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Intracellular Expression of Single Chain Antibodies Reverts ErbB-2 Transformation*

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We report a novel approach for specific *in vivo* inactivation of the ErbB-2 receptor tyrosine kinase and suppression of ErbB-2-induced transformation. Genes encoding single chain antibodies that specifically bind to the extracellular domain of human ErbB-2 were constructed and expressed intracellularly in NIH/3T3 fibroblasts transformed by activated ErbB-2. The single chain antibodies are derived from monoclonal antibodies FRP5 and FWP51 (Harwerth, I. M., Wels, W., Marte, B. M., and Hynes, N. E. (1992) *J. Biol. Chem.* 267, 15160–15167) and are composed of heavy and light chain variable domains connected by a flexible peptide linker. The antibodies were provided with: 1) an N-terminal hydrophobic leader sequence to target their synthesis to the lumen of the endoplasmic reticulum, and 2) a C-terminal retention signal to prevent secretion. When expressed in ErbB-2-transformed cells, the single chain antibodies bound to the receptor and prevented its transit through the endoplasmic reticulum. This resulted in the functional inactivation of the receptor and reversion of the transformed phenotype. This is the first demonstration of a targeted and stable inactivation of a cellular oncoprotein via intracellular antibody expression. The use of such a strategy represents a simple and powerful approach to study the *in vivo* function of receptors and other cellular proteins.

The *erb*B-2 gene encodes a 185-kDa transmembrane glycoprotein that is a member of the subclass I, epidermal growth factor receptor-related tyrosine kinases (1–4). Amplification and/or overexpression of *erb*B-2 is observed in tumors arising at many sites including breast and ovary where it correlates with an unfavorable patient prognosis (5–10). The oncogenic potential of the ErbB-2 protein can be activated by different mechanisms including point mutation (11–14) and overexpression (15, 16). ErbB-2 as a target for cancer therapy is an area of intense research. ErbB-2-specific monoclonal antibodies (17–21) and recombinant immunotoxins (22) both of which inhibit *in vitro* or *in vivo* growth of ErbB-2 overexpressing tumor cells have been described. Here we present a novel approach using intracellular expression of single chain antibodies (scFv) to suppress ErbB-2 transformation.

The concept of using antibodies to alter cellular physiology was initially tested in yeast where it was shown that expression and assembly in the cytoplasm of functional intact antibodies (23) and Fab fragments (24) was possible. Recent technological advances have made possible the cloning and expression of Fv molecules, the smallest high affinity binding domain of an antibody (25–27). cDNAs specific for the variable domains of Immunoglobulin heavy and light chains are cloned by polymerase chain reaction and joined via an oligonucleotide linker to yield a gene encoding a single chain antibody. Stable alteration of a cellular phenotype by intracellular scFv expression in a mammalian system has been described recently. A single chain antibody directed against the human immunodeficiency virus-1 envelope protein was expressed in the endoplasmic reticulum and shown to inhibit the processing of the envelope protein precursor (28).

We report here on the expression in mammalian cells of scFvs directed against human ErbB-2. ScFvs derived from mAbs FRP5 and FWP51, which bind to the extracellular domain of the receptor (20, 21) were used for this purpose. The scFvs were targeted to the lumen of the ER[1] of NIH/3T3 fibroblasts transformed by oncogenically activated ErbB-2. We show that both scFvs were stably expressed at high levels and functional in binding and inhibiting the ER transit of ErbB-2. Retention of the receptor in the ER caused its functional inactivation and reversion of the transformed phenotype.

EXPERIMENTAL PROCEDURES

Cloning and Construction of the Single Chain Antibodies—RNA from hybridoma cells producing mAbs FRP5 and FWP51 (20, 21) was reverse transcribed and the heavy chain (VH) and the light chain (VL) variable domain cDNAs were isolated by polymerase chain reaction, sequenced, and used to construct genes encoding the single chain antibodies scFv FRP5 and scFv FWP51, as described (22, 29, 30). In order to obtain genes for intracellular expression in mammalian cells, cDNAs encoding scFvs-5S, 51S, 5R, and 51R were constructed. Two pairs of complementary oligonucleotides encoding sequences specific for a human Immunoglobulin heavy chain signal peptide (VH 71–5′CL) (31) were designed. The oligonucleotides were assembled, ligated, and cloned as a *Hin*dIII/*Pst*I fragment into pWW15 (29), upstream of the scFv FRP5 (22, 29, 30) and scFv FWP51 (30) cDNAs. To produce scFv-5S and scFv-51S, the proteins were tagged with a C-terminal FLAG epitope (DYKD). To produce scFv-5R and scFv-51R an additional EL was added to the C terminus. The DYKD and DYKDEL peptides were encoded by synthetic oligonucleotides that were assembled into the cDNAs as *Bgl*II/*Xba*I fragments. The resulting open reading frames were flanked by upstream *Hin*dIII and *Eco*RI sites and downstream *Sal*I and *Xba*I sites. The cDNAs were cloned into the retroviral vector pBabePuro (32) as *Eco*RI/*Sal*I fragments.

