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Sequence-specific Peptide Aptamers, Interacting with the Intracellular Domain of the Epidermal Growth Factor Receptor, Interfere with Stat3 Activation and Inhibit the Growth of Tumor Cells*

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Receptor tyrosine kinases of the epidermal growth factor (EGF) receptor family regulate essential cellular functions such as proliferation, survival, migration, and differentiation but also play central roles in the etiology and progression of tumors. We have identified short peptide sequences from a random peptide library integrated into the thioredoxin scaffold protein, which specifically bind to the intracellular domain of the EGF receptor (EGFR). These molecules have the potential to selectively inhibit specific aspects of EGF receptor signaling and might become valuable as anticancer agents. Intracellular expression of the aptamer encoding gene construct KDI1 or introduction of bacterially expressed KDI1 via a protein transduction domain into EGFR-expressing cells results in KDI1·EGF receptor complex formation, a slower proliferation, and reduced soft agar colony formation. Aptamer KDI1 did not summarily block the EGF receptor tyrosine kinase activity but selectively interfered with the EGF-induced phosphorylation of the tyrosine residues 845, 1068, and 1148 as well as the phosphorylation of tyrosine 317 of p46 Shc. EGF-induced phosphorylation of Stat3 at tyrosine 705 and Stat3-dependent transactivation were also impaired. Transduction of a short synthetic peptide aptamer sequence not embedded into the scaffold protein resulted in the same impairment of EGF-induced Stat3 activation.

Signal transduction pathways and their components involved in the regulation of cellular proliferation have been studied in great detail, and aberrations have been detected in tumor cells, which are causally involved in malignant transformation. Enhanced epidermal growth factor receptor (EGFR)[1] expression and activation has been found in a variety of human tumor cells, and high levels were found to correlate with an unfavorable prognosis and a high incidence of metastasis (for review see Refs. 1 and 2).

The EGFR family of receptor tyrosine kinases consists of the epidermal growth factor (EGF) receptor EGFR, ErbB2/Neu, ErbB3, and ErbB4 and binds a large family of EGF-like ligands (3). The N-terminal extracellular domain consists of 622 amino acids and has two cysteine-rich regions that form the ligand binding domain. The cytoplasmic domain contains a kinase region and a C-terminal tail that has several tyrosine phosphorylation sites. Upon binding of ligand to the extracellular domain, the receptors homo- or heterodimerize, bringing their kinase domains into close contact. This leads to autophosphorylation of specific tyrosine residues (Tyr-992, Tyr-1068, Tyr-1086, Tyr-1148, and Tyr-1173) in the cytoplasmic tail (4–7) that serve as docking sites for SH2- and PTB domain containing signaling molecules, which activate intracellular signaling pathways (reviewed in Refs. 2 and 8). A major signaling route of the EGFR family is the Ras/Raf-mitogen-activated protein kinase (MAPK) pathway (9). EGF can also activate the transcription factor Stat3 (10). After EGF stimulation the cytoplasmic kinase c-Src is recruited to the receptor, where it phosphorylates the kinase domain at Tyr-845 and thereby hyperactivates the EGFR (11–13) and other signaling molecules like Jak2 and Stat3 (14, 15), Shc and components of the cytoskeleton and the endocytic machinery (16). Activation of these pathways results in proliferation, differentiation, and/or oncogenesis of epithelial cells, neuronal cells, and fibroblasts (17). The expression of EGFR in tumor cells can be correlated with a poor response to treatment, disease progression, and shortened survival periods (18).

Conventional tumor therapy often results in severe side effects due to the high toxicity of the drugs toward normal cells. New, selectively acting anticancer agents that differentiate between malignant and non-malignant cells constitute a major advance circumventing unwanted side effects. Although the EGFR is also expressed in non-malignant cells, the high expression levels in a certain subset of tumors and the absence of a physiological role in the adult body make the receptor a promising target for a more specific and selective tumor therapy. The inhibition of the EGFR can interfere with the growth of tumors and has led to the design of new drugs (18). Diverse strategies and reagents have been pursued for targeting the EGFR for cancer therapy, including monoclonal antibodies,

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[1] The abbreviations used are: EGFR, epidermal growth factor receptor; KD, kinase domain; Trx, thioredoxin; PTD, protein transduction domain; PTB, phospho tyrosine binding; SH2, Src homology; Stat, signal transducers and activators of transcription; Jak, Janus kinase; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; FCS, fetal calf serum; CMV, cytomegalovirus; DTT, dithiothreitol; PBS, phosphate-buffered saline; XTT, sodium 3′-[1-(plenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate.

ligand-linked immunotoxins, tyrosine kinase inhibitors, and antisense molecules (19, 20).

With the selection of EGFR-specific peptide aptamers we have added a new approach to the inhibition of EGFR-mediated signaling and cellular transformation. Peptide aptamers are peptides of random sequence integrated into and displayed by the bacterial thioredoxin (Trx) protein as a platform. Specifically binding peptide aptamers can be selected from combinatorial libraries in which the variable peptide sequence is conformationally constrained. Selections of specific binders are carried out *in vivo* by an adaptation of the yeast two-hybrid system (21). The interaction between a specific peptide sequence inserted into the active loop of the scaffold protein thioredoxin A and the target protein domain of interest restores the activity of a Gal4 transcription factor and allows the growth of yeast cells under selection. Several examples have been described in which aptamers were used to interfere with specific protein interactions *in vivo* and thus elicit distinct cellular phenotypes (22–28).

To evaluate the effects of the EGFR binding peptide aptamers on cellular growth parameters we used two experimental strategies. The first one is based on the introduction of a gene construct, encoding the peptide aptamer, into the cells of interest and observing the consequences of endogenous gene expression. The second strategy relies on the bacterial expression of the aptamer fusion protein and introduction into target cells via a linked protein transduction domain (PTD). We made use of a transduction domain consisting of nine L-arginine residues (9R), which allows a fast and efficient cellular uptake of linked proteins (29–31). This sequence represents the optimized version of the PTD of the human immunodeficiency virus TAT protein (32, 33). Other protein transduction domains have been identified in the Antennapedia protein from *Drosophila* and in the herpes simplex virus VP22 protein (34, 35). The exact mechanism of protein transduction across membranes remains unknown; however, the mechanism does not seem to be receptor-mediated and is energy-independent (35).

We isolated peptide aptamers from a high complexity random peptide library that specifically bind to the intracellular kinase domain of the EGF receptor (EGFR). Expression upon gene transfer of the aptamer encoding construct, KDI1, into EGFR-expressing cells or KDI1 protein transfer mediated by a PTD yielded consistent results with respect to the capability to interfere with EFGR action. The aptamer inhibited EGF-induced phosphorylation at tyrosine residues 845, 1068, and 1148 of the EGFR, interfered with the activation of p46 Shc, tyrosine phosphorylation of Stat3, and thereby suppressed proliferation and transformation. A short synthetic peptide comprising the aptamer sequence and a PTD also interfered with EGF-dependent Stat3 activation.

EXPERIMENTAL PROCEDURES

Antibodies and Growth Factors—Recombinant human EGF was from R&D Systems. Antibodies used were: thioredoxin (Sigma), EGFR sc-03 (Santa Cruz Biotechnologies), EGFR sc-120 (Santa Cruz), ErbB2 C-18 (Santa Cruz), Stat3 C-20 (Santa Cruz), phosphorylation site-specific antibodies were from New England BioLabs/Cell Signaling.

Cell Lines and Cell Culture—Herc cells (NIH 3T3/EGFR), the SKBR3 breast carcinoma cell line, and the A431 vulval carcinoma cell line were maintained in Dulbecco's modified Eagle's medium containing 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin. Herc Trx, KDI1, KDI3, and KDI4 cell lines were generated by stable introduction of pRc/CMV VP22-Trx-based vectors (25) encoding for the peptide aptamer or the thioredoxin protein without a peptide insert using LipofectAMINE2000 (Invitrogen). Stably transfected cell lines were maintained in Dulbecco's modified Eagle's medium/10% FCS/1 mg/ml G418. Prior to growth factor stimulation the cells were kept for 18 h in serum-free medium. Renca EGFR and Renca-ErB2 cells (36) were kept in RPMI medium supplemented with 10% FCS, 0.25 mg/ml Zeocin, and 0.48 mg/ml G418.

Peptide Aptamer Screening—The aptamer screening was done in the yeast strain KF1 (MATα *trp1–901 leu2–3*, *112 his 3–200 gal4D gal80D LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ SPAL10-URA3*) (25), which contains three selectable marker genes under the transcriptional control of Gal4 binding sites. As bait, the first part of the kinase domain of the EGFR, containing amino acids 688–821, was fused to the Gal4-DNA binding domain into the vector pPC97 (pPC97 EGFR KDI). A randomized 20-mer peptide aptamer library was used as published in a previous study (25). Briefly, a randomized 60-mer oligonucleotide was inserted into the bacterial thioredoxin A open reading frame, resulting in an aptamer expression library of $\sim 2 \times 10^8$ different clones. KF1 transformants expressing the bait constructs and the library were selected for growth in the absence of adenine, uracil, and histidine. The interaction was verified by retransformation and mating.

