PROCEDURES

Expression, Purification, and Biotin-Conjugation of p53 Protein. Recombinant human p53 was produced in insect cells using the baculovirus system and purified as reported previously with some modifications.[24] Briefly, insect cells harvested 48 h post infection were lysed. The cleared whole cell lysate was then loaded onto a Q-Sepharose column (GE Healthcare, Munich, Germany) and bound p53 was eluted with 400 mM NaCl. Fractions containing p53 were pooled, diluted in 20 mM HEPES-KOH, pH 7.4, and injected onto a Heparin-Sepharose column (GE Healthcare, Munich, Germany). p53 was eluted by a linear NaCl gradient and concentrated to 1 mg/mL using Vivaspin concentration tubes (Vivascience, Göttingen, Germany).

Purified p53 was labeled with biotin using the amine-reactive linker sulfo-NHS-Biotin (Pierce, Bonn, Germany) at a 2.5-fold molar excess in PBS, pH 7.4, for 30 min at RT. Biotinylated p53 was desalted with Zeba spin columns (Pierce, Bonn, Germany) and successful biotin modification was confirmed by SDS-PAGE followed by Western blot analysis using a streptavidin−peroxidase conjugate (Roche, Mannheim, Germany).

Determination of the Number of Biotin Molecules per p53 Protein. To assess the number of biotin moieties conjugated to p53, a standard assay based upon 4′-hydroxyazobenzene-2-carboxylic acid (HABA) was used.[25] HABA forms a yellow−orange complex with avidin that absorbs light at 500 nm. After addition of the biotinylated protein sample the HABA dye is released from the complex due to its lower affinity for avidin, resulting in a decrease in absorbance. This change in absorbance is directly proportional to the number of biotin molecules attached to the protein. The HABA assay was performed in a 96-well format in triplicates. Briefly, 160 μL of Tris-buffered saline (TBS) pH 7.4 was added into each well and supplemented with 20 μL of HABA/avidin premix (Pierce, Bonn, Germany). After recording the absorbance at 500 nm, 20 μL of biotin-conjugated p53 (1 mg/mL) diluted in TBS-buffer was added and mixed well by pipetting up and down. Subsequently, the reduction of absorbance at 500 nm was measured that allowed for calculating the number of biotin molecules per p53 according to the manufacturers' instructions. As positive control, horseradish peroxidase (HRP) conjugated with 1.2 mol of biotin per protein was used (Pierce, Bonn, Germany).

p53 DNA Binding Assay. The specific DNA-binding of biotin-p53 was assessed using an electrophoretic mobility shift assay (EMSA). To this end, an oligonucleotide duplex containing a p53-binding site of the MDM2 promotor was used as described previously.[26] Binding of p53 to the oligonucleotide duplex leads to the formation of higher molecular weight DNA-p53 complexes with reduced electrophoretic mobility. Increasing amounts of biotin-p53 (0−1000 ng of protein) were preincubated in DNA-binding buffer for 10 min. After addition of the biotin-end-labeled oligonucleotide duplex (6 nM), the DNA binding of biotin-p53 was allowed to occur for an additional 20 min. The reaction was terminated by addition of EMSA loading buffer on ice. Samples were then subjected to a 6% (w/v) native PAGE and transferred onto a positively charged nylon membrane (GE Healthcare, Munich, Germany) by semidry blotting. Subsequently, the membrane was fixed for 60 min at 90 °C and blocked with 2% (w/v) BSA in PBS-T. Free biotinylated oligonucleotide duplex and DNA-biotin-p53 complexes were detected with streptavidin-peroxidase (1:15 000) using enhanced chemiluminescence.

Expression and Purification of C2IN-Streptavidin and C2IIa. C2IN-streptavidin was expressed and purified as described previously.[12] In brief, *E. coli* Rosetta transformed with pGEX2T-C2IN-streptavidin were grown in LB-medium to an optical density of 0.6. Isopropyl-β-D-thiogalactopyranoside was then added to induce protein expression and cultures were incubated at 16 °C for 20 h. Bacterial cultures were harvested by centrifugation for 10 min at 4 °C at 4400 × *g*, resuspended in buffer containing 0.1% (v/v) Triton X-100 and disrupted by sonication. After centrifugation (20 000 × *g*, 15 min, 4 °C), the supernatant was incubated with glutathione-agarose beads (Macherey-Nagel, Düren, Germany) for 2 h at room temperature. After centrifugation for 5 min at 1000 × *g*, the beads were thoroughly washed and resuspended in PBS with thrombin (15 NIH units/L culture) to cleave off the GST-tag. Following 1 h incubation, the glutathione-agarose beads were pelleted by centrifugation (10 000 × *g*, 1 min) and the supernatant containing C2IN-streptavidin was collected. Remaining thrombin was eliminated by incubation with benzamidine beads (GE Healthcare, Munich, Germany) for 10 min and collected. Isolated C2IN-streptavidin was analyzed for homogeneity by SDS-PAGE followed by Coomassie blue staining. For immunoblot detection, an anti-C2IN-antibody raised in rabbits was used.[11]

