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Tissue-Specific Targeting of Retroviral Vectors Through Ligand-Receptor Interactions

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The development of retroviral vectors that target specific cell types could have important implications for the design of gene therapy strategies. A chimeric protein containing the polypeptide hormone erythropoietin and part of the *env* protein of ecotropic Moloney murine leukemia virus was engineered into the virus. This murine virus became several times more infectious for murine cells bearing the erythropoietin receptor, and it also became infectious for human cells bearing the erythropoietin receptor. This type of tissue-specific targeting by means of ligand-receptor interactions may have broad applications to a variety of gene delivery systems.

Mammalian retrovirus vectors commonly used for gene transfer are classified on the basis of their host range as either ecotropic, which only infect murine cells, or amphotropic, which infect both murine and nonmurine cells. The host range is determined primarily by the binding interaction between viral envelope glycoproteins and specific proteins on the host cell surface that act as viral receptors (*1*). In murine cells, an amino acid transporter serves as the receptor for the envelope glycoprotein gp70 of ecotropic Moloney murine leukemia virus (Mo-MuLV) (*2*). The receptor for the amphotropic Mo-MuLV has recently been cloned and shows homology to a phosphate transporter (*3*). Because the transporters are widely distributed among various tissues, these retrovirus vectors can infect virtually all cell types and therefore are not tissue-specific.

It has previously been shown that the host range of viruses can be altered by pseudotyping (*4*). However, the alternative envelope proteins used in such experiments were derived from naturally occurring viral sequences such as those of gibbon ape leukemia virus, avian leukosis virus, and the human immunodeficiency virus, and hence the resultant pseudotyped virions were still limited by the host range of the naturally occurring virus. In some cases, it has been shown that viral targeting can be achieved by ligand-receptor interactions, mediated by bivalent antibodies linked by biotin-streptavidin (*5*) or by chemical modification with lactose to produce an asialoglycoprotein (*6*). These manipulations, which involve modifications to the virus after its production, usually result in low infection efficiency. A recombinant virus containing in its envelope a sequence encoding a single-chain antibody variable region has been shown to bind to a solid matrix containing the appropriate polypeptide antigen, and the bound viruses, as expected, were infectious for NIH 3T3 cells (*7*). However, direct infection of target cells by the virus through antigen-antibody interaction was not demonstrated. In this study, we engineered an ecotropic virus to bear a chimeric envelope-ligand protein on its surface. This virus not only showed enhanced infectivity for murine cells that bear the appropriate receptor but could also cross species and specifically infect the appropriate receptor-bearing human cells.

We introduced the polypeptide hormone erythropoietin (EPO) into the ecotropic Mo-MuLV envelope. A portion of the Mo-MuLV envelope gene (*env*) (*8*) encoding the NH_2-terminal end of gp70 was removed and replaced, in frame, with sequences coding for EPO (*9*); this construct was designated pEPOenv Δ5923. Portions of the gp70 sequence that encode the *env* signal peptide at the NH_2-terminus, as well as the cysteine residues in the COOH-terminal region that participate in sulfhydryl bonding with the inner envelope subunit p15, were left intact. This EPO-*env* hybrid construct was cotransfected into ψ2 packaging cells (*10*) with the plasmid pFR400, which contains the meth-

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otrexate (MTX)-resistant dihydrofolate reductase gene (*dhfr*) (*11*). The transfected cells were selected with increasing concentrations of MTX, and subclones were isolated and screened by protein immunoblot analysis of whole-cell lysates; an EPO-specific monoclonal antibody (mAb) and a polyclonal *env*-specific antiserum (anti-*env*) were used. Several of the subclones transfected with pEPOenv Δ5923 showed the same 70-kD band, which reacted both with the EPO mAb and the anti-*env*, which indicates coexpression of both epitopes in the same protein (*12*). One subclone, designated ψEPOenv 8, was chosen for further characterization.