GST Pull-down Assay—The peptide aptamer coding sequence was subcloned in a GST expression vector (pGEX 4T3, Amersham Biosciences, Freiburg, Germany). The peptide aptamer fusion proteins were expressed in *Escherichia coli* BL21 (Stratagene) lysed in a GST lysis buffer (50 mM Tris, pH 7.9, 250 mM NaCl, 1% Triton X-100, 1 mM DTT, protease inhibitors) and immobilized on glutathione-Sepharose beads (Amersham Biosciences). 500 μg of cell lysate of EGFR- or ErbB2-expressing cell lines and binding buffer (50 mM Tris, pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 0.1% Nonidet P-40, 0.3 mM DTT, protease inhibitors) was added. Complex formation was performed overnight at 4 °C. The beads were washed with binding buffer, and the bound complex was eluted by boiling in SDS loading buffer. Two-thirds of the eluate was loaded on a low percentage gel with subsequent Western blotting. As input control one-third of the eluate was loaded on a high percentage gel, which was stained with Coomassie Blue.

Bacterial Expression and Purification of PTD Peptide Aptamers—A PTD of nine arginine residues was fused to the C terminus of the peptide aptamers, which were inserted into the pET30$^+$ vector (Novagen). Expression of the aptamer was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 4.5 h at room temperature. Purification of the aptamer under denaturing conditions was done as published before (36). Briefly the bacteria were lysed in 8 M urea, purified via a Ni^{2+}-Sepharose column (Amersham Pharmacia), and eluted with 250 mM Imidazole in 4 M urea. Purified proteins were renatured by dialysis against PBS containing 400 mM L-arginine and subsequently against PBS.

Immunofluorescence Staining—Cells were grown on coverslips and fixed with 95% methanol, followed by permeabilization with 0.1% Tween 20 in PBS. Cells were washed, blocked (0.5% fish gelatin, 0.1% oval albumin in PBS) and incubated overnight at 4 °C with primary antibodies. After extensive washing, incubation with fluorescence-labeled secondary antibodies (Molecular Probes) was performed.

Immunoprecipitation and Western Blot Analysis—Cells were solubilized in Triton extraction buffer (50 mM Tris, pH 7.5, 5 mM EGTA, 150 mM NaCl, 1% Triton X-100, protease inhibitors) and incubated on ice for 10 min. The lysates were clarified by centrifugation at 16,000 $\times$ *g* for 10 min. For immunoprecipitation, equal amounts of protein were incubated with a specific antibody for 2 h on ice. Immune complexes were collected with protein A-Sepharose (Amersham Biosciences) and washed with extraction buffer and once with TNE-T buffer (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 5 mM EDTA, 1% Triton X-100). Bound proteins were eluted by boiling them in sample buffer. Total cell lysates or immunoprecipitates were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. After blocking in 5% milk in TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), membranes were probed with specific antibodies and proteins were visualized with peroxidase-coupled secondary antibodies using the ECL system (Amersham Biosciences). In some experiments the filters were stripped in SDS buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8) for 30 min at 60 °C, washed with TBS-T, blocked, and reprobed with specific antibodies.

Proliferation and Soft Agar Assay—Cell numbers were quantified using the XTT-based proliferation kit II (Roche Applied Science). For proliferation assays 5×10^3 cells were seeded in a 96-well plate, and cell numbers were quantified over a certain time period. For soft agar assays 2.5×10^3 Herc cells were seeded in 0.3% soft agar in a 96-well plate, over a bottom layer of 0.5% agar. The agar was covered with a medium layer containing three times the final EGF concentration.

Luciferase Reporter Assays—2×10^5 cells were plated into 12-well dishes. After 24 h, the cells were transfected with 1 μg of α-macroglobulin promoter reporter plasmid, 20 ng of β-galactosidase expression plasmid, and 0.5 or 1 μg of pRc/CMV VP22-Trx-based vectors encoding

for the peptide aptamer or the thioredoxin protein without a peptide insert. The transfection was done with LipofectAMINE (Invitrogen) according to the manufacturer's protocol. After 24 h the cells were starved in serum-free medium and induced for 15h with 50 ng/ml EGF. Cells were harvested by adding 100 μl of lysis buffer (1% Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT) for 10 min on ice. The lysate was clarified by centrifugation for 10 min at 16,000 $\times$ *g*. 20 μl of lysate was measured using a luminometer (Berthold) by injecting luciferin reagent (25 mM glycylglycine, pH 7.8, 5 mM ATP, pH 7.8, 330 mM beetle luciferin). The triplicate samples were normalized for the β-galactosidase activity, which was measured by incubating 3 μl of lysate with 33 μl of reaction buffer (100 mM N_2HPO_4, 1 mM $MgCl_2$, 1$\times$ Galacton (Tropix)) for 30 min on ice. The galactosidase activity was measured in a luminometer by injection of amplifier (10% Emerald (Tropix), 0.2 M NaOH).

Peptide Synthesis—KDI1 peptide was synthesized by Coring System Diagnostix, Gernsheim, Germany using standard Fmoc (*N*-(9-fluorenyl) methoxycarbonyl) solid phase chemistry.

RESULTS

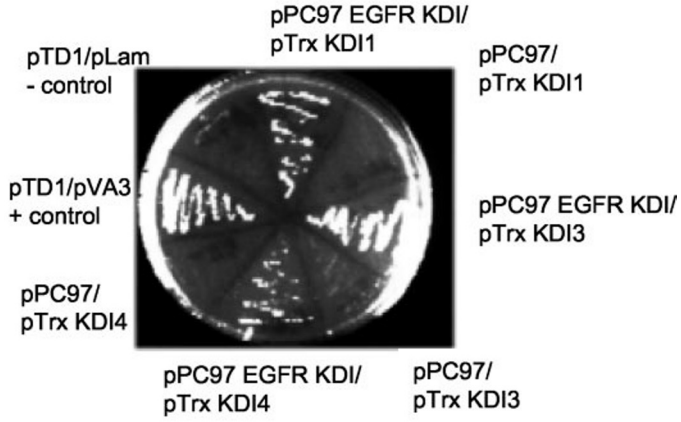
Isolation of Peptide Aptamers Specifically Interacting with the Kinase Domain of the EGFR—In a yeast two-hybrid assay, we isolated peptide aptamers that specifically interact with the intracellular kinase domain of the EGFR from a random peptide library of high complexity. The peptide aptamer interaction screen was performed with the yeast strain KF1, which contains three selectable growth markers: GAL2-Ade2, GAL1-HIS3, and SPO13-URA3 (25). The N-terminal part of the EGFR kinase domain (amino acid positions 688–821), comprising the lysine residue known to be responsible for binding of ATP, served as bait. As prey constructs we used a library in which 20-mer peptides of randomized sequence had been integrated into the bacterial thioredoxin (trxA) protein. This protein serves as a scaffold to present the peptide sequence in a constrained conformation. The screening of 10^7 transfected yeast clones led to the isolation of three different transformants exhibiting growth of the yeast cells in the absence of adenine. These clones also grew in the absence of histidine and uracil. The aptamer-encoding plasmids were cloned, and the sequences of the peptide inserts were determined (Table I). A sequence analysis did not reveal similarities with known proteins.