SDS-PAGE and Immunoblot Analysis. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Whatman, Dassel, Germany) with a wet-blot

chamber (GE Healthcare, München, Germany). 5 % (w/v) nonfat dry milk in PBS containing Tween-20 [0.1% (v/v), PBS-T] was used to block the membrane for 1 h at room temperature (RT). Subsequently, the membrane was probed with the respective primary antibody diluted in PBS-T for 1 h at RT. After washing the membrane 3 times with PBS-T, it was incubated with the appropriate secondary antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h. After further washing steps, proteins were detected by chemiluminescence using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, Schwalbach, Germany).

Overlay Blot Analysis. Increasing amounts of C2IN-streptavidin or C2IN (5 up to 50 pmol) were separated by SDS-PAGE and transferred on a nitrocellulose membrane. After a blocking step, the membrane was incubated with Biotin-p53 in PBS-T (2.5 μg/mL). To disrupt unspecific protein binding, the membrane was washed thoroughly with PBS-T containing 500 mM NaCl followed by three washing steps in PBS-T. Bound Biotin-p53 was visualized with a streptavidin−POD conjugate diluted 1:2000 in PBS-T and subsequent chemiluminescence detection. Densitometric evaluation of three independent experiments was performed by *Adobe Photoshop CS3* software and analyzed by *GraphPad Prism 4* software.

Surface Plasmon Resonance (SPR). SPR studies were performed on a Biacore 2000 system with a CM5 sensor chip (GE Healthcare, Munich, Germany). C2IN-streptavidin in sodium acetate buffer, pH 5.0, was immobilized on the sensor chip via amine coupling, resulting in a final response level of 750 RU. An activated reference cell without immobilized ligand was used to subtract buffer refractive effects and as a control for unspecific binding. Biotin-labeled p53 protein was injected at room temperature using the following concentrations: 0.975 nM, 1.95 nM, 3.9 nM, 7.8 nM, 15.6 nM, 31.2 nM, and 62.5 nM. The flow rate was set to 30 μL/min with the following parameters: 2 min contact time, 30 min dissociation time. Dissociation was carried out in HBS buffer, pH 7.4, without a regeneration step. Baseline levels were stable throughout all cycles. Binding curves obtained were analyzed using *BIAevaluation* software 3.2.

Cell Culture. HeLa cervix carcinoma and African Green Monkey kidney (Vero) cells were cultured at 37 °C and 5% (v/v) CO_2 in minimum essential medium (MEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), L-glutamate (2 mM), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were routinely trypsinized and reseeded three times per week. Human promyelocytic leukemia cells (HL-60) were maintained at 37 °C and 5% (v/v) CO_2 in RPMI 1640 medium containing 10% (v/v) heat-inactivated FCS and antibiotics. Cells were grown to a density of (0.3−2.0) × 10^6 cells/mL.

Flow Cytometry. 4 μg C2IN-streptavidin was preincubated with 4 μg biotin-p53 for 30 min to allow for conjugation, followed by addition of 8 μg C2IIa and further incubation for 30 min on ice. Vero cells grown in 6 cm dishes overnight were then treated for 5 h with C2-streptavidin transporter (2 μg/mL C2IN-streptavidin +4 μg/mL C2IIa) conjugated to biotin-p53 (2 μg/mL). As controls, cells were left untreated or incubated with biotin-p53 (2 μg/mL) in the absence of the transporter. Subsequently, cells were washed twice with PBS, detached by trypsin, and harvested by centrifugation. Cells were resuspended in PBS supplemented with Pronase (1 μg/mL) to digest cell membrane-associated, noninternalized complex and incubated in this medium for 5 min on ice. Proteolysis was terminated by addition of protease inhibitor cocktail and cells were pelleted by centrifugation (10 000 × *g*, 30 s). Cells were then fixed in 2% paraformaldehyde (PFA), washed in FACS buffer and permeabilized in 0.4% Triton X-100. Cells were blocked in 5% nonfat dry milk in PBS-T followed by incubation with an anti-p53-antibody (DO-1; Santa Cruz Biotechnology, Heidelberg, Germany). After several washing steps, cells were incubated with an Alexa488-coupled secondary antibody (Invitrogen, Karlsruhe, Germany) and finally resuspended in PBS containing 2 mM EDTA. Cells were analyzed by flow cytometry using a FACS Canto II device (BD Biosciences, San Jose, USA). Alexa488-positive cells were assessed with *FACS Diva* software (BD Biosciences, San Jose, USA).

