family proteins contain common structural motifs (Sakai *et al.*, 1994; Ishino *et al.*, 1995; Alexandropoulos and Baltimore, 1996; Law *et al.*, 1996; Minegishi *et al.*, 1996). It has been reported that some classes of Tax-binding proteins have coiled-coil domain, through which they associate with the same domain of Tax (Chun *et al.*, 2000). Unexpectedly, SR showed the strongest affinity for Tax, while SD and HLH only displayed some affinity. The significance of the interaction between Tax and these domains, especially in tyrosine phosphorylation of Cas-L, remains unclear. This important issue will be a main focus in future experiments.

To explore the effect of Tax-Cas-L interaction, we next analysed Tax-dependent transcriptional activation. Tax is a potent transactivator of numerous host genes besides being a cis-activator of the HTLV-I long terminal repeat. Among them, we compared the effect of Cas-L on NF-κB (Tax-dependent and Tax-independent) and TRE, and our results indicated that Cas-L may selectively interfere with Tax-mediated transactivation of NF-κB pathway. Due to methodological limitations involving the use of patient samples and HTLV-I infected cells that endogenously co-express Cas-L and Tax, we employed Jurkat T cells, which do not have detectable Tax expression and low level of Cas-L expression. Since the luciferase assay is a transient expression system, there is a possibility that Cas-L-mediated inhibition of NF-κB transactivation by Tax might reflect an artifact of overexpression. However, based on the findings that overexpression of Cas-L did not significantly alter other stimulatory signals that also induce NF-κB pathway (PMA, TNF-α), we believe that the observed phenomenon may still be biologically relevant.

Evaluating potential mechanisms by which Cas-L partially suppresses the Tax-mediated transactivation of NF-κB, we presented data suggesting that subcellular distribution of Tax was altered by the co-expression of Cas-L. Another possibility is that Cas-L may interact with other components of the NF-κB signaling pathway. Among them, IKK-α and IKK-β are known to possess HLH, through which they may associate with Cas family proteins. The possible interaction between Cas family members and IKK proteins and its impact on Tax-mediated transactivation remain to be solved in future.

In the clinical setting, our results that Cas-L might inhibit Tax-mediated transactivation of NF-κB appear to be contrary to the findings that Tax-mediated induction of Cas-L might be responsible for the invasive behavior of leukemic cells in ATL.

Our working hypothesis is that Cas-L may be a negative feedback regulator of the Tax/NF-κB system, potentially analogous to its role in the TGF-β/Smad system (Liu *et al.*, 2000). In the TGF-β/Smad system, Cas-L inhibits Smad3-mediated signaling by TGF-β by acting as a cytosolic sequestering protein for Smad3. On the other hand, it has been shown recently that TGF-β induces Cas-L (Zheng and McKeown-Longo, 2002).

Another possible interpretation is that induction of Cas-L by Tax might be a *double-edged sword*, since suppression of NF-κB-mediated transcription acts against leukemogenesis, whereas enhancement of cell motility contributes to the invasive nature of leukemic cells, which results in the complex clinical course of ATL as described above. Indeed, the clinical course of ATL is atypical in that the duration from viral infection to the clinical manifestation of leukemia often spans several decades.

In light of the fact that NF-κB is constitutively active in HTLV-I-transformed cells and ATL-derived T cell lines, which show also elevated Cas-L expression and Tax, it is possible that Cas-L-mediated suppression of transactivation by Tax might be overcome by unknown mechanisms during leukemogenesis. The discrepancy remains to be solved as a future question. Understanding the ontogeny of expression of Tax and Cas-L *in vivo* remains a difficult but critical point for evaluating the role of Cas-L in cellular transformation.

Together with previous results, the present findings suggest that Cas-L protein plays a crucial role in the pathophysiology of ATL. Additional investigations focusing on these new findings may lead to the eventual development of possible therapeutic applications of Cas-L gene products in HTLV-I-related diseases in the future.

Materials and methods

Antibodies and reagents

Anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). MAbs against FAK, Pyk2/CAKβ, p130Cas, and paxillin were obtained from Transduction Laboratories (Lexington, KY, USA). A rabbit polyclonal Ab (pAb) against Cas-L was described previously (Ohashi *et al.*, 1998). All chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise stated. Mab against Tax (MI73) was kindly provided by Dr S. Yamaoka (Tokyo Medical and Dental University, Japan). Mab against c-myc tag (9E10) was produced from the hybridoma obtained from American Type Culture Collection (Manassas, VA, USA).

Plasmids

pCDSRα-CasL, pCDSRα-c-myc-Cas-L, pMT3-c-myc-Cas-L, pCDRSRα-p130Cas were described elsewhere (Tachibana *et al.*, 1997). The expression construct pCDSRα-c-myc-CasLΔSH3 lacks aa 1–60, pCDSRα-c-myc-CasLΔSD lacks aa 63–401, pCDSRα-c-myc-CasLΔC lacks aa 406–834 of Cas-L, respectively. In pCDSRα-c-myc-CasLF, Y629 and Y631 were mutated into F. pH2Rneo, pH2Rneo-wtTAX and pH2Rneo-40MTAX were kindly provided by Dr S Yamaoka (Tanaka *et al.*, 1990).

Cell lines, clinical samples from ATL patients and HTLV-I infected individuals

JPX-9 cells and JPX-M cells, kindly provided by Dr K Sugamura (Tohoku University School of Medicine), are derivatives of a human T cell line Jurkat, and have a stably integrated Tax gene under the control of a metallothionein

promoter (Nagata *et al.*, 1989). For the induction of Tax, the cells were cultured with 10 μM of $CdCl_2$.

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy volunteer donors, HTLV-I infected individuals, and patients with ATL by Ficoll-Hypaque density-gradient centrifugation, and washed with PBS. The diagnosis of ATL was based on clinical features, hematologic findings, and the presence of anti-HTLV-I antibodies in patient sera. All clinical samples were collected after informed consent was obtained.

Retroviral gene transfer

pMX-IRES-GFP and Plat-E were kindly provided by Dr T Kitamura (The Institute of Medical Science, The University of Tokyo, Japan) (Morita *et al.*, 2000). C-myc-tagged Cas-LΔSD, Cas-LΔC, and FAKCT (aa 709–1052) were subcloned into pMX-IRES-GFP. Plat-E cells were transfected using the Fugene6 reagent (Roche Applied Science, Indianapolis, IN, USA) with 1 μg of each retroviral vector. In total, 48 h post-transfection, the viral supernatant was used to infect PT-67 amphotropic packaging cell line (BD Biosciences Clontech, Palo Alto, CA, USA). After establishing PT-67 derived cell line stably producing each retrovirus, the supernatant was collected and used to infect JPX-9 cells at 32°C. After appropriate time period, GFP-positive cells were sorted by EPICS ELITE (Beckman Coulter, Inc., Tokyo, Japan).

Migration assays

Migration of JPX-9 cells was assayed using Transwell™ inserts with polycarbonate filter as described previously (Ohashi *et al.*, 1999). CS-1/GST fusion protein was described previously (Iwata *et al.*, 2002). The Transwell chambers containing 100 μl of 10^6 cells were inserted into wells filled with 600 μl of 0.6% BSA RPMI1640. After incubation at 37°C, the filters were stained with May-Giemsa solutions. The number of migrated cells to the lower side of the filters was counted microscopically in five high-power fields per insert at $\times 400$ magnification. Each experiment was performed in triplicate wells.

Immunoprecipitation and immunoblotting

For immunoprecipitation, cells were lysed in 1% Triton X-100 lysis buffer (Iwata *et al.*, 2002). Briefly, cellular lysates were incubated with appropriate first Ab at 4°C overnight, and then with protein A sepharose beads for 4 h. The beads were washed with 1% Triton X-100 lysis buffer, and heated to 95°C in the SDS–PAGE loading buffer. The supernatants were loaded onto 8% SDS–PAGE gels, electro-transferred onto PVDF membranes. After blocking, membranes were incubated with the primary Ab, then washed and incubated with HRP-conjugated anti-mouse IgG Ab. The membranes were developed by the enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ, USA).

Yeast two-hybrid screening

The two-hybrid analysis was carried out essentially as described previously (Durfee *et al.*, 1993), using pACTII (for GAL4 activator domain) and pBTM116 (for LexA DNA-binding domain). cDNA encoding full-length Cas-L was cloned into pBTM116. The resulting plasmid, pBTM116-Cas-L, was used as bait in a two-hybrid screen of a cDNA library of human HTLV-I infected T cell line (SLB-I) (BD Biosciences Clontech) in *S. cerevisiae* L40. Positive yeast clones were selected for histidine prototrophy and expression of β-galactosidase. Plasmids containing cDNA clones were rescued from yeast and characterized by DNA sequencing.

Immunofluorescence and confocal microscopy

Transfected cells cultured on the glass coverslips were fixed by immersion in 3.7% PBS-paraformaldehyde solution for 10 min, and permeabilized with PBS-0.1% Triton X-100 for 10 min. After blocking with 1% BSA-PBS, slips were incubated with the primary antibodies for 1 h, then washed and incubated with Cy3-, Texas Red- or FITC-conjugated appropriate second antibodies for one additional hour. After washing, slips were mounted and observed using an argon–krypton laser confocal microscope (FV500, OLYMPUS, Tokyo, Japan).

GST-pull down assay

The coding regions for Cas-L SH3 (aa 1–77), SD (substrate domain) (aa 62–359), SR (serine-rich region) (aa 350–639), CC (coiled-coil domain) (aa 635–700), HLH (helix–loop–helix domain) (aa 678–775), or CC/HLH/CT (c-terminal region) (aa 635–834) were PCR-amplified and subcloned into the pGEX-2T vector. GST-SRF contains aa 350–639 of Cas-L with Y629F and Y631F mutation. GST or GST-Cas-L domains expressed in *E. coli* were purified by adsorption to glutathione sepharose 4B beads. Those proteins preimmobilized on 5 μl of 50% slurry of the brads were incubated with cellular lysates from Tax-transfected 293T cells (50 μg total protein) for 4 h at 4°C. Following incubation, the bound matrices were washed with 1% Triton X-100 lysis buffer, and then subjected to 12% SDS–PAGE and immunoblotting, as described.

Transfection and luciferase assay

Transfection and Luciferase reporter assay were performed as described previously with some modification (Iwata *et al.*, 2002). Briefly, Dual-Luciferase™ Reporter Assay System (Promega, Madison, WI, USA) was used to measure Luciferase activity expressed by the experimental plasmids pNF-κB-Luc (Stratagene, La Jolla, CA, USA) and pHTLV-Luc (TRE-Luc). pNF-κB-Luc has $5\times$ tandem repeat of κB enhancer element and pHTLV-Luc contains 1–650 bp of HTLV-I LTR (U3-R part of U5). For the internal control, pRL-TK was employed. The enzyme activities of firefly luciferase and Renilla luciferase were measured by luminometer (Model TD-20/20, Turner Design, Inc., Sunnyvale, CA, USA).

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Regulation of p38 Phosphorylation and Topoisomerase IIα Expression in the B-Cell Lymphoma Line Jiyoye by CD26/Dipeptidyl Peptidase IV Is Associated with Enhanced *In vitro* and *In vivo* Sensitivity to Doxorubicin

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Abstract

CD26 is a M_r 110,000 surface-bound glycoprotein with diverse functional properties, including having a key role in normal T-cell physiology and the development of certain cancers. In this article, we show that surface expression of CD26, especially its intrinsic dipeptidyl peptidase IV (DPPIV) enzyme activity, results in enhanced topoisomerase IIα level in the B-cell line Jiyoye and subsequent *in vitro* sensitivity to doxorubicin-induced apoptosis. In addition, we show that expression of CD26/DPPIV is associated with increased phosphorylation of p38 and its upstream regulators mitogen-activated protein kinase kinase 3/6 and apoptosis signal-regulating kinase 1 and that p38 signaling pathway plays a role in the regulation of topoisomerase IIα expression. Besides demonstrating that CD26 effect on topoisomerase IIα and doxorubicin sensitivity is applicable to cell lines of both B-cell and T-cell lineages, the potential clinical implication of our work lies with the fact that we now show for the first time that our *in vitro* results can be extended to a severe combined immunodeficient mouse model. Our findings that CD26 expression can be an *in vivo* marker of tumor sensitivity to doxorubicin treatment may lead to future treatment strategies targeting CD26/DPPIV for selected human cancers in the clinical setting. Our article thus characterizes the biochemical linkage among CD26, p38, and topoisomerase IIα while providing evidence that CD26-associated topoisomerase IIα expression results in greater *in vitro* and *in vivo* tumor sensitivity to the antineoplastic agent doxorubicin. (Cancer Res 2005; 65(5): 1973-83)

Introduction

CD26 is a M_r 110,000 type II cell surface glycoprotein with diverse functional properties, which is widely expressed on various tissues, including lymphocytes, with its extracellular domain encoding a membrane-associated dipeptidyl peptidase IV (DPPIV) activity that cleaves selected biological factors to alter their functions (1). It plays an important role in T-cell biology through its physical and functional association with molecules involved in T-cell signal transduction processes (1–6). Recent findings suggest that CD26/DPPIV has a role in the development of certain neoplasms, being overexpressed in certain aggressive T-cell malignancies (7, 8), B-chronic lymphocytic leukemia (9), and thyroid carcinoma (10). On the other hand, loss or decreased surface expression of CD26/DPPIV is found in prostate cancer (11), colorectal carcinoma (12), and melanomas (13). Meanwhile, investigators have shown that DPPIV expression in melanoma and non–small cell lung carcinoma leads to inhibition of tumorigenicity, whereas DPPIV expression in ovarian carcinoma cells reduces i.p. dissemination of carcinoma cells and prolongs survival time (14–16). Topoisomerase IIα is an intracellular protein with a key role in proliferation and is a target for various antineoplastic agents (17). We found recently that CD26/DPPIV expression on the T-cell line Jurkat is associated with increased topoisomerase IIα level, leading to a concomitant enhancement in *in vitro* sensitivity to topoisomerase II inhibitors (18–20).