TABLE I
Peptide sequence of isolated peptide aptamers specifically interacting with the EGF receptor

KDI 1	–Trx-VFGVSWVVGFWCQMHRRLVC-Trx–
KDI 3	–Trx-TFSPVVLLYRLFLATVLIWA-Trx–
KDI 4	–Trx-LSFQLSPLSLVHRWGTTVDV-Trx–

To confirm the specificity of the interaction between peptide aptamer sequences and the EGF receptor tyrosine kinase domain, the rescued yeast expression plasmids were retransformed into bait-expressing yeast cells. The peptide aptamers were again able to support yeast cell growth in the selection medium, *i.e.* they interacted with the KDI bait construct (Fig. 1, *A* and *B*). No interaction was observed when the Gal4 DNA binding domain without EGFR sequences was used as a bait construct (Fig. 1*A*). In addition, mating of peptide aptamer (KDI1, -3, or -4)- or thioredoxin-expressing clones with yeast cells transformed with different unrelated bait constructs such as the kinase domains of the ErbB2 (ErbB2 KDI and II) or IGF1 receptor (IGFIR K_D) were performed (Fig. 1*B*). With the exception of aptamer KDI3, which showed binding to the homologue part of the kinase domain of the ErbB2 receptor (ErbB2 KDI), the aptamers did not show any interaction with

A



B

bait constructs	peptide aptamers Trx	KDI1	KDI3	KDI4
EGFR KDI	-	+++	+++	++
EGFR KDII	-	-	-	-
EGFR KDIII	-	++	++	+
ErbB2 KDI	-	-	+	-
Erb2 KDII	-	-	-	-
IGFR KD	-	-	-	-

FIG. 1. **Interaction of the isolated peptide aptamers with growth factor receptor tyrosine kinase domains in yeast cells.** *A*, KF1α strains expressing the individual aptamers (encoded by pTrx KDI1, pTrx KDI3, and pTrx KDI4) were mated with KF1a clones transformed with the bait construct pPC97 EGFR KDI (amino acid positions 688–821) or the empty plasmid alone (pPC97). The positive control, the large T-antigen (encoded by pTD) interacted with p53 (encoded by pVA3), whereas no background interaction was found with human lamin C (pLam). *B*, KF1α strains expressing the individual aptamers (KDI1, -3, and -4) or the thioredoxin control protein (*Trx*) were mated with KF1a clones transformed with different bait constructs, such as the N- or C-terminal part of the EGFR kinase domain (EGFR KDI and KDII), the whole kinase domain (EGFR KDIII), or the kinase domains of the ErbB2 receptor (ErbB2 KDI and KDII) or IGFI receptor (IGFR KD), respectively. With the exception of KDI3, no unspecific interaction with any of the unrelated bait proteins was found. All aptamers showed a specific interaction with the EGFR KDI bait protein.

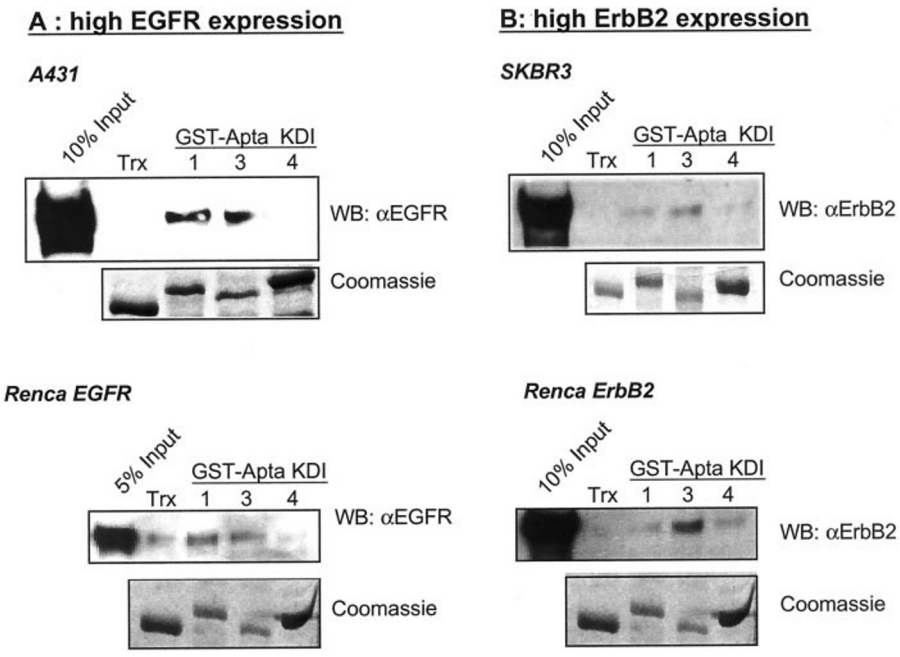


FIG. 2. **Selected peptide aptamers bind to the EGFR *in vitro*.** Thioredoxin and peptide aptamers were expressed as GST fusion proteins and immobilized on glutathione-Sepharose beads. After incubation with lysates from different EGFR (*A*) or ErB2 overexpressing cells (*B*), bound receptor was detected by SDS-PAGE and Western blotting. The thioredoxin (*Trx*) wild type protein without a peptide insert served as a negative control. Equal amounts of peptide aptamers were detected in a Coomassie Blue gel. Aptamers KDI1 and -3 interacted strongly with the EGFR. Aptamer KDI4 bound with a weaker affinity to the receptor, whereas aptamer KDI3 displayed binding to the close family member ErbB2.

the C-terminal 130 amino acids of the EGFR kinase domain KDII or with the kinase domain of the IGFR. The thioredoxin protein did not exert background binding to any of the tested bait proteins (Fig. 1*B*).

Furthermore, the binding specificity of the isolated peptide aptamers was verified in GST pull-down experiments (Fig. 2). GST-aptamer fusion proteins were expressed in bacteria and purified, and equal amounts of GST-aptamer proteins were immobilized on glutathione beads. Lysates from different cell lines that express the EGFR or the ErbB2 receptor were prepared and incubated with the GST-aptamers bound to the beads. After washing the beads, retained receptors were eluted and visualized by SDS-PAGE and Western blotting. The thioredoxin protein without peptide insert served as a negative control and did not interact with the EGFR or the ErbB2 receptor. Using cell lysates from two different EGFR-expressing cell lines in these pull-down assays revealed that aptamers KDI1 and KDI3 interacted strongly with the EGFR. Aptamer KDI4 bound with a lower affinity to the receptor (Fig. 2*A*). Aptamer KDI3 displayed some cross reactivity with the related ErbB2 receptor (Fig. 2*B*). This cross reactivity was not entirely unexpected, because the two receptors exhibit about 80% sequence homology within their kinase domains. Therefore, this clone was not used for subsequent experiments to assess specific inhibition on the EGF receptor. The highest specificity was observed for KDI1 and therefore was the best candidate for inhibitory interference with the EGF receptor. We assume that the three isolated peptide aptamers recognize different epitopes within the 130-amino acid sequence used as bait construct. Because the same results were obtained with different cell lines, it has to be assumed that these pull-down assays truly represent the binding affinity and specificity of the three aptamers.

The specificity of the interaction also could be observed in co-immunoprecipitation studies. Herc cells, NIH3T3 cells expressing the human EGFR, were transiently transfected with expression constructs of the peptide aptamers. Cell lysates were prepared and the EGFR was immunoprecipitated with a specific antibody. The presence of the aptamer in the immune complex was analyzed by SDS-PAGE and Western blotting (Fig. 3). Aptamer KDI1 and KDI4, transiently expressed in Herc cells (Fig. 3*B*) were found in the immunoprecipitates,

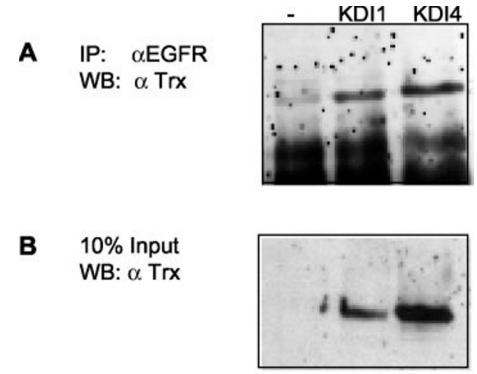


FIG. 3. **Selected aptamers are co-precipitated with the EGFR in transiently transfected Herc cells.** Peptide aptamers were transiently transfected into Herc cells (NIH3T3/EGFR). 48 h post-transfection lysates were prepared and the receptor was precipitated with an EGFR-specific antibody. The complexes were subjected to Western blotting, where the bound aptamers were detected with a thioredoxin-specific antibody. Untransfected cells (−) served as a negative control. Both expressed aptamers interacted with the EGFR, whereas aptamer KDI3 could not be sufficiently expressed transiently.

indicating interaction between the aptamer and the receptor (Fig. 3*A*). No expression of aptamer KDI3 was detected in transiently transfected Herc cells.

Intracellular Detection of Peptide Aptamers upon Expression of Transfected Gene Constructs and Delivery of Recombinant Proteins via Protein Transduction—To assess the biological activities of the EGFR binding peptide aptamers, we used two experimental strategies. First, we transfected peptide aptamer-encoding gene constructs into Herc cells and derived stably expressing lines. The second strategy is based on the expression of the peptide aptamer fusion proteins in bacteria, purification of the proteins, and introduction of the proteins into target cells via a protein transduction domain (PTD). For this purpose the peptide aptamers were fused to a PTD consisting of nine L-arginine residues. This sequence allows a fast and efficient uptake of linked proteins into mammalian cells (31). The recombinant proteins were expressed in *E. coli*, purified under denaturing conditions via their histidine tags, and refolded *in vitro*.