Confocal Microscopy. A complex consisting of C2-streptavidin (2 μg/mL C2IN-Streptavidin +4 μg/mL C2IIa) and biotin-p53 (2 μg/mL) was prepared as described above and added to HeLa cells grown on coverslips. Furthermore, cells were either incubated only with biotin-p53 or left untreated. After incubation for 5 h, the medium was removed and cells were washed twice with PBS. Subsequently, cells were fixed with 4% (w/v) PFA, permeabilized with 0.4% (v/v) Triton X-100, and blocked with 5% (w/v) dry-milk powder in PBS-T. Biotin-p53 was then visualized with a monoclonal p53 antibody together with an appropriate Alexa488-conjugated secondary antibody (1:400). Endosomes were stained by a transferrin-Alexa647 conjugate (Invitrogen, Karlsruhe, Germany) used at 20 μg/mL. Cells were mounted on microscope slides with Prolong Gold Antifade Solution (Invitrogen, Karlsruhe, Germany) and analyzed by confocal microscopy with a Zeiss Axiovert 200 M microscope equipped with a LSM510 Meta laser scanning device (Zeiss, Oberkochen, Germany). Z-Stack images were acquired in optical sections of 1 μm and processed with *ImageJ* (NIH, USA).

Digitonin-Based Cell Fractionation. Preparation of cytosolic fractions of Vero and HeLa cells was performed as described previously.[12,27] Briefly, cells were seeded in 12-well plates and incubated with C2-streptavidin (2 μg/mL C2IN-Streptavidin +4 μg/mL C2IIa) and biotin-p53 (2 μg/mL) for the indicated time points. The medium was then removed and cells were washed 3 times with PBS. Subsequently, the cells were permeabilized by incubation with digitonin (Sigma-Aldrich, Deisenhofen, Germany) for 5 min at RT. Cells were incubated for an additional 25 min on ice to obtain the cytosolic supernatant. Afterward, the supernatant was carefully collected and the extracted cells were scraped off. Both fractions were subjected to SDS-PAGE followed by Western Blot analysis. Biotin-p53 was detected either with a monoclonal p53 antibody and the appropriate secondary antibody or with a streptavidin−peroxidase conjugate. To confirm equal protein loading, the cytosolic marker protein Hsp90 was detected with a monoclonal anti-Hsp90 antibody (Santa Cruz, Heidelberg, Germany). The endosomal protein Rab5 was visualized by a monoclonal antibody (Santa Cruz Biotechnolgy, Heidelberg, Germany) to check for cross-contamination of the cytosolic fraction with endosomal vesicles.

Reproducibility of the Experiments and Statistics. All experiments were performed independently at least twice. Results from representative experiments are shown. Values ($n \geq 3$) are presented as means ± standard errors of the means (SEM) using *GraphPad Prism4* Software.

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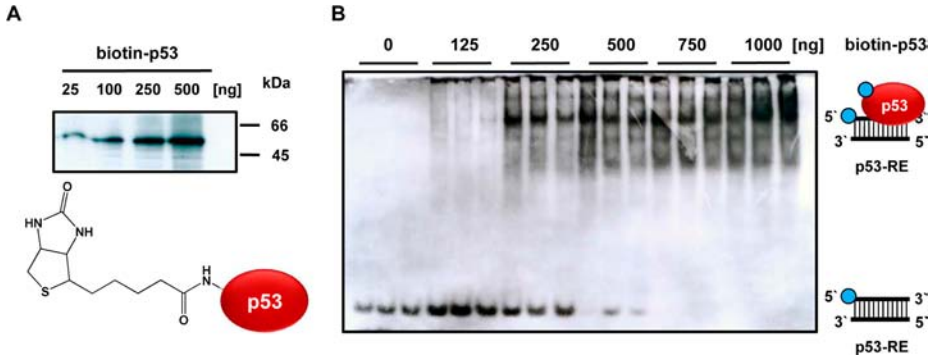

Figure 1. Analysis of biotin-p53 and its DNA binding activity. A. Biotin-labeling of p53. Recombinant human p53 expressed in insect cells was biotinylated using the amino-reactive linker sulfo-NHS-biotin. Biotin-p53 was then analyzed by SDS-PAGE and subsequent immunoblotting with a streptavidin-POD conjugate. B. DNA-binding of biotinylated p53. The specific DNA-binding activity of biotin-p53 was determined using an electrophoretic mobility shift assay with a biotin-end-labeled oligonucleotide duplex harboring the p53 responsive element of the MDM2 promotor (p53-RE). The formed complex was separated by native PAGE and visualized by streptavidin-POD. Blue circles represent biotin residues.

