Bcl-w Promotes Gastric Cancer Cell Invasion by Inducing Matrix Metalloproteinase-2 Expression via Phosphoinositide 3-Kinase, Akt, and Sp1

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Abstract

Given a previous report that Bcl-w is expressed in gastric cancer cells, particularly in those of an infiltrative morphology, we investigated whether Bcl-w expression influences the invasiveness of gastric cancer cells. To accomplish this, Bcl-w was overexpressed in adherent types of gastric adenocarcinoma cell lines, and this was found to result in an increase in their migratory and invasive potentials. These effects were not induced when Bcl-2 was overexpressed in the same cell types. Consistently, Bcl-w, but not Bcl-2, overexpression increased matrix metalloproteinase-2 (MMP-2) expression, and synthetic or natural inhibitors of MMP-2 abolished Bcl-w–induced cell invasion. Bcl-w overexpression also activated phosphoinositide 3-kinase (PI3K), Akt, and Sp1, and the blocking effects of each of these components using pharmacologic inhibitors, dominant-negative mutants, or small interfering RNA abolished the ability of Bcl-w to induce MMP-2 and cell invasion. The inhibition of PI3K/Akt signaling also prevented Sp1 activation. Overall, our data suggest that Bcl-w, which was previously shown to enhance gastric cancer cell survivability, also promotes their invasiveness by inducing MMP-2 expression via the sequential actions of PI3K, Akt, and Sp1. (Cancer Res 2006; 66(10): 4991-5)

Introduction

Cancer cells display many abnormal properties, which can reduce the efficacy of anticancer treatments. Resistance to apoptosis is one such example and is a major problem encountered during chemo- and radiotherapy. It is widely accepted that the up-regulation of prosurvival proteins can provide cancer cells with such resistance. The invasion and subsequent metastasis of cancer cells is a major additional reason for the failure of cancer therapy. Matrix metalloproteinases (MMP) that degrade the extracellular matrix are frequently up-regulated in cancer cells and seem to facilitate cancer cell invasion (1, 2). Given the importance of cancer cell survival and invasion, the determination of their relationships seems to be essential for a better understanding of tumor biology and for the development of new treatment strategies. However, relations between the two are poorly characterized.

Bcl-w is a prosurvival member of the Bcl-2 protein family (3) but has received scant attention as compared with other Bcl-2 family members. Consequently, only limited information is available about its physiologic role and modes of action. Recent reports suggest that Bcl-w can be up-regulated in certain tumors. For example, Bcl-w expression was detected in gastric cancer samples obtained from patients in a cancer cell–specific manner (4), suggesting that Bcl-w is involved in the survival of gastric cancer cells. The ability of Bcl-w to protect gastric cancer cells from death stimuli has been directly shown using Bcl-w–overexpressing cell lines (4). Cancer cell–specific Bcl-w expression was also reported using samples from colorectal cancer patients (5). Therefore, Bcl-w seems to be important in cancer, at least in the gastrointestinal tract. Interestingly, pathologic analyses of gastric tumor samples revealed that Bcl-w expression is tightly associated with the infiltrative (diffuse) types of the tumor (4). This observation raised the possibility that the role of Bcl-w in gastric cancer is not confined to the survival of cancer cells but that Bcl-w can also promote their invasiveness. The present study was undertaken to investigate this possibility and to identify the cellular components involved.

Materials and Methods

Antibodies and inhibitors. Antibodies used were anti-Bcl-w, anti-PTEN, anti-Sp1, anti-Akt, and anti-phospho-Akt (purchased from Santa Cruz Biotechnology, Santa Cruz, CA); anti-MMP-2 and anti-MMP-9 (Calbiochem, La Jolla, CA); anti–tissue inhibitor of metalloproteinase (TIMP)-1 and anti-TIMP-2 (Chemicon, Temecular, CA); anti-Bcl-2 (PharMingen/Transduction Laboratories, San Diego, CA); and anti–p85 subunit of phosphoinositide 3-kinase (PI3K; Upstate Biotechnology, Lake Placid, NY). Calbiochem provided the recombinant TIMP-2 protein and all the synthetic inhibitors used in this study.

Cell culture and transfection. SNU-484 and SNU-638 cells, which were established from gastric adenocarcinoma patients with the same pathologic type (poorly differentiated; ref. 6), were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and gentamicin (50 μg/mL). Expression constructs were prepared using pcDNA vectors and transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfected cells were selected by using 2 mg/mL G418 sulfate. Heterogeneous populations of stably transfected cells were used in this study to avoid any possible clonal variations. Sp1 small interfering RNA (purchased from Santa Cruz Biotechnology) was introduced into cells using Lipofectamine 2000.