Cell Culture—NIH/ErbB-2 cells are a clone of NIH/3T3 cells (clone no. 3.7) expressing an oncogenically activated human *erb*B-2 under the control of the SV40 early promoter (21). NIH/3T3 and NIH/ErbB-2* cells were maintained in Dulbecco's modified Eagle's medium containing 8% fetal calf serum. For the latter, the medium was supplemented with 0.5 mg/ml G-418.

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[1] The abbreviations used are: ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorting; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet-derived growth factor; scFv, single chain antibody; VH, heavy chain variable domain; VL, light chain variable domain.

Retroviral Gene Transfer—Ecotropic virus was prepared basically as described (33). The amphotropic packaging cell line PA317 (34) was transfected with 10 μg each of pBabePuro/scFv-5S, 51S, 5R, 51R, and empty vector using the calcium phosphate precipitation method. After 24 h, conditioned medium containing transiently produced virus was harvested and used for infection of the ecotropic packaging cell line ΩE (32) in the presence of 8 μg/ml polybrene. Two days after infection, the cells were placed in 2 μg/ml puromycin (Fluka). Virus-containing medium collected from pools of puromycin-resistant ΩE was used to infect NIH/3T3 and NIH/ErbB-2* cells, and after 3 days the cells were placed in 2 μg/ml puromycin. Pools of puromycin-resistant cells were analyzed in all the experiments.

Western Blotting—Cell lysates were prepared by adding 500 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM EGTA) supplemented with protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (2 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM sodium molybdate, 20 μM phenyl arsine oxide) to cells in a 10-cm dish and incubating on ice for 10 min. The lysates were clarified by centrifugation at 10,000 × *g* for 10 min. 50 μg of protein were separated by SDS-PAGE, transferred to polyvinylidenedifluoride membranes, and assayed by Western blot analysis with either an anti-scFv antiserum (29), 21N antiserum against ErbB-2 (35), or an anti-phosphotyrosine monoclonal antibody (36). Bound antibody was detected with horseradish peroxidase-coupled anti-rabbit or anti-mouse polyclonal antibodies using enhanced chemiluminescence (Amersham).

Immunoprecipitations—For the detection of scFv proteins in culture supernatants, conditioned medium was harvested, a polyclonal rabbit anti-mouse IgG (ICN Immunobiologicals) was added and allowed to bind for 1 h on ice after which the immune complexes were collected by the addition of protein A-Sepharose. Bound proteins were released by boiling in sample buffer and analyzed by Western blotting with an anti-scFv antiserum (29). For coimmunoprecipitation of scFvs with ErbB-2*, the receptor from 250 μg of protein was immunoprecipitated with the 21N antiserum (35) and analyzed by Western blotting with an anti-scFv antiserum (29).

Cell Growth Assays—To monitor growth, 3×10^3 cells in culture medium were plated/well of a 96-well dish and growth was monitored after 24, 48, and 72 h. To examine growth induction by PDGF and basic fibroblast growth factor, the cells were placed 24 h in serum-free medium. Then, they were either induced with 20 ng/ml PDGF or 50 ng/ml basic fibroblast growth factor, or grown in the absence of factors. Growth induction as compared with the untreated cells was measured after 2 days. The experiments were performed using the Cell Titer 96™ kit (Promega), and all points were prepared in triplicate.

Immunocytochemistry—For indirect immunofluorescence, cells were grown 2 days on chamber slides (Nunc), fixed 30 min with 3.7% formaldehyde, and permeabilized with 0.5% Triton X-100 for 3 min. Staining for scFvs was done using a scFv-specific antiserum (29) in combination with a rhodamine-linked anti-rabbit polyclonal antibody (Sigma). Staining for ErbB-2 was done using mAb FSP77 (20) in combination with a fluorescein-linked anti-mouse polyclonal antibody (Amersham). The cells were mounted and observed by fluorescence microscopy.