RESULTS

Biotin-Conjugation of p53 Does Not Interfere with Its DNA-Binding Activity. We have previously generated a novel C2-streptavidin delivery system that promotes the uptake of small biotinylated compounds such as biotin-fluorescein into mammalian cells. To address the question as to whether C2-streptavidin mediates the cellular uptake of biotin-labeled proteins, we selected p53 tumor suppressor protein as an attractive target for delivery studies. First, p53 protein was produced in insect cells infected with a genetically engineered p53 baculovirus. The protein was purified using a two-step protocol via anion exchange chromatography followed by heparin affinity chromatography. This method resulted in recombinant protein of sufficient yield and purity, as revealed by Coomassie blue staining (data not shown). Thereafter, purified protein was reacted with sulfo-NHS-biotin to attach biotin to primary amino groups of p53. The biotinylated p53 (biotin-p53) was then analyzed by SDS-PAGE followed by immunoblot detection with a streptavidin−peroxidase conjugate, showing efficient biotin modification, which is crucial for later internalization studies with the C2-streptavidin transporter (Figure 1 A). Furthermore, a HABA assay was performed to assess the number of biotin moieties introduced per p53 molecule by measuring the absorbance of an avidin/HABA complex before and after addition of biotin-p53. The p53 protein was modified with 2.2 biotin groups per molecule (Table 1), while the positive control horseradish peroxidase displayed 1.6 biotin groups per protein (1.2 biotin groups as determined by the manufacturer). To rule out an effect of biotinylation on the specific DNA-binding activity of p53, we used an electrophoretic mobility shift assay (EMSA) that relies on the binding of p53 to a biotinylated oligonucleotide duplex harboring the p53 binding site of the MDM2 promotor. Addition of biotin-p53 resulted in the formation of DNA-p53 complexes in a concentration-dependent manner (Figure 1B). Of note, the DNA-binding of biotin-labeled p53 was comparable to that of unmodified p53 (data not shown).

Table 1. Degree of Biotinylation Assessed by HABA Assay

protein	biotin groups per protein
Biotin-p53	2.2 ± 0.2
Biotin-HRP	1.6 ± 0.1

Interaction of Biotin-p53 with the C2-Streptavidin Delivery System. Next, we studied the noncovalent binding of biotin-p53 to C2IN-streptavidin using an overlay blot technique. C2IN-streptavidin and the negative control C2IN were subjected to SDS-PAGE followed by transfer on a nitrocellulose membrane. After incubation with biotin-p53 (2.5 μg/mL; 57 nM) and several washing steps, including high salt to disrupt nonspecific electrostatic interactions, the bound biotin-p53 was visualized by streptavidin-POD. Biotin-p53 specifically interacted with immobilized C2IN-streptavidin starting at 5 pmol C2IN-streptavidin, but displayed only weak binding even to high quantities of C2IN (Figure 2A). Densitometric evaluation of three independent experiments revealed a clear concentration-dependent binding of biotin-p53 to C2IN-streptavidin, which is crucial for subsequent delivery studies (Figure 2B). Furthermore, SPR studies were performed to determine the binding kinetics and affinity. To this end, C2IN-streptavidin was covalently immobilized on CM5 sensor chips by amine coupling and increasing concentrations of biotin-p53 were injected in the flow cell. The binding was monitored in real-time and binding curves obtained were fitted with a 1:1 Langmuir binding model, providing a K_D value of 3.9 × 10^{-9} M (Figure 2C, Table 2). To correct for nonspecific binding and buffer changes, an activated reference cell containing no immobilized C2IN-streptavidin was used.

Taken together, biotin-p53 displayed specific binding to C2IN-streptavidin with an affinity in the low nM range. The latter is several orders of magnitude lower than the affinity of biotin for wild-type streptavidin[13] and may, therefore, allow for intracellular dissociation of biotin-p53 from its streptavidin-containing transport unit.

C2-Streptavidin-Mediated Uptake of Biotin-p53 into Vero Cells. Following successful conjugation, the C2-streptavidin-mediated internalization of biotin-p53 was initially assessed in Vero cells by flow cytometry. To this end, cells were harvested 5 h after treatment by trypsinization and subjected to Pronase digestion to remove cell-membrane bound, non-internalized protein. Intracellular human p53 was then detected by immunofluorescence staining and measured by flow cytometry, revealing efficient internalization in a C2-streptavidin-dependent manner (Figure 3A). Conversely, biotin-p53 on its own showed only little uptake, as confirmed by the low frequency of Alexa488-positive cells, which was within the range of the frequency observed for untreated control cells. To

