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A Dominant Inhibitory Mutant of the Type II Transforming Growth Factor β Receptor in the Malignant Progression of a Cutaneous T-Cell Lymphoma

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In many cancers, inactivating mutations in both alleles of the transforming growth factor β (TGF-β) type II receptor (TβRII) gene occur and correlate with loss of sensitivity to TGF-β. Here we describe a novel mechanism for loss of sensitivity to growth inhibition by TGF-β in tumor development. Mac-1 cells, isolated from the blood of a patient with an indolent form of cutaneous T-cell lymphoma, express wild-type TβRII and are sensitive to TGF-β. Mac-2A cells, clonally related to Mac-1 and isolated from a skin nodule of the same patient at a later, clinically aggressive stage of lymphoma, are resistant to TGF-β. They express both the wild-type TβRII and a receptor with a single point mutation (Asp-404→Gly [D404G]) in the kinase domain (D404G-TβRII); no TβRI or TβRII is found on the plasma membrane, suggesting that D404G-TβRII dominantly inhibits the function of the wild-type receptor by inhibiting its appearance on the plasma membrane. Indeed, inducible expression, under control of a tetracycline-regulated promoter, of D404G-TβRII in TGF-β-sensitive Mac-1 cells as well as in Hep3B hepatoma cells results in resistance to TGF-β and disappearance of cell surface TβRI and TβRII. Overexpression of wild-type TβRII in Mac-2A cells restores cell surface TβRI and TβRII and sensitivity to TGF-β. The ability of the D404G-TβRII to dominantly inhibit function of wild-type TGF-β receptors represents a new mechanism for loss of sensitivity to the growth-inhibitory functions of TGF-β in tumor development.

Transforming growth factor β (TGF-β) is a multifunctional polypeptide that regulates cell proliferation and differentiation; loss of growth inhibition by TGF-β is thought to contribute to development of many types of tumors (7, 10, 15, 27, 29, 33, 35, 48). TGF-β triggers these diverse pathways by binding to the high-affinity cell surface receptors TβRI and TβRII. Molecular cloning of TβRI and TβRII as well as of receptors for activins and bone morphogenic proteins revealed a family of proteins with a cysteine-rich extracellular domain, a single transmembrane segment, and a cytoplasmic segment with a serine/threonine kinase domain (5, 8, 25, 31, 39). A functional signaling complex consists of both TβRII and TβRI (47). TGF-β1 binds directly to TβRII, which then causes TβRI to be recruited into the complex and become transphosphorylated by TβRII (46). TβRI is unable to bind TGF-β1 in the absence of TβRII. In contrast, only a preformed complex of TβRII and TβRI can bind TGF-β2, while either receptor alone cannot bind this ligand (37). The majority of cell surface TβRII exists as a homodimer in the presence or absence of ligand (13) and can form hetero-oligomers of undetermined composition with TβRI. A truncated mutant of TβRII, lacking the Ser/Thr kinase domain, blocks TGF-β-dependent transcription in cardiac myocytes (2).

Retinoblastoma cells, which are resistant to growth inhibition by TGF-β, do not express TGF-β receptors on their cell surface (21). Mutations in the TβRII gene correlate with loss of sensitivity to TGF-β. For example, colon cancer cells with microsatellite instability (RER+ cells) harbor mutations in the TβRII gene (28). These mutations are clustered within small repeated sequences in the TβRII gene and are accompanied by loss of TβRII surface expression and usually with a decrease in TβRII mRNA transcripts. While deletions and insertions in simple repeated sequences occur throughout the genome of RER+ tumors (17), the inactivation of TβRII is likely to be a critical step in tumor progression, rather than a simple correlate of the RER+ phenotype (28). Gastric cancer cells showed truncations or undetectable levels of TβRII in resistant cells (35), and a TGF-β-resistant hepatoma cell line (Hep3B-TR) has lost both alleles of the TβRII gene (16).

Several dominant-negative mutants of growth factor receptors have been characterized. Most show alterations in their cytoplasmic domains, causing interference with ligand-induced receptor oligomerization essential for signal transduction; examples include the epidermal growth factor receptor (19), platelet-derived growth factor receptor (40), erythropoietin receptor (43), and c-Kit (38). All such dominant-negative mutations result in the inability of these cells to grow in response to the respective growth factor.

Here we report the identification of a dominant inhibitory mutation in the cytoplasmic domain of TβRII, isolated from a TGF-β-resistant human cutaneous T-cell lymphoma cell line. In contrast to those in other growth factor receptors, this dominant inhibitory mutant causes a growth advantage by inducing resistance to TGF-β-mediated growth inhibition. The mutant receptor was expressed, together with its wild-type counterpart, only in an advanced malignant tumor. A clonally related TGF-β-sensitive cell line isolated from the same patient at an earlier, clinically indolent disease stage showed expression only of the functional wild-type (wt) TβRII. Unlike

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dominant inhibitory mutants of growth factor receptors, which inhibit activation of the cell surface ligand-induced oligomer, the dominant inhibitory TβRII described here inhibits cell surface expression of wt TβRII. This is the first description of a dominant inhibitory TβRII isolated from a TGF-β-resistant tumor cell line.

MATERIALS AND METHODS

Antisera and proteins. The specific TβRII antiserum was described by Moustakas et al. (34); the anti-TβRI serum was described by Franzen et al. (8), and the anti-hemagglutinin serum (12CA5) was purchased from Boehringer Mannheim; TGF-β1 was supplied by Celtrix Laboratories (Palo Alto, Calif.) and was radioiodinated as described previously (42); anti-TGF-β neutralizing antibodies were purchased from R&D Systems, Minneapolis, Minn.

Retroviral vectors. The LXSN retroviral vector and the SV40-y-A-MLV vector (32) were a gift from K. Luo, Whitehead Institute. To construct the SFG ttcE$^-$ retroviral vector, the transactivator tTA (12) was cloned into the retrovirus SFG under the control of a modified Moloney murine leukemia virus long terminal repeat. The vector also contained the gene of interest (here D404G-TβRII [TβRII with a change of Asp-404 to Gly]) regulated by the cytomegalovirus promoter preceded by *tet* operator sites (26).

Cell culture. COS 7 cells (CRL1651; American Type Culture Collection, Rockville, Md.) were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum (FCS), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 1 mM glutamine (Gibco BRL, Gaithersburg, Md.). Mac-1 and Mac-2A as well as stable cell lines derived from them were grown in RPMI containing 15% FCS, 1 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Bing cells (36) produce retroviruses that are helper free and infect a wide variety of mammalian cells, including human T cells; they were grown in Dulbecco modified Eagle medium containing 10% FCS, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 1 mM glutamine. Hep3B cells (16) as well as stable cell lines derived from them were grown in minimal essential medium containing 10% FCS, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 1 mM glutamine. All cells were cultured at 37°C in a 5% CO_2 atmosphere.