Flow Cytometric Analysis—Cells were trypsinized and counted prior to staining. 5×10^5 cells were washed with 2 ml of FACS buffer (phosphate-buffered saline containing 0.1% sodium azide and 1% bovine serum albumin) and resuspended in 50 μl of FACS buffer containing 20 μg/ml fluorescein isothiocyanate-coupled mAb FSP77 (20). Following incubation on ice for 1 h, the cells were washed twice with 2 ml of FACS buffer, resuspended in 300 μl of FACS buffer, and analyzed for their fluorescence in a Becton-Dickinson FACScan.

Soft Agar Growth—To examine anchorage-independent growth, 5×10^4 cells were plated in duplicate in 6-cm dishes in 6 ml of culture medium supplemented with 0.35% noble agar overlying a 0.7% agar layer. The plates were incubated 14 days at 37 °C after which colonies were stained by adding 2 ml of phosphate-buffered saline containing 0.5 mg/ml nitro blue tetrazolium for 24 h. Colonies were counted using an Artek 880 colony counter (Dynatech Laboratories, Inc.).

RESULTS

Construction and Expression of scFvs FRP5 and FWP51—Hybridoma cells producing mAbs FRP5 and FWP51 (20, 21) which bind to the extracellular domain of human ErbB-2 were used to construct genes encoding scFvs, as described previously (22, 29, 30). For expression in eukaryotic cells, two versions of

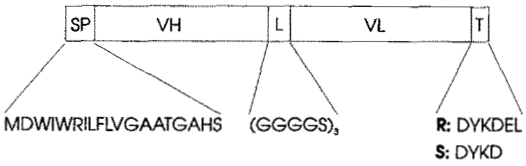

FIG. 1. **Diagram of the secreted (*S*) and ER-retained (*R*) forms of scFvs FRP5 and FWP51.** The N-terminal signal peptide (*SP*), the heavy chain variable domain (*VH*), the linker peptide (*L*), the light chain variable domain (*VL*), and the C-terminal tags (*T*) are indicated. *DYKD*: FLAG epitope; *DYKDEL*: FLAG epitope/ER retention signal.

predicted to encode secreted proteins, whereas the scFv-5R and scFv-51R cDNAs encode variants which are expected to be localized to the lumen of the ER (Fig. 1). The scFv proteins have an N-terminal Ig heavy chain-derived signal peptide which directs them to the secretory compartment of the cell, the same compartment through which ErbB-2 passes on its way to the plasma membrane. The FLAG epitope tag DYKD is present at the C terminus of all scFv proteins. The scFv-R proteins have an additional EL at their C terminus, thereby providing these versions with the ER retention signal, KDEL. This sequence is predicted to cause the retention of soluble proteins in the lumen of the ER (37).

We investigated effects of intracellular scFv expression in ErbB-2-transformed fibroblasts. NIH/3T3 clone 3.7 expresses an oncogenically activated form of the receptor, designated ErbB-2*, that carries a single amino acid substitution (valine to glutamic acid) in the transmembrane domain (21). The kinase activity of ErbB-2* is constitutive and ligand-independent (14). This cell line, referred to as NIH/ErbB-2*, shows the typical phenotype of transformed fibroblasts, as judged by morphology, focus formation, ability to undergo anchorage-independent growth, and tumor formation in nude mice (21).

We have used retroviral gene transfer to express the scFvs FRP5 and FWP51 in control NIH/3T3 and in NIH/ErbB-2* cells. First, we examined expression and localization of the scFvs. In both cell lines the proteins were produced at high levels as shown by Western blotting with a scFv-specific antiserum. The scFvs targeted for secretion were found in the conditioned medium of the cells (Fig. 2*A*), whereas the scFvs targeted to the lumen of the ER were found intracellularly (Fig. 2*B*). Both in control NIH/3T3 and in NIH/ErbB-2*, the scFv-5R was detected as a double band (Fig. 2*B*, *lanes 3* and *8*). The upper band corresponds to a *N*-glycosylated form and was not detected after treatment of extracts with peptidyl-*N*-glycosidase F^2. In extracts of NIH/ErbB-2* cells, more of the secreted scFvs was detected than in extracts of control NIH/3T3. This difference is probably due to scFvs bound to the ErbB-2* on the cell surface.

Intracellular expression or secretion of scFv proteins did not affect the general metabolism of the cells. Morphology and growth rate of scFv expressing and vector control NIH/3T3 cells was indistinguishable, indicating that the antibodies were nontoxic to the cells (Fig. 3).