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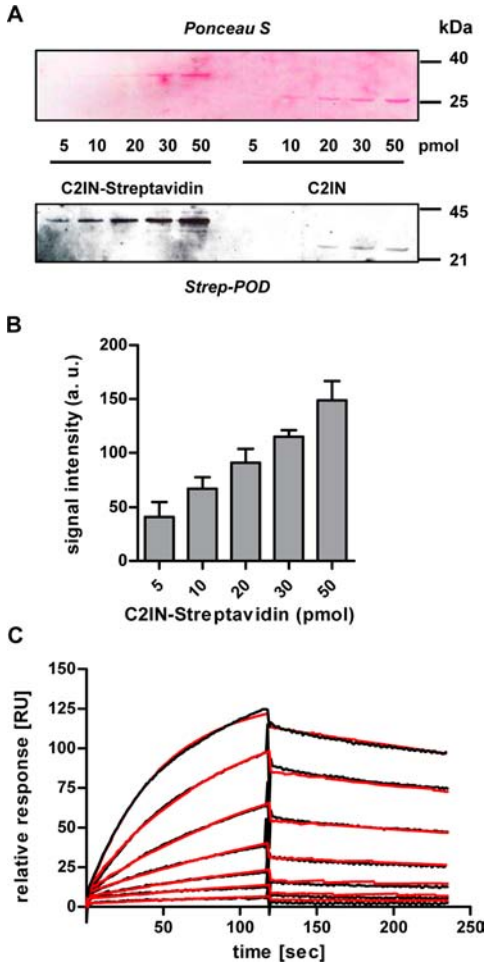

Figure 2. Interaction of Biotin-p53 and C2-streptavidin delivery system. A. Overlay blot binding assay. Increasing amounts of C2IN-streptavidin and C2IN used as negative control were subjected to SDS-PAGE and transferred on a nitrocellulose membrane. Protein loading was visualized by Ponceau S staining (upper panel). Following incubation with 2.5 μg/mL biotin-p53, nonspecifically interacting biotinylated protein was removed by high-salt washes and bound biotin-p53 was detected using streptavidin-POD (lower panel). B. Quantification of biotin-p53 binding to C2IN-Streptavidin. Signal intensity was determined using *Adobe Photoshop CS3* software and is given as mean ± SEM of three independent experiments. C. Real-time surface plasmon resonance. To assess the binding kinetics, C2IN-streptavidin was covalently immobilized on a Biacore CM5 sensor chip and biotin-p53 was injected at concentrations ranging from 1 nM up to 62.5 nM. Concentration-dependent binding was monitored (black curves) and fitted with a Langmuir 1:1 binding model (red curves).

Table 2. Binding Kinetics of Biotin-Conjugated p53 to C2IN-Streptavidin Obtained by Real-Time SPR Analysis

k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)	χ^2
3.1×10^5	1.2×10^{-3}	3.9×10^{-9}	1.53
$\pm 0.1 \times 10^5$	$\pm 0.2 \times 10^{-3}$	$\pm 0.5 \times 10^{-9}$	

dissect the intracellular fate of delivered biotin-p53, digitonin-based cell fractionation was used.[12,27] Cells were treated for up to 24 h with C2-streptavidin conjugated biotin-p53 and various controls. The fractions obtained by cell fractionation were then separated by SDS-PAGE followed by Western Blot analysis. C2-streptavidin specifically promoted the uptake of biotin-p53 into the cytosol of Vero cells, with a maximum after 24 h (Figure 3B). A time-dependent accumulation of biotin-p53 was also observed in extracted cells, which reflects membrane-bound and vesicular protein. Biotin-p53 also bound to the cells in the absence of the transporter, but did not translocate into the cytosol. Rab 5, an early endosomal marker protein,[28] was visualized only in extracted cells, excluding a carryover of early endosomal vesicles into the cytosolic fraction.

Collectively, these results demonstrate for the first time that the C2-streptavidin transporter mediates the uptake of a biotinylated cargo protein, i.e., biotin-53, into the cytosol of mammalian cells.

Delivery of Biotin-p53 into Human Tumor Cells. As a next step, we were interested to see whether C2-streptavidin could also direct the uptake of biotin-p53 into human tumor cell lines. Therefore, HeLa cervix carcinoma cells were treated for 5 h with biotin-p53 conjugated to C2-streptavidin and cell fractionation was performed as described above. Biotin-p53 was efficiently internalized into HeLa cells in a C2-streptavidin-dependent way, with a pronounced translocation into the cytosol (Figure 4A). In contrast to Vero cells, biotin-p53 was almost undetectable in extracted cells without prior conjugation to the transporter. Please also note that HeLa cells exhibit only very low levels of endogenous wild-type p53, since its proteasomal degradation is enhanced by the viral E6 oncoprotein.[29] In addition, we performed confocal microscopy of HeLa cells that had been incubated for 24 h with biotin-p53 attached to C2-streptavidin (Figure 4B). Z-stack images were recorded in 1 μm optical sections and images containing the nucleus, as evidenced by DIC, were evaluated for biotin-p53 staining using a monoclonal p53 antibody. Internalized biotin-p53 showed predominantly cytoplasmic localization, in line with the findings obtained by cell fractionation. C2-streptavidin also promoted the entry of biotin-p53 into HL-60 leukemia cells as monitored by confocal microcopy (data not shown).