Binding and cross-linking of radiolabeled TGF-β1 to cell surface receptors followed by specific immunoprecipitation. Binding of ^{125}I-TGF-β1 and cross-linking with disuccinimidyl suberate were done as described previously (25). Cells were lysed in 10 mM Tris (pH 7.5)–1 mM EDTA (pH 7.5)–1% Triton X-100. Cell lysates (1 ml from 10^7 cells) were incubated with 10 μl of specific antiserum for 4 h at 4°C, then 50 μl of protein A-Sepharose was added for 90 min, and the precipitate was collected by centrifugation and washed twice with lysis buffer (10 mM Tris [pH 7.5], 1 mM EDTA [pH 7.5], 1% Triton X-100) and once with phosphate-buffered saline. TGF-β receptors were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by exposure to X-ray films or to a phosphor imager screen for quantification in a Fuji Phosphoimager.

RNA extraction and Northern (RNA) blot analysis. RNA was extracted from exponentially growing cells as described previously (25), and 4 μg of poly(A)$^+$ RNA was resolved on a 1% agarose–2.2 M formaldehyde gel before being blotted onto a nylon membrane (Hybond N; Amersham). The filter was hybridized in buffer containing 50% formamide at 42°C for 16 h. Blots were washed at 55°C in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and exposed to XAR film at −70°C.

Retroviral infection for the generation of stable Mac-2A cell lines expressing wt TβRII. Ten micrograms of LXSN-TβRII-HA DNA was cotransfected with 3 μg of SV40-y-A-MLV into COS 7 cells by a DEAE-dextran method (1). Two days after transfection, the COS cells were overlaid with 2×10^5 Mac-2A cells in the presence of 5 μg of Polybrene per ml. Selection for neomycin resistance was started 2 days later by addition of 1 mg of G418 per ml. The first cell pool was analyzed after 3 weeks by binding and cross-linking with ^{125}I-TGF-β1. By limiting dilution, 38 different G418-resistant cell clones were obtained and characterized.

Retroviral infection for the generation of stable Mac-1 cell lines and Hep3B cell lines expressing D404G-TβRII. The retroviral construct SFG ttcE$^-$ D404G-HA was transfected into Bing cells, generating recombinant amphotropic murine leukemia viruses as described previously (26, 36). Bing cells were cultured in the presence of tetracycline (1 μg/ml) starting 1 day prior to transfection. On day 2 after transfection, Mac-1 cells (cultured for 24 h in the presence of tetracycline) were overlaid on the Bing cell monolayer in the presence of 8 μg of Polybrene per ml. For infection of Hep3B cells, the virus supernatant containing 8 μg of Polybrene per ml was added to Hep3B cells. The cells were cultured for 24 h in the presence of 1 μg of tetracycline per ml; the medium (containing tetracycline) was changed after 4 h of infection.

After 48 h of cocultivation, Mac-1 cells were transferred into flasks with fresh tetracycline-containing medium. The medium (containing tetracycline) was changed every 3 days. Upon depletion of tetracycline from the system, tTA (a fusion protein of the *tet* repressor and VP16) binds to the operator sites linked to the cytomegalovirus promoter and activates transcription of D404G-TβRII. In the presence of tetracycline, the tTA dissociates from its binding sites, and depending on the nature of the minimal promoter and its site of integration in the genome, the transcription unit is silenced. Tissue specificity of the system is achieved by selection of a suitable expression signal for expression of the tTA (11, 12). All experiments were done on pools of infected cells, since the retroviral construct does not carry any selectable marker.

Growth inhibition assay. (i) Clonogenic assay in methylcellulose (T cells). Cells in log phase were harvested; at this time, the viability was greater than 95%. They were adjusted to a concentration of 1×10^5 to 2×10^5 cells per ml. One hundred microliters of cells was mixed with 0.9 ml of 0.9% methylcellulose containing 5% FCS in Iscove's modified Dulbecco's medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). Mixtures were set up in triplicate with and without added recombinant human TGF-β1 (1 ng/ml). One milliliter of the mixture of methylcellulose and cells was added into a well of a 24-well tissue culture plate. Triplicates were plated and incubated in a humidified incubator at 37°C in 5% CO_2–95% air. After 10 to 14 days, wells were scored for colony formation by visualization in an inverted microscope. Aggregates of 20 or more cells were scored as a colony.

(ii) [^{3}H]thymidine incorporation (T cells). Cells were washed two times in serum-free RPMI 1640 medium (GIBCO, Gaithersburg, Md.) and plated at a concentration of 10^5 cells per well in flat-bottom wells of 96-well microtiter plates in 200 μl of serum-free RPMI 1640 medium in the presence or absence of 10 μg of an anti-TGF-β neutralizing antibody per ml. Cultures were incubated overnight at 37°C in a humidified 5% CO_2 atmosphere. Four identical sets of cultures were established. Each set consisted of triplicate wells containing or lacking the anti-TGF-β neutralizing antibody. At the end of the overnight culture period, wells from all sets of cultures were mixed with 0.5 mCi of tritiated thymidine and FCS to bring the total volume to 250 μl per well and the FCS to 5%. Sets of cultures were harvested at 4, 8, 16, and 32 h after the addition of tritiated thymidine and FCS, and incorporated radioactivity was determined by liquid scintillation counting. Incorporated radioactivity was plotted against time after the addition of tritiated thymidine and FCS.

(iii) Clonogenic assay for stable Hep3B cell lines. Hep3B(D404G^{tet}) and Hep3B(TβRII^{tet}) cells which were cultured without tetracycline for 6 days were plated at 10^5 cells per 10-cm-diameter dish. Cells were incubated with 24 pM TGF-β1 for 6 days, and the medium was changed after 3 days. Colonies of 50 or more cells were counted on day 6.

RESULTS

Absence of surface TβRII and TβRI in Mac-2A, a TGF-β-resistant cutaneous T-cell lymphoma line. Ki (CD30)+ cutaneous T-cell lymphomas are slowly progressive tumors in which initial spontaneous regression is often observed. Rarely, after repeated cycles of regression, an aggressive lymphoma emerges. The Mac-1 cell line, generated from the blood of a patient with an early stage of cutaneous T-cell lymphoma whose skin lesions regressed spontaneously, is sensitive to TGF-β. The cell line contains 5 to 10% large Reed-Sternberg-like cells and a majority of smaller Sézary-like cells with convoluted nuclei (18). Mac-2A was derived from a nonregressing skin nodule from the same patient after progression to a Ki1+ anaplastic large-cell lymphoma; the cell line contains mostly large anaplastic cells, many of which resemble Reed-Sternberg cells. Mac-1 and Mac-2A are clonally related, as revealed by a common balanced translocation [t(8,9)(p22;p24)] and the same T-cell receptor α-chain rearrangements (18). Neither cell line exhibits the t(2:5) translocation or its molecular equivalent. Human T lymphocytes produce TGF-β, which autoinhibits cell growth.