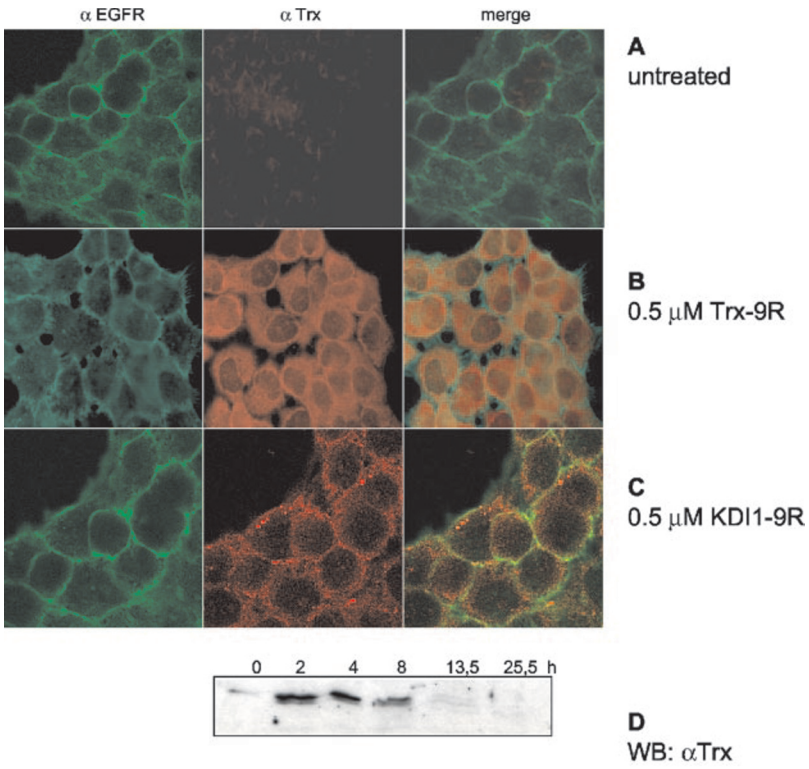


FIG. 4. **Bacterially expressed peptide aptamers transduce fast and efficiently into target cells.** A431 cells were left untreated (*A*), or were treated with 0.5 μM of the thioredoxin protein (*B*) or the aptamer (*C*). After 12 h the cells were subjected to immunofluorescence analysis using anti-EGFR and anti-thioredoxin antibodies, followed by anti-mouse-fluorescein isothiocyanate and anti-rabbit-rhodamine secondary antibodies. Representative pictures of the aptamer/EGFR interaction are shown. In contrast to the thioredoxin protein, which was localized in the cytoplasm (*B*), aptamer KDI1 was recruited preferentially to the membrane, where it interacted with the receptor (*C*). At different time points after transduction cell lysates were prepared and subjected to SDS-PAGE and Western blotting. The presence of the aptamer was detected with a thioredoxin antibody (*D*).

After addition of the recombinant aptamers to the growth medium, the peptide aptamers were taken up rapidly and efficiently into the cells (Fig. 4). The thioredoxin protein without an integrated aptamer sequence served as a control and was found to be located mainly in the cytoplasm (Fig. 4*B*); aptamer KDI1 was found preferentially associated with the plasma membrane, indicating interaction with the EGFR (Fig. 4*C*). Similar observations were made with transduced aptamers KDI3 and KDI4 (data not shown). Additionally, the uptake and the stability were detected by Western blotting. The intracellular presence of the aptamer was detectable after several minutes (data not shown) and lasted for at least 8 h as detected by Western blotting (Fig. 4*D*) and for up to 24 h in immunofluorescence experiments (data not shown).

As shown by us and others (*e.g.* Refs. 31 and 37) the transduction process is fast and efficient. The protein transduction domain does not seem to interfere with the interaction between the peptide aptamer and the receptor (Fig. 4*C*) and allows the introduction of the aptamer into the target cells in a transient and dose dependent fashion.

Aptamer KDI1 Reduces Proliferation and Soft Agar Colony Formation of Herc Cells—EGFR-transfected NIH3T3 cells (Herc cells) respond to EGF treatment with proliferation and anchorage-independent growth (38). We investigated if the intracellular binding of the peptide aptamers to the EGF receptor can cause interference with receptor functions and influence the phenotype of these cell lines. Monitoring the proliferation of Herc cells over a period of 72 h revealed that Herc cells stably expressing the peptide aptamer KDI1 proliferate significantly slower than cells expressing the thioredoxin protein without aptamer insert or cells expressing aptamers KDI3 or KDI4 (Fig. 5*A*). These results could be obtained with different stably expressing clones.

NIH3T3 cells overexpressing the EGFR can grow as colonies in soft agar and exhibit characteristics of transformation in the presence of EGF. We investigated if the expression of the aptamers could interfere with this phenotype. We compare the number of colonies formed by control cells and cells expressing the aptamer proteins in the presence and absence of EGF. We found that cells expressing KDI1 formed fewer colonies in the presence of EGF when compared with wild type Herc cells or cells expressing the thioredoxin framework protein only (Fig. 5*B*). This is consistent with the experiments described above measuring the kinetics of growth. The aptamers KDI3 and KDI4 did not influence growth or colony formation.

To correlate the growth inhibitory effects of KDI1 we introduced the aptamers into A431 cells by protein transduction. Uptake of KDI1–9R resulted in a dose-dependent growth inhibition. 2 μM KDI1–9R completely blocked cell division (Fig. 5*C*, *right panel*). Trx-9R served as a control and exhibited only weak effects on cell growth (Fig. 5*C*, *left panel*).

Aptamer KDI1 Interferes with EGFR Phosphorylation at Tyrosine Residues 845, 1068, and 1148—We investigated the mechanism by which aptamer KDI1 interferes with EGF-dependent proliferation. Ligand activation of the receptor involves dimerization, activation of the intrinsic tyrosine kinase activity, and phosphorylation of tyrosine residues Tyr-992, Tyr-1068, Tyr-1086, Tyr-1148, and Tyr-1173. They serve as binding sites for effector molecules, activating downstream signaling cascades. We followed the phosphorylation of these tyrosine residues upon EGF stimulation of cells with specific antibodies. Upon EGF stimulation the receptor becomes phosphorylated at all of these tyrosine residues (Fig. 6). In cells transduced with 1 μM aptamer KDI1 the phosphorylation of tyrosine 1068 and 1148 is reduced, compared with control cells (Fig. 6, *B* and *C*). Phosphorylation of tyrosines 992, 1086, and 1173 was also investigated and was not found to be influenced by the presence of the aptamer (data not shown).

The EGF receptor is not only phosphorylated by its intrinsic kinase activity but is also phosphorylated at tyrosine 845 and 1101 by the cytoplasmic kinase c-Src. This is associated with the modulation of receptor activity (13). Upon EGF stimulation