*Subcellular Localization and Association of scFvs and ErbB-2**—We next examined the subcellular distribution of the scFv-R proteins and ErbB-2* in NIH/ErbB-2* cells expressing scFv-5R and scFv-51R. In both cell lines, immunofluorescence using a scFv-specific antiserum revealed the staining of a tubular network throughout the cytoplasm, typical of ER resident proteins. Staining of control NIH/ErbB-2* cells with a mAb specific for human ErbB-2 revealed that the major part of the receptor was located intracellularly (Fig. 4*A*). Since it has been shown that most of the constitutively activated ErbB-2 is degraded before reaching the plasma membrane (38), we assume that this is the reason for the weak plasma membrane staining.

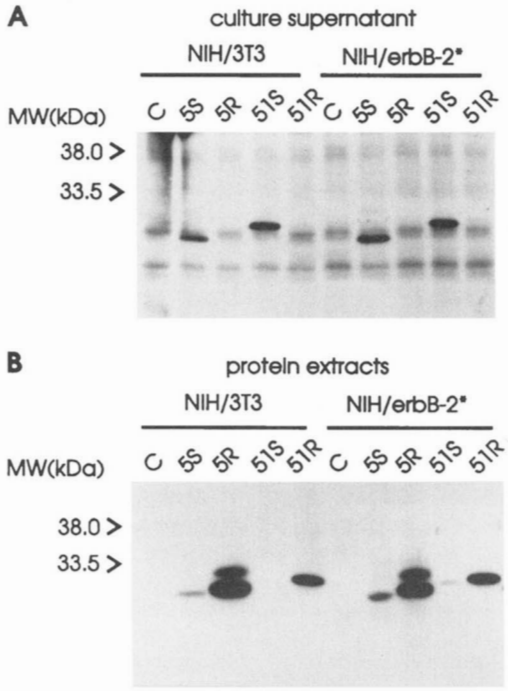

FIG. 2. **Expression of scFvs FRP5 and FWP51 in control NIH/3T3 and in NIH/ErbB-2* cells.** *A*, secretion of the scFvs. Conditioned medium of cells infected with vector control, scFv-5S, scFv-5R, scFv-51S, and scFv-51R virus was collected, immunoprecipitated with rabbit anti-mouse IgG (ICN Immunobiologicals), subjected to 15% SDS-PAGE, and analyzed by Western blotting with a scFv-specific antiserum (29). *B*, intracellular expression of scFvs. Virus-infected cells were lysed in Triton X-100 lysis buffer, and 50 μg of protein were subjected to 15% SDS-PAGE and analyzed by Western blotting as above.

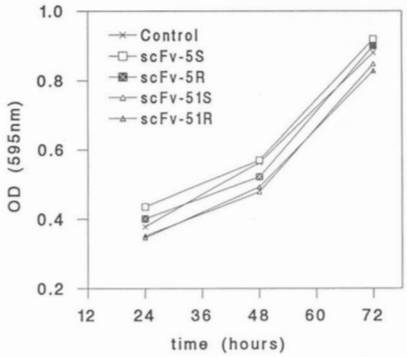

FIG. 3. **Growth of NIH/3T3 cells infected with vector control, scFv-5S, scFv-5R, scFv-51S, and scFv-51R virus.** Cell growth was monitored at the indicated times using the Cell Titer 96™ kit.

was an increased ER staining of the NIH/ErbB-2* cells expressing scFv-5R and scFv-51R with the ErbB-2 specific mAb (Fig. 4*A*). This suggests that there was colocalization of ErbB-2* and antibodies in the lumen of the ER. As an additional indication for a physical interaction *in vivo*, the scFvs could be coimmunoprecipitated with the receptor from total extracts of NIH/ErbB-2* cells using an ErbB-2 specific antiserum (Fig. 4*B*). However, it is noteworthy that in the case of the scFv-5R apparently only the unglycosylated protein bound to the receptor. A flow cytometric analysis revealed that the amount of ErbB-2* on the cell surface was dramatically decreased in the cells expressing scFv-5R and scFv-51R (Fig. 4*C*). This demonstrates that there was a stable association of the scFvs with ErbB-2* within the lumen of the ER which inhibited transit of the receptor to the cell surface.