The TGF-β1-sensitive Mac-1 cell line expresses all three principal TGF-β receptors, TβRI, TβRII, and TβRIII (β-glycan), a proteoglycan with a small cytoplasmic domain without any signaling motif (18). In contrast, the TGF-β-resistant Mac-2A cell line was reported to exhibit increased cell surface expression of TβRIII but to have lost cell surface expression of TβRII and TβRI (18). We confirmed the loss of TβRII and TβRI with a more sensitive method in which radiolabeled TGF-β1 was bound and cross-linked to surface receptors and then subjected to immunoprecipitation with specific antisera against TβRII and TβRI. Mac-2A cells completely lack surface expression of TβRII (Fig. 1A). Mac-1 cells, in contrast, exhibited cell surface expression of both TβRI and TβRII (Fig. 1A). Mac-2A cells, compared with Mac-1 cells, exhibited increased binding to TβRIII (18); a similar increase in TβRIII was also

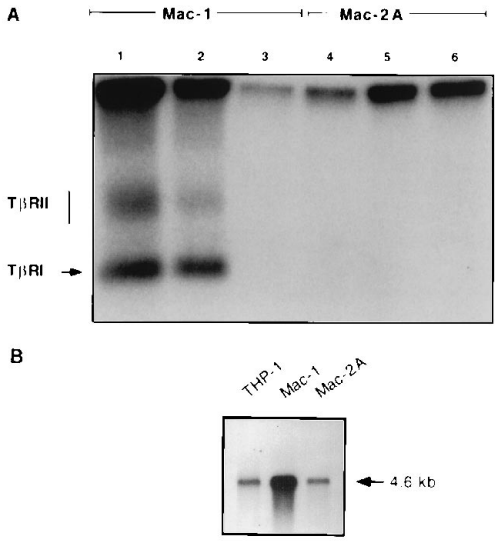


FIG. 1. Expression of TGF-β receptors on Mac-1 and Mac-2A cells. (A) Iodinated TGF-β1 (200 pM) was bound and chemically cross-linked to Mac-1 (lanes 1 to 3) and Mac-2A cells (lanes 4 to 6). Cell lysates were immunoprecipitated with anti-TβRII (lanes 1 and 4), anti-TβRI (lanes 2 and 5), and anti-ActRIB (lanes 3 and 6) antibodies. The position of ligand-bound TβRII and TβRI is indicated. (B) Northern blot analysis on poly(A)$^+$ RNA isolated from Mac-1, Mac-2A, and THP-1 cells (human leukemic promonocytes). A full-length cDNA probe of the human TβRII was hybridized to 4 μg of poly(A)$^+$ RNA. The position of TβRII mRNA is indicated with an arrow. Equal loading was confirmed by probing the blot with actin cDNA (data not shown). The level of TβRII mRNA in THP-1 cells is comparable to that of Mac-2A cells; THP-1 cells do express TβRII on their surface (data not shown).

detected in Hep3B-TR hepatoma cells, which have lost sensitivity to TGF-β by a deletion of both copies of the TβRII gene (16).

Northern blot analysis showed that Mac-2A cells expressed about 40% as much TβRII mRNA as did Mac-1 cells (Fig. 1B; equal loading was confirmed by probing the blot with actin cDNA [data not shown]). Although TβRII mRNA is present in Mac-2A cells, no TβRII protein is expressed on the plasma membrane.

Identification of a point mutation in one copy of the TβRII gene in Mac-2A cells. Using reverse transcription-PCR (RT-PCR) on poly(A)$^+$ RNA from Mac-2A cells, as well as high-stringency hybridization screening of a Mac-2A oligo(dT)-primed plasmid cDNA library, we cloned the TβRII receptor from Mac-2A cells. After sequencing the full-length product of 10 independent clones, we identified six encoding the wild-type receptor as well as four encoding a mutant TβRII with a single point mutation at nucleotide 1214 (A to G) causing a change of aspartate 404 to glycine (Fig. 2). This D-to-G mutation is localized in subdomain VII of the serine/threonine kinase domain. D-404 is conserved among all species from which the TβRII has been cloned (mink, rat, pig, and human) and in TβRI and ActRIb (Fig. 2). Using *Apa*I digestion of an RT-PCR product (below), we confirmed that Mac-2A cells express wt and mutant receptor in a 1:1 ratio (data not shown).

Stable Mac-2A cell lines overexpressing wt TβRII exhibit surface expression of TβRII and TβRI and show normal TGF-β-induced growth inhibition. Since Mac-2A cells express both the wt and the mutant TβRII, yet no TβRII is found on the cell surface, it would appear that D404G-TβRII blocks the appearance of the wt TβRII on the plasma membrane. To study this further, Mac-2A cells were infected with a recombinant retrovirus expressing the wt TβRII cDNA under the control of a murine retroviral long terminal repeat (LXSN) (32). Using this system, we selected for high-level expression of the wt receptor. Binding and cross-linking of ^{125}I-TGF-β1 to individual clones of neomycin-resistant infected cells (Fig. 3) showed that both TβRII and TβRI are expressed on the cell surface of infected cells but not on nontransfected Mac-2A cells. As expected, expression of transfected wt TβRII in Mac-2A cells restored binding to endogenous TβRI, since TβRI cannot bind TGF-β unless TβRII is expressed on the cell surface.

We tested TGF-β-induced growth inhibition in infected Mac-2A cells by both colony formation in methylcellulose (18) and [^{3}H]thymidine incorporation into DNA. Both assays (Fig. 4 and data not shown) showed that cell pools as well as individual clones expressing wt TβRII respond to TGF-β by inhibition of cell growth. They all show the same sensitivity to TGF-β as Mac-1 cells, an inhibition of colony formation by 65 to 80% (Fig. 4). Thus, overexpression of transfected wt TβRII in Mac-2A cells restores growth regulation by TGF-β via a functional cell surface complex of TβRII and TβRI. This finding suggests that the only defect in TGF-β signaling in Mac-2A cells resides in the type II receptor.

The D404G-TβRII is defective in signaling growth inhibition and cell surface expression. To investigate the ability of D404G-TβRII to signal growth inhibition, we expressed this receptor in Hep3B-TR cells (16), which are cells unaffected by TGF-β as a result of the loss of both TβRII alleles. We confirmed that stable expression of wt TβRII in Hep3B-TR cells restored TGF-β-mediated growth inhibition (16). In contrast, no line of Hep3B-TR cells stably expressing D404G-TβRII was growth inhibited by TGF-β1 (data not shown). Thus, D404G-TβRII is defective in at least one crucial step in TGF-β-mediated signal transduction.