The family of mitogen-activated protein kinases (MAPK) plays a very important role in the signal pathways of cell proliferation, differentiation, survival, and apoptosis (21). Three major molecules belong to this family: extracellular signal-regulated kinase (ERK) 1/2 (p44/p42), c-Jun NH_2-terminal kinase (JNK/stress-activated protein kinase), and p38 MAPKs. In general, the ERK pathway mediates primarily cell growth and survival signals and promotes induction of cell differentiation under certain circumstances. On the other hand, both JNK and p38 pathways, which comprise the stress-activated protein kinase family, generally mediate proapoptotic, growth inhibitory signals and proinflammatory responses. However, p38 also induces antiapoptotic, proliferative, and cell survival signals under certain conditions (22, 23). Of note is the fact that certain antineoplastic agents, such as doxorubicin and cisplatin, induce p38-mediated apoptosis (23, 24). CD26/DPPIV is also associated with p38 signaling in certain instances. Inhibition of DPPIV enzyme activity resulted in p38 activation, leading subsequently to transforming growth factor-β1 expression and secretion (25). Meanwhile, ERK was phosphorylated and activated in CD26 Jurkat transfectant following treatment with anti-CD26 antibody (26).

Extending our previous findings in this study, we use the Burkitt B-cell lymphoma line Jiyoye to characterize the effect of CD26 expression on topoisomerase IIα and p38. We show that CD26 expression on CD26 Jiyoye transfectants is associated with enhanced topoisomerase IIα level and increased sensitivity to the antineoplastic agent doxorubicin. We also show that CD26 expression results in increased p38 phosphorylation, associated with increased phosphorylation of the upstream regulators MAPK

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kinase (MKK) 3/6 and apoptosis signal-regulating kinase 1 (ASK1). Inhibition of p38 phosphorylation decreases topoisomerase IIα expression, suggesting a role for p38 in the regulation of topoisomerase IIα. Finally, studies using a severe combined immunodeficient (SCID) mouse xenograft model with CD26 Jiyoye transfectants show that CD26 expression is associated with enhanced survival following treatment with low doses of doxorubicin. Our data thus characterize the biochemical linkage among CD26, p38, and topoisomerase IIα while suggesting a potential role for CD26 in the clinical setting in the treatment of selected malignancies.

Materials and Methods

Animals, Cells, and Reagents. Human Burkitt B-cell lymphoma cell line Jiyoye and human anaplastic large T-cell lymphoma cell line Karpas-299 were obtained from American Type Culture Collection (Rockville, MD). Jiyoye cells were maintained in culture medium, which consisted of RPMI 1640 supplemented with 20% FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO_2. Karpas-299 cells were maintained in RPMI 1640 supplemented with 10% FCS and penicillin-streptomycin at 37°C and 5% CO_2. Female CB-17 SCID mice were obtained from Taconic Farms, Inc. (Germantown, NY) at 3 weeks of age and were housed in microisolator cages, and all food, water and bedding were autoclaved before use. Annexin V-FITC, anti-poly(ADP-ribose) polymerase, phycoerythrin-conjugated anti-CD19, and phycoerythrin-conjugated anti-CD8 were from BD PharMingen (San Diego, CA); FITC-conjugated anti-CD26 was from Caltag (Burlingame, CA); anti-actin was from Sigma Chemical Co. (St. Louis, MO); anti-topoisomerase IIα was from Roche (Indianapolis, IN); antibodies against p38, phospho-p38, ERK1/2 (p44/p42 MAPK), phospho-ERK1/2, JNK, phospho-JNK, MKK3, phospho-MKK3/MKK6, ASK1, and phospho-ASK1 (Ser^{83} and Ser^{967}) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The p38 inhibitor SKF86002 was from Calbiochem (La Jolla, CA). Substrate for DPPIV, Gly-Pro-*p*-nitroanilide-tosylate, was purchased from WAKO (Osaka, Japan). Doxorubicin was purchased from Calbiochem and was dissolved in sterile PBS. All oligonucleotides were synthesized with Invitrogen (Carlsbad, CA).

Establishment of CD26 Transfectants. The CD26 cDNA insert was prepared from the plasmid pSRa-26 as described previously (27). 5′ Flanking region of CD26 (28) was extended and amplified by the PCR used with primers Ad1 (CCCGGGTCTGCCTGCGCTCCTTCTCTGAACGCTCACTTCCGAGGAGACGCCGACGATGAAGACACC) and R3 (GCGCGGTACCCTAAGGTAAAGAGAAACATTG). Through site-directed gene mutagenesis method (29), mutant CD26 containing an alanine at the putative catalytic Ser^{630} was prepared with primers Ad1, R3, and SA (AATTTGGGGCTGGGCATATGGAGGGTACGT), resulting in a mutant CD26 positive-DPPIV negative (S630A; ref. 30). After the sequences were confirmed, CD26 or CD26S630A fragment was inserted into retroviral vector pLNCX2 containing a neomycin-selection marker, which was obtained from Clontech Laboratories, Inc. (Palo Alto, CA). To generate the recombinant, the dualtropic retroviral packaging cell line GP2-293 was transfected by Plus Reagent (Invitrogen) and LipofectAMINE reagent (Invitrogen) with p10A1 (Clontech Laboratories) and recombinant vectors as per manufacturer's protocol. Seventy-two hours after transfection, the supernatants containing retrovirus expressing CD26 or CD26S630A were collected, filtered through a 0.45 μm syringe filter, and used to transduce target cells. To transduce Jiyoye cells, viral supernatant was added with polybrene (final concentration 8 μg/mL, Sigma Chemical) and the cells were incubated at 37°C for 24 hours; then, the medium was replaced with fresh medium containing G418 (1.5 mg/mL, Life Technologies, Grand Island, NY).

Small Interfering RNA Studies. To design target-specific small interfering RNA (siRNA) duplexes, we selected sequences of the type AA (N19; N, any nucleotide) from the open reading frame of CD26 mRNA (accession no. NM 001935) by Dharmacon siDESIGN Center (Lafayette, CO). We selected the target sequence from 1,768 to 1,786 downstream of the start codon of CD26 mRNA. Inserted siRNA oligonucleotide of pSilencerRetroQ vector (Clontech Laboratories) was designed according to manufacturer's protocol. The inserted sequence was as follows: sense GATCCGATCATGCATGCAATCAACTTCAAGAGAGTTGATTGCATGCATGATCTTTTTTGGAAG [sense siRNA (CD26-siRNA)] and antisense AATTCTTCCAAAAAAGATCATGCATGCAATCAACTCTCTTGAAGTTGATTGCATGCATGATCG. Moreover, missense siRNA [mis-siRNA (mis-CD26-siRNA)] at 3 nt was prepared to examine nonspecific effects of siRNA duplexes. Inserted sequence was as follows: sense GATCCGATCTTGCAAGCAAACAACTTCAAGAGAGTTGTTTGCTTGCAAGATCTTTTTTGGAAG and antisense AATTCTTCCAAAAAAGATCTTGCAAGCAAACAACTCTCTTGAAGTTGTTTGCTTGCAAGATCG. These sense and antisense primers were hybridized and then inserted into pSilencerRetroQ vector. After all sequences were confirmed, CD26-siRNA retrovirus was produced by the same method as above, and Karpas-299 cells were transduced and selected with puromycin (0.4 μg/mL, Clontech Laboratories).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay. Cell growth assay was done as described previously (31). Cells were incubated in 96-well plates in the presence of culture medium alone or culture medium with doxorubicin at the indicated concentrations for a total volume of 100 μL (50,000 cells per well). After 72 hours of incubation at 37°C, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (25 μL) was added to the wells at a final concentration of 1 mg/mL. The 96-well plates were then incubated for 2 hours at 37°C followed by the addition of 100 μL extraction buffer. After overnight incubation at 37°C, absorbance measurements at 570 nm were done, with SE of the triplicate well being <15%.

Cytotoxicity index was calculated as follows:

$$\text{Cytotoxicity index (\% of control)} = 1 - \frac{A_{570\ nm}\ \text{of treated cells}}{A_{570\ nm}\ \text{of control cells}} \times 100\%$$

Immunofluorescence. All procedures were carried out at 4°C and flow cytometric analyses were done (FACScan, Becton Dickinson, San Jose, CA) as described previously (32). Cells were stained with FITC-conjugated anti-CD26 antibody and washed twice with PBS and then with goat anti-mouse IgG FITC (Coulter, Fullerton, CA). Cells were then washed twice with PBS before flow cytometric analysis. Negative control samples were stained with second antibody alone.

Annexin V/Propidium Iodide Assays. Exposure of phosphatidylserine residues was quantified by surface Annexin V staining as described previously (33). Briefly, cells were washed in binding buffer [10 mmol/L HEPES (pH 7.4), 2.5 mmol/L $CaCl_2$, 140 mmol/L NaCl], resuspended in 100 μL, and incubated with 0.5 μL/mL Annexin V-FITC and 2.5 μg/mL propidium iodide (PI) for 15 minutes in the dark. Cells were then washed again and resuspended in 400 μL binding buffer; then, flow cytometric analysis was done. A total of 10,000 cells were acquired per sample and data were analyzed using CellQuest software (BD PharMingen). Cells in early stages of apoptosis were Annexin V positive, whereas cells that were Annexin V and PI positive were in late stages of apoptosis (34).

SDS-PAGE and Immunoblotting. After incubation at 37°C in culture medium, Jiyoye-vector control, Jiyoye-wild-type (wt) CD26 transfectant, and Jiyoye-SACD26 transfectant were harvested, washed with PBS, and lysed in lysis buffer consisting of 1% NP40, 0.5% deoxycholate, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, 10 μg/mL aprotinin, 50 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, and 1 μg/mL pepstatin. After incubating on ice for 5 minutes, nuclei were removed by centrifugation and supernatants were collected as whole cell lysates. Sample buffer (4×) consisting of 20% glycerol, 4.6% SDS, 0.5 mol/L Tris (pH 6.8), 4% β-mercaptoethanol, and 0.2% bromophenol blue was added to the appropriate aliquots of supernatants. After boiling, protein samples were submitted to SDS-PAGE analysis on appropriate gel under standard conditions using mini-Protein II system (Bio-Rad, Richmond, CA). For each experiment, each lane was loaded with equal amount of protein. For immunoblotting, the proteins were transferred onto nitrocellulose (Immobilon-P, Millipore, Billerica, MA).

After blocking for 1 hour at room temperature or overnight at 4°C in blocking solution consisting of 5% bovine serum albumin or 5% dry milk in 0.1% Tween 20-TBS, membranes were blotted with the appropriate primary antibodies diluted in blocking solution for 1 hour at room temperature or overnight at 4°C. Membranes were then washed with Tween 20-TBS, and appropriate secondary antibodies diluted in Tween 20-TBS were then applied for 1 hour at room temperature. Secondary antibody was goat anti-rabbit or goat anti-mouse horseradish peroxidase conjugates (DAKO, Kyoto, Japan). Membranes were then washed with Tween 20-TBS, and proteins were detected using an enhanced chemiluminescence system according to the manufacturer's instructions (Pierce, Rockford, IL). Membranes were exposed to Hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ).

DPPIV Enzyme Activity Assay. As described previously (16), DPPIV enzyme activity was measured spectrophotometrically using Gly-Pro-*p*-nitroanilide-tosylate, a substrate for DPPIV. A 1× PBS-washed whole cell suspension was prepared, and 5×10^5 cells were resuspended in 200 μL PBS into 96-well plate; then, Gly-Pro-*p*-nitroanilide-tosylate was added at a final concentration of 0.24 mmol/L. The absorption was measured at 405 nm using microplate spectrophotometer (BIO-TEK Instruments, Inc., Winooski, VE) twice just before the addition of the substrate and after 60-minute incubation at 37°C. DPPIV enzyme activity was calculated from the increase of absorption between 0 and 60 minutes.