Invasion and migration assays. Tumor cell invasion was measured by examining cell invasion through Matrigel-coated polycarbonate filters using modified Boyden chambers (Corning, Corning, NY). Briefly, cells (2×10^5) in 200 μL of medium were seeded onto the upper portion of a chamber; the lower compartment was filled with 1 mL of serum-free media supplemented with 0.1% bovine serum albumin. After incubation for 24 hours at 37°C, the cells that migrated to the lower surface of the filter were fixed and stained using Diff-Quick kit (Fisher Scientific, Pittsburgh, PA) and then counted under a microscope (7). Migration assays were done using the same

procedure but with uncoated polycarbonate filters. Results were analyzed for statistical significance using Student's *t* test. Differences were considered significant at $P < 0.05$.

Gelatin zymography. This was used to analyze the activities of secreted MMP-2 and MMP-9. Conditioned media were prepared by incubating cells in serum-free media for 24 hours. Where indicated, media were supplemented with specific inhibitors. Equal volumes of conditioned media were subjected to 10% SDS-PAGE containing 0.1% gelatin. Gels were stained and gelatinase activities were visualized as clear bands (7).

Western blot analysis. Proteins either in conditioned media or in cell lysates, prepared using a previously described method (8), were separated by SDS-PAGE, and electrotransferred to Immobilon membranes (Millipore, Bedford, MA), which were subsequently blotted using the indicated antibodies and visualized by the enhanced chemiluminescence detection system (Amersham, Uppsala, Sweden).

Reverse transcription-PCR. Total RNA was extracted using TRIzol (Invitrogen). RT reaction was done using total RNA as a template and a RT-for-PCR kit (Promega, Madison, WI). PCR amplification was carried out with the following primers: MMP-2, 5′-caggctcttctcctttcacaac-3′ and 5′-aagccacggcttggtttcctc-3′; TIMP-1, 5′-ggggcttcaccaagacctacac-3′ and 5′-aagaaagatgggagtgggaaca-3′; TIMP-2, ccacccagaagaagagcctgag-3′ and 5′-tggacccatgggatgactgttt-3′; and actin, 5′-gcccagagcaagagaggcat-3′ and 5′-ggccatctcttgctcgaagt-3′.

Electrophoretic mobility shift assay. The preparation of nuclear extracts and subsequent electrophoretic mobility shift assay (EMSA) were done as previously described (9). Briefly, 5 μg of nuclear extracts were incubated for 30 minutes with 35 pmol of the ^{32}P end-labeled Sp1-specific oligonucleotide 5′-attcgatcggggcggggcgagc-3′ in 10 μL of a binding buffer (9). The Sp1-DNA complex was separated from free oligonucleotide on a 5% native polyacrylamide gel. The specificity of binding was confirmed by competition with unlabeled oligonucleotides.

PI3K assay. Cells were lysed as previously described (10). Equal amounts of the lysate proteins were immunoprecipitated with anti-p85. The immune complexes were washed and resolved in a reaction buffer containing [γ-^{32}P]ATP (10). L-α-Phosphatidylinositol (Sigma, St. Louis, MO; 5 mg/mL) was used to initiate the kinase reactions. After 20-minute incubation, the reactions were quenched by adding 1 mol/L HCl and analyzed by TLC (10).

Results

Bcl-w enhances SNU-484 cell invasion. To investigate whether Bcl-w influences the invasiveness of gastric cancer cells, Bcl-w was overexpressed in SNU-484 cells (Fig. 1*A*). This treatment efficiently reduced the cell death induced by γ-irradiation (2.5-10 Gy; data not shown), thus confirming the prosurvival role of Bcl-w in SNU-484 cells. Interestingly, Bcl-w overexpression also promoted their migration and invasion, as analyzed on uncoated and Matrigel-coated membrane filters, respectively (Fig. 1*B*).