To develop a system in which expression of D404G-TβRII could be reversibly induced or repressed, we inserted D404G-TβRII into a retroviral vector (SFG ttcE$^-$) (26) and generated a stable Mac-1 line [Mac-1(D404G^{tet})]. In this vector, D404G-TβRII expression is regulated by tetracycline via a tetracycline-repressible cytomegalovirus promoter and the constitutive expression of the tTA transactivator (12). We analyzed surface expression of TGF-β receptors in Mac-1(D404G^{tet}) cells after induction of D404G-TβRII. D404G-TβRII, which was tagged with a hemagglutinin epitope (45), could never be detected on the surface of Mac-1(D404G^{tet}) cells by binding and cross-linking to ^{125}I-TGF-β1 and immunoprecipitation with an anti-HA antibody. In contrast, wt TβRII, similarly epitope tagged, could readily be detected by this technique in similarly transfected Mac-1 cells (data not shown). This result indicates that intracellular maturation of D404G-TβRII to the cell surface is blocked.

Expression of D404G-TβRII in Mac-1 cells results in loss of growth inhibition by TGF-β. To demonstrate that D404G-TβRII, when expressed in Mac-1 cells at levels comparable to those of the endogenous wt TβRII (Fig. 5D), inhibits the function of wt TβRII, we measured growth inhibition by [^{3}H]thymidine incorporation. Cells were starved for 10 h and then labeled with [^{3}H]thymidine for 16 h in the absence or presence of TGF-β. In the presence of TGF-β, Mac-2A cells incorporated the same amount of [^{3}H]thymidine as in the absence of TGF-β. In contrast, incorporation of [^{3}H]thymidine by Mac-1 cells was inhibited in the presence of TGF-β (Fig. 5A). Next, duplicate populations of Mac-1(D404G^{tet}) cells were incubated for 4 days in the absence of tetracycline (to induce D404G^{tet}) or in the presence of tetracycline. The cells were then starved for 10 h and labeled with [^{3}H]thymidine in the presence or absence of TGF-β. As shown in Fig. 5A, upon expression of D404G-TβRII (i.e., without tetracycline), Mac-1(D404G^{tet})

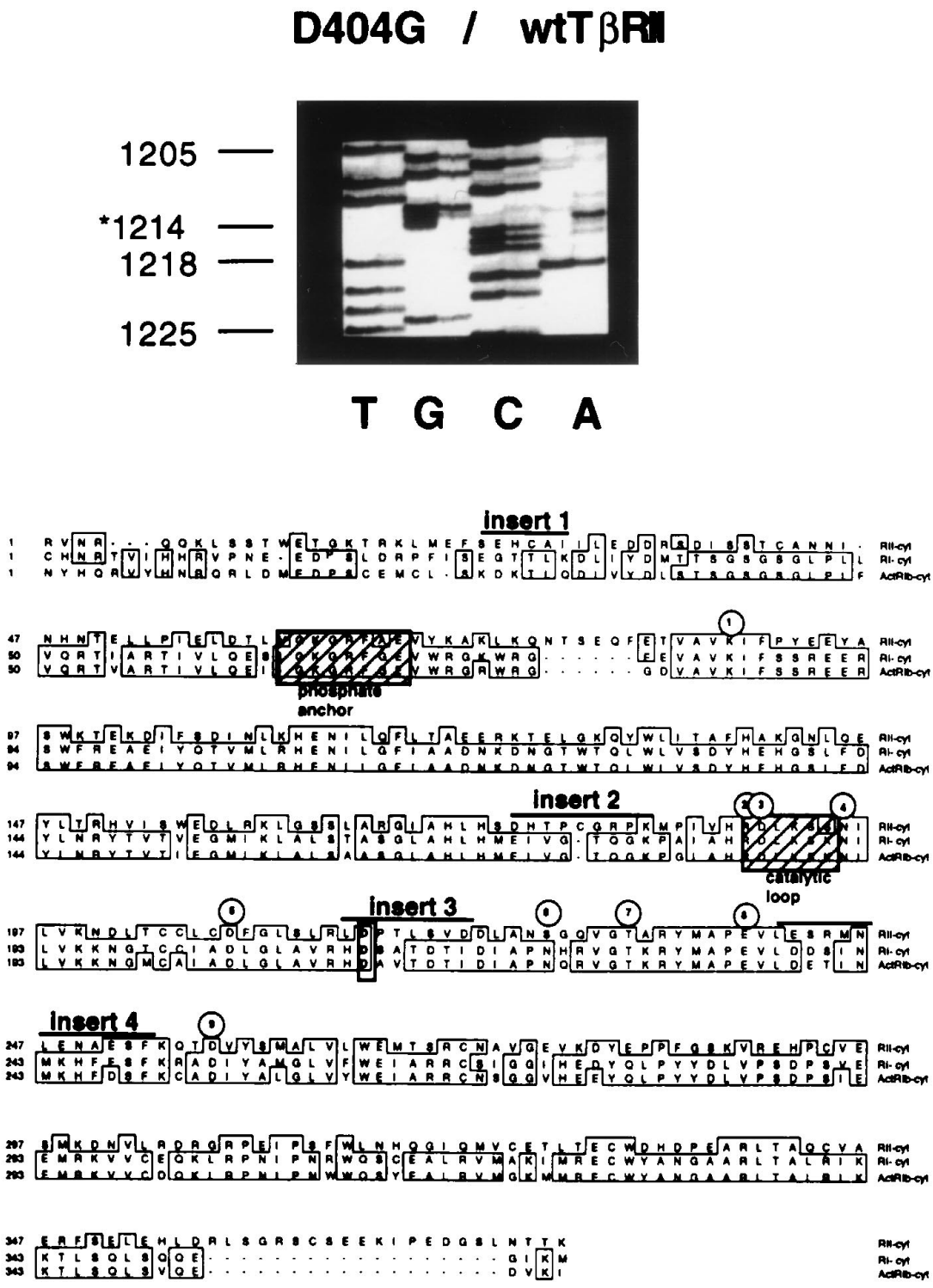


FIG. 2. Sequence analysis of D404G-TβRII, a mutant TβRII isolated from Mac-2A cells. The sequence of wt TβRII differs from that of the original cloned TβRII (called 3FF) (25) in three amino acids (S66P, A439V, and E554K); these amino acid changes have not been detected in any other cell line besides HepG2. 3FF and wt TβRII are equivalent in the ability to bind TGF-β and to signal (data not shown). A single point mutation at nucleotide position 1214 (A to G), the D404G change, was found in four cDNAs from Mac-2A cells. Sequence comparison of wt TβRII and the D404G mutant, both cloned from Mac-2A cells, is shown at the top. Inserts 1 to 4 refer to insertions in TβRII when the sequence was aligned with that of PK-A; alignment was done with PK-A since this was the first Ser/Thr kinase whose three-dimensional structure was determined. The position of D-404 is indicated with a box at the beginning of insert 3 of TβRII. The circled numbers indicate amino acids which are structurally and functionally crucial in the kinase domain of PK-A (22): 1, Lys-72 is essential for kinase activity; 2, Arg-165 is involved in p+1 peptide binding; 3, Asp-166 interacts with Asn 171; 4, Asn-171 contacts Asp-166 and Asp-184; 5, Asp-184 interacts with Lys-72, Asn-171, and Asp-166, and it communicates between the small and large lobes of the kinase; 6, Thr-197 is the autophosphorylation site in PK-A; 7, Thr-201 is close to the p+1 peptide binding site and close to Asp-166; 8, Glu-208 makes an ion pair to Arg-280 and stabilizes the p+1 peptide binding site; 9, Asp-220 stabilizes the catalytic loop. All of these amino acids are conserved in the TβRII except that Thr-197, the autophosphorylation site in PK-A, is a serine in TβRII. D-404 is located in that part of the receptor kinase where, by analogy with PK-A, the substrate binds.