Antibody expression did not affect the ER transit of other

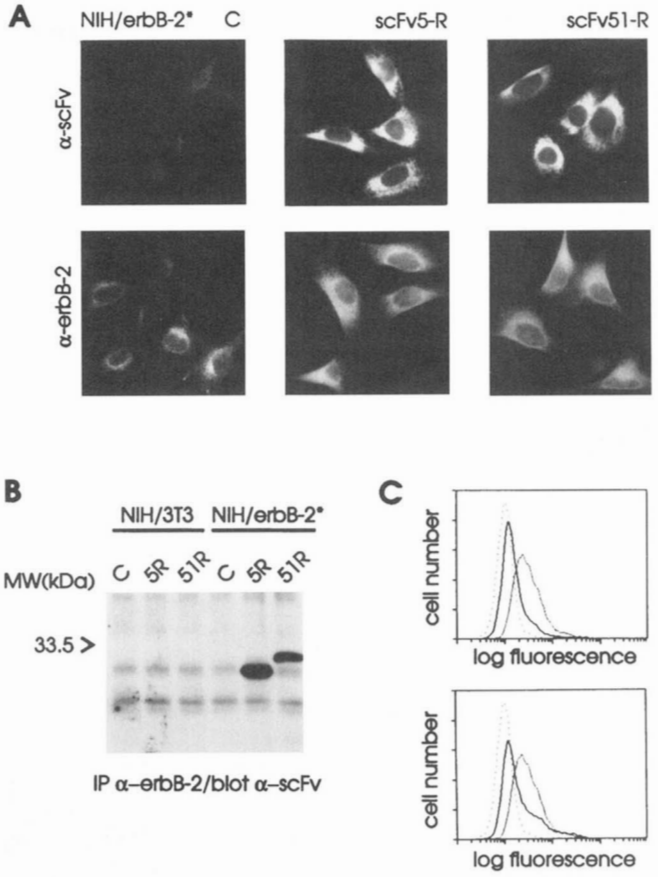

FIG. 4. **Subcellular localization and association of scFvs and ErbB-2* in NIH/ErbB-2* cells.** *A*, immunofluorescence of scFv expressing NIH/ErbB-2* cells. Cells infected with scFv-5R, scFv-51R, and vector control virus were stained for expression of scFvs using a scFv-specific antiserum (29) and for human ErbB-2* using mAb FSP77 (20). *B*, coimmunoprecipitation of scFvs and ErbB-2*. Control NIH/3T3 and NIH/ErbB-2* cells infected with scFv-5R, scFv-51R, and vector control virus were lysed in Triton X-100 lysis buffer. ErbB-2* was immunoprecipitated from 250 μg of lysate the with 21N antiserum (35), subjected to 15% SDS-PAGE, and analyzed by Western blotting with a scFv-specific antiserum (29). *C*, cell surface staining for ErbB-2*. NIH/ErbB-2* cells infected with scFv-5R (*solid line*, *upper graph*), scFv-51R (*solid line*, *lower graph*), and vector control virus (*narrow dotted lines*), as well as control NIH/3T3 cells (*wide dotted lines*) were stained using mAb FSP77 (20) and subjected to flow cytometric analysis.

growth factor, stimulated growth of the scFv-5R and scFv-51R expressing NIH/3T3 cells to an extent similar to that seen for the vector control cells (Table I), showing that the effect of the scFv-R proteins is specific for ErbB-2.

ErbB-2 Function in scFv Expressing Cells*—To assess the effects of the scFv-mediated ER retention on ErbB-2*, we analyzed whole cell lysates by Western blotting (Fig. 5). An analysis using an ErbB-2-specific antiserum revealed that the ER-retained ErbB-2* showed an increased mobility on SDS-PAGE (Fig. 5*A*), suggesting that there are changes in its post-translational modifications. ErbB-2 is a glycoprotein (4, 38) which undergoes *N*-linked glycosylation in the ER followed by modification and extension of the carbohydrate side chains in the Golgi apparatus. Thus it is likely that glycosylation of the ER-retained ErbB-2* is impaired which may account for the different apparent molecular weight. The level of ErbB-2* was strongly elevated in the scFv-5R and scFv-51R expressing cells (Fig. 5*A*). This accumulation in the ER might be due to an increased rate of synthesis or a decreased rate of degradation of the protein. A Western analysis using a phosphotyrosine-specific mAb showed that the total phosphotyrosine content of

TABLE I
Growth stimulation by PDGF and basic fibroblast growth factor in scFv expressing NIH/3T3 cells

NIH/3T3 cells infected with vector control virus, scFv-5R virus, and scFv-51R virus were serum-starved for 24 h prior to induction by PDGF (20 ng/ml) or basic fibroblast growth factor (50 ng/ml) for 2 days. The percentage of growth stimulation (± S.D.) in comparison to untreated cells was calculated and is given below.