Finally, colocalization studies with markers of endocytosis were conducted in Vero cells where introduced biotin-p53 displayed some overlap with a transferrin-Alexa647 conjugate, a marker for clathrin-mediated endocytosis.[30] The specific C2-streptavidin-dependent entry of biotin-p53 was confirmed by omitting the transporter (Figure 5, middle panel). Moreover, biotin-p53 also partially colocalized to Rab5 as observed by confocal microscopy of Vero cells (data not shown). Taken together, these results imply that the C2-streptavidin-mediated delivery of biotin-p53 into cells occurs via the C2 toxin-specific uptake mechanism.

■ DISCUSSION

In this study, we have used the C2-streptavidin delivery system to internalize biotin-labeled p53 protein into different mammalian cell lines including human tumor cells. Recombinant human p53 was successfully labeled with 2 biotin moieties per protein, not affecting its vital DNA-binding activity as demonstrated by gel-shift analysis. To circumvent the stochastic chemical modification of p53, an elegant approach resting on site-selective labeling via biotin ligase could be used in the future.[31] We then studied the noncovalent interaction of biotin-p53 and the C2IN-streptavidin moiety of the transporter using overlay blot technique and real-time SPR measurements, demonstrating a strong and specific, concentration-dependent binding. The K_D-value of the C2IN-streptavidin/biotin-p53 interaction was calculated on the basis of the binding kinetics obtained by SPR and revealed a high binding affinity in the low nanomolar range (3.9 nM). This is somewhat higher than that

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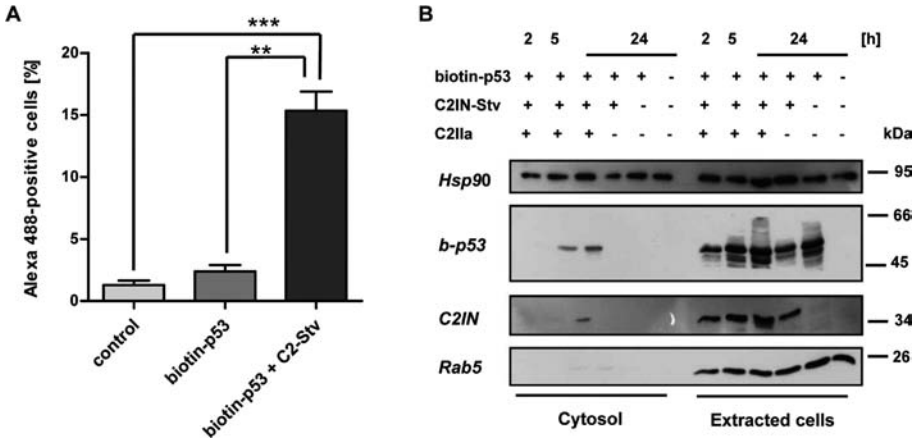

Figure 3. C2-streptavidin-mediated delivery of biotin-p53 into Vero cells. A. Flow cytometry of biotin-p53 internalization. Vero cells were treated with a complex of C2-Streptavidin (C2-Stv) and biotin-p53 for 5 h, trypsinized, and fixed. Intracellular biotin-conjugated p53 was stained with a monoclonal antibody in conjunction with an Alexa488-coupled secondary antibody and analyzed by flow cytometry. Data obtained were processed by *FACS Diva* software and represent mean ± SEM of three independent experiments. **, $p < 0.005$; ***, $p < 0.001$. B. Delivery of biotin-p53 into the cytosol of Vero cells. Cells were incubated with C2-Stv and biotin-p53 over a period of 24 h. Subsequently, cell fractionation was performed and samples obtained (cytosol vs extracted cells) were subjected to SDS-PAGE followed by Western Blot analysis. Biotin-p53 was detected by means of a streptavidin-POD conjugate. Equal protein loading was verified by anti-Hsp90 immunoblotting. The early endosomal marker Rab5 was used as quality control for the cell fractionation.