Preparation of Nuclear Extracts for Detection of Topoisomerase IIα Protein Level. For detection of topoisomerase IIα by immunoblotting, isolation of nuclear fractions from Jiyoye-CD26 transfectants was prepared as follows. In brief, 10×10^6 cells were harvested and allowed to swell for 15 minutes on ice in cytoplasmic extraction buffer (10 mmol/L HEPES, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine). Then, NP40 (final concentration 0.3%) was added to the cell suspension and vortexed for 10 seconds. After 2 minutes of centrifugation at 16,000 × *g*, the supernatant was removed. The pellet was then incubated with nuclear extraction buffer (20 mmol/L HEPES, 400 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine) for 30 minutes on ice with intermittent vortexing. The suspension was centrifuged at 16,000 × *g* for 5 minutes, and the supernatant was saved as the nuclear extract. SDS-PAGE and immunoblotting were then done on the nuclear extracts. Each lane was equally loaded with 10 μg protein.

***In vivo* Experiments.** All mice were pretreated by i.p. route with 0.2 mL anti-asialo-GM1 polyclonal antisera 25% (v/v, WAKO) 1 day before tumor transplant to eliminate host natural killer cell activity and facilitate tumor engraftment (35). On day 0, 7×10^6 Jiyoye-wtCD26 transfectant cells or Jiyoye-vector control cells were then inoculated by i.p. injection. Following tumor cell inoculation, SCID mice then received saline or doxorubicin in saline by i.p. injection at 0.5 mg/kg on days 1 and 15. Tumor bearing mice were monitored for tumor development and progression, and moribund mice were euthanized, with necropsies being done for evidence of tumors. In addition, mice with visible or palpable tumors measuring 15 mm at its smallest dimension were euthanized, with necropsies done to minimize suffering to the mice.

Results

Expression of CD26/DPPIV and Topoisomerase IIα in Jiyoye-CD26 Transfectants. Following transfection of the human Burkitt B-cell lymphoma cell line Jiyoye with the retroviral vector pLNCX2 as described in Materials and Methods, CD26/DPPIV status is evaluated. Parental Jiyoye cells and pLNCX2-only Jiyoye transfectants (Jerome-vector control) do not express detectable amount of CD26 as determined by cell surface staining. Meanwhile, Jiyoye-wtCD26 transfectants have high level of CD26 surface expression, and Jiyoye-S630A (SACD26) transfectants express the catalytically inactive variant of CD26 (Fig. 1*A*). On the other hand, only the Jiyoye-wtCD26 transfectants express DPPIV enzyme activity, with Jiyoye-vector control and Jiyoye-SACD26 transfectants having no detectable DPPIV activity (Fig. 1*B*). Consistent with our previous findings that CD26 expression is associated with increased topoisomerase IIα level in CD26 transfectants of the T-cell leukemia line Jurkat (19, 20), Jiyoye-wtCD26 transfectants also express higher level of topoisomerase IIα than Jiyoye-vector control or Jiyoye-SACD26 transfectants (Fig. 1*C*). By demonstrating that CD26 expression, particularly its DPPIV enzyme activity, is associated with enhanced topoisomerase IIα expression in the B-cell line Jiyoye, our findings indicate that a relationship between these key proteins is potentially found in a wide variety of tumor types. Furthermore, our data suggest a potential role for CD26/DPPIV in the treatment of malignancies of both B-cell and T-cell lineages.

Enhancement of Doxorubicin-Mediated Apoptosis in Jiyoye-CD26 Transfectants. To elucidate the potential consequence of the CD26-topoisomerase IIα association, we investigated the effect of CD26/DPPIV surface expression on doxorubicin sensitivity of Jiyoye-CD26 stable transfectants. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide uptake assays show that Jiyoye-wtCD26 transfectants display significantly increased sensitivity to doxorubicin compared with Jiyoye-vector control. In addition, Jiyoye-SACD26 transfectant, with CD26 mutated at the DPPIV catalytic site (S630A), is less sensitive to doxorubicin than Jiyoye-wtCD26 transfectants, consistent with the key role played by the DPPIV enzyme activity in increasing topoisomerase IIα level and subsequent drug sensitivity (Fig. 2*A*). Meanwhile, Annexin V-PI assays show greater doxorubicin-induced apoptosis for Jiyoye-wtCD26 transfectants than Jiyoye-vector control cells or Jiyoye-SACD26 transfectants (Fig. 2*B*). Furthermore, Western blot analyses show that Jiyoye-wtCD26 transfectants exhibit greater poly(ADP-ribose) polymerase cleavage with doxorubicin treatment than Jiyoye-vector cells (Fig. 2*C*) Taken together, these data show that surface expression of CD26/DPPIV on the B-cell lymphoma line Jiyoye directly enhances cellular sensitivity to doxorubicin and drug-induced apoptosis.

Effect of CD26/DPPIV Surface Expression on the p38 Signaling Pathway. Because CD26 signaling involves MAPK in certain experimental conditions (25, 26), we evaluated the status of p38, ERK, and JNK in Jiyoye-CD26 transfectants. Figure 3 shows that Jiyoye-wtCD26 transfectants exhibit greater phosphorylation of p38 compared with Jiyoye-vector or Jiyoye-SACD26 transfectant in the absence of any extrinsic stimulation. In contrast to p38, there is no difference in the phosphorylation status of ERK and JNK among the cells incubated in culture medium, confirming that p38 is selectively phosphorylated in the presence of CD26, particularly its DPPIV enzyme activity.

To further confirm our findings with the Jiyoye-CD26 transfectants that CD26 presence enhances p38 phosphorylation, we established Karpas-299-CD26-siRNA as described in Materials and Methods. Whereas parental Karpas-299 cells have high level of CD26 surface expression, as shown previously (35), Karpas-299-CD26-siRNA cells exhibit low level of CD26 and a concomitant decrease in DPPIV enzyme activity (Fig. 4*A* and *B*). Importantly, Karpas-299-CD26-siRNA transfectants display decreased p38 phosphorylation level compared with parental Karpas-299 and missense Karpas-299-CD26-siRNA (Karpas-299-mis-CD26-siRNA) transfectants with unaltered CD26/DPPIV levels (Fig. 4*C*). Together, our findings show a clear association between CD26/DPPIV expression and increased p38 phosphorylation.

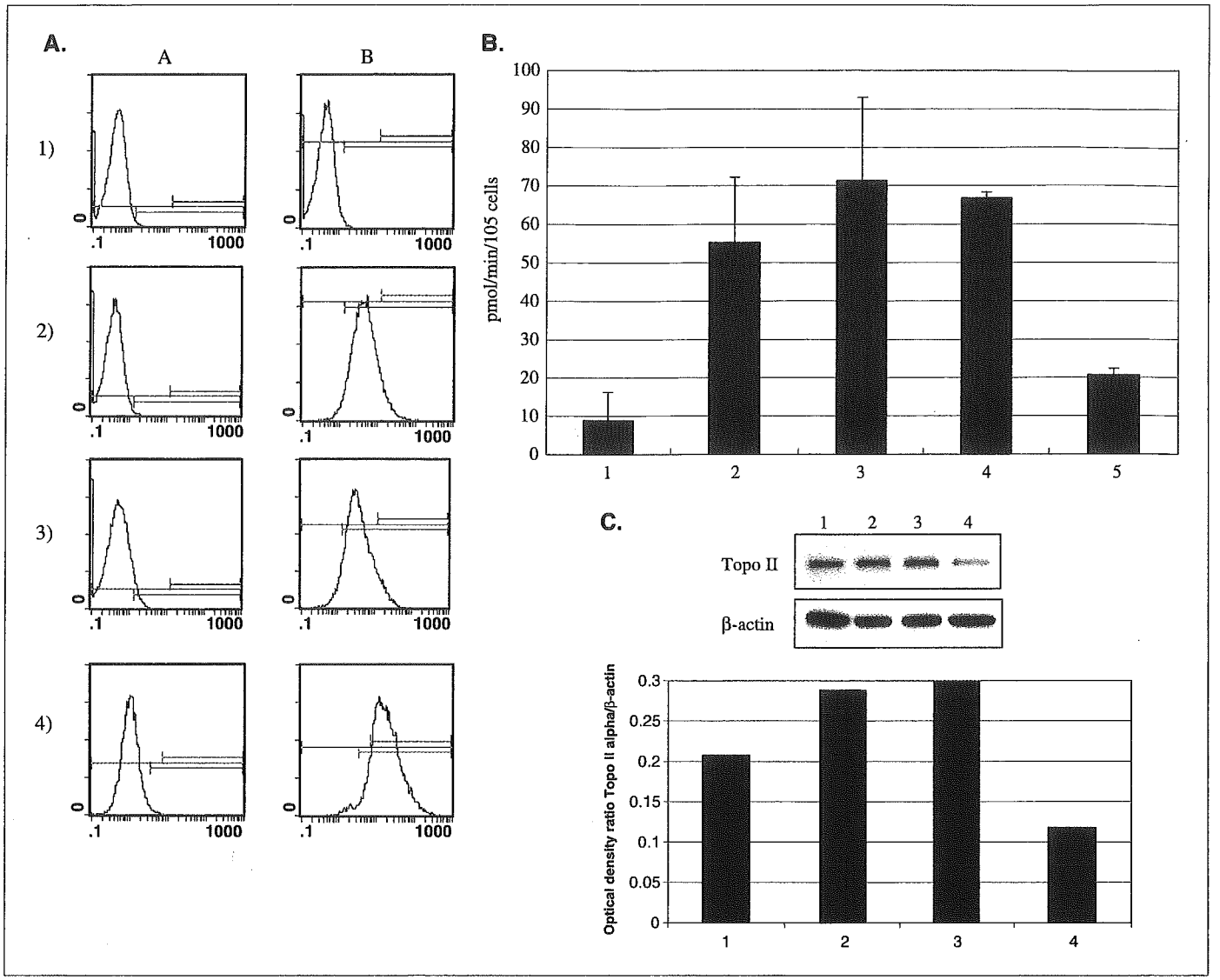

Figure 1. CD26 expression and DPPIV activity on Jiyoye-CD26 transfectants. *A*, CD26 expression on Jiyoye-CD26 transfectants. Jiyoye cells were evaluated for CD26 expression by flow cytometry as described in Materials and Methods. *1)*, Jiyoye-vector control; *2)*, Jiyoye-wtCD26-1; *3)*, Jiyoye-wtCD26-2; *4)*, Jiyoye-SACD26 transfectant (*A*, negative control; *B*, anti-CD26 antibody). Representative of three different experiments. *B*, DPPIV activity on Jiyoye-CD26 transfectants. Jiyoye cells were evaluated for DPPIV activity as described in Materials and Methods. *1)*, Jiyoye-vector control; *2)*, Jiyoye-wtCD26-1; *3)*, Jiyoye-wtCD26-2; *4)*, Jiyoye-wtCD26-3; *5)*, Jiyoye-SACD26 transfectant. *Columns*, means of three separate experiments. *C*, topoisomerase IIα expression on Jiyoye-CD26 transfectants Jiyoye cells were incubated in culture medium, and nuclear extracts were collected for immunoblotting studies to evaluate topoisomerase IIα protein levels, with β-actin as controls, as described in Materials and Methods. Each lane was equally loaded with 10 μg protein. *Lane 1*, Jiyoye-SACD26; *lane 2*, Jiyoye-wtCD26-1; *lane 3*, Jiyoye-wtCD26-2; *lane 4*, Jiyoye-vector. Representative of three different experiments.

The p38 signaling pathway is activated by various stress agents. To further characterize the effect of CD26/DPPIV on p38-mediated signaling, we stimulated Jiyoye-CD26 transfectants with UVC irradiation and phorbol 12-myristate 13-acetate (PMA). As shown in Fig. 5*A*, whereas Jiyoye-wtCD26 transfectants have greater level of baseline p38 phosphorylation than Jiyoye-vector control or Jiyoye-SACD26 transfectant, as shown above, Jiyoye-wtCD26 transfectants and Jiyoye-vector control exhibit enhanced p38 phosphorylation when stimulated with both UVC irradiation and PMA. Interestingly, Jiyoye-SACD26 transfectant displays enhanced p38 phosphorylation only when stimulated with UVC irradiation but not PMA, suggesting that different signaling events are involved for CD26/DPPIV-associated p38 phosphorylation by different stimuli. Time course studies show that PMA does not induce p38 phosphorylation in Jiyoye-SACD26 cells across the range of times tested, indicating that there is a true attenuation of phosphorylation and not merely a temporal shift in phosphorylation (Fig. 5*B*).