NIH/3T3	PDGF	Basic fibroblast growth factor
Control	10.8 (±0.6)	12.6 (±1.9)
scFv-5R	13.8 (±1.5)	15.5 (±1.6)
scFv-51R	10.3 (±1.0)	12.4 (±0.4)

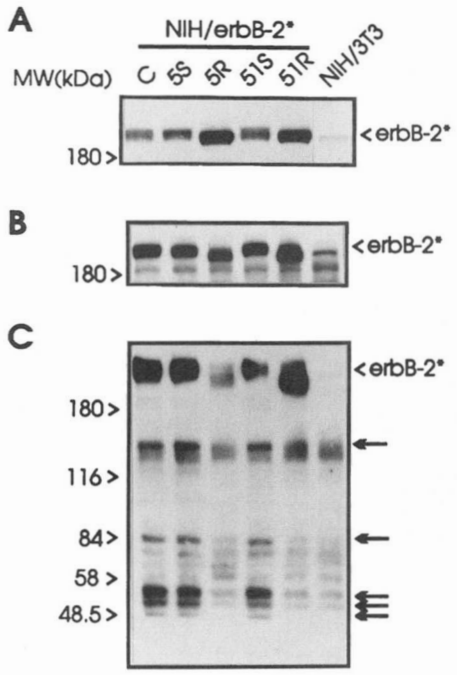

FIG. 5. **Phosphotyrosine analysis and level of ErbB-2* in NIH/ErbB-2* cells.** *A*, Western blot analysis of ErbB-2*. NIH/ErbB-2* cells infected with vector control, scFv-5S, scFv-5R, scFv-51S, and scFv-51R virus, as well as control NIH/3T3 cells were lysed in Triton X-100 lysis buffer, 50 µg of total protein were separated on 7.5% SDS-PAGE, and the ErbB-2* content was analyzed by Western blotting with the 21N antiserum (35). *B*, phosphotyrosine content of ErbB-2*. 50 µg of total protein were subjected to 9% SDS-PAGE and analyzed by Western blotting with a phosphotyrosine specific mAb (36). *C*, pattern of phosphotyrosine containing proteins. 50 µg of total protein were subjected to 5–12% gradient SDS-PAGE and analyzed by Western blotting with a phosphotyrosine specific mAb (36). *Arrows* indicate bands that show different intensity in the individual cell lines.

(Fig. 5*B*). Due to the strongly increased level of ErbB-2*, the receptor appears to be phosphorylated at a much lower stoichiometry in the cells expressing the retained versions of the scFvs, indicating a decrease in its kinase activity. It should be noted that the phosphotyrosine content of ErbB-2* was lower in cells expressing scFv-5R than in cells expressing scFv-51R, while the ErbB-2* level was slightly higher. Most importantly, compared to the control-infected NIH/ErbB-2* cells and to cells expressing the secreted scFvs, the intensity of bands with apparent molecular masses of 145, 84, 56, 52, and 48 kDa was markedly reduced in cells expressing scFv-5R and scFv-51R (Fig. 5*C*). The pattern of phosphotyrosine-containing proteins in these cells was very similar to the one in control NIH/3T3 cells. These observations suggest that the retention of ErbB-2* in the ER led to its functional inactivation.

Reversion of the Transformed Phenotype—Retention of ErbB-2* in the ER resulted in a drastic change in the morphol-

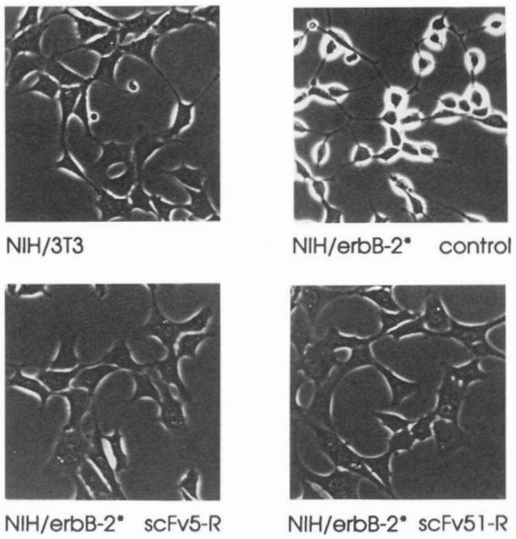

FIG. 6. **Morphological reversion of cell transformation.** The morphology of NIH/ErbB-2* cells infected with scFv-5R, scFv-51R, and vector control virus, as well as control NIH/3T3 cells is shown.