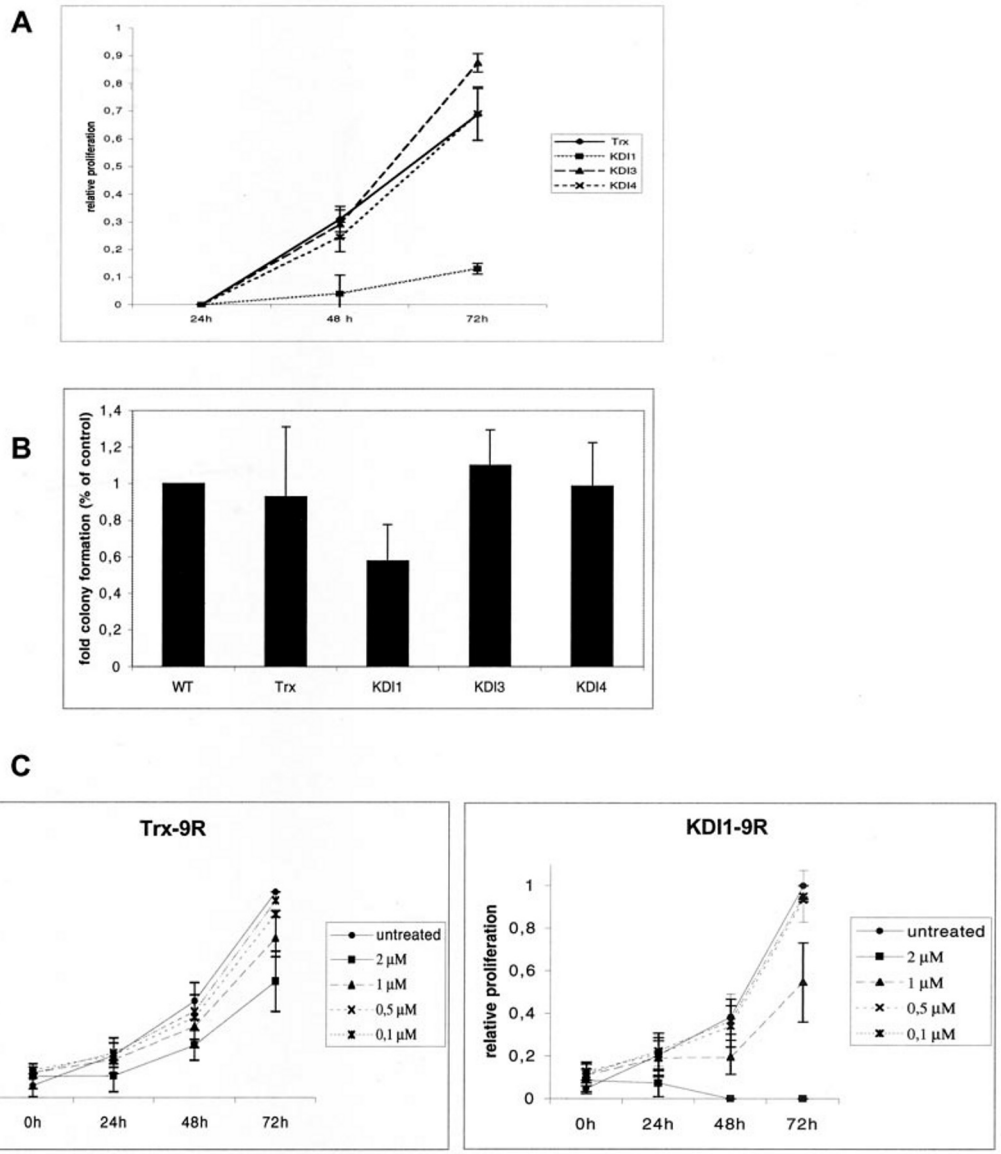


FIG. 5. **KDI1 reduces the EGF-dependent proliferation and transformation in fibroblasts stably expressing the aptamers.** *A*, to monitor the impact of the aptamers on cell proliferation, equal cell numbers of stable cell lines were seeded in a multititer plate. After the indicated time points cell numbers were quantified using XTT. The *error bars* represent the ± S.D. of three independent experiments each done in triplicates. KDI1-expressing cells proliferate significantly slower than control cells ($p < 0.05$). *B*, the transformation capacity of the cell lines was assayed by seeding the cells in 0.3% soft agar, over a bottom layer of 0.5% soft agar. The colony formation was induced with 30 ng/ml EGF. The cell number was quantified after 5 days. The -fold colony induction was calculated and normalized by the number of wild type cells. Values are expressed as the mean ± S.D. of triplicates from four independent experiments. Wild type cells and cells expressing thioredoxin or aptamer KDI3 and KDI4 respond to EGF treatment with increased proliferation and a strong colony formation in soft agar. In cell expressing KDI1 this response is significantly inhibited ($p < 0.05$). *C*, A431 cells were treated with the indicated concentration of Trx-9R or KDI1–9R. After the indicated time points cell numbers were quantified using XTT. The *error bars* represent the ± S.D. of triplicates of three independent experiments. Although the control protein (Trx-9R) only has a weak growth inhibitory effect, high concentrations of peptide aptamer KDI1 strongly reduce proliferation ($p < 0.01$).

c-Src is rapidly recruited to the receptor, where it phosphorylates the tyrosine residues 845 and 1101 and associated signaling proteins (15). In control cells tyrosine 845 is readily phosphorylated upon EGF addition, but after addition of 1 μM KDI1–9R to the medium and transduction of this protein into Renca-EGFR cells, the phosphorylation of tyrosine 845 was strongly suppressed (Fig. 6*A*). The result from three independent protein transduction experiments were scanned and quantified (Fig. 6*D*). The transduced peptide aptamer repressed the activation of these tyrosine residues to ~50%.

We monitored the receptor activation level in cells stably transfected with gene constructs encoding the aptamer KDI1 or the thioredoxin control protein (data not shown). The inhibitory effect on the tyrosine phosphorylation of the EGF receptor was much weaker. A strong inhibition of phosphorylation to at least 50% was only observed on tyrosine 845 (data not shown). These results suggest that aptamer KDI1 binds to a site within the kinase domain of the EGF receptor that does not entirely block its ability to phosphorylate tyrosine residues but interferes with a specific subset of tyrosines and the activation of tyrosine 845 by c-Src.

Binding of KDI1 to the EGFR Impairs the Activation of Shc and Stat3 but Not That of Erk1/2—Activation of the EGF receptor is accompanied by the initiation of several signaling cascades that involve protein kinases and phosphatases, which finally regulate the activity of distinct transcription factors and

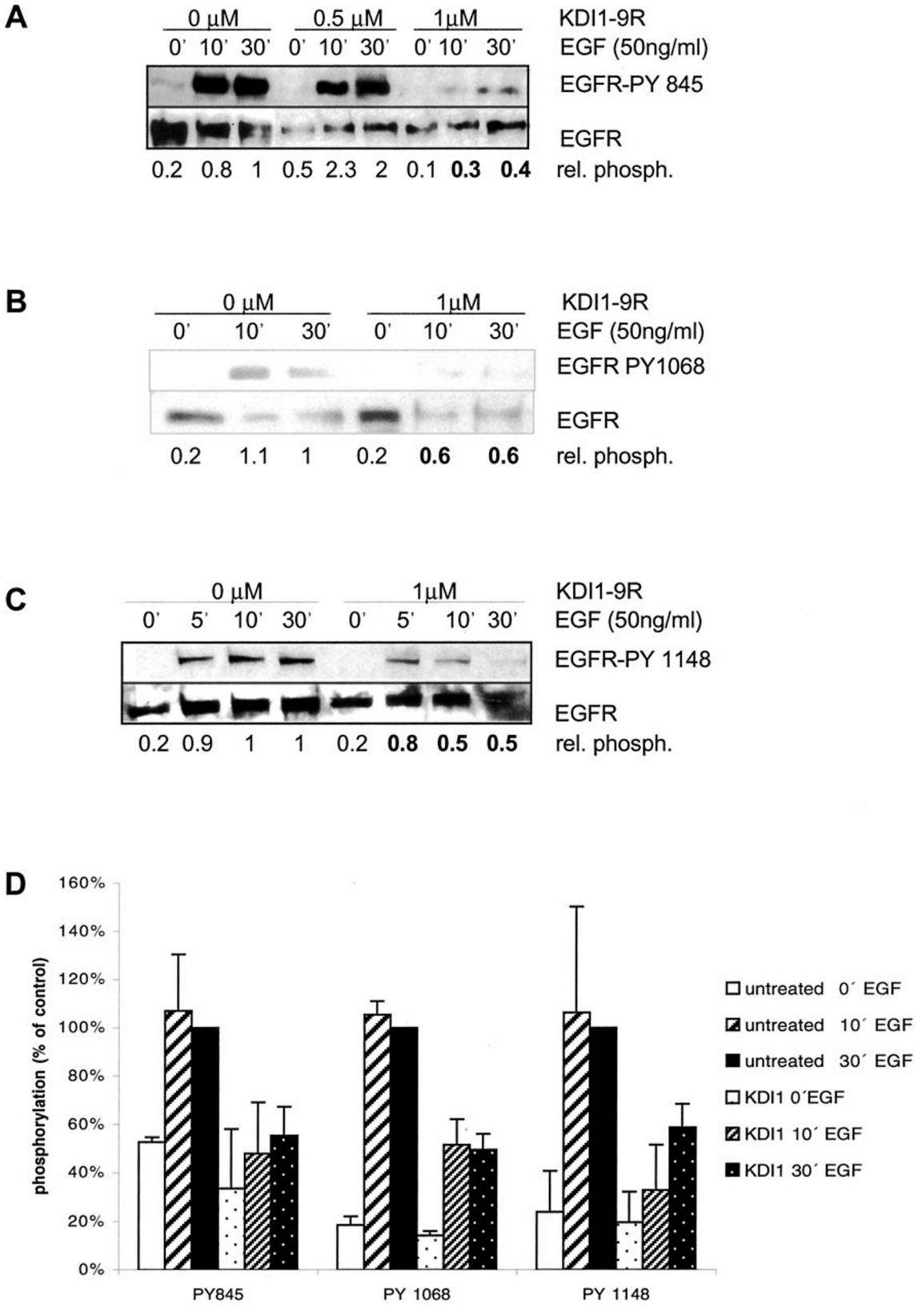


FIG. 6. **Aptamer KDI1 reduces the activation of the EGFR.** Renca-EGFR cells were starved overnight, and 2 h before induction (50 ng/ml EGF) they were transduced with 0.5 or 1 μM KDI1–9R. Cell lysates were prepared and subjected to Western blotting. The activation status of the receptor was monitored using antibodies specific for defined phosphorylated tyrosine residues. The blots were stripped and incubated with an EGFR-specific antibody. In cells exposed to aptamer KDI1 the phosphorylation was reduced at tyrosines 845 (*A*), 1068 (*B*), and 1148 (*C*). Reprobing the blots with an EGFR-specific antibody revealed that equal protein amounts were loaded in each lane. The relative phosphorylation, as indicated *under each panel*, was quantified using TINA software. The inhibitory effects of peptide aptamer KDI1 on each tyrosine residues is expressed as mean ± S.D. of three independent protein transduction experiments (*D*).

their target genes. We investigated the possibility that the peptide aptamer KDI1 interferes with individual EGFR-mediated signaling events and thus exerts growth inhibitory effects. The adaptor proteins Shc and Grb, *e.g.* are recruited to the phosphorylated EGF receptor, bind to Sos and thus induce Ras, which in turn activates the serine/threonine kinase Raf. The activation of Raf activates the MAPK pathway (39).