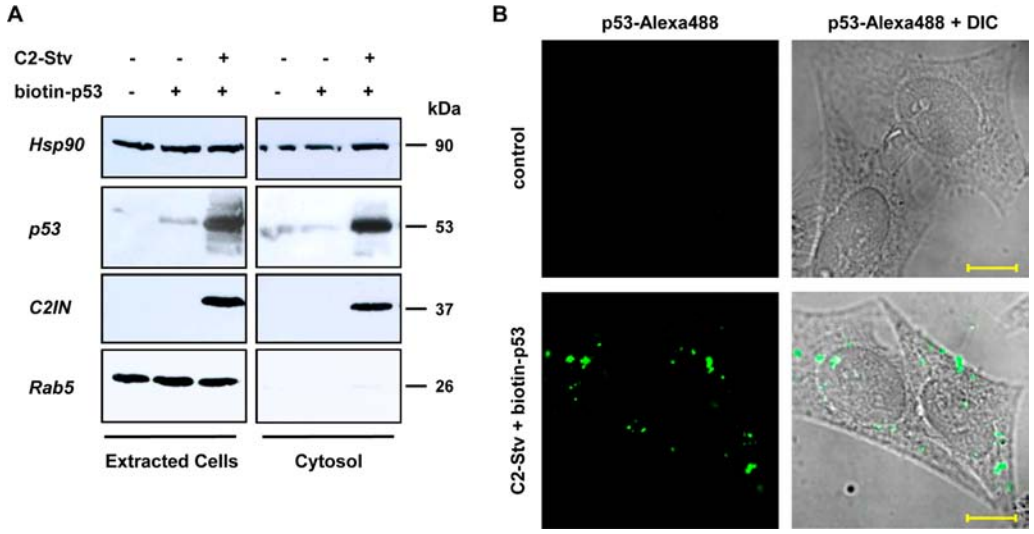

Figure 4. Delivery of biotin-p53 into HeLa cervix carcinoma cells. A. Cell fractionation of biotin-p53-treated HeLa cells. Cells were incubated with biotin-p53 conjugated to C2-Streptavidin (C2-Stv) for 5 h and then processed for cell fractionation. The fractions obtained were analyzed by SDS-PAGE and subsequent immunoblot detection. Hsp90 was visualized as loading control and Rab5 was used to rule out contamination of cytosol by early endosomes. B. Confocal microscopy of biotin-p53 uptake. Cells were treated with biotinylated p53 and C2-Stv and analyzed by confocal microscopy. Detection of biotin-p53 was performed with a monoclonal p53 antibody followed by Alexa488 conjugated secondary antibody (green). Z-Stack images were recorded in 1 μm optical sections and processed by *ImageJ*. Representative images thereof are shown. Scale bar: 10 μm.

reported for the dimeric streptavidin−biotin interaction (K_D = 150 nM) or the binding of biotinylated clostridial C3 toxin to C2IN-streptavidin (K_D = 750 nM).[12,32] One explanation could be an elevated degree of biotinylation, slowing down the dissociation of biotin-p53 from C2-streptavidin. Indeed, p53 was modified on average with 2.2 biotin moieties as revealed by HABA assay. Other reasons may also contribute to the observed increase in binding affinity, such as an improved surface accessibility of biotin groups on p53 or electrostatic interactions between p53 and C2-streptavidin. This increased binding affinity may reduce the intracellular release of biotin-p53 from C2-streptavidin by endogenous free biotin that is produced in cells by biotinidase.[33] To enhance the intracellular dissociation from the transporter, p53 could be conjugated in future approaches with disulfide-containing biotin linkers that are cleaved under the reducing conditions met intracellularly as described previously.[34]

The specific C2-streptavidin-dependent uptake of biotin-p53 was demonstrated in different cell lines and represents to our knowledge the first evidence of intracellular delivery of p53 protein in a noncovalent manner using biotin-streptavidin technology. Biotin-p53 was delivered into the cytosol only in the presence of C2IIa as revealed by cell fractionation, highlighting the specificity of the uptake mechanism. Interestingly, biotin-p53 was stable over 24 h in the cytosol and not subjected to proteasomal degradation. Upon internalization, biotin-p53 was shown to reside mainly in the cytoplasm with some overlap to endosomal vesicles, whereas it was barely