2*-transformed cells reverted to the more flattened appearance of normal fibroblasts (Fig. 6). In addition, while the control-infected cells and those expressing the secreted scFvs formed foci at a high efficiency, the cells expressing the ER-retained scFvs failed to do so.[2] To further investigate the changes in the transformed phenotype of NIH/ErbB-2* cells, we analyzed their anchorage-independent growth in soft agar. ER expression of both scFvs dramatically reduced colony formation as compared to the control-infected cells and to cells expressing the scFv-51S. Expression of the scFv-5S caused up to 44% inhibition on soft agar growth (Table II). Anchorage-independent growth of cells expressing the scFv-5R was similar to normal NIH/3T3,[2] indicating complete reversion of the transformed phenotype.

DISCUSSION

Our results show the stable inactivation of an oncoprotein, accompanied by reversion of a transformed phenotype, via intracellular single chain antibody expression. As a model system we have chosen the mAbs FRP5 and FWP51 which bind to the extracellular domain of human ErbB-2. We have studied effects of ER lumenal expression of scFvs FRP5 and FWP51 in NIH/ErbB-2* fibroblasts transformed by point-mutated, activated human ErbB-2. We have shown that both scFvs, which recognize different epitopes on the ErbB-2 extracellular domain, were very potent in affecting the ER transit and transforming ability of ErbB-2*. Our results demonstrate (i) the feasibility of expressing functional antibodies intracellularly, (ii) the possibility of inhibiting the transit of an integral membrane protein through the ER by means of scFv expression, and (iii) that retention of a constitutively active receptor tyrosine kinase in the ER interferes with its transforming ability.

We have observed that single chain antibodies directed to the secretory pathway are found in the conditioned medium, whereas scFvs containing a C-terminal ER retention signal are stably retained in the ER, irrespective of coexpression of ErbB-2*. This is in contrast to a recent publication showing that a single chain antibody against gp160, the human immunodeficiency virus-1 envelope protein precursor, which was designed for secretion, was stably retained in the ER, whereas a variant carrying an additional C-terminal ER retention signal, yielded an unstable protein that was rapidly degraded if not coex-

TABLE II
Soft agar growth of scFv expressing NIH/ErbB-2 cells*

NIH/ErbB-2* cells infected with vector control, scFv-5S, scFv-5R, scFv-51S, and scFv-51R virus were plated in culture medium supplemented with 0.35% agar. After 14 days viable cells were stained and colonies >50 and >200 μm were counted using an Artek colony counter. The experiment was repeated three times in duplicate, and the colony numbers of one typical experiment (± S.D.) are shown below.

NIH/ErbB-2*	>50 μm	inhibition	>200 μm	% inhibition
Control	239 (±7.1)		71 (±0.0)	
scFv-5S	163 (±7.8)	32	40 (±4.2)	44
scFv-5R	11 (±0.7)	96	4 (±0.7)	95
scFv-51S	231 (±9.9)	3	68 (±2.8)	4
scFv-51R	59 (±0.7)	76	14 (±2.8)	80

pressed with its binding partner (28). Single chain antibodies consist of Immunoglobulin heavy and light chain variable domains and are, therefore, variable in sequence. This is likely to cause biochemical differences between individual scFv proteins which might explain their different *in vivo* behavior.

ER expression of both scFv-5R and -51R led to a strongly elevated level of ErbB-2*. This could either be due to an increased synthesis rate or a decreased turnover rate of ErbB-2*. We favor the latter explanation since in the transfectants, ErbB-2* expression is under the control of the SV40 early promoter making transcriptional or translational control unlikely. Due to its constitutive kinase activity, the half-life of ErbB-2* is short, approximately 1.5 h, while the normal ErbB-2 has a half-life of more than 7 h (14). Compared to the transformed control cells, the ER-retained ErbB-2* has, on a molar basis, lower amounts of phosphotyrosine, suggesting that its kinase activity is decreased which may in turn alter the kinetics of turnover. Indeed, the level of ErbB-2* found in scFv-5R and scFv-51R-expressing cells suggests that there is a close link between half-live and kinase activity. In the scFv-5R expressing cells where ErbB-2* kinase activity was inhibited to a greater extent than in the scFv-51R expressing cells, higher levels of ErbB-2* were found. In this context it is noteworthy that in cell lines expressing normal ErbB-2, scFv-mediated ER retention of the receptor did not affect its phosphorylation or result in an increased level of ErbB-2.[2]