We followed the activation of Shc and Erk1/2 in Renca-EGFR cells transduced with high concentrations of the recombinant KDI1 protein (KDI1–9R). The adaptor protein Shc occurs in three isoforms (p66, p52, and p46), resulting from differential splicing. In Renca-EGFR cells only the smallest form was detectable (Fig. 7*A*). p46 shows a basal tyrosine phosphorylation at position 317 even in the absence of EGF. This is enhanced by EGF stimulation. The phosphorylation of p46 in Renca-EFGR cells can be strongly suppressed by exposure to 1 μM of KDI1–9R (Fig. 7*A*).

We also measured the activation of the MAP kinase p42/p44. The induction of phosphorylation (PT202/PY204) was very similar in control cells and cells transduced with the KDI1–9R protein (Fig. 7*B*). We also found that in Herc cells Akt activation is not affected by KDI1 expression when compared with Trx-expressing cells (data not shown). We conclude that, although Shc seems to be somewhat impaired in its activation by KDI1, activation of p42/p44 MAPK and Akt kinase are unaffected.

EGF binding to the receptor also results in the activation of members of the Stat family of transcription factors (15). The activation of Stat3 as a consequence of EGFR activation has been consistently observed in cells with a high proliferative index (10). Stat3 is activated through phosphorylation of tyrosine residue 705, which results in its dimerization and translocation into the nucleus and transcription of target genes. In Herc cells maintained in low concentrations of serum and in the absence of EGF, Stat3 is not phosphorylated on tyrosine 705. Upon addition of EGF the phosphorylation of Stat3 is strongly induced (Fig. 8). The activation of Stat3 was much weaker in cells expressing KDI1 (Fig. 8*A*) and completely abolished in cells transduced with 1 μM of the KDI1–9R recombinant protein (Fig. 8*B*). Phosphorylation of Stat3 at Tyr-705 promotes activation through homo-dimerization and the transcriptional induction of target genes. We measured the ability of EGF to induce Stat3-dependent transcription and the ability of the KDI1 peptide aptamer to interfere with this process. For this purpose a Stat3-dependent reporter gene construct and different amounts of aptamer or Trx expression plasmids were transiently transfected into Herc cells that were subsequently treated with EGF. The transcriptional induction of the luciferase construct in KDI1-transfected cells was reduced by 40–60%, in a dose-dependent manner, when compared with Herc cells or Herc Trx cells (Fig. 9). This is consistent with the reduced tyrosine phosphorylation of Stat3 in these cells upon EGF treatment. In addition, gel shift assays were performed with lysates from cell lines expressing peptide aptamer KDI1 or Trx. KDI1 caused a reduction of phosphorylation of Stat3 resulting in a reduced DNA binding ability (data not shown). These results argue that interference of the aptamer KDI1 with the receptor has a strong impact on the activation of the Stat3 pathway and might account for the growth inhibitory effect.

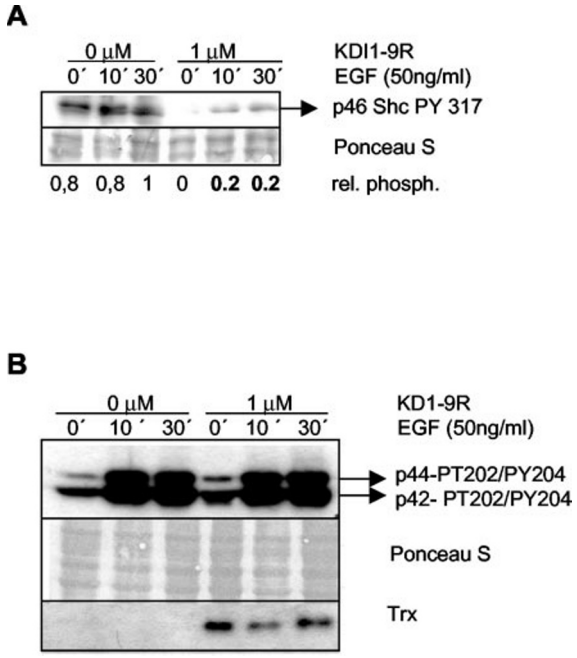


FIG. 7. **Interference of aptamer KDI1 influences the activation of Shc, but not of Erk1/2 or Akt.** Renca-EGFR cells were starved overnight, and 2 h before induction (50 ng/ml EGF) they were transduced with 1 μM KDI1–9R. Cell lysates were prepared and subjected to Western blotting. The activation of Shc (*A*) and the MAPK Erk1/2 (*B*) was detected with phosphorylation-specific antibodies. The membrane was stained with Ponceau S solution to monitor equal protein loading in each lane. *B*, the transduction efficiency was followed by staining the membrane with a thioredoxin-specific antibody. Quantification of the relative phosphorylation is indicated *under each blot*. *A*, p46 Shc showed a basal phosphorylation in the absence of EGF, which was enhanced by EGF stimulation. Renca cells transduced with 1 μM KDI1–9R do not show any basal activation of p46, which also cannot be induced by EGF, whereas in untransduced cells Shc is strongly phosphorylated (*A*). The exposure to aptamer KDI1 does not influence the activation of p42/p44 MAPK (*B*). The blots are representatives of two independent experiments.

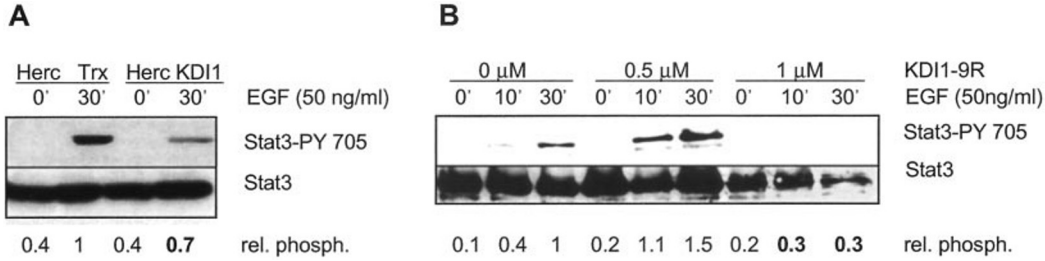


FIG. 8. **Interference of aptamer KDI1 influences the EGF-dependent activation of Stat3.** Stably transfected Herc Trx and Herc KDI1 (*A*) cell lines were starved overnight and induced with 50 ng/ml for the indicated time periods. Renca-EGFR cells (*B*) were starved overnight, and 2 h before induction (50 ng/ml EGF) they were transduced with 0.5 or 1 μM KDI1–9R. Cell lysates were prepared and subjected to Western blotting. The activation of Stat3 was detected with an antibody specific for the phosphorylated tyrosine residue 705. Membranes were stripped and incubated with a Stat3 antibody. Quantification of the relative phosphorylation is indicated *under each blot*. In serum-starved, unstimulated cells Stat3 was not phosphorylated. Upon addition of EGF to control cells the phosphorylation can be strongly induced. In Herc KDI1 cells the activation of Stat3 was strongly reduced (*A*) and could be completely blocked by transduction of 1 μM KDI1–9R into Renca-EGFR cells (*B*). Reprobing with a Stat3-specific antibody revealed that Stat3 levels were equal in each lane. The blots are representatives of three independent experiments.