CD26/DPPIV Effect on Upstream Regulators of p38 in Jiyoye-CD26 Transfectants. Phosphorylation of p38 is regulated by several upstream proteins, including MKK3/MKK6. To further delineate the effect of CD26/DPPIV on p38 signaling pathway, we investigated the status of upstream regulators of p38 in Jiyoye-CD26 transfectants. Specifically, Western blot analyses with anti-phospho-MKK3/MKK6 antibody show that Jiyoye-wtCD26 transfectants have higher level of MKK3/MMK6 phosphorylation compared with Jiyoye-vector control and Jiyoye-SACD26 transfectants (Fig. 6). However, there is no detectable difference in

phosphorylation of MKK4 in these cells (data not shown), which has been described previously to contribute to p38 phosphorylation (36). We also evaluated the status of ASK1, which has a role in stress-induced apoptosis and has been found to be an upstream regulator of MKK3/MKK6 (37). Figure 6 shows that ASK1 is overexpressed in Jiyoye-wtCD26 transfectants compared with Jiyoye-vector control and Jiyoye-SACD26 cells. Interestingly, there is a higher level of phospho-ASK1 (Ser^{83}) in Jiyoye-wtCD26

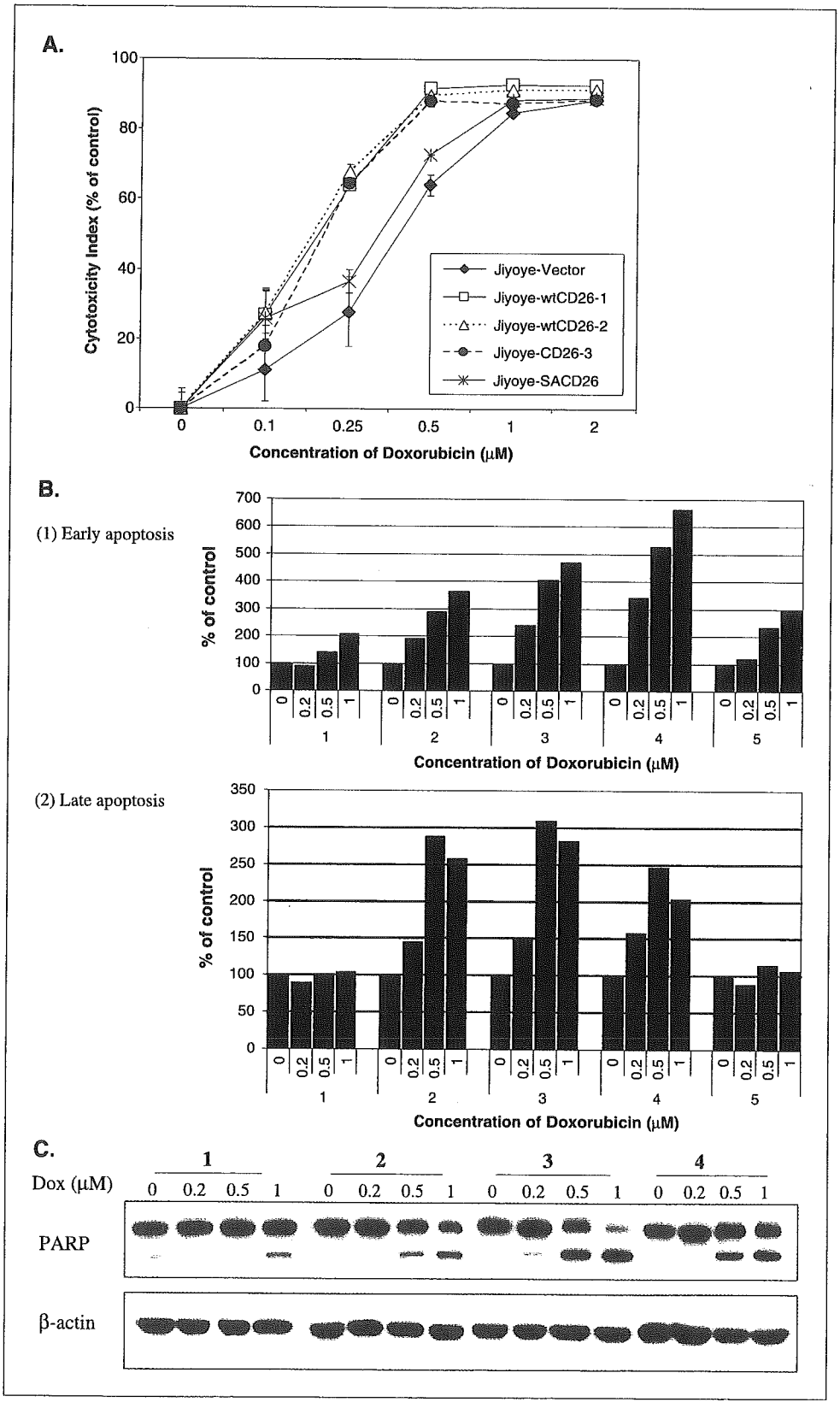

Figure 2. Effect of CD26 expression on doxorubicin-mediated growth inhibition and apoptosis. *A,* Jiyoye-CD26 transfectants were incubated at 37°C in culture medium alone or culture medium containing doxorubicin at the indicated concentrations, and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide uptake assay was done as described in Materials and Methods. *Points,* means of three separate experiments. *B,* Jiyoye-vector, Jiyoye-wtCD26 transfectants, and Jiyoye-SACD26 transfectants were incubated at 37C in culture medium alone or culture medium containing doxorubicin for 48 hours at the concentrations indicated. Cells were then harvested, and Annexin V-PI assays were done as described in Materials and Methods. Cells in early stages of apoptosis were Annexin V positive, whereas Annexin V– and PI-positive cells were in late-stage apoptosis. Representative of three independent experiments. *Y axis,* % of control was calculated as follows: % of control = treated cells / nontreated cells × 100. *Group 1,* Jiyoye-vector control; *group 2,* Jiyoye-wtCD26-1; *group 3,* Jioye-wtCD26-2; *group 4,* Jiyoye-wtCD26-3; *group 5,* Jiyoye-SACD26. *C,* Jiyoye-CD26 transfectants were incubated at 37°C with medium containing doxorubicin (*Dox*) for 48 hours at the indicated doses. Cells were then harvested and whole cell lysates were obtained. Following SDS-PAGE of lysates, immunoblotting studies for poly(ADP-ribose) polymerase (*PARP*) and β-actin were done as described in Materials and Methods. The cleaved product of poly(ADP-ribose) polymerase was detected at 85 kDa. Each lane was loaded with 30 μg protein. *Group 1,* Jiyoye-vector control; *group 2,* Jiyoye-wtCD26-1; *group 3,* Jiyoye-wtCD26-2; *group 4,* Jiyoye-wtCD26-3. Representative of three different experiments.

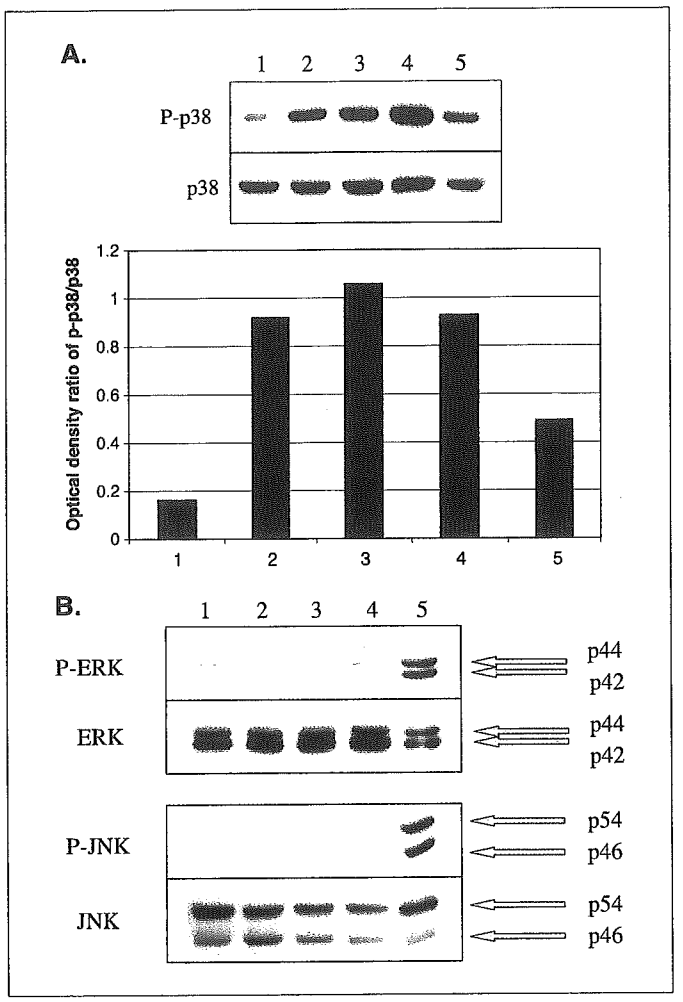

Figure 3. Increased phosphorylation of p38 on Jiyoye-CD26 transfectants. After 24 hours of incubation with culture medium, Jiyoye-vector control, Jiyoye-wtCD26 transfectants, and Jiyoye-SACD26 transfectant were harvested and whole cell lysates were obtained as described in Materials and Methods. Phosphorylation of p38, ERK, and JNK was determined with the use of phosphospecific antibodies. As positive control of phospho-ERK, parental Jiyoye cells were incubated in culture medium containing 100 nmol/L PMA for 30 minutes. As positive control of phospho-JNK, parental Jiyoye cells were washed by PBS and irradiated with UVC (254 nm: UVG-54, UVP inc. CA) for 5 minutes. After irradiation, culture media were immediately added to the cells followed by incubation for 30 minutes. Cells were then harvested and whole cell lysates were obtained with the same method as above. Each lane was loaded with 100 μg protein. *A*, p38 phosphorylation. *Lane 1*, Jiyoye-vector control; *lane 2*, Jiyoye-wtCD26-1; *lane 3*, Jiyoye-wtCD26-2; *lane 4*, Jiyoye-wtCD26-3; *lane 5*, Jiyoye-SACD26. *B*, ERK and JNK phosphorylation. *Lane 1*, Jiyoye-vector control; *lane 2*, Jiyoye-wtCD26-1; *lane 3*, Jiyoye-wtCD26-2; *lane 4*, Jiyoye-wtCD26-3; *lane 5*, positive control (*top*, ERK: PMA stimulation; *bottom*, JNK: UVC irradiation). Representative of three different experiments.

cells, whereas no significant level of phospho-ASK1 (Ser^{967}) is detected. Taken together, our findings suggest that there is an increase in the absolute number of ASK1 molecules phosphorylated at Ser^{83} in Jiyoye-wtCD26 transfectants. Meanwhile, the elevated phospho-ASK1 (Ser^{83}) level seen in conjunction with a similar increase in overall ASK1 level, along with the lack of change in phospho-ASK1 (Ser^{967}), indicates that the specific phosphate content of each ASK1 molecule is likely unchanged following CD26/DPPIV expression. Our data suggest that ASK1 and its specific phosphorylation at Ser^{83} serve as an up-regulator of CD26/DPPIV-associated p38 phosphorylation in these Jiyoye-wtCD26 transfectants.

Effect of p38 Inhibition on Topoisomerase IIα Expression. To determine the relationship between p38 phosphorylation and topoisomerase IIα expression, we evaluated topoisomerase IIα level following inhibition of p38 phosphorylation by its specific inhibitor SKF86002 (23, 38) in Jiyoye-vector controls and Jiyoye-wtCD26 transfectants. Of note is the fact that treatment with the p38 inhibitor at the indicated concentration and time course did not affect cell viability or cell cycle status (data not shown). As shown in Fig. 7, treatment with the p38 inhibitor decreases p38 phosphorylation, associated with markedly decreased topoisomerase IIα expression in both Jiyoye-vector cells and Jiyoye-wtCD26 transfectants. We found that inhibition of topoisomerase IIα expression consistently lags behind the inhibitory effect of SKF86002 on p38 phosphorylation. Whereas p38 phosphorylation is decreased from 6 to 24 hours after SKF86002 treatment, with recovery seen by 48 hours post-treatment, decreased topoisomerase IIα expression is clearly detected 48 hours post-treatment. Similar results were obtained with the p38 inhibitor SB203580 (data not shown). The fact that inhibition of p38 phosphorylation leads temporally to decreased topoisomerase IIα expression strongly suggests that p38 signaling pathway is involved in the regulation of topoisomerase IIα. Furthermore, our data show that p38-mediated regulation of topoisomerase IIα is independent of CD26 presence.