Why are the NIH/ErbB-2* cells expressing scFv-5R and scFv-51R reverted? There are differences in the post-translational modifications, activity, and subcellular location of ErbB-2* in the reverted cells. As discussed above, the ER-retained ErbB-2* in the reverted cells has, on a molar basis, lower amounts of phosphotyrosine suggesting that its kinase activity is decreased. The lower activity may in turn be due to the immature, under-glycosylated state of the ErbB-2* precursor. It has been described that the ErbB-2* precursor is less phosphorylated, and thus presumably less active, than the mature protein (38). Significantly, in the reverted cells ErbB-2* is no longer present on the plasma membrane, but accumulates in the ER. These differences may all contribute to reversion of the transformed phenotype. In the revertants a number of cellular proteins display a lower level of phosphotyrosine, a level comparable to that seen in control NIH/3T3 cells. This may be due to the lower ErbB-2* kinase activity and/or to the fact that the ER-located receptor cannot interact with crucial substrates. It is possible that one or more of these proteins is a key substrate for cellular transformation.

Although ER expression of both scFvs reverted transformation, the two proteins were not equally efficient. The scFv-5R protein inhibited soft agar growth more strongly than the scFv-51R protein. This may be partly due to the fact that the loss of cell surface ErbB-2* was more complete in the scFv-5R expressing cells, as the FACS analysis showed. However, the scFv FRP5 appears to have additional inhibitory effects. First, the ER-located ErbB-2* displayed a lower phosphotyrosine content in the scFv-5R, than in the scFv-51R expressing cells. Retention of proteins in the lumen of the ER is mediated by a receptor-like protein recognizing the KDEL peptide present on the C terminus of ER resident proteins and is achieved by their continual retrieval from the *cis*-Golgi (or a pre-Golgi) compartment (37, 39, 40). In the case of scFv-5R expressing cells, the final configuration of the transiently formed, trimeric complex consisting of the KDEL receptor, scFv, and ErbB-2* might interfere with dimerization and kinase activity. Second, binding of the the scFv FRP5, but not the scFv FWP51, *per se* seems to affect the activity of ErbB-2*. The secreted form of the scFv FRP5 also repressed the soft agar growth of NIH/ErbB-2* cells, without affecting ErbB-2* receptor trafficking[2] or phosphotyrosine content, possibly by altering the receptor conformation. It was noted in a recent publication (41) that an ErbB-2-specific mAb was growth inhibitory, without affecting ErbB-2 tyrosine phosphorylation. This suggests that for the inhibition of growth, antibody induced effects such as alteration of receptor conformation could be as important as effects on kinase activity.

It has been suggested that constitutively active receptor tyrosine kinases, such as ErbB-2* or the PDGF receptor in *sis*-transformed cells, might at least partially exert their transforming activity intracellularly, in the ER and/or the Golgi compartment (38, 42). The experiments presented in this paper show that the intracellular retention of an activated ErbB-2* causes reversion of transformation. Cells expressing the secreted version of scFv FWP51 are not affected in their growth, indicating that the binding of scFv FWP51 does not affect receptor activity *per se*. In contrast, cells expressing the scFv-51R are inhibited by 80% in their soft-agar growth. Thus, we conclude that the activated ErbB-2* must be on the cell surface, or at least in a cellular compartment distinct from ER and *cis*-Golgi, to cause transformation.

The data presented here are the first demonstration of a targeted and stable inactivation of a cellular oncoprotein via intracellular antibody expression. The potential for inactivating malignant cell growth by a gene therapy approach has implications for the treatment of cancer. In a more general sense, our results and the recent work of other groups (28, 43) illustrate how potent engineered antibodies are in altering the *in vivo* function of selected cellular or viral target proteins. This approach should also be useful in studying complex receptor-ligand systems such as the one that has become apparent in the epidermal growth factor receptor-related family of growth factor receptors (44). It should be possible to retain individual members of the receptor family in the ER and analyze effects of ligands on the altered cells. Moreover, expression of scFvs in other subcellular compartments should allow the interference with different classes of proteins. In particular, the intracellular expression of highly specific kinase inhibitory scFvs should prove to be a simple and powerful approach to study the complex network of signal transduction in eukaryotic cells, providing an attractive alternative to chemical inhibitors, dominant kinase-negative mutants, or microinjection of antibodies. We conclude that single chain antibody-mediated "immunointerference" represents a simple and powerful approach to study the *in vivo* function of receptors and other cellular proteins.

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