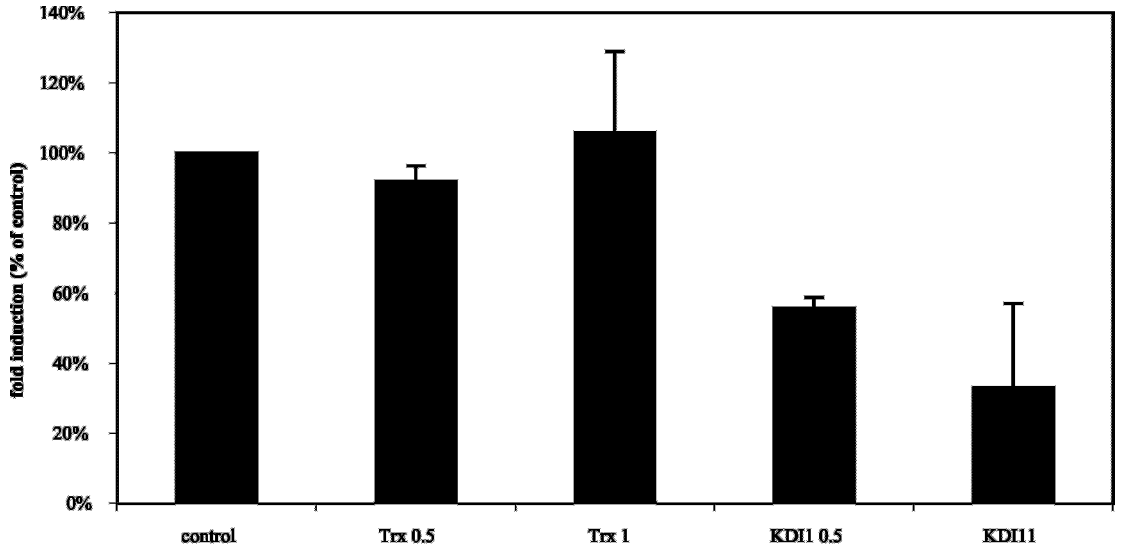


FIG. 9. **Expression of KDI1 reduces the transactivation capacity of STAT3.** Herc cells were transiently transfected with a Stat3 luciferase reporter construct, a β-galactosidase expression plasmid, and 0.5 or 1 μg of KDI1 or Trx expression plasmids. 24 h after transfection the cells were starved in serum-free medium and induced with EGF (50 ng/ml) for 15 h. The luciferase activity was measured and normalized with β-galactosidase activity. The *graph* represents four independent experiments each done in triplicate. In control cells the transcriptional activity was greatly enhanced by EGF stimulation. Although the thioredoxin control protein does not affect the transactivation by Stat3, peptide aptamer inhibited Stat3-dependent transactivation significantly ($p < 0.05$).

A Short Synthetic Peptide Comprising the Peptide Aptamer Sequence and a PTD Inhibits EGF-dependent Stat3 Activation—To verify the specific mechanism of aptamer action, a biotin-tagged peptide consisting of the 20-mer aptamer sequence, flanked by three amino acids of the thioredoxin scaffold protein and a PTD consisting of four arginine residues was synthesized (named pepKDI1–4R) (Fig. 10*A*). After incubation of cells with the biotin-tagged peptide, we found that the peptide translocates into target cells (Fig. 10*B*). We assayed the potential of the peptide pepKDI1–4R to interfere with EGF-induced Stat3 phosphorylation. In the presence of pepKDI1–4R the EGF-induced phosphorylation is greatly reduced, when compared with control cells (Fig. 10*C*). These data suggest that the presence of the thioredoxin scaffold protein is not necessarily required for the inhibitory function of the peptide aptamer.

DISCUSSION

The majority of intracellular signaling events are based on specific interactions of proteins. The targeted interference with such specific interactions could become very valuable for studying and understanding these events, but also for the design and the validation of drugs. Peptide libraries of large complexities can be produced by genetic means and provide the basis for the selection of specific binders with inhibitory properties to distinct domains of signaling components. We have employed a variation of the yeast-two hybrid selection system to isolate three peptide aptamers specifically interacting with the intracellular kinase domain of the EGF receptor and tested their ability to interfere with the growth promoting functions of the receptor.

The interaction between the peptide aptamers and the EGFR was verified by different methods such as re-transformation in yeast cells, GST pull-down experiments, co-immunoprecipitation and co-localization in immunofluorescence studies. In yeast mating experiments the aptamers showed specific interaction with the kinase domain of the EGFR but no interaction with the Gal4 DNA binding domain alone (Fig. 1*A*) or other unrelated bait constructs such as the kinase domains of ErbB2 or IGFI receptor (Fig. 1*B*). Only aptamer KDI3 has a low affinity for the ErbB2 receptor. These interactions were confirmed in GST pull-down assays using cell extracts from EGFR- or ErbB2-expressing cell lines (Fig. 2). These assays also allow an evaluation of aptamer binding specificity. Only one of the three aptamers showed a slight interaction with the ErbB2 receptor. This result was not unexpected as the two receptors exhibit about 80% homology in their kinase domains. Because we wanted to isolate an EGFR-specific inhibitor, we excluded this aptamer from further investigations. Co-immunoprecipitation studies confirmed the interactions of aptamer KDI1 and KDI4 with the EGFR upon intracellular expression. It was not possible to express high levels of KDI3 in cultured cells. This might be due to a double inhibitory effect of KDI3 on EGFR and ErbB2 signal transduction, because this aptamer showed binding to both receptors (see above). Aptamer KDI4 interacted only weakly with the receptor in GST pull-down studies, but strong interaction was found upon cellular expression and co-precipitation. This discrepancy might be due to differences in the experimental setup. In the GST pull-down the bead-bound aptamer needs high binding affinities to pull the receptor out of a whole cell lysate. However, in a co-immunoprecipitation from cells expressing the aptamer less stringent conditions allow the weak interaction between KDI4 and the receptor still to be detected by Western blotting. In summary, KDI4 showed the lowest binding affinity to the EGF receptor, whereas KDI1 represents the most promising of the isolated candidates, because it displays a high and specific binding affinity toward the EGF receptor. KDI3 also interacts with ErbB2, so that its inhibitory effect on EGFR/ErbB2 heterodimer signaling needs to be assessed in a different study.

We employed two approaches to assess the biological activities of the peptide aptamers, the derivation of cell lines stably expressing the peptide aptamers, and the introduction of bacterially produced proteins into cells via a fused protein transduction domain. In the latter approach, we observed a time- and dose-dependent uptake of the purified aptamers proteins, and a preferential localization with the membrane-bound receptor (Fig. 4). Our immunofluorescence experiments showed that the efficiency of protein transduction reaches 100%. Intracellular concentrations of the peptide aptamer protein in a

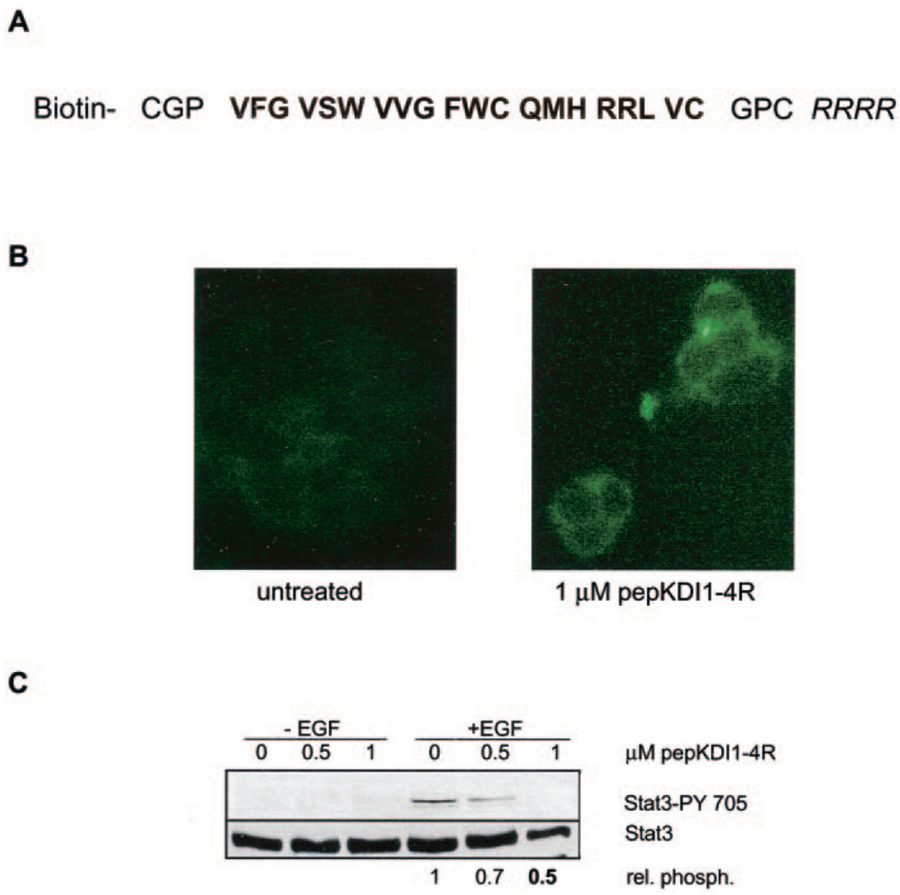


FIG. 10. **A linear peptide shows the same inhibitory capacity as the peptide aptamer from which its sequence is derived.** *A*, a linear peptide, named pepKDI1–4R, containing the 20-mer aptamer sequence of KDI1, flanked by a 3-amino acid motif of the thioredoxin protein (*CGP*) and 4-arginine PTD was synthesized. A Biotin tag for detection was included. The cysteine residues flanking the aptamer sequence might build a disulfide bond, leading to a constrained peptide as displayed by the thioredoxin scaffold protein. *B*, Herc cells were transduced with 1 μM of the peptide. After 3 h the cells were subjected to immunofluorescence using fluorescein isothiocyanate-coupled streptavidin. *C*, Herc cells were starved overnight and treated with the indicated concentrations of the pepKDI1–4R peptide for 1.5 h. After induction with 50 ng/ml EGF for 30 min, the cells were harvested, and cell lysates were prepared. 40 μg of total protein was separated by SDS-PAGE and subjected to Western blotting. Activation of Stat3 was followed using a phospho-specific Stat3 antibody. After stripping, the membrane was incubated with a Stat3 antibody. Quantification of the relative phosphorylation is indicated. The blot is representative of three independent experiments. Transduction of the pepKDI1–4R peptide inhibits EGF-induced Stat3 activation in a similar manner as the aptamer displayed by the scaffold protein.