Increased Survival of Doxorubicin-Treated SCID Mice Bearing Jiyoye-wtCD26 Cells. Extending our *in vitro* findings, we investigated the effect of CD26 expression on overall survival in doxorubicin-treated SCID mice inoculated with Jiyoye-vector control or Jiyoye-wtCD26 transfectants. Jiyoye-wtCD26 transfectants or Jiyoye-vector control cells (7×10^6 cells per mouse) were implanted by i.p. injection into the SCID mice on day 0; then, once per day on days 1 and 15, animals were treated by i.p. injection with saline alone or doxorubicin at a dose of 0.5 mg/kg of body weight per injection. As shown in Fig. 8, most of the SCID mice inoculated with Jiyoye-vector control cells and treated with saline (line 1) developed tumors and then were subsequently euthanized with large tumor burden as per protocol requirements. Mice injected with Jiyoye-vector cells and treated with low-dose doxorubicin (line 2) had similar survival as those treated with saline control ($P = 0.50325$), indicating that the low-dose doxorubicin treatment did not have a statistically significant effect on tumor growth. Meanwhile, mice inoculated with Jiyoye-wtCD26 cells and treated with saline alone (line 3) exhibited in general the same survival rate as those injected with Jiyoye-vector cells and treated with saline or doxorubicin. Although there seemed to be a trend for a slight enhancement in survival among saline-treated mice inoculated with Jiyoye-wtCD26 cells (line 3) compared with Jiyoye-vector cells (line 1), the difference was not statistically significant ($P = 0.10576$). Importantly, mice inoculated with Jiyoye-wtCD26 cells and treated with doxorubicin (line 4) had a marked survival advantage compared with saline-treated mice injected with the same transfectants (line 3), which is statistically significant ($P = 0.00612$). In summary, our data show that SCID mice inoculated with Jiyoye-vector cells did not exhibit survival difference when treated with either saline or low-dose doxorubicin. However, for SCID mice inoculated with Jiyoye-wtCD26 transfectants, those treated with low-dose doxorubicin showed statistically significant difference in survival compared with those treated with saline alone. These *in vivo* results therefore extend our *in vitro* findings by demonstrating that the presence of CD26 renders tumor cells more sensitive to the antineoplastic agent doxorubicin, leading to

enhanced survival of treated animals. Our findings also suggest that treatment strategies regulating CD26 expression may be considered in the future for selected neoplasms in the clinical setting.

Discussion

In this study, we show that surface expression of CD26, especially its intrinsic DPPIV enzyme activity, results in enhanced topoisomerase IIα level in the B-cell line Jiyoye and subsequent sensitivity to doxorubicin-induced apoptosis, thus expanding our previous published work with the T-cell line Jurkat. In addition, our article is the first to show that expression of CD26/DPPIV is associated with increased p38 phosphorylation and that p38 signaling pathway plays a role in the regulation of topoisomerase IIα expression. The connection between CD26 and the p38 signaling pathway is shown in two experimental systems, one in which CD26 is overexpressed in Jiyoye transfectants and another in which CD26 expression is decreased by target-specific siRNAs in the T-cell line Karpas-299. Besides demonstrating that CD26 effect on topoisomerase IIα and doxorubicin sensitivity is applicable to cell lines of both B-cell and T-cell lineages, the potential clinical implication of our work lies with the fact that we now show for the first time that our *in vitro* findings can be extended to animal studies. Our findings that CD26 expression can be an *in vivo* marker of tumor sensitivity to doxorubicin treatment may lead to future treatment strategies targeting CD26/DPPIV for selected human cancers in the clinical setting.

MAPKs include three subfamilies: ERK, JNK/stress-activated protein kinase, and p38. Activation of the MAPK signaling pathways regulates various cellular processes, including apoptosis, proliferation, or differentiation, with the p38 signaling pathway being activated by various stress agents. In this article, we show that the presence of CD26/DPPIV results in enhanced phosphorylation of p38 in two experimental systems: the B-cell line Jiyoye in which CD26 is overexpressed and the T-cell line Karpas-299 in which CD26 expression is reduced. Previous work has shown that antibody binding to CD26 molecules expressed on the surface of CD26-Jurkat transfectants results in tyrosine phosphorylation and activation of such signaling molecules as ERK, $p56^{lck}$, $p59^{fyn}$, ZAP-70, c-Cbl, and PLC. In addition, anti-CD26 antibody-induced phosphorylation of ERK leads to expression of $p21^{Cip1}$ (26, 39). Our work is the first to clearly show that surface expression of the CD26 molecule itself is linked to increased p38 phosphorylation. Furthermore, our data suggest that upstream regulators of p38, including MKK3/MKK6 and ASK1, particularly when phosphorylated at residue Ser^{83}, is linked to the CD26/DPPIV-associated p38 signaling pathway in these Jiyoye-wtCD26 transfectants. ASK1 plays a important role in cell death induced by several stimuli, including genotoxic stress (40) and tumor necrosis factor-α (41). Meanwhile, data from Mabuchi et al. suggested that ASK1 may have a key role in determining the balance between tumor survival and apoptosis in cancer treatment (42). By affecting ASK1 phosphorylation status,

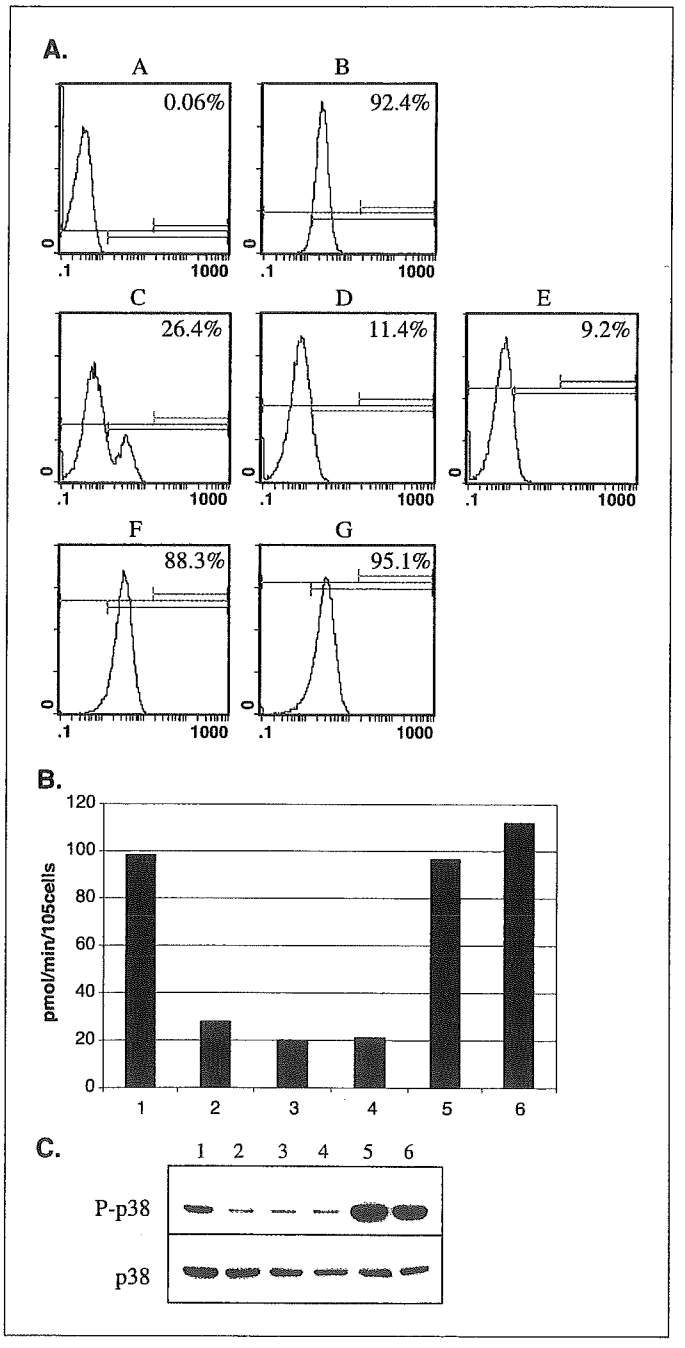

Figure 4. CD26 expression, DPPIV activity, and p38 status on Karpas-299-CD26-siRNA cells. *A*, CD26 expression on parental Karpas-299, Karpas-299-CD26-siRNA, and Karpas-299-mis-CD26-siRNA cells. Karpas-299 cells were evaluated for CD26 expression by flow cytometry as described in Materials and Methods. Amount of CD26-positive cells is presented as percentage of total cells. *A*, negative control; *B*, parental Karpas-299; *C-E*, Karpas-299-CD26-siRNA cells 1-3, respectively; *F* and *G*, Karpas-299-mis-CD26-siRNA cells 1 and 2, respectively. Representative of three different experiments. *B*, DPPIV enzyme activity on parental Karpas-299, Karpas-299-CD26-siRNA, and Karpas-299-mis-CD26-siRNA cells. Various Karpas-299 cells were evaluated for DPPIV enzyme activity as described in Materials and Methods. *Lane 1*, parental Karpas-299; *lanes 2-4*, Karpas-299-CD26-siRNA cells 1-3, respectively; *lanes 5* and *6*, Karpas-299-mis-CD26-siRNA cells 1 and 2, respectively. Representative of three different experiments. *C*, phosphorylation status of p38 on parental Karpas-299, Karpas-299-CD26-siRNA, and Karpas-299-mis-CD26-siRNA cells. After 24 hours of incubation with culture medium, parental Karpas-299 cells, Karpas-299-CD26-siRNA cells, and Karpas-299-mis-CD26-siRNA cells were harvested and whole cell lysates were obtained as described in Materials and Methods. Phosphorylation status of p38 was determined by Western blot analysis. Each lane was loaded with 100 μg protein. *Lane 1*, parental Karpas-299; *lanes 2-4*, Karpas-299-CD26-siRNA cells 1-3, respectively; *lanes 5* and *6*, Karpas-299-mis-CD26-siRNA cells 1 and 2, respectively. Representative of three different experiments.

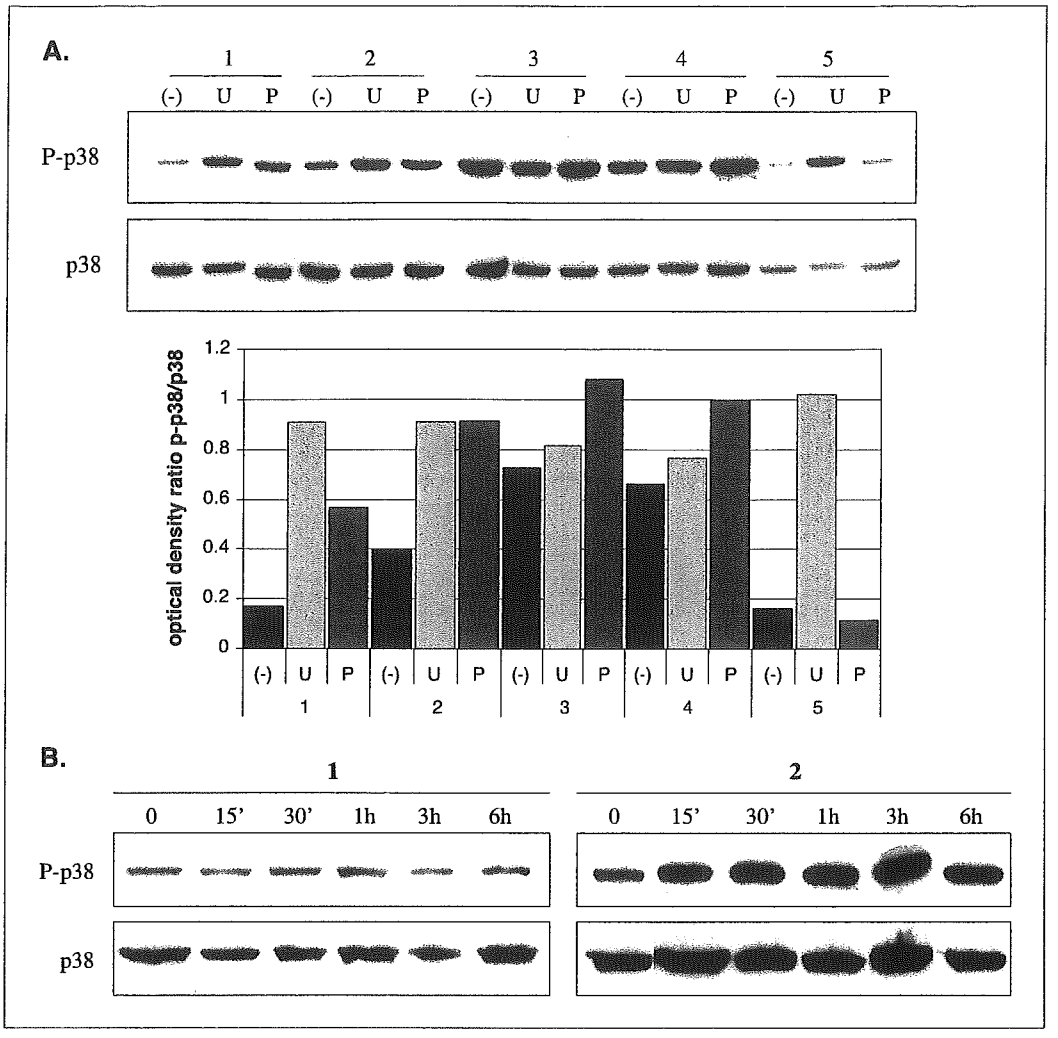

Figure 5. Effect of UVC irradiation and PMA stimulation on p38 phosphorylation in CD26 Jiyoye transfectants. *A,* After 24 hours of incubation with culture medium, Jiyoye-vector, Jiyoye-wtCD26 transfectants, and Jiyoye-SACD26 transfectant were stimulated with UVC irradiation (254 nm, 5 minutes) or PMA stimulation (100 nmol/L, 30 minutes). Cells were then harvested and whole cell lysates were obtained as described in Materials and Methods. Each lane was loaded with 100 μg protein. *(-),* nontreatment; *U,* UVC irradiation; *P,* PMA stimulation. *Group 1,* Jiyoye-vector control; *group 2,* Jiyoye-wtCD26-1; *group 3,* Jiyoye-wtCD26-2; *group 4,* Jiyoye-wtCD26-3; *group 5,* Jiyoye-SACD26. Representative of three different experiments. *B,* cells were treated with PMA (100 nmol/L) at the indicated times, and p38 phosphorylation was detected as described above. *Group 1,* Jiyoye-SACD26; *group 2,* Jiyoye-wtCD26-1. Representative of three different experiments.