Bcl-w induces MMP-2 expression. This finding raised the possibility that Bcl-w enhances the activities of MMPs. Given the frequent up-regulations of MMP-2 and MMP-9 in various cancer cell types (1, 2), we compared the activities of these MMPs in control and Bcl-w-overexpressing cells by using zymography assays. The data shown in Fig. 1*C* show that Bcl-w overexpression significantly enhanced MMP-2 activity, which might reflect increased MMP-2 expression. Indeed, Bcl-w overexpression resulted in an elevation in MMP-2 protein (Fig. 1*D*) and of its mRNA (Fig. 1*E*), as shown by Western blotting and reverse transcription-PCR (RT-PCR), respectively. In contrast, Bcl-w did
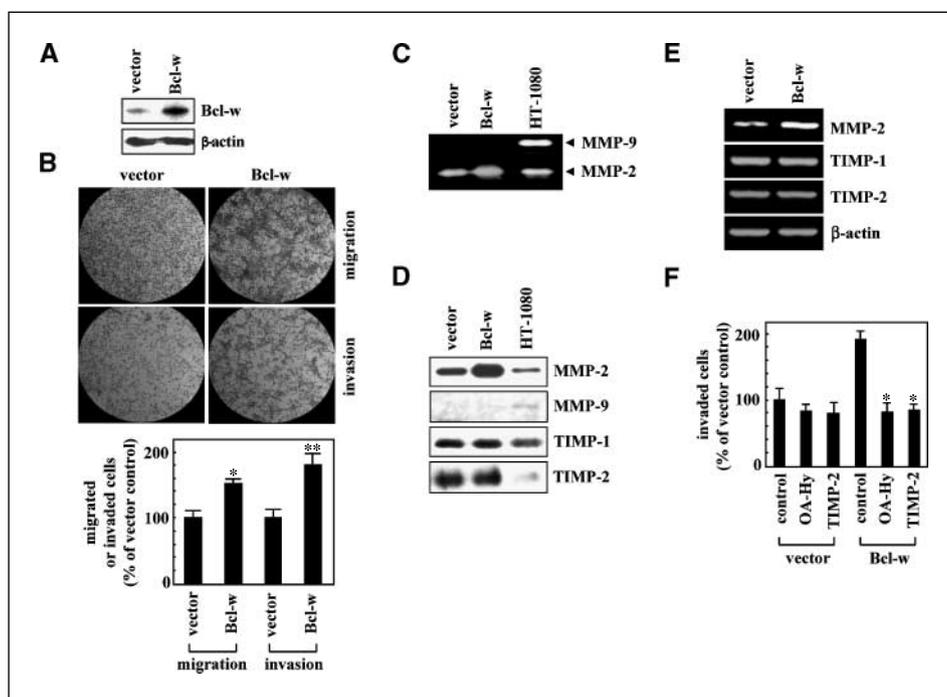

Figure 1. Bcl-w promotes the migration and invasiveness of SNU-484 gastric cancer cells by inducing MMP-2. SNU-484 cells were transfected with empty pcDNA vector or a vector containing Bcl-w cDNA. *A,* Bcl-w expressions were compared by Western blotting using β-actin as a loading control. *B, top,* transfectants were seeded onto either uncoated or Matrigel-coated polycarbonate filters to analyze their migratory and invasive potentials, respectively. Cells were then incubated for 24 hours in modified Boyden chambers and the number of cells that migrated through filters were stained and counted under a light microscope. *Bottom, columns,* mean of triplicate experiments; *bars,* SD. *, $P < 0.05$; **, $P < 0.005$, statistically different from controls. *C,* conditioned media were prepared by incubating control and Bcl-w transfectants in serum-free media for 24 hours. MMP-2 and MMP-9 activities in samples were analyzed by gelatin zymography. HT-1080 fibrosarcoma cells were used as a positive control. *D,* levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 in conditioned media were compared by Western blotting. *E,* cellular levels of MMP-2, TIMP-1, and TIMP-2 mRNAs were analyzed by RT-PCR using β-actin as a control. *F,* Matrigel invasion assays were done in the presence or absence of the MMP-2 inhibitors, OA-Hy (20 μmol/L) and TIMP-2 (0.1 μmol/L). *, $P < 0.05$, versus untreated Bcl-w control ($n = 3$).