To demonstrate that the chimeric envelope protein was transported to and oriented correctly in the cell membrane, we analyzed ψEPOenv 8 cells by fluorescence-activated cell sorter (FACS) analysis. A biphasic pattern of fluorescence was observed with antiserum to EPO (anti-EPO). One peak was shifted (Fig. 1A), and the shift was abolished in competition experiments with an excess of EPO peptide (Fig. 1B). We sorted the cells showing high fluorescence intensity to obtain a monophasic population of cells expressing the EPO epitope at high concentrations (Fig. 1C). The monophasic shift in fluorescence could also be abolished by competition with soluble EPO peptide (*12*). One subcloned packaging cell line showing the largest shift in fluorescence, designated ψEPOenv 8.1, was selected for further studies. Southern (DNA) blot analysis showed that this cell line, as expected, carried the 2.4-kb Eco RI restriction fragment containing the chimeric EPO-*env* sequence (*12*).

Virus-producing cell lines were then generated by infection of these packaging cells with virion-containing cell culture medium harvested after transient transfection of wild-type PA317 packaging cells (*13*) with pCRIP-SVlac, a packageable, replication-defective viral vector (*14*) that contains both the gene for neomycin resistance and the gene for β-galactosidase (β-Gal). G418-resistant subclones of ψEPOenv 8.1 were retested for expression of the EPO-*env* proteins by protein immunoblot and FACS analysis, and a positive cell line designated ψEPOenv 8.1.8 was obtained. Viruses produced by this cell line should be coated with wild-type ecotropic envelope proteins, derived from the parental ψ2 cell line, as well as the chimeric EPO-*env* proteins, derived from the stably transfected construct.

To show that the virions produced by these producer cell lines do contain the chimeric envelope protein, culture medium from these cell lines was fractionated by sucrose density gradient centrifugation. The fractions were subjected to protein immunoblot analysis with antisera specific for EPO, *env*, or total Mo-MuLV. The same 70-kD band was detected by anti-EPO as well as anti-*env*, predominantly in two fractions that also contained other components of the Mo-MuLV virus, such as the p15 transmembrane subunit of the envelope and the 30-kD *gag* proteins (Fig. 2, A through C). This demonstrates that the chimeric EPO-*env* proteins were indeed incorporated into intact virions released by the producer cells into the medium.

To test whether the EPO-*env*–containing viruses had increased affinity for cells with