CD26/DPPIV may therefore play a potential role in key aspects of tumor biology.

Earlier work has linked constitutive p38 phosphorylation and activation to apoptosis as well as changes in cell growth status under certain experimental conditions. For example, constitutive p38 activation is associated with spontaneous apoptosis of human neutrophils; however, inhibition of p38 by its specific inhibitor and antisense RNA delays spontaneous apoptosis (43). Meanwhile, constitutive activation of p38 in B-cell tumors, including chronic lymphocytic lymphoma, diffuse large B-cell lymphoma, and follicular lymphoma, contributes to B-cell tumor growth (44). Our study links expression of CD26, particularly its DPPIV enzyme activity, to constitutive p38 phosphorylation. However, we did not detect appreciable difference in cell viability as assayed by trypan blue uptake or Annexin V-PI studies among cells differing in CD26 expression (data not shown). Whereas the presence of an intact CD26/DPPIV results in the greatest levels of p38 phosphorylation and topoisomerase IIα expression, we consistently find that Jiyoye transfectants expressing the catalytically inactive variant of CD26 still have slightly higher levels of p38 phosphorylation and topoisomerase IIα expression than Jiyoye-vector control (Figs. 1*C* and 3*A*). These findings suggest that CD26 is linked to signaling pathways independent of its peptidase activity.

Our data also show that Jiyoye cells transfected with a mutant CD26 missing the DPPIV enzyme activity (Jiyoye-SACD26 transfectant) have enhanced p38 phosphorylation only when stimulated with UVC irradiation but not when stimulated with PMA. Although the mechanisms behind this observation remain to be elucidated, several potential explanations may be considered. DPPIV activity may be associated with signaling pathways that play a role in p38 phosphorylation mediated by PMA but not by UV irradiation, and the absence of DPPIV enzyme activity may lead to the lack of engagement of these signaling pathways necessary for PMA-induced p38 phosphorylation. Regarding this point, previous work has shown that the inhibition of DPPIV enzymatic activity in T cells induces an inhibitory signaling process mainly transmitted by tyrosine kinases, resulting in the inhibition of PMA-induced $p56^{lck}$ hyperphosphorylation (45). It is also possible that phorbol esters and UV irradiation engage different downstream signals to phosphorylate p38 that are differentially associated with CD26 and its intrinsic DPIPV enzyme activity. Previous work has shown that p38 activation is differentially regulated by PMA and UV irradiation in other experimental conditions (46). Furthermore, UV irradiation induces the activation of all p38 isoforms, whereas PMA stimulation activates only the p38 γ and δ isoforms (38). Our results also show a connection between p38 and topoisomerase IIα, as inhibition of p38 phosphorylation by a specific p38 inhibitor reduces topoisomerase IIα expression. The fact that decreased topoisomerase IIα level is seen 48 hours after

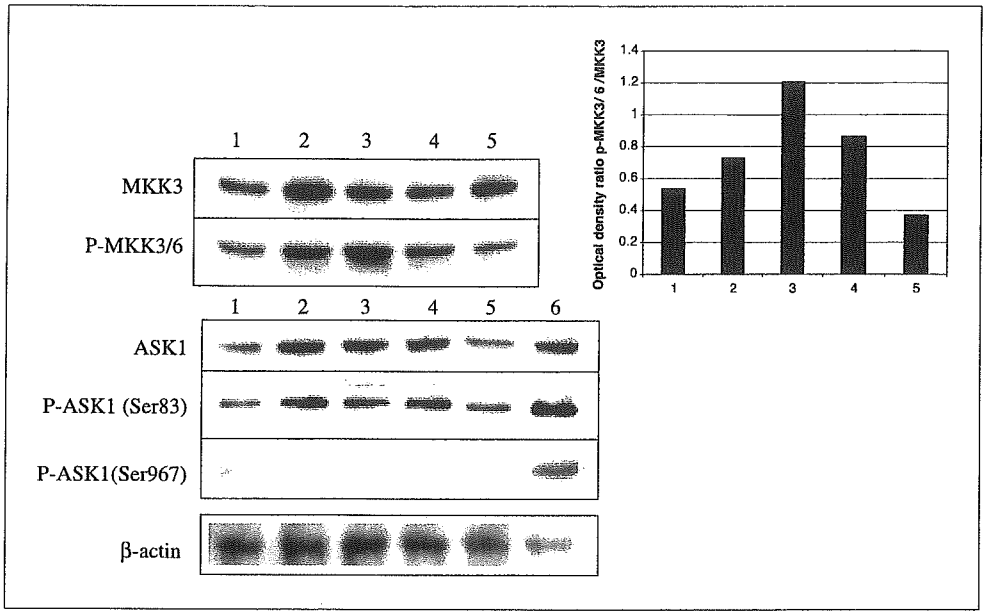

Figure 6. Constitutive p38 phosphorylation and its correlation with MKK3/MKK6 and ASK1 phosphorylation in CD26 Jiyoye transfectants. After 24 hours of incubation with culture medium, Jiyoye-Vector, Jiyoye-wtCD26 transfectants, and Jiyoye-SACD26 transfectant were harvested and whole cell lysates were obtained as described in Materials and Methods. Each lane was loaded with 100 μg protein. MKK3/MKK6 and ASK1 phosphorylation status was determined with the use of phosphospecific antibodies. For ASK1, specific antibodies can detect phosphorylation at Ser^{83} and Ser^{967}. *Lane 1,* Jiyoye-vector; *lane 2,* Jiyoye-wtCD26-1; *lane 3,* Jiyoye-wtCD26-2; *lane 4,* Jiyoye-wtCD26-3; *lane 5,* Jiyoye-SACD26; *lane 6,* UVC irradiation 5 minutes (for positive control). Representative of three different experiments.

treatment with the p38 inhibitor, whereas inhibition of p38 phosphorylation is seen earlier at 6 hours post-treatment, also suggests that p38 signaling pathway has a role in regulating topoisomerase IIα expression. The fact that p38 regulates topoisomerase IIα expression in both Jiyoye-vector controls and Jiyoye-wtCD26 transfectants indicates that this is a CD26-independent process. Furthermore, our data show that the increase in topoisomerase IIα associated with the ectopic expression of CD26 is controlled by existing p38-linked pathways regulating topoisomerase IIα expression. To our knowledge, our work is the first to show a potential connection between these two intracellular proteins, including the potential regulation of topoisomerase IIα level by p38.

Meanwhile, our data showing that the expression of CD26, especially its intrinsic DPPIV enzyme activity, is associated with enhanced topoisomerase IIα level and increased doxorubicin sensitivity in the B-cell lymphoma line Jiyoye extend our previous findings with the T-cell line Jurkat (19, 20). Whereas CD26 role in normal T-lymphocyte physiology is well established and its involvement in selected T-cell tumors is being elucidated (1, 7, 8, 47), CD26 function in B cells has not been well studied. Our work therefore suggested that CD26/DPPIV effect on topoisomerase IIα and subsequent doxorubicin sensitivity is not restricted only to tumors of T-cell lineage but is also applicable potentially to other lymphoid malignancies. Recently, topoisomerase IIα expression on malignant tumors has been found to correlate response to treatment of malignant tumors and longer patient survival, including breast cancer and Hodgkin's disease (48, 49). In addition, Walker and Nitiss show that an increase in topoisomerase IIα gene copy number is associated with cancers that have increased sensitivity to topoisomerase II inhibitors, such as doxorubicin (50). Importantly, we show for the first time that our *in vitro* results can be extended to and confirmed in animal studies. Specifically, the presence of CD26 renders tumor cells more sensitive to doxorubicin, resulting in statistically significant survival advantage. SCID mice injected with Jiyoye control cells treated with low-dose doxorubicin did not show any significant difference in survival compared with those treated with saline, whereas SCID mice inoculated with Jiyoye-wtCD26 transfectants showed significantly greater survival when treated with low-dose doxorubicin than with saline alone. Interestingly, our *in vivo*

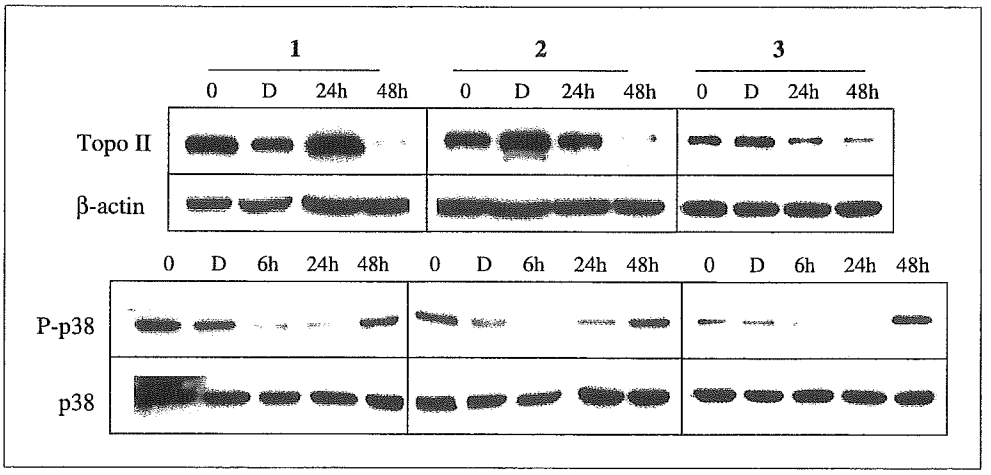

Figure 7. Effect of inhibition of p38 phosphorylation on topoisomerase IIα expression. Jiyoye-wtCD26 transfectants and Jiyoye-vector controls were incubated in culture medium alone or culture medium containing SKF86002 (20 μmol/L) for either 24 or 48 hours. Cells were then harvested and nuclear extracts were obtained. Following SDS-PAGE of lysates, immunoblotting studies for topoisomerase IIα (*Topo II*) or β-actin were done as described in Materials and Methods. Each lane was loaded with 20 μg protein. *D,* DMSO control. *Group 1,* Jiyoye-wtCD26-1; *group 2,* Jiyoye-wtCD26-2; *group 3,* Jiyoye-vector controls. At various times, an aliquot was removed and stained with trypan blue dye to evaluate cell viability with a hemocytometer. All experiments had >95% cell viability. Representative of three different experiments.