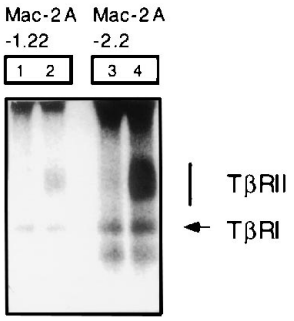


FIG. 3. Cell surface expression of TGF-β receptors in stable Mac-2A cell lines expressing wt TβRII. Binding and cross-linking of ^{125}I-TGF-β1 to stable Mac-2A cell lines overexpressing wt TβRII is shown for two individual cell clones (1.22 and 2.2). The positions of ligand-bound TβRII and TβRI are indicated. Binding and cross-linking were followed by immunoprecipitation with anti-TβRI (lane 1 and 3) or anti-TβRII (lane 2 and 4) antiserum, as described in the legend to Fig. 1.

cells exhibited no growth inhibition in response to TGF-β, while in the presence of tetracycline, they were growth inhibited by TGF-β to a level similar to that of Mac-1 cells, i.e., ~38%. (Because of the inducible nature of the tetracycline system, we cannot use the colony formation assay [Fig. 4], which shows the expected greater sensitivity of Mac-1 cells to growth inhibition by TGF-β.)

The time course with which TGF-β resistance is induced is shown in Fig. 5B. In this study, Mac-1(D404G^{tet}) cells were withdrawn from tetracycline for up to 7 days, and the response to TGF-β was measured as described above. Within 24 h of tetracycline depletion, Mac-1(D404G^{tet}) cells became resistant to TGF-β.

When complete TGF-β resistance was induced, readdition of tetracycline restored sensitivity to growth inhibition by TGF-β. Four days after readdition of tetracycline, Mac-1(D404G^{tet}) cells recovered sensitivity to TGF-β1 (Fig. 5B). Thus, sensitivity to the antiproliferative action of TGF-β was restored by switching off the expression of the dominant negative D404G-TβRII.

Induction of D404G-TβRII causes reduction of the signaling TGF-β receptor complex on the surface of Mac-1(D404G^{tet}) cells. Induction of D404G-TβRII transcription in Mac-1 (D404G^{tet}) cells by withdrawal of tetracycline caused a rapid decrease in cell surface expression of TβRII (Fig. 5C). The small amount of TβRII and TβRI remaining after 3 days of removal of tetracycline (Fig. 5C) probably represents surface expression by uninfected cells; we note that Mac-1(D404G^{tet}) is a population of selected virus-infected cells, not a clonal cell line. The decrease in surface expression of TβRII correlated with the loss in responsiveness to TGF-β seen after induction of D404G-TβRII (Fig. 5B). As in Mac-2A cells, from which D404G-TβRII was cloned, expression of this dominant inhibitory TβRII results in loss of the signaling TGF-β receptor complex. With readdition of tetracycline (i.e., repression of D404G-TβRII transcription) to Mac-1(D404G^{tet}) cells, there was a recovery of growth inhibition by TGF-β (Fig. 5B). Concomitantly, there was an increase of TβRII on the cell surface in a complex with TβRI (Fig. 5C). We conclude that induction of D404G-TβRII causes loss of wt TβRII from the cell surface and blocks responsiveness to the growth-inhibitory action of TGF-β.

To estimate the ratio of D404G-TβRII and endogenous wt TβRII in Mac-1(D404G^{tet}) cells expressing the mutant TβRII (i.e., in the absence of tetracycline), we isolated poly(A)$^+$ RNA from these cells after 4 days of cultivation without tetracycline. By RT-PCR, we amplified the 488-bp fragment spanning nucleotides 1057 to 1546 in the TβRII coding sequence. Because of the A1214G point mutation, an *Apa*I restriction site is present in the mutant but not in the wt TβRII cDNA. The amplified fragment was digested with *Apa*I; as measured by the relative amounts of the cut (representing D404G-TβRII) and uncut (representing wt TβRII) fragments, Mac-1 (D404G^{tet}) cells express the mutant and endogenous wt TβRII in a ratio of 1:1 (Fig. 5D). Using the same RT-PCR technique, we showed that the nontransfected Mac-2A cells from which D404G was isolated express wt and mutant receptors in the same ratio (1:1) (data not shown). As a control, Mac-1 cells generate a PCR fragment resistant to cutting by *Apa*I (Fig. 5D). Because of the PCR amplification, it is not possible to compare the amounts of the wt TβRII mRNA in the two cell lines. However, since both the wt TβRII and the D404G-TβRII mRNAs are expressed at the same level in Mac-1 (D404G^{tet}) cells (in the absence of tetracycline), we can conclude that D404G-TβRII indeed acts as a dominant inhibitor of endogenous TβRII.

Expression of D404G-TβRII in Hep3B cells results in loss of growth inhibition by TGF-β. To study the effect of D404G-TβRII in another cell line highly responsive to TGF-β1, we generated pools of Hep3B cells (16) stably infected with SFG ttcE$^-$ containing either D404G-TβRII or wt TβRII. In these cells, D404G-TβRII or wt TβRII expression is repressed by tetracycline. To analyze the effects of TGF-β1 on the growth of these cells, 10^5 cells were added to 10-cm-diameter plates and cultured for 6 days. In the absence of TGF-β, and in the presence or absence of tetracycline, both Hep3B(D404G^{tet}) and Hep3B(TβRII^{tet}) cells formed confluent monolayers. In the presence of TGF-β1, and in the presence or absence of tetracycline, neither parental Hep3B cells nor Hep3B(TβRII^{tet})