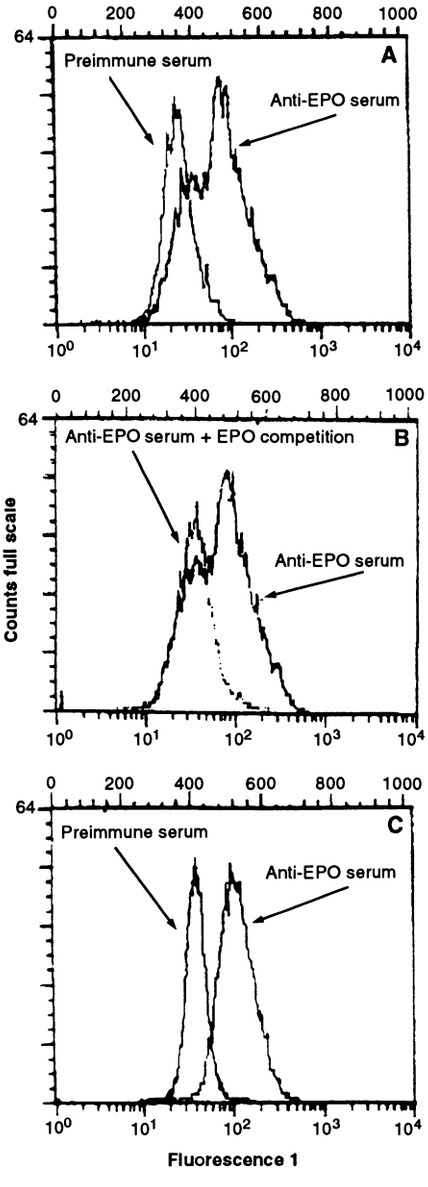

Fig. 1. FACS analysis of packaging cell lines. Subclones of ψ2 packaging cells that had been stably transfected with the chimeric envelope construct pEPOenv Δ5923 (*18*) were initially screened for expression of the protein by protein immunoblot (*19*). Positive subclones were grown to confluence on 10-cm plates, detached and dispersed with PBS + 2 mM EDTA, and preincubated with normal goat serum in PBS + 2% BSA for 30 to 60 min on ice. After washing with PBS + 2% BSA, the cells were incubated with preimmune rabbit serum (CalTag) or with a polyclonal EPO-specific rabbit antiserum (no. 8C295, Amgen) in PBS + 2% BSA for 30 to 60 min, washed again, incubated with fluorescein-conjugated goat antiserum to rabbit IgG (CalTag), washed, and subjected to flow cytometry after addition of propidium iodide (*20*). (**A**) Before sorting, the ψEPOenv 8 cell line reacted with preimmune and EPO-specific antiserum. (**B**) ψEPOenv 8 cells reacted with EPO-specific antiserum, with and without competition by excess soluble EPO. (**C**) After sorting, the ψEPOenv 8.1 cell line, a subclone isolated after sorting of the high-intensity fluorescence population, reacted with preimmune and EPO-specific antiserum.

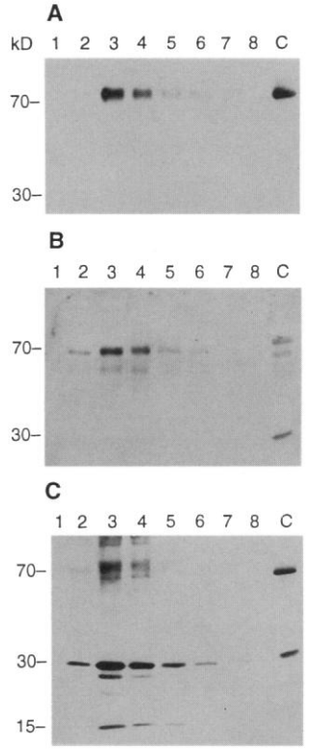

Fig. 2. Sucrose density gradient fractionation of virus particles isolated from overnight cell culture medium of producer cell line ψEPOenv 8.1.8. Viral particles from producer cell line ψEPOenv 8.1.8 (*21*) were purified by centrifugation at 150,000*g* through 2 ml of 20% sucrose onto a 1-ml 60% sucrose cushion in an SW40 rotor for 1 hour at 4°C. Virus particles removed at the interface were diluted to 3 ml, layered on top of a 9-ml 20 to 45% sucrose gradient, and centrifuged at 150,000*g* overnight in an SW40 rotor at 4°C. Eight 1-ml fractions (lanes 1 to 8) collected from the bottom of this gradient were each diluted 1:3 before pelleting at 190,000*g* in an SW50 rotor for 1 hour at 4°C. Pelleted samples were lysed in lysis buffer, boiled, and analyzed by protein immunoblot (*19*) with the use of either (**A**) EPO-specific antibody, (**B**) *env*-specific antibody, or (**C**) total MLV-specific antibody. The control lane (lane C) contains a sample of whole-cell lysate from cell line ψEPOenv 8.1.8.

the EPO receptor, we created an EPO receptor–containing target cell line by stably transfecting wild-type NIH 3T3 cells with a complementary DNA (cDNA) encoding the EPO receptor (*15*). Subclones were screened by ^{125}I-labeled EPO radioligand binding assay, and the subclones showing the largest amounts of ^{125}I-EPO binding, corresponding to approximately 10,000 receptors per cell, were chosen for use in infection experiments. The wild-type and EPO receptor–containing NIH 3T3 target cells were infected with the CRIP-SVlac vector, packaged by wild-type ψ2 cells or produced by the ψEPO-env 8.1.8 producer cell line. The EPO-*env* virus showed a 6.1-fold increase in efficiency on NIH 3T3 + EPO receptor target cells as compared with ψ2 wild-type virus. This increased infection efficiency was mediated through the EPO ligand-receptor interaction, because the increase in infection events was abolished in a dose-dependent manner by addition of soluble EPO peptide at the time of infection (Fig. 3A).

Wild-type ecotropic viruses normally do not infect human cells. To test whether the ecotropic virus pseudotyped by the chimeric envelope would infect human cells bearing the EPO receptor, we next tested ψ2-, PA317-, and ψEPOenv 8.1.8–packaged CRIP-SVlac virus for its ability to infect human cells with or without the EPO receptor. As expected, ψ2-packaged virus, which infects NIH 3T3 cells in the presence or absence of the EPO receptor, did not infect HEL (human erythroleukemia) or HeLa (human cervical carcinoma) cells, whereas PA317-packaged virus infected all of the cell lines. The ψEPOenv 8.1.8–packaged ecotropic virus specifically infected the erythroid cell line HEL, which bears the EPO receptor, but did not infect HeLa cells, which do not bear the EPO receptor (Fig. 3B). We also found that this virus infected the human erythroid cell line K562 but not the human lymphocytic cell line Raji (*12*). The HEL and K562 cells contained approximately 1000 and 800 receptors per cell, respectively, as measured by ^{125}I-EPO binding. Because the ecotropic virus bearing the chimeric EPO-*env* envelope infected HEL and K562 cells as effectively as did the PA317-packaged amphotropic virus, the ligand-receptor interaction appears to have been highly efficient and did not require large numbers of receptors on the target cell surface.

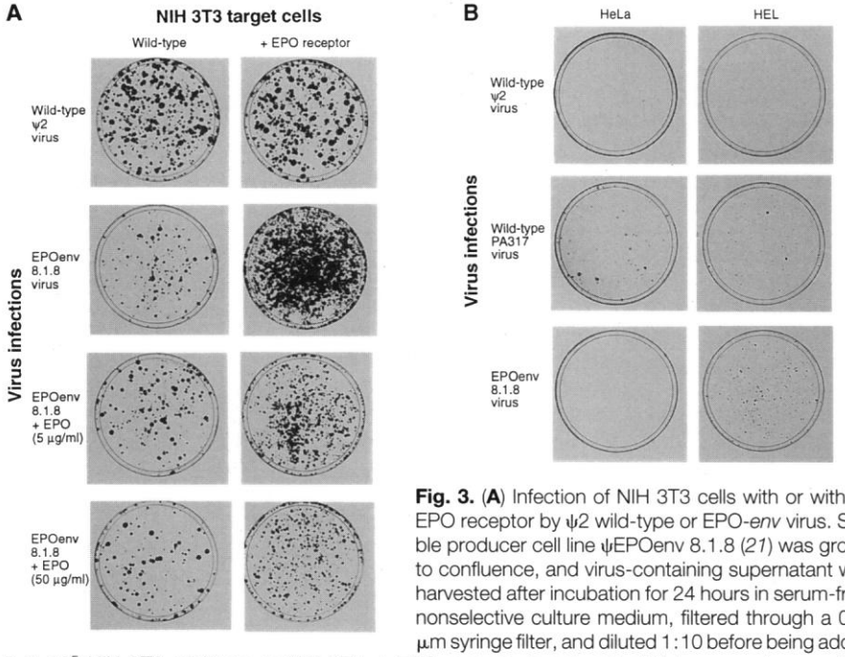

Fig. 3. (**A**) Infection of NIH 3T3 cells with or without EPO receptor by ψ2 wild-type or EPO-*env* virus. Stable producer cell line ψEPOenv 8.1.8 (*21*) was grown to confluence, and virus-containing supernatant was harvested after incubation for 24 hours in serum-free, nonselective culture medium, filtered through a 0.2-μm syringe filter, and diluted 1:10 before being added to 5 × 10^5 NIH 3T3 wild-type or NIH 3T3 + EPO receptor target cells (*22*) in serum-free medium containing polybrene (8 μg/ml). Wild-type ψ2 cells stably expressing CRIP-SVlac virus after infection by means of PA317-derived vectors were used for production of ψ2 wild-type control virus. For competition studies, 5 to 50 μg of soluble EPO (Amgen) was also added to the target cell plates at the time of infection. The infected target cells were trypsinized 24 hours later, replated at 1:50 dilution in duplicate, and selected with G418 (400 μg/ml) for 12 days, after which the plates were fixed with methanol and acetone (1:1), and the colonies were visualized by Giemsa staining and counted (*23*). (**B**) Infection of human cell lines with or without EPO receptor. Virus-containing supernatant was harvested from producer cell line ψEPOenv 8.1.8 (*20*) at confluence after overnight incubation in serum-free nonselective medium, filtered through a 0.2-μm syringe filter, and added without dilution to 5 × 10^5 HEL cells, which express EPO receptor (*21*), or HeLa cells, which do not express EPO receptor, in medium containing polybrene (8 μg/ml). Negative control infections were done with virus similarly harvested from wild-type ψ2 producer cells, and positive control infections were done with wild-type PA317 cells expressing CRIP-SVlac virus at a titer of 6.8 × 10^2 per milliliter, diluted 1:10. The infected target cells were replated 24 hours later at a 1:5 dilution and selected with G418 (400 to 1000 μg/ml), after which the plates were fixed, and the surviving cells were visualized by Giemsa staining.

Our present studies thus directly demonstrate enhanced ecotropic viral infection of murine cells expressing the appropriate receptor for EPO and, more strikingly, ligand-receptor–mediated, cell-specific, cross-species infection of EPO receptor–bearing human erythroid cells. Infection of earlier hematopoietic progenitors could conceivably be achieved by targeting c-Kit or CD34 (*16*). A retrovirus vector with tissue tropism for this specific subpopulation of target cells could potentially be of use in gene therapy for red blood cell disorders such as sickle cell anemia and thalassemias, as well as in developmental studies of erythroid cell differentiation. In principle, the approach demonstrated here should be broadly applicable, allowing production of a wide range of viruses that bear ligands for specific receptors and thus can deliver genes to specific tissues or organs for the treatment of genetic diseases and cancer.

Note added in proof: Since this manuscript was first submitted, Valsesia-Wittmann *et al.* (*17*) have reported infection of mammalian cells by avian retroviruses through introduction of an integrin sequence into the envelope.

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18. Plasmid EPOenv Δ5923 was constructed by use of unique restriction sites at position 5923 (Bst EII) and position 6537 (Bam HI) of the Mo-MuLV *env* gene to delete the intervening envelope sequence and allow the EPO sequence to be inserted, in frame, at the NH_2-terminal end of gp70. The EPO cDNA sequence coding for the mature 166–amino acid peptide hormone, without the 27–amino acid signal peptide, was used to replace the Mo-MuLV *env* sequences that had been deleted. An EPO sequence with compatible ends was created by polymerase chain reaction (PCR)–mediated mutagenesis, with 5′-GGCCTC-CCAGTGGTAACCGCCCCACCACGC-3′ as the 5′ primer and 5′-GGACACTCCTGGGATCCTGTCCC-CTGTCCT-3′ as the 3′ primer, followed by restriction digest of the PCR product with Bst EII and Bam HI, so that it could be inserted by means of the appropriate restriction sites while maintaining the proper reading frame. We also made a second construct containing EPO in the central portion of gp70 (between positions 6257 and 6761 of the *env* gene), directly overlapping a proline-rich hypervariable region. Although a few subclones expressed this construct at the protein level, as shown by protein immunoblot analysis, none expressed it on the surface of the packaging cells, as shown by FACS analysis. This lack of cell-surface expression was presumably due to retention in the endoplasmic reticulum (*12*).
19. To create packaging cell lines expressing the recombinant envelope, 1×10^6 ψ2 cells, grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% penicillin-streptomycin, were cotransfected with 20 μg of pEPOenv Δ5923 and 1 μg of pFR400 by means of the calcium phosphate precipitation method (Gibco). The medium was changed 16 to 24 hours later and was subsequently selected with gradually increasing concentrations of MTX (Sigma) from 0.2 to 10 μM, at which point subclones were isolated from the surviving colonies and screened by protein immunoblot. Subclones were grown to confluence in 10-cm plates, washed with phosphate-buffered saline (PBS), and lysed in 4% SDS, 10% glycerol, 10% β-mercaptoethanol, and 50 mM tris (pH 6.7) and boiled for 10 min. The samples were subjected to 8% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose (Hybond ECL, Amersham). The filters were blocked in TBST buffer [0.02% Tween-20, 150 mM NaCl, and 50 mM tris (pH 7.5)] with 5% dried milk and incubated with a primary EPO mAb (Genzyme), then washed in TBST buffer with 0.5% dried milk and incubated with a secondary horseradish peroxidase–conjugated goat antibody to mouse immunoglobulin G (IgG) (CalTag, San Francisco, CA). After being washed again, the filters were incubated in ECL chemiluminescent immunodetection reagents (Amersham) and exposed to film. The same specific 70-kD band was also observed when the filters were reblocked and reprobed with a polyclonal goat anti-*env* (Microbiological Associates, Rockville, MD) as the primary antibody, followed by horseradish peroxidase–conjugated swine antibody to goat IgG (CalTag) as the secondary antibody.
20. Polyclonal antiserum to EPO no. 8C295 (Amgen) and fluorescein-conjugated secondary antibodies (CalTag) were used for flow cytometry, carried out with a Becton Dickinson FACScan and FACS IV, operated by P. Dazin.
21. Virus-producing cell lines were created by transient transfection of amphotropic packaging cell line PA317 with pCRIP-SVlac (which contains the gene for neomycin resistance and the gene for β-Gal and is a modification of a retroviral vector plasmid provided by R. Scharfmann) by means of the calcium phosphate precipitation method. The medium was changed after 24 hours, and the virus-containing medium was filtered through a 0.2-μm filter into medium containing polybrene (8 μg/ml) and then used to infect wild-type ψ2 as well as ψEPOenv 8.1 packaging cells 48 hours after transfection. The infected packaging cells were subsequently replated at low density and selected in G418 (400 μg/ml) (Gibco), and individual G418-resistant colonies were isolated. Subcloned colonies were retested for expression of the EPO epitope by protein immunoblot and FACS analysis. Amphotropic, wild-type virus-producing cells were created by a similar strategy that used transient transfection of wild-type ψ2 cells with pCRIP-SVlac to generate virus for infection of PA317 packaging cells, followed by G418 selection.
22. NIH 3T3 cells were cotransfected with pXM EPO-R and pFR400 and selected in increasing concentrations of MTX ranging from 0.2 to 10 μM. Expression of the EPO receptor was assayed by binding of ^{125}I-EPO peptide (Amersham); target cells were grown to confluence in 24-well plates and subsequently incubated in DMEM with 2% bovine serum albumin (BSA) and approximately 10^6 cpm of ^{125}I-EPO, with or without excess cold EPO, for 90 min at 37°C in a humidified incubator, washed with PBS, lysed in 1N NaOH, and counted in a gamma counter. The approximate number of EPO receptors per cell was estimated on the basis of the specific activity of the ^{125}I-EPO and the approximate number of cells contained within the wells. HEL and K562 cells were similarly assayed for ^{125}I-EPO binding, except that cells were incubated with ^{125}I-EPO in suspension and were spun for 5 min at 1000 rpm in a Beckman desktop centrifuge to pellet the cells after each wash.
23. The ψ2 wild-type control virus showed titers of 3.1×10^4 per milliliter and 2.8×10^4 per milliliter on NIH 3T3 wild-type and NIH 3T3 + EPO receptor target cells, respectively. The ψ2 wild-type virus titers were not affected by the presence or absence of EPO. The EPO-*env* virus showed a titer of 1.8×10^4 per milliliter on NIH 3T3 wild-type cells and 1.2×10^5 per milliliter on NIH 3T3 + EPO receptor cells without EPO competition. With EPO competition, the EPO-*env* virus titers on NIH 3T3 wild-type cells were not significantly affected; however, the titers on NIH 3T3 + EPO receptor cells were drastically reduced, to 6.2×10^4 per milliliter with the addition of 5 μg of EPO per milliliter and 5.5×10^4 per milliliter with the addition of 50 μg of EPO per milliliter. Two variables were then taken into account in interpreting these results and determining the efficiency of infection. First, differences in initial plating density and growth rates of the two target cell lines were corrected for by comparison of the number of G418-resistant NIH 3T3 + EPO receptor colonies with the number of G418-resistant NIH 3T3 wild-type colonies after infection by ψ2 wild-type virus. Second, differences in the overall titers of the wild-type and EPO-*env* viruses were corrected for by comparison of the number of G418-resistant NIH 3T3 wild-type colonies after infection with ψ2 virus with that of NIH 3T3 wild-type colonies after infection with EPO-*env* virus. Thus, the relative infection efficiency of EPO-*env* virus on NIH 3T3 + EPO receptor cells as compared with that of ψ2 wild-type virus was derived by the following formula: relative infection efficiency = (number of G418-resistant NIH 3T3 + EPO receptor colonies per number of G418-resistant NIH 3T3 wild-type colonies) after EPO-*env* virus infection ÷ (number of G418-resistant NIH 3T3 + EPO receptor colonies per number of G418-resistant NIH 3T3 wild-type colonies) after ψ2 wild-type virus infection. The relative infection efficiency thus derived, expressed as the mean ± SE, was 6.1 ± 1.2 (n = 6).
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Interaction of the p53-Regulated Protein Gadd45 with Proliferating Cell Nuclear Antigen

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GADD45 is a ubiquitously expressed mammalian gene that is induced by DNA damage and certain other stresses. Like another p53-regulated gene, *p21$^{WAF1/CIP1}$*, whose product binds to cyclin-dependent kinases (Cdk's) and proliferating cell nuclear antigen (PCNA), *GADD45* has been associated with growth suppression. Gadd45 was found to bind to PCNA, a normal component of Cdk complexes and a protein involved in DNA replication and repair. Gadd45 stimulated DNA excision repair in vitro and inhibited entry of cells into S phase. These results establish *GADD45* as a link between the p53-dependent cell cycle checkpoint and DNA repair.

An important cellular response to DNA damage is the arrest of cell cycle progression at G_1 and G_2 checkpoints, which presumably allows time for DNA repair before entry into S and M phase, respectively (*1*). The p53 tumor suppressor is required for one such G_1 checkpoint in mammalian cells (*2*, *3*). After genotoxic stress, p53 functions as a transcription factor and transactivates effector genes such as *GADD45* and *p21$^{WAF1/CIP1}$*, although both of these genes can be induced by other pathways (*3*, *4*). p21$^{Waf1/Cip1}$ inhibits the kinase activity of multiple Cdk complexes, which may be one mechanism by which it suppresses cellular growth (*5*, *6*), and it inhibits the ability of PCNA to activate

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