Figure 2. Role of Sp1 in Bcl-w–induced invasion. *A*, EMSA for Sp1 was done using nuclear extracts prepared from the indicated SNU-484 transfectants. *B*, SNU-484 transfectants were treated with serum-free media containing the indicated concentrations of mithramycin A (*MTM*) or curcumin (*CCM*). Gelatin zymography and Matrigel invasion assays were done after incubating for 24 hours. *, $P < 0.001$, versus untreated Bcl-w control ($n = 5$). *C*, Sp1 small interfering RNA (*siRNA*) was introduced into the indicated SNU-484 transfectants, and after 24 hours of incubation, cellular levels of Sp1 were compared by Western blotting using α-tubulin as a loading control. Gelatin zymography and Matrigel invasion assays were done using small interfering RNA–treated and untreated cells. *, $P < 0.005$, versus untreated Bcl-w control ($n = 5$).
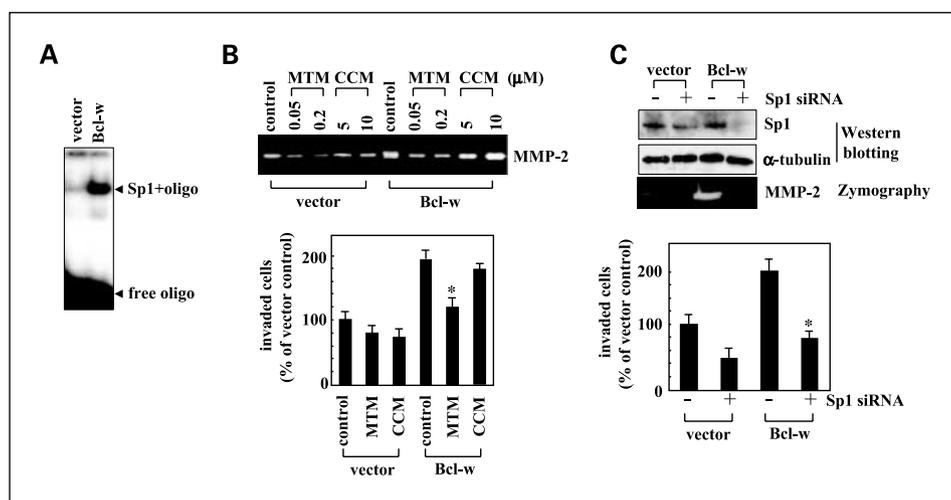
not significantly alter the protein and mRNA levels of TIMP-2, an endogenous inhibitor of MMP-2. The activity of MMP-9 (Fig. 1*C*) and its protein (Fig. 1*D*) and mRNA levels (data not shown) were barely detectable, regardless of Bcl-w overexpression. TIMP-1, an inhibitor of MMP-9, was found to be expressed in SNU-484 cells, but its protein and mRNA levels were not significantly influenced by Bcl-w overexpression. Therefore, Bcl-w seems to specifically up-regulate MMP-2.

To determine the role of MMP-2 in this system, an invasion assay was done in the presence of the synthetic MMP-2 inhibitor OA-Hy. Under this condition, Bcl-w failed to promote cell invasion and similar results were obtained using recombinant TIMP-2 protein (Fig. 1*F*), suggesting that MMP-2 activity is required for Bcl-w–induced cell invasion. Overall, Bcl-w seems to promote SNU-484 cell invasion by increasing MMP-2 expression.

Sp1 is involved in MMP-2 induction. Given that the transcription factor Sp1 supported the constitutive MMP-2 expression in astroglioma cells (11), we next investigated whether Sp1 is involved in Bcl-w–induced MMP-2 expression. Bcl-w overexpression indeed promoted Sp1 activation analyzed by EMSA (Fig. 2*A*). Moreover, mithramycin A (0.05-0.2 μmol/L), a Sp1-specific inhibitor, abolished Bcl-w–induced MMP-2 activity and cell invasion (Fig. 2*B*), and similar effects were induced when cellular Sp1 levels were reduced by RNA interference (Fig. 2*C*). In contrast, curcumin (5-10 μmol/L), an activator protein 1 (AP-1) inhibitor, did not significantly influence these abilities of Bcl-w (Fig. 2*B*). Therefore, Sp1 seems to mediate the ability of Bcl-w to induce MMP-2 and cell invasion.

PI3K and Akt act upstream of Sp1. The PI3K/Akt pathway has been suggested to participate in tumor cell invasion under other experimental conditions (12, 13). In the present study, Bcl-w overexpression also elevated PI3K activity (Fig. 3*A*) and Akt phosphorylation (Fig. 3*B*), suggesting that Bcl-w can activate the PI3K/Akt pathway. Moreover, Bcl-w failed to increase MMP-2 activity and cell invasion when SNU-484 cells were treated with 10 μmol/L LY294002, an inhibitor of PI3K (Fig. 3*C*). Such effects were not induced by 25 μmol/L of either of PD 98059, SB 203580, or SP 600125, which inhibit extracellular signal–regulated kinase, p38 kinase, and c-Jun NH₂-terminal kinase, respectively (data not shown). These findings suggest that PI3K has a specific role in Bcl-w–induced invasion. To further confirm this possibility, PTEN, a tumor suppressor protein that can block

PI3K-induced signaling, was cotransfected with Bcl-w. This treatment, like LY294002, suppressed the ability of Bcl-w to increase MMP-2 activity and cell invasion (Fig. 3*D*). Furthermore, the effects of PTEN were mimicked by the dominant-negative mutant of Akt (Akt-M), suggesting that PI3K and Akt are involved in Bcl-w–induced MMP-2 expression and invasion. Moreover, the coexpression of either PTEN or Akt-M with Bcl-w also abolished Bcl-w–induced Sp1 activation (Fig. 3*E*), suggesting that PI3K and Akt act upstream of Sp1. Similarly, it has been proposed that Akt induces vascular endothelial growth factor