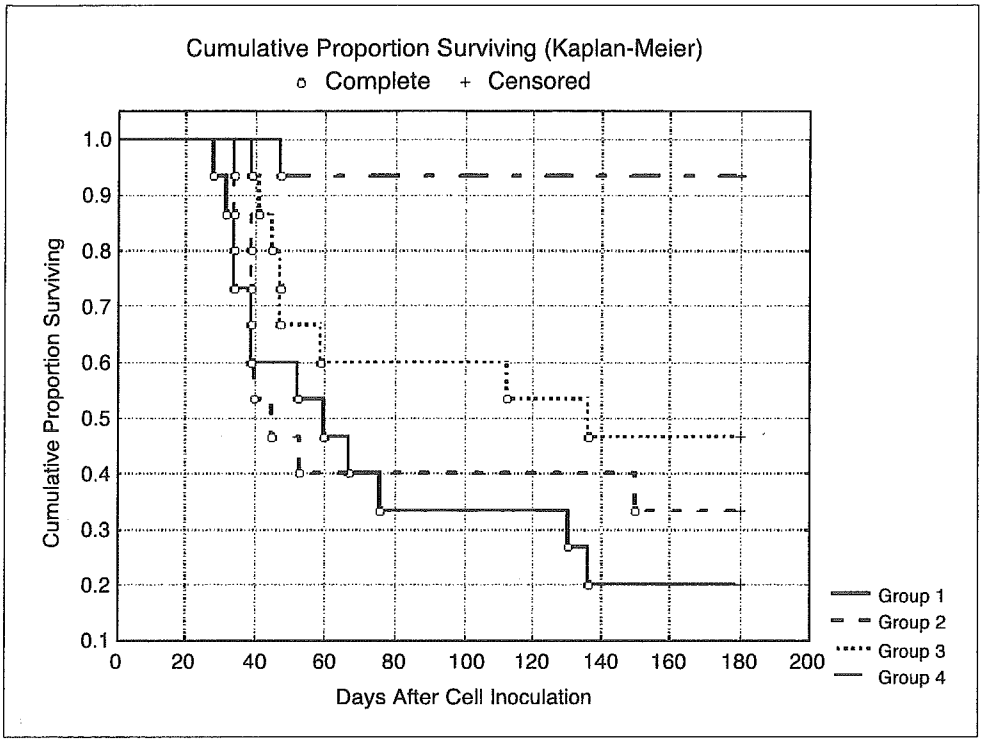

Figure 8. Enhanced survival of Jiyoye-CD26 transfectant-bearing SCID mice after doxorubicin treatment. SCID mice were injected with Jiyoye-vector control cells or Jiyoye-wtCD26-1 cells (7×10^6 cells per mouse) on day 0 and treated with saline or doxorubicin 0.5 mg/kg) on days 1 and 15 as described in Materials and Methods. Each treatment group consisted of 15 mice. *Group 1,* Jiyoye-vector control treated with saline alone; *group 2,* Jiyoye-vector control treated with doxorubicin; *group 3,* Jiyoye-wtCD26 transfectant treated with saline alone; *group 4,* Jiyoye-wtCD26 transfectant treated with doxorubicin. Analysis for statistically significant differences in survival was done using a log-rank test (*P*s: 1 versus 2 = 0.50325, 1 versus 3 = 0.10576, 1 versus 4 = 0.00009, 2 versus 3 = 0.25056, 2 versus 4 = 0.00070, and 3 versus 4 = 0.00612).

studies also suggested that the presence of CD26 itself enhances survival, although the difference in survival between the Jiyoye-wtCD26 group treated with saline alone and the Jiyoye-vector control group treated with saline alone did not reach statistical significance in our experiments. Although our studies may have been underpowered to detect this difference, this potential effect resulting from CD26 expression may indicate that CD26 presence itself can modulate tumor engraftment or tumorigenicity of the transplanted cells. Regarding this point, other groups have shown that CD26/DPPIV expression in melanoma, lung carcinoma, and ovarian carcinoma inhibits tumorigenicity and prolongs survival time (14–16). Taken together, our findings thus have potential implications in the clinical setting, suggesting that future treatment strategies that involve CD26/DPIV may be effective for selective neoplasms of both B-cell and T-cell lineages.

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CD26$^+$ T cells in the pathogenesis of asthma

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Asthma is a chronic inflammatory disease of the small airways, being clinically characterized by reversible airway obstruction and bronchial hyperreactivity [1]. These clinical features result from a chronic inflammation of the airways, caused by a migration of leucocytes and an increase of inflammatory mediators in the bronchial wall [2]. This pathological reaction in asthma is thought to arise from a complex interaction between genes and the environment.

Although allergens and infection appear to be environmental modifiers of asthma [3,4], it is now estimated that at least a dozen polymorphic genes regulate asthma and control the chronic inflammatory response and production of immunoglobulin E (IgE), cytokines and chemokines. By genetic-linkage analysis on 460 pairs of siblings from asthmatic families in the USA and UK, Van Eerdewegh *et al.* [5] identified a locus on the short arm of chromosome 20 which was linked to asthma and bronchial hyperreactivity. They identified the *ADAM-33* gene as significantly associated with asthma. ADAMs are a subfamily of metalloproteinases expressed on the cell surface, and have proteolytic functions such as shedding tumour necrosis factor receptors, and other cell-surface cytokines, adhesion molecules, and growth factors and receptors that are involved in inflammation, cell proliferation, and cell death. *ADAM-33* is expressed by lung fibroblasts and bronchial smooth muscle cells, and is suspected to be associated with small-airway remodeling in patients with asthma.

Regarding immunological aspects of asthma genes, the human homologue of *Tim1* has been identified as an asthma susceptibility gene [6]. *Tim1* lies at chromosome 5q33.2, a region that has been repeatedly linked to asthma, and codes for the cellular receptor for hepatitis A virus [6]. The *Tim1* gene product is expressed on T cells and appears to regulate the production of interleukin-4 (IL-4) in T cells by affecting CD4+ T cell differentiation, the development of T_H2 cells and development of airway hyperreactivity. As observed in the *Tim1* hypothesis, the inappropriate T_H2 response causes pulmonary inflammation, airway eosinophilia, and airway hyperreactivity to a variety of specific and nonspecific stimuli that result in the clinical symptoms of asthma.

In the T_H1-T_H2 paradigm, T_H1 cell responses are thought to protect against asthma, by dampening the activity of T_H2 responses [7–9]. However, other investigators have shown that T_H1 cells may exacerbate asthma, as human asthma is associated with the production of IFN-γ which appears to contribute to pathogenesis of asthma [10,11]. Allergen-specific T_H1 cells, when adoptively transferred into naïve recipients, migrate to the lungs but fail to counterbalance T_H2 cell-induced airway hyperreactivity. Instead, allergen-specific T_H1 cells cause severe airway inflammation [12]. Moreover, Dahl *et al.* [13] showed virus-induced T_H1-dependent enhancement of allergic pulmonary inflammation via T_H1-polarized dendritic cells (DCs). Thus, although T_H2 cells play an important role in the pathogenesis of asthma, the binary T_H1-T_H2 paradigm cannot explain all the immunological processes that occur in asthma. These processes in asthma may be much more complex than is hypothesized by a T_H1-T_H2 paradigm. In this regard, Kruschinski *et al.* [14], in this issue of *Clinical and Experimental Immunology*, show a critical role for CD26$^+$ T cells, which are T_H1 cells, in asthma, by examining CD26-deficient and CD26-reduced rats in ovalubmin (OVA)-induced asthma models. They show in their article that the decrease in T cell recruitment to the airway observed in CD26^{low} and CD26-deficient rats is associated with significantly reduced OVA-specific IgE-titres. They suggest a role for CD26$^+$ T cells in the pathogenesis of asthma by means of T cell migration and T cell-dependent IgE production in the airway.

CD26/dipeptidyl peptidase IV (DPPIV) is a 110-kDa cell surface glycoprotein that belongs to the serine protease

family [15]. It is expressed on a variety of tissues including T lymphocytes, endothelial and epithelial cells. It is composed of a short cytoplasmic domain of 6 amino acids, a transmembrane region of 22 amino acids, and an extracellular domain of 738 amino acids, with DPPIV activity which selectively removes the N-terminal dipeptide from peptides with proline or alanine in the second position [16]. Possible substrates of DPPIV include several critical cytokines and chemokines. Activity of CCL5 (RANTES, regulated on activation, normal T cell expressed and secreted) is altered by the enzymatic cleavage of DPPIV, as CD26-processed CCL5(3–68) has a more than 10 times lower chemotactic potency for monocytes and eosinophils. CCL5(3–68) also has impaired binding and signalling properties through CCR1 and CCR3, but remains fully active on CCR5, leading to T_H1 polarization [17,18]. Other important chemokines that appear to be substrates of the enzymatic activity of DPPIV include CCL11 (eotaxin), CCL22 (macrophage-derived chemokine), CXCL10 and CXCL11 (interferon inducible chemokines), and other chemokines [19,20]. Besides its ability to regulate the effect of biological factors through its enzymatic activity, CD26/DPPIV has an essential role in human T cell physiology.

Originally characterized as a T-cell differentiation antigen, CD26 is preferentially expressed on a specific population of T lymphocytes, the subset of CD4+ memory T cells, and is up-regulated after T cell activation [21,22]. As well as its enhanced expression on activated T cells, various lines of evidence have converged to demonstrate that CD26 is functionally associated with T cell signal transduction processes, which are capable of transmitting signals relating to T cell activation [22,23]. In addition CD26 serves as a functional collagen receptor with a role in T cell activation, as well as having a potential role in thymic ontogeny [24]. The enzymatic activity of CD26 appears to be very important in enhancing cellular responses to external stimuli. For example, Jurkat cells transfected with wild type CD26 consistently demonstrated greater activation than parental CD26 nega-

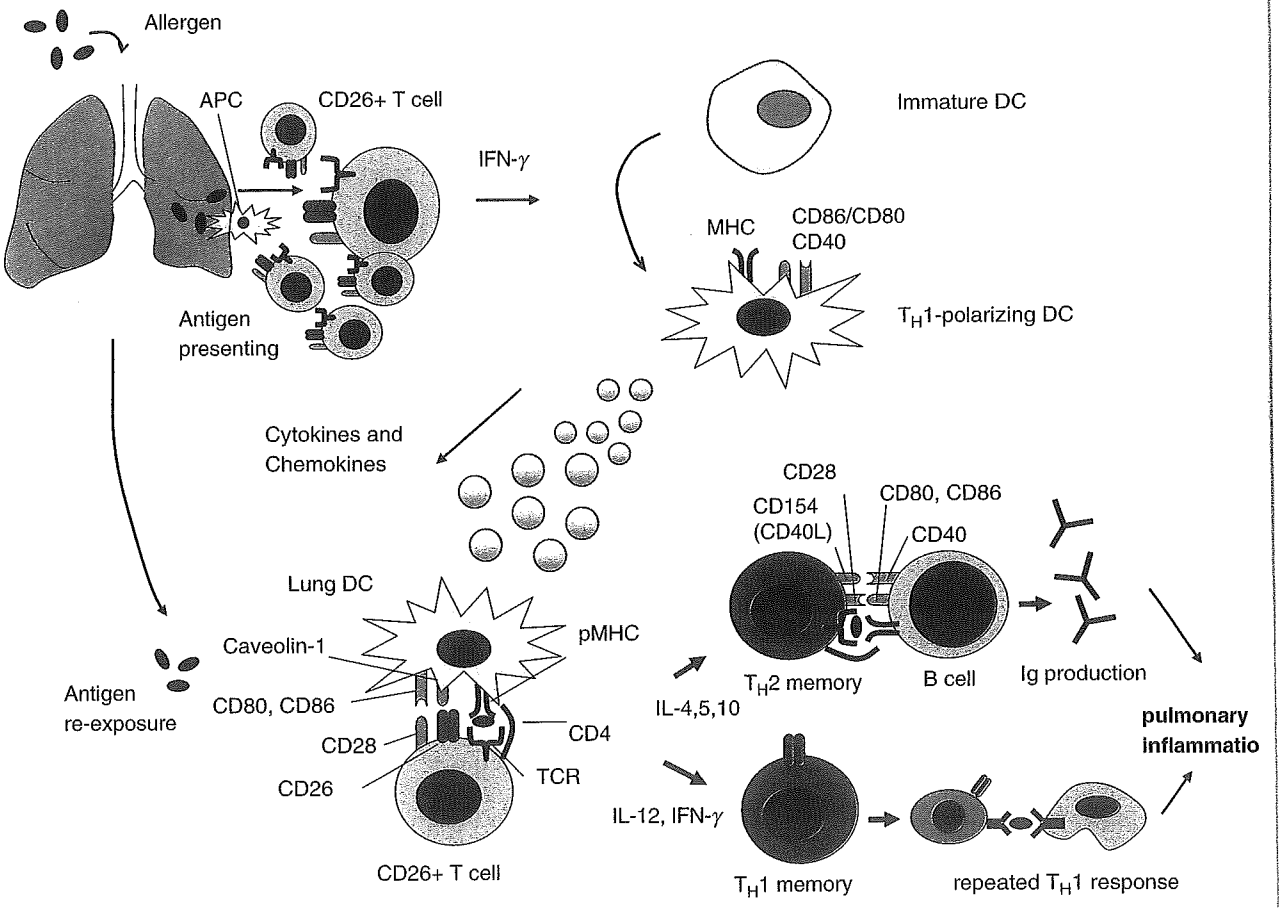

Fig. 1. T_H1-dependent enhancement of allergic pulmonary inflammation. Initially, antigens such as sensitizing-allergens stimulate $CD26^+$ T cells via antigen presenting cells (APC), and subsequently generate $CD26^+$ T_H1 response to produce IFN-γ and stable T_H1-polarizing DCs. Later, these DCs are capable of augmenting both T_H1 and T_H2 controlled immune responses in allergen-induced pulmonary inflammation, partly via interaction of CD26 on T cells and caveolin-1 on APC loaded with sensitized allergens. Ig, immunoglobulin; MHC, major histocompatibility complex; pMHC, peptide-loaded major histocompatibility complex.

tive Jurkat or cells transfected with CD26 mutated at the DPPIV enzymatic site [25].