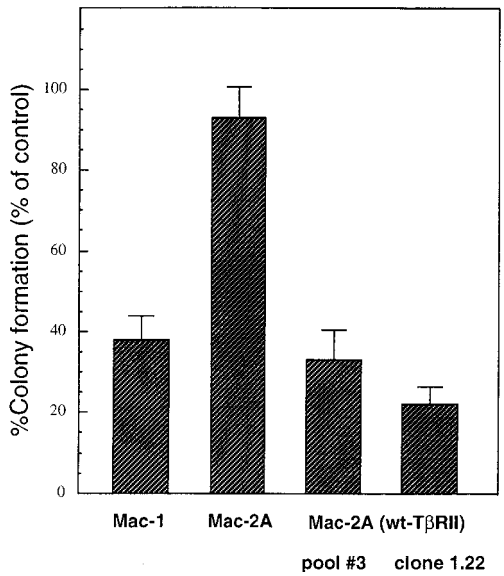
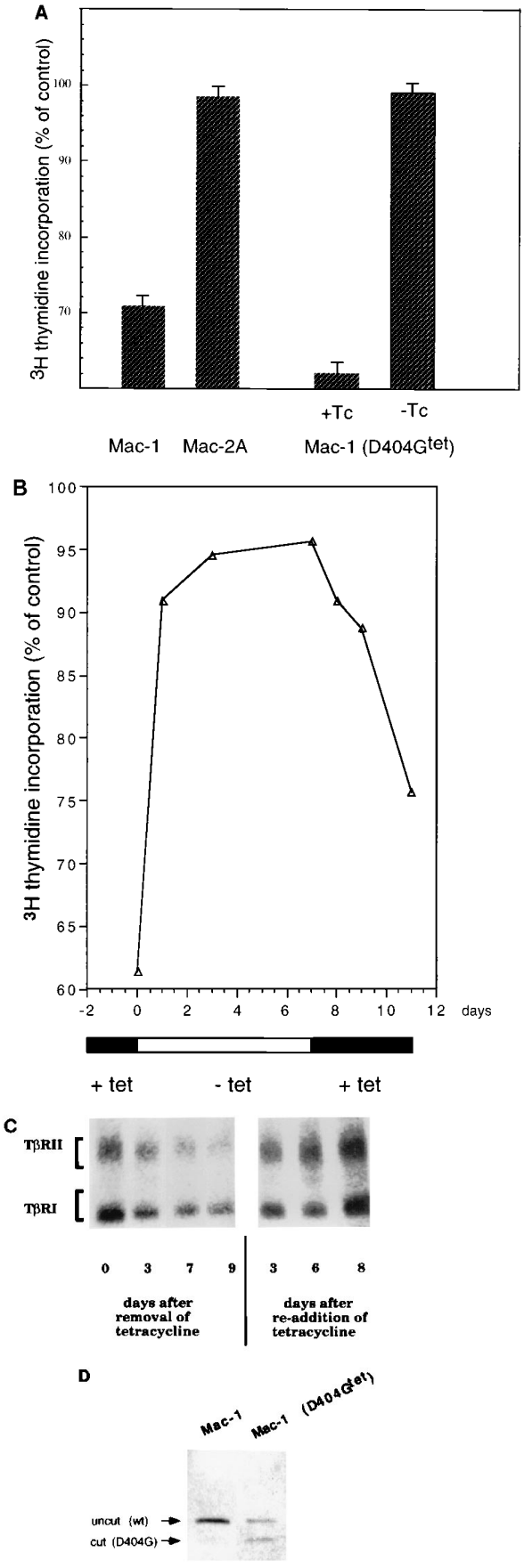


FIG. 4. Growth inhibition by TGF-β1 of Mac-1, Mac-2A, and Mac-2A lines stably expressing wt TβRII (pool 3 and clone 1.22). Inhibition of colony formation in response to TGF-β1 was measured after 10 to 14 days of incubation in 0.9% methylcellulose. Colonies of 20 or more cells were counted. Bars show standard deviations of four experiments. Percent colony formation compared with controls in the absence of TGF-β (100%) is shown. Pool 3 refers to a pool of neomycin-resistant Mac-2A cells selected after infection with the retrovirus LXSN-TβRII-HA, which expresses wt TβRII. Clone 1.22 refers to an individual cell clone from this pool which also expresses wt TβRII (Fig. 3, lanes 1 and 2).



cells were able to grow; no colonies were visible, and the culture dish contained only scattered cells (Fig. 6B). In contrast, in the absence of tetracycline, some Hep3B(D404G^{tet}) cells were able to grow. The plate was ~20% confluent and contained more than 600 colonies with more than 50 cells per colony; examples are shown in Fig. 6A. This result shows that expression of D404G-TβRII causes loss of sensitivity to the antiproliferative action of TGF-β1 in the TGF-β-sensitive Hep3B hepatoma cell line. D404G-TβRII acts as a dominant inhibitor of wt TβRII, not only in T cells, from which it was isolated from, but also in other highly sensitive cell lines such as Hep3B.

DISCUSSION

The Mac-1 and Mac-2A cell lines were generated from the same patient at different stages of development of a Ki1$^+$ anaplastic large-cell lymphoma. Mac-1 cells were isolated from an early stage at which time the subcutaneous tumors were indolent and slowly regressed. Mac-1 cells are sensitive to the growth-inhibitory actions of TGF-β and express on their surface substantial amounts of all three high-affinity TGF-β receptors, the proteoglycan TβRIII as well as the signaling receptor serine/threonine kinases TβRII and TβRI (18). Since TGF-β is secreted by most types of tissues, it is reasonable to suppose that the spontaneous regression of tumors at this stage is due, at least in part, to TGF-β released by the tumor or by neighboring cells. Mac-2A cells were isolated from a later malignant tumor; they do not express TβRII or TβRI on their surface and are resistant to the antiproliferative effect of

FIG. 5. Expression of D404G-TβRII in Mac-1 cells abolishes growth inhibition by TGF-β1 and surface expression of TGF-β receptors. (A) Growth inhibition by TGF-β1 of Mac-1 and Mac-2A cells and a stable Mac-1 line expressing D404G-TβRII under the control of a tetracycline-regulated promoter. After 10 h of starvation, cells were labeled with [^{3}H]thymidine for 16 h in the absence or presence of TGF-β1. The values plotted are the results from four experiments, each done in triplicate. In the presence of tetracycline (+Tc), Mac-1(D404G^{tet}) cells do not express D404G-TβRII; Mac-1(D404G^{tet}) were withdrawn from tetracycline (−Tc) for 4 days, causing induction of D404G-TβRII. Bars indicate [^{3}H]thymidine incorporation in the absence of TGF-β1, relative to control incorporation in the absence of TGF-β1. Standard errors are indicated. The 100% values correspond to about 50,000 cpm of ^{3}H incorporated in the absence of TGF-β1 and were similar (±10%) in all cell lines. (B) Time course of acquisition of TGF-β resistance in Mac-1(D404G^{tet}) cells. Mac-1(D404G^{tet}) cells were withdrawn from tetracycline (tet) for 0, 1, 3, and 7 days, and growth inhibition was assayed by using [^{3}H]thymidine incorporation. Growth in the presence of TGF-β1 is plotted as percentage of the value in the absence of TGF-β. The 100% values correspond to about 50,000 cpm of ^{3}H. At day 7, tetracycline was added back to the culture, and the growth response was measured by using the same assay after an additional 1, 2, and 4 days (days 8, 9, and 11). (C) Cell surface expression of TβRII in Mac-1(D404G^{tet}) cells after tetracycline depletion. Mac-1 (D404G^{tet}) cells were withdrawn from tetracycline for 0, 3, 7, and 9 days, and 10^7 cells were used for binding and cross-linking with ^{125}I-TGF-β1. The same assay was done with 10^7 tetracycline-withdrawn Mac-1(D404G^{tet}) cells to which tetracycline was added back at day 9, after another 3, 6, and 8 days in the presence of tetracycline (days 12, 15, and 17). Cell lysates were immunoprecipitated with anti-TβRII antiserum and analyzed by SDS-PAGE. The positions of ligand-bound TβRII and TβRI are indicated. Mac-1(D404G^{tet}) is a pool, not a clonal cell line, and the small amount of cell surface TβRII and TβRI remaining after 3 days of growth in the absence of tetracycline may represent cells that were not infected by the retrovirus and do not express the mutant D404G receptor (data not shown). (D) RT-PCR followed by *Apa*I digestion to estimate the ratio of D404G-TβRII and endogenous TβRII in Mac-1(D404G^{tet}) cells. Poly(A)$^+$ RNA was isolated from Mac-1 cells and Mac-1(D404G^{tet}) cells which were withdrawn from tetracycline for 4 days. One microgram of poly(A)$^+$ RNA was used for RT-PCR to amplify the 488-bp fragment of the TβRII coding sequence from nucleotides 1057 to 1546. The fragment was subsequently digested with *Apa*I, which cuts at position 1214 in the mutant but not in the wt TβRII cDNA. The restriction digest was run on a 1.2% agarose gel and stained with ethidium bromide. The sizes of the uncut wt TβRII and cut D404G-TβRII fragments are indicated with arrows.