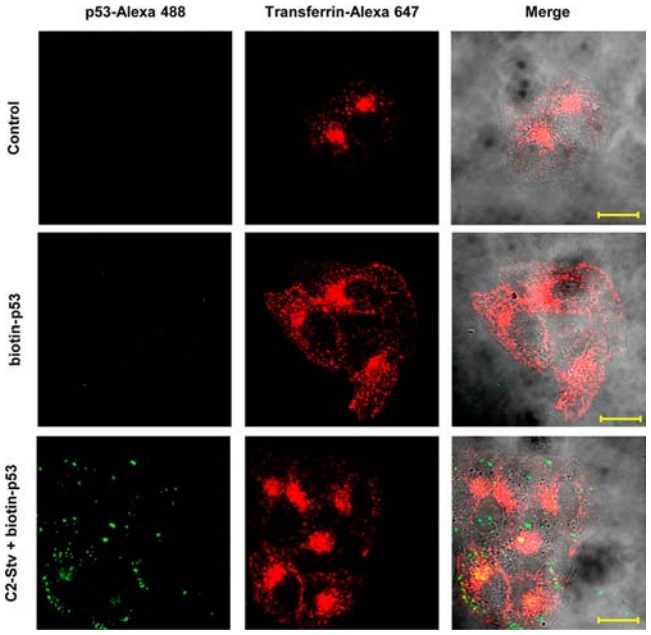

Figure 5. Colocalization of biotin-p53 and early endosomes. Cells were treated with biotin-p53 complexed to the C2-streptavdin (C2-Stv) delivery system. As controls, cells were left untreated or incubated in the absence of transporter. Clathrin-dependent endocytosis was monitored with a transferrin-Alexa 647 conjugate. Images were acquired by confocal microscopy of optical 1 μm sections and processed by *ImageJ*. Representative images thereof are shown. Scale bar: 10 μm.

observed in the nucleus. The intracellular trafficking of wild-type p53 is tightly controlled by post-translational modifications and protein interactions. Cytoplasmic p53 is transported to the perinuclear region by dynein,[35] where it binds to importin facilitating its subsequent nuclear translocation.[36] Persistent association of biotin-p53 with the C2-streptavidin transporter may interfere with this nuclear import process, which could be eliminated introducing cleavable linkers as mentioned before.

The C2-streptavidin delivery system rests on the binding of the C2IIa moiety to its cell surface receptor,[3] which is found in all mammalian cells tested, and on its subsequent endocytotic uptake. C2IIa harbors four distinct protein domains: D1′, D2, D3, and D4. Domain D1′ serves as a docking platform for C2I and C2IN-streptavidin, respectively, while D2 is important for the generation of the pore lumen.[37] The C-terminal domain D4 mediates the binding of C2IIa to the cellular receptor and its deletion abrogates the receptor binding of C2IIa, yet retaining its capability to form oligomers and pores.[38] Thus, it is conceivable to replace or modify the receptor binding domain of C2IIa by specific targeting moieties to enable a selective delivery into tumor cells of interest. A similar approach has very recently been reported for the binding/translocation component of the binary anthrax toxins, protective antigen (PA),[39] which is structurally and functionally similar to C2IIa.[1] Cancer cells are known to overexpress a variety of cellular receptors, including EGF-, folate-, and somatostatin-receptors,[40−42] which allow for specific targeting. It should be feasible to attach these targeting groups to the C2IIa binding/translocation component for preferential association with receptor-positive cells.

The data presented demonstrate that biotin-p53 is internalized efficiently by C2-streptavidin into cultured tumor cells. Yet, *in vivo* medical application of the system may require further optimization steps. One potential drawback of streptavidin is its bacterial origin and intrinsic immunogenicity, in particular, in settings with repetitive cycles of administration. Nevertheless, streptavidin and mutants thereof have been successfully used in cancer radioimmunotherapy, leading to a significant increase of tumor-to-normal tissue ratio.[43] A conceivable strategy to reduce the immunogenicity of streptavidin represents the site-directed mutagenesis of amino acid residues critically involved in its immune response.[44] Another obstacle in a clinical setting might be the endogenous level of biotin in blood serum originating from dietary sources and in tissue that may compete with the binding of biotin-p53 to C2-streptavidin and interfere with complex stability. Previous work using wild-type streptavidin and biotinylated vasoactive intestinal peptide demonstrated stability of the complex in the bloodstream *in vivo*.[45] In this regard, the increased binding affinity of biotin-p53 to C2-streptavidin observed should be beneficial by impairing its dissociation in the bloodstream. Another important issue to be addressed for future *in vivo* studies is the method of application. The most obvious route of administration is the intratumoral injection of the C2-streptavidin transporter conjugated with biotin-p53, which could be envisioned for solid tumors and tested in a xenograft mouse model using HeLa cells. For treatment of hematological malignancies an intravenous administration is mandatory, which requires an improved C2-streptavidin system with regard to its immunogenicity and targeting as pointed out above.

We have previously shown that C2-streptavidin promotes the uptake of biotinylated low molecular weight compounds, such as biotin-fluorescein, into various cell lines. Using biotinylated p53 as cargo we successfully demonstrated its ability to function as a protein shuttle, which broadens its applicability. It is noteworthy that C2-streptavidin failed to direct the internalization of biotinylated C3 toxin into Vero cells (J. Fahrer, unpublished data), which displayed an approximately 100-fold lower affinity for the transporter *in vitro*. This indicates that successful delivery by C2-streptavidin may depend on the physicochemical properties of its protein cargo, such as three-dimensional structure, accessibility of biotin groups, and electrostatic interactions, all of which are important parameters for successful translocation through the C2IIa-pore.

In summary, we used the C2-streptavidin delivery system to effectively transduce p53 into the cytosol of mammalian cells in a noncovalent manner, which may be useful for further cell-biological and biomedical applications.

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The authors declare no competing financial interest.

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