CD26 expression is tightly regulated on human T lymphocytes, with its density being markedly elevated following T cell activation [21,26]. At the resting state in the peripheral blood, CD26 is preferentially expressed on the helper/memory T cell population [21]. High CD26 cell surface expression is correlated with the production of T_H1-like cytokines by T-cell clones, and CD26 expression is induced by stimuli that favour the development of the T_H 1 response [27,28]. CD26 is able to conduct IL-2-dependent comitogenic signals in conjunction with activation through the CD3/T cell receptor complex or the CD2 pathway of mature human T lymphocytes when crosslinked with solid-phase immobilized antibodies [22].

Meanwhile, recombinant soluble CD26/DPPIV molecule up-regulates expression of CD86 on antigen presenting cells (APC), leading to greater APC–T cell interaction and enhanced T cell proliferation, with important implications for immunoregulation [29]. More recently, T cell proliferation via CD26 driven by recall antigens such as tetanus toxoid is mediated by means of caveolin-1 on APC, leading to up-regulation of the costimulatory molecule CD86 [30]. Thus, $CD26^+$ T cells play a critical role in inflammation responding to recall antigen.

Of clinical relevance, patients with autoimmune diseases such as Graves' disease and rheumatoid arthritis have been found to increase numbers of $CD26^+$ T cells in inflamed tissues such as thyroid and synovial fluids [26,31]. In addition, enhancement of CD26 expression in these autoimmune diseases may correlate with disease severity [32,33]. Moreover, we have shown that T cells migrating through endothelial cell monolayers *in vitro* express high levels of CD26 [34], and the fact that chemokines play a key role in T cell migration supports the notion that CD26/DPPIV may interact with chemokines [18–20]. These findings imply that $CD26^+$ T cells play a role in the inflammation process and subsequent tissue damage not only in autoimmune diseases, but also in asthma.

In the context that $CD26^+$ T cells preferentially play an important role in allergic pulmonary inflammation, it is hypothesized that allergens such as OVA incite a robust T_H1-type cytokine response, such as IFN-γ, in the lung, promoting the development of durable T_H1-polarizing DCs, partly via a CD26–caveolin-1 interaction, subsequently leading to enhancement of CD28-CD86 costimulation (Fig. 1). These DCs support subsequent immunity in a T_H2-dependent process of allergen-induced pulmonary inflammation, and so enhance both T_H1 and T_H2 immune cytokines and IgE production.

Interestingly, a recent report on single-nucleotide polymorphism in asthmatic patients revealed that polymorphism of DPP-10, a CD26/DPPIV-like peptidase, was observed [35]. Further elucidation on DPPs and asthma will open an avenue for our understanding the pathophysiology of asthma.

Although the precise effects rely on the exact timing, intensity and dose of cytokines and antigens as well as how these interactions alter T helper regulatory cells and cytokines, $CD26^+$ T cells play an important role in asthma and targeting CD26/DPPIV may contribute to the elucidation of the pathophysiology and therapeutic means to treat asthma, and other inflammatory disorders.

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The Distinct Agonistic Properties of the Phenylpyrazolosteroid Cortivazol Reveal Interdomain Communication within the Glucocorticoid Receptor

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Recent structural analyses of the nuclear receptors establish a paradigm of receptor activation, in which agonist binding induces the ligand binding domain (LBD)/activation function-2 helix to form a charge clamp for coactivator recruitment. However, these analyses have not sufficiently addressed the mechanisms for differential actions of various synthetic steroids in terms of fine tuning of multiple functions of whole receptor molecules. In the present study, we used the glucocorticoid receptor (GR)-specific agonist cortivazol (CVZ) to probe the plasticity and functional modularity of the GR. Structural docking analysis revealed that although CVZ is more bulky than other agonists, it can be accommodated in the ligand binding pocket of the GR by reorientation of several amino acid side chains but without major alterations in the active conformation of the LBD. In this induced fit model, the phenylpyrazole A-ring of CVZ establishes additional contacts with helices 3 and 5 of the LBD that may contribute to a more stable LBD configuration. Structural and functional analysis revealed that CVZ is able to compensate for the deleterious effects of a C-terminal deletion of the LBD in a manner that mimics the stabilizing influence of the F602S point mutation. CVZ-mediated productive recruitment of transcriptional intermediary factor 2 to the C-terminally deleted LBD requires the receptor's own DNA binding domain and is positively influenced by the N-terminal regions of GR or progesterone receptor. These results support a model where ligand-dependent conformational changes in the LBD play a role in GR-mediated gene regulation via modular interaction with the DBD and activation function-1. (*Molecular Endocrinology* 19: 1110–1124, 2005)

GLUCOCORTICOIDS ARE PRODUCED in the adrenal cortex under the strict control of the hypothalamus-pituitary-adrenal axis and exert a variety of biological actions including the regulation of glucose and lipid metabolism, electrolyte balance, and modulation of the immune, cardiovascular, and central nervous system (1, 2). Multiple compounds with glucocorticoid activity including dexamethasone (DEX), prednisolone, and cortivazol (CVZ) are widely used as an antiinflammatory and/or immunosuppressive agents (3). At pharmacological doses, however, patients often suffer from side effects of glucocorticoids, the molecular basis for which have not been fully clarified. Indeed, dissociation of their therapeutic effects and adverse reactions is still one of the most challenging clinical issues to be solved (4, 5).

Glucocorticoids act by the binding to their cognate receptor, the glucocorticoid receptor (GR), the prototypic member of the nuclear receptor superfamily, which also includes the receptors for the mineralocorticoids (MR), estrogens, progestins (PR), and androgens (AR), as well as those for peroxisome proliferators, vitamin D, and thyroid hormones (6, 7). Phylogenetic and sequence analysis indicate that the GR, MR, PR, and AR form a subfamily of oxosteroid receptors (6, 7). Like most nuclear receptors, the GR is a modular protein that is organized into three major domains: an N-terminal regulatory domain harboring a strong transcriptional activation function (AF)-1, a central DNA binding domain (DBD), and a C-terminal ligand binding domain (LBD) (6, 8). The LBD harbors a second AF-2 directly regulated by ligands. Agonist binding to the LBD induces the reorientation of a critical α-helix (AF-2 helix) and the formation of a binding pocket for a family of coactivator proteins that play

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Abbreviations: AF, Activation function; AR, androgen receptor; CVZ, cortivazol; DBD, DNA binding domain; DEX, dexamethasone; GFP, green fluorescent protein; GR, glucocorticoid receptor; GRE, glucocorticoid response element; hsp90, heat shock protein 90; LBD, ligand binding domain; MR, mineralocorticoid receptor; NF-κB, nuclear factor-κ B; NLS, nuclear localization signal; PR, progesterone receptor; SDS, sodium dodecyl sulfate; TIF, transcriptional intermediary factor.

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essential roles in transactivation (9, 10). Among nuclear receptors, the AF-2 pocket is highly conserved, whereas the molecular size and amino acid composition of AF-1 is much more diverse (6, 9). In the absence of ligand, the GR is retained in the cytoplasm in association with chaperone proteins such as heat shock protein 90 (hsp90) (11, 12). Hormone binding initiates the release of the chaperone proteins and translocation of the receptor into the nucleus where GR binds to DNA promoter elements termed glucocorticoid response element (GRE) from which it can either activate or repress transcription depending on the context of the target promoter (6, 8). In addition, the GR also interacts with other transcription factors such as nuclear factor-κB (NF-κB) to repress their transcriptional activities. This GR-mediated repression has been postulated to be one of the major mechanisms for the therapeutic antiinflammatory and immunosuppressive activities of glucocorticoids (13, 14).

Recent crystallographic analyses of the nuclear receptors have clarified the relationship between receptor structure and function and established a paradigm of receptor activation. The GR LBD, similar to other nuclear receptor LBDs, is composed of α-helices and β-strands folded into a three-layer helical sandwich. The ligand binding pocket is composed of residues from helices 3, 4, 5, 6, 7, 10, and the AF-2 helix as well as residues from β-strands between helices 5 and 6. Following AF-2 helix is an extended strand that forms a conserved β-sheet with a β-strand between helices 8 and 9. This C-terminal β-strand also appears to play an important role in receptor activation by stabilizing AF-2 helix in an active conformation (15, 16). Many AF-2 coactivators for the GR have been identified to date, including steroid receptor coactivator-1, transcriptional intermediary factor (TIF) 2/GR-interacting protein-1 and cAMP response element binding protein-binding protein/p300 (17–21). These coactivators directly associate with the GR LBD via their LXXLL motif. For example, the LLRYLL sequence in the TIF2 forms a two-turn α-helix that orients the hydrophobic leucine side chains into a groove formed in part by the AF-2 helix and residues from helices 3, 3′, 4, and 5. The N- and C-terminal ends of the coactivator helix are clamped by Glu-755 from the AF-2 helix and Lys-579 in helix 3, respectively (15). Mutations that disrupt either the first (Glu-755) or the second (Arg-585 and Asp-590) charge clamp dramatically reduce activation mediated by the GR LBD, demonstrating that they are critical for transactivation function of the GR (15). On the other hand, AF-1 coactivators have only recently been described. For example, basal transcription factors including TBP and TFIID have been shown to associate with the AF-1 of GR (22, 23). TSG101 and DRIP150 have also been reported to interact with GR AF-1 and regulate GR function in a reciprocal manner; GR transcriptional activities are repressed by TSG101 but enhanced by DRIP150 (24). These cofactors are shown to interact with distinct regions of AF-1 (22–24). Although we now have at hand a large number of regulatory proteins that interact directly or indirectly with the various modular domains of nuclear receptors, how ligands differentially regulate the functional interplay between them remains poorly understood.

The phenylpyrazologlucocorticoid CVZ is a unique synthetic glucocorticoid agonist with complex binding properties and is more potent than DEX (25). We previously demonstrated that CVZ selectively binds to the GR but not to the MR and, based on two criteria, we proposed that the functional interaction of CVZ with the GR LBD is different from that of DEX. Firstly, deletion of the last 12 amino acids of GR severely compromises DEX but not CVZ binding and secondly, the point mutant L753F, in which Leu-753 in AF-2 is substituted to Phe, can efficiently recruit TIF2 to the LBD when bound to CVZ but not when bound to DEX (26). These results prompted us to propose that occupancy of the GR LBD by CVZ might lead to a more stable active conformation that can tolerate the disrupting effects of LBD mutations and may have unique effects on the structure and function of the whole GR molecule. In the present study, we explore the distinct properties of CVZ-bound GR by modeling its structure, analyzing the influence of both destabilizing and stabilizing LBD mutations, and probing the role played by other receptor domains. We provide evidence that the CVZ-specific LBD conformation allows efficient TIF2 recruitment to the receptor at least in part through intrareceptor communication between the LBD and DBD, as well as through collaboration with N-terminal sequences.

RESULTS

Docking Model of CVZ-Bound GR LBD

Because we and others showed that CVZ-bound GR has distinct functional characteristics when compared with DEX-bound GR (see introductory section), we first examined whether CVZ could be accommodated into the classical ligand binding pocket of the GR LBD or whether a distinct binding mode must be invoked. For this purpose, we modeled the three-dimensional structure of CVZ-bound GR using the coordinates of the crystal structure between the F602S mutant LBD and DEX (15). In brief, we docked CVZ *in silico* into the ligand binding pocket of the GR LBD by superimposing its steroid backbone with that of DEX. The calculated volume of DEX and CVZ is 386 Å^3 and 541 Å^3, respectively, reflecting that CVZ has a bulky phenylpyrazole ring attached to the A-ring of steroid backbone as well as a C21-acetoxyl group at the D-ring (Fig. 1A). The estimated volume of the ligand binding pocket of the GR LBD is 600 Å^3, which appears to be large enough for binding either ligand. Energy minimization of the CVZ/GR LBD complex suggested that favorable configurations between GR and CVZ could be reached by the induced fit mechanism. The resultant model for CVZ-GR LBD is shown in Fig. 1B. CVZ,