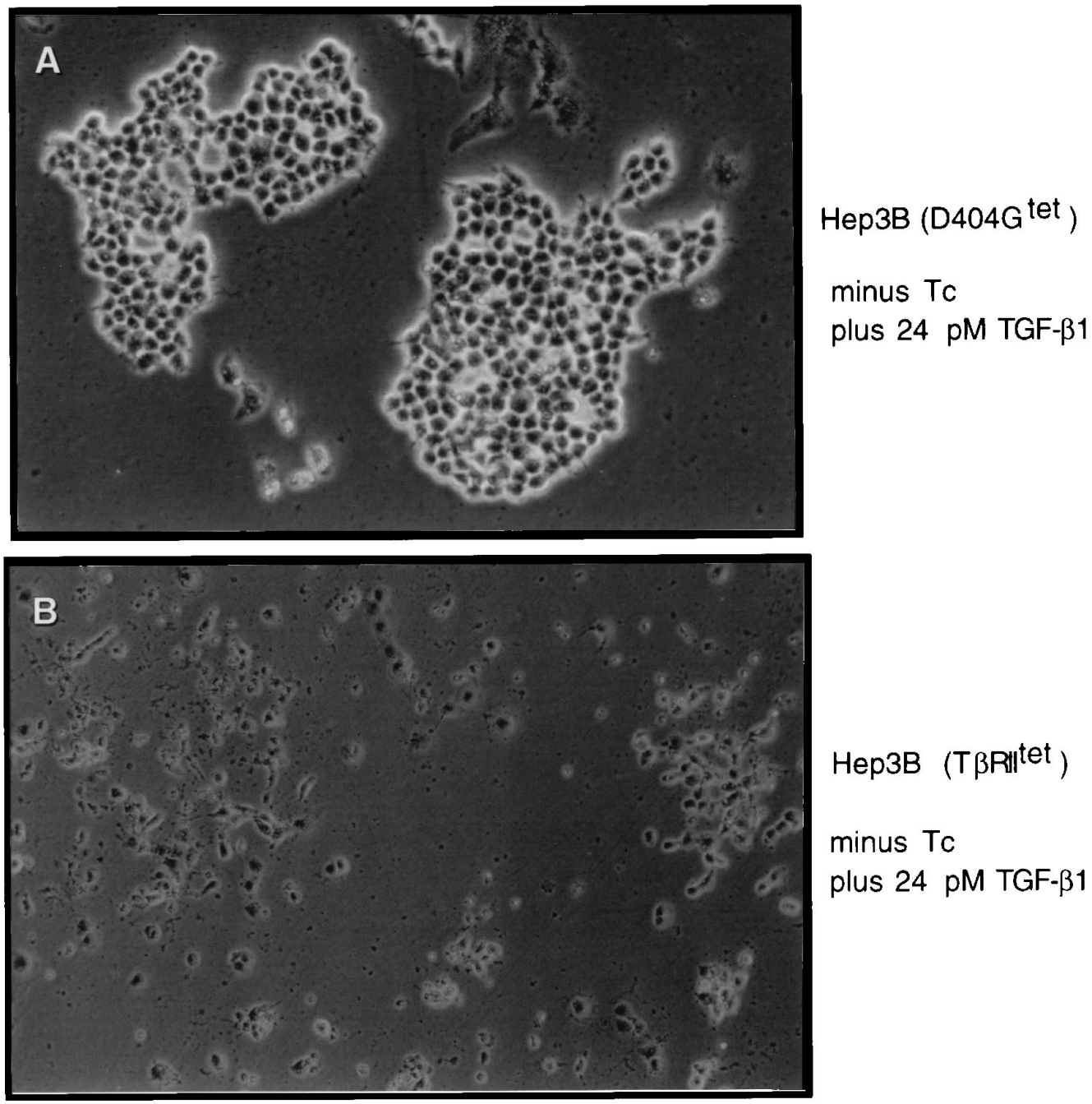


FIG. 6. Expression of D404G-TβRII in Hep3B cells abolishes growth inhibition by TGF-β1. Hep3B(D404G^{tet}) represents a pool of TGF-β-sensitive Hep3B cells stably expressing transfected D404G-TβRII under the control of a tetracycline (Tc)-regulated promoter. Hep3B(TβRII^{tet}) is a corresponding pool of stable Hep3B cells expressing the wt TβRII under the control of a tetracycline-regulated promoter. In the absence of tetracycline, Hep3B(D404G^{tet}) cells express D404G-TβRII, and Hep3B(TβRII^{tet}) express wt TβRII in addition to the endogenous receptor. To analyze the effects of TGF-β1 on the growth of these cells, 10^5 cells were added to 10-cm-diameter plates and cultured for 6 days in the presence of 24 pM TGF-β1. A representative view of clones of TGF-β resistant Hep3B(D404G^{tet}) cells is shown in panel A; about 20% of the petri plate is confluent, and it contained more than 600 colonies with more than 50 cells per colony. A representative view of the plate containing the corresponding Hep3B(TβRII^{tet}) cells is shown in panel B; only a few scattered cells and no colonies are visible. In the absence of TGF-β, and in the presence or absence of tetracycline, both Hep3B(D404G^{tet}) and Hep3B(TβRII^{tet}) cells formed confluent monolayers (not shown).

TGF-β. Here we showed that Mac-2A cells contain a near-normal amount of mRNA for TβRII and express both wt TβRII and a mutant TβRII with a single point mutation, D404G, in the kinase domain. D404G-TβRII acts as a dominant inhibitor of wt TβRII and blocks its appearance on the plasma membrane. Since TβRI is unable to bind and be cross-linked to TGF-β in the absence of TβRII, no TβRI can be detected on the surface of Mac-2A cells.