proper conformation can be achieved to mediate biological responses (40). Protein transduction is therefore a suitable method to introduce inhibitory molecules into target cells. Recent studies showed that a PTD consisting of nine D-arginine residues shows even better transduction efficiencies than the L-amino acids and that the degradation of the recombinant protein is decreased (41). Although such a PTD could be linked chemically to the peptide aptamer protein, the genetic fusion and the ease of recombinant protein production are practical advantages.

All isolated peptide aptamers interacted with the EGFR, but only one aptamer, KDI1, also interfered with its function. Aptamer KDI1 reduced proliferation (Fig. 5, *A* and *C*) and focus formation in soft agar (Fig. 5*B*); in contrast, the other aptamers or the thioredoxin protein were not able to do so. Aptamer KDI1 interferes with the proliferative function of the receptor in a similar way as the small molecular weight EGFR inhibitor PKI-166 (42). However, KDI1 does not totally block EGF-induced proliferation as PKI-166 does. This might be due to the fact that the stably transfected clone was selected for growth in the presence of the aptamer. For this reason a total inhibition of EGF-induced proliferation might not yield single clones expressing the aptamer protein. However, the growth inhibitory effect of aptamer KDI1 is not due to unspecific clonal effects within the stable expressing cell lines, because protein transduction of the aptamer into EGFR-expressing cells inhibited cell proliferation in a dose-dependent manner (Fig. 5*C*). These results clearly exclude that the inhibitory effect is due to unspecific effects of the peptide aptamer. Aptamers KDI3 and KDI4 did not interfere with the described phenotypes. This is most likely due to the site of EGFR interaction or additionally in the case of KDI4 due to weaker binding affinities as seen in the GST pull-down studies. We assume that the individual binding sites of the three aptamers differ within the 130-amino acid region employed in their selection in the yeast two-hybrid system. These two aptamers probably interact with sites that are not important for the recruitment or phosphorylation of signaling components. This assumption is consistent with a study, where it was shown that aptamers selected for interaction with cdk2 interact with different distinct domains of the target protein (22).

We investigated the phosphorylation pattern of the receptor to obtain information about the molecular mechanisms by which aptamer KDI1 inhibits cell proliferation. In the presence of aptamer KDI1, the EGF-induced phosphorylation of tyrosine residues 845, 1068, and 1148 of the receptor (Fig. 6) and that of Shc was reduced (Fig. 7), and the activation of Stat3 was inhibited (Figs. 8 and 9). No adverse effects on the activation of the p42/p44 MAPKs were found (Fig. 9). Comparable results were obtained with cell lines stably expressing the aptamer (data not shown) and with cells transduced with the recombinant KDI1 protein. However, the biochemical effects were much stronger if the aptamer was applied via a PTD. This might be due to higher intracellular concentrations of the peptide aptamer at the time point of the experiment, which cannot be achieved by stable transfection of the construct.

A strong inhibition was found for the phosphorylation of tyrosine residue 845 (Fig. 6). This residue is located in the kinase domain of the receptor, but is not part of the sequence that was used for the selection of the aptamer. Tyrosine 845 is not phosphorylated via the autocatalytic activity of the receptor but is phosphorylated by the action of c-Src (13).

The aptamer also had a negative effect on Shc, especially on the p46 isoform (Fig. 7). Shc preferentially interacts with the tyrosine residues 1148 and 1173 of the receptor and becomes phosphorylated (43). The binding of the aptamer impeded phos-

phorylation of the tyrosine residue 1148, and we suppose that this is the reason why Shc could not be recruited to the receptor to become phosphorylated.

The aptamer did not affect activation of the MAPKs p42/44, which become activated after recruitment of Grb·SOS complexes to the phosphorylated residues 1068, 1086, and 1173 of the EGFR. Although tyrosine phosphorylation at 1068 was reduced, the two other sites are probably sufficient for the activation of the MAPK pathway. Redundancy might also explain the lack of an effect on MAPK activation exerted by the inhibition of Shc activation. The MAPK pathway can be activated through binding of Grb·SOS to tyrosine 317 of the Shc protein. Although the tyrosine phosphorylation on residue 317 of p46 Shc was reduced, this did not interfere with the activation of p42/44. This might be due to the function of the two other Shc isoforms, which are still fully activated. The binding of the aptamer to the receptor also had a strong effect on the activation of Stat3 (Fig. 8). Phosphorylation at tyrosine 705 and transactivation of a Stat3-dependent reporter gene were inhibited.

Because interference of the aptamer KDI1 with the receptor had an inhibitory effect on the activation of signaling molecules such as the EGFR, Shc, and Stat3 that are also regulated by the cytoplasmic kinase c-Src, we raise the question whether this effect is indirect and involves the function of c-Src. The cytoplasmic tyrosine kinase c-Src is the cellular homologue of the viral oncoprotein v-Src encoded by the chicken Rous sarcoma virus. Src kinases play important roles transmitting mitogenic signals from receptor tyrosine kinases (44), and a high coincidence of overexpression of the EGFR and c-Src was found in several tumors. c-Src phosphorylates tyrosine 845 of the EGFR and stabilizes the active conformation of the kinase domain (11, 12). This causes the enhancement of receptor activity (13). Stat3 is crucially regulated by c-Src. Fibroblasts, stably transfected with c-Src show constitutive Stat3 activity (45). The current model suggests that Stat3·Jak2 complexes are pre-associated with the EGFR in the absence of ligand. Upon receptor activation, Src is recruited to the receptor and phosphorylates Stat and Jak proteins (15). Tyrosine residues 1068 und 1086 were identified as Stat3 binding sites to the receptor (46). This suggests that the reduced activation of Stat3 in the presence of aptamer might possibly be due to two reasons, first the reduced receptor recruitment and activation of c-Src and second the reduced recruitment of Stat3 to the receptor.

The Shc adaptor molecule is also a substrate for c-Src kinase. Tyrosine residues 239 and 240 of Shc can be phosphorylated by Src (43), and Src only interacts with the two larger isoforms of Shc, but not with p46 (43, 47). We only observed an effect on the small isoform. We are currently investigating whether the binding of the aptamer interferes with the activity of c-Src.

We also investigated the properties of a short synthetic peptide lacking the scaffold protein. Such a molecule might offer advantages for the *in vivo* application of this class of signaling inhibitors and for the structural determination of its binding ability. Although it was reported that PTDs consisting of longer arginine stretches confer better transduction efficiencies (31), in this context a protein transduction sequence of four arginine residues was sufficient to promote transduction of the peptide into target cells (Fig. 10*B*). Recent publications argue that the scaffold protein displaying the peptide aptamer sequence can be a major determinant for binding of peptide aptamers to their target structures. Transfer of peptide moieties from a Trx-based scaffold to alternative scaffold proteins, such as green fluorescent protein or staphylococcal nuclease, resulted often in a loss of their function (48). In our synthetic peptide, pepKDI1–4R, the aptamer sequence is flanked by three residues of the thioredoxin protein sequence. These sequences comprise a cysteine residue on either side. This might allow the formation of a di-sulfide bond and results in a constrained protein confirmation comparable to the one formed in the intact thioredoxin formation.

In summary, we isolated a peptide aptamer, which acts as a specific inhibitor of EGFR signal transduction. This molecule might have interesting applications in basic research and cancer therapy. Although it is generally assumed that proteins are difficult to utilize as therapeutics, the present study shows that aspects not accessible to inhibition of enzymatic functions can be targeted by peptide aptamers. In addition, the structure of the interaction domain might provide leads for the design of synthetic compounds with optimal pharmaceutical properties.

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