All defects in TGF-β signaling in Mac-2A cells are caused by the D404G mutation, since overexpression of wt TβRII in these cells restores growth sensitivity to TGF-β as well as expression of both TβRI and TβRII on the plasma membrane.

To show the dominant inhibitory properties of the mutant D404G-TβRII, we expressed it in the parental TGF-β-sensitive Mac-1 cells under control of a tetracycline-repressible promoter and also in the TGF-β-sensitive Hep3B hepatoma cells. Induction of D404G-TβRII in both cells caused loss of cell surface expression of TβRII, concomitant loss of cell surface TβRI, and loss of sensitivity to the growth-inhibitory actions of TGF-β. Induction of D404G-TβRII in Mac-1 cells mimicked the properties of the malignant Mac-2A cells. In contrast, when expression of D404G-TβRII was inhibited, sensitivity to TGF-β was rapidly restored concomitant with reappearance of cell surface TβRII and TβRI. The D404G mutation in TβRII defines a novel mechanism by which malignant cells become resistant to the growth-inhibitory action of TGF-β by means of a mutation in only one of the two TβRII alleles.

The properties of the D404G mutation are consistent with the fact that wt TβRII forms homodimers on the plasma membrane in the absence or presence of the TGF-β ligand (13). We do not know where in the cell these dimers are formed; one possibility is that this occurs in the endoplasmic reticulum. The mutant D404G-TβRII might bind to wt TβRII in an intracellular compartment, presumably the endoplasmic reticulum, and block maturation of the wt receptor to the cell surface. Others have reported that TβRII in malignant T cells of patients with Sézary syndrome shows defects in trafficking to the plasma membrane, suggesting this as a significant contribution to the development of the disease (4). Golgi complex-processed TβRII, including the proportion that is on the plasma membrane, has a half-life of about 90 min (44a). It is possible that D404G-TβRII has a shorter half-life and that interaction of the D404G-TβRII and wt receptors on the plasma membrane induces endocytosis and/or degradation of the wt receptor. The latter explanation is consistent with our finding (not shown) that, when overexpressed in COS cells, both the wt and D404G-TβRII receptors appear on the cell surface.

TβRII forms hetero-oligomeric complexes with TβRI that are essential for TGF-β signaling (47, 50). These may form only on the plasma membrane or also inside the cell. However, at least some of the hetero-oligomeric receptor complexes are formed only on the plasma membrane after TGF-β1 is bound to TβRII, causing TβRI to be recruited into the signaling complex (47). Other hetero-oligomeric complexes composed of TβRI and TβRII exist on the plasma membrane in absence of ligand; such preformed receptor complexes are able to bind TGF-β2 (37), while either the TβRII or TβRI receptor expressed in the absence of the other cannot bind this ligand (24).

The TβRII homodimer is a constitutively active kinase which undergoes *cis* autophosphorylation (6); when TβRI is recruited into a signaling complex, TβRI becomes phosphorylated by the TβRII receptor kinase (46). While this *trans* phosphorylation is thought to be important for TGF-β signaling and activation of TβRI for further signal transduction, there is no direct evidence for this presumption. We showed that the autophosphorylation activity in vivo of the D404G receptor kinase is normal, as is the ability of the D404G-TβRII to *trans* phosphorylate TβRI (data not shown).

The serine/threonine kinase domain of TβRII shows sequence homology to protein kinase A (PK-A), whose three-dimensional structure is known (22). The principal difference is that, relative to PK-A, TβRII contains four inserts that probably form loops in the three-dimensional structure. One of these inserts, insert 3, is localized, in PK-A, to the substrate binding site between kinase domains VII and VIII. D404, which is mutated to glycine in D404G-TβRII, is localized immediately N terminal to insert 3 in the Ser/Thr kinase domain of TβRII and thus presumably is near the substrate binding site. D404 is conserved between species and is also conserved in close relatives of TβRII, including TβRI (8) and ActRIb (5). As noted in Fig. 2, several other conserved amino acids that are critical for PK-A kinase function are located in this part of the molecule. Several conserved charged residues in this part of the TβRII kinase have been mutated to uncharged ones; most resulted in altered protein structure and loss of response to TGF-β (3). Several of these, when overexpressed, inhibited the signaling by wt TβRII, a property similar to that of the D404G mutant that we described here.

Loss of growth regulation by TGF-β is common in tumorigenesis; in all cases so far analyzed, this is due to loss of functional cell surface TβRII as a result of mutation in or loss of both genes encoding this protein. During the evolution of many tumors, chromosomal rearrangements that lead to loss of heterozygosity and result in homozygosity of the mutant gene occur. Tumor cell genomes have been screened for loci that repeatedly suffer from loss of heterozygosity during tumor progression (44). One such genetic locus associated with tumor progression is 3p21-p22 (20). Homozygous deletions at 3p21-p22 suggested that a tumor suppressor gene in this region is associated with lung carcinogenesis (49). The gene for the TβRII maps to chromosomal position 3p22, suggesting it is mutated in a variety of tumors (30). Together, the evidence indicates that the TβRII gene is a tumor suppressor gene which, like other such genes, encodes a growth-inhibitory protein whose loss during tumorigenesis leads to deregulated cell proliferation (14).

In addition to TβRII, eight tumor suppressor genes have been identified in a variety of tumor cell types (14). Of these, three encode nuclear proteins (p53, RB, and WT-1) and four encode cytoplasmic signal transduction molecules (DCC, APC, NF-1, and NF-2) (for a review, see reference 14). Only the *VHL* gene product is thought to be localized on the cell membrane. The well-characterized gene Rb is typical of most tumor suppressor genes in that both Rb alleles are inactivated in retinoblastomas (9). In contrast, in many types of tumors, only one of the two p53 alleles is mutated (23); such p53 alleles encode p53 proteins with point mutations that interfere with the function of wt p53 by destabilizing the normal protein structure. In these respects, TβRII is similar to p53, in that TβRII forms homo-oligomers, and as we have shown, the D404G mutation in TβRII generates a dominant-negative effect over its wt counterpart.

Many dominant inhibitory mutations in receptor protein-tyrosine kinases have been described. Different ones inhibit the function of the corresponding wt receptor by blocking intermolecular autophosphorylation after ligand-induced dimerization, by competition for downstream effector molecules, or by a combination of these mechanisms (19, 40, 41). D404G-TβRII is the first known mutation in TβRII to arise during malignant progression of a tumor. This is a late event in development of a Ki1+ large-cell lymphoma and appears to cause the tumor to escape the growth-inhibitory properties of TGF-β secreted by adjacent cells. The D404G-TβRII mutation interrupts the growth-inhibitory cascade at the level of the cell surface receptor expression, upstream of other tumor suppressor signal transduction proteins such as p53 or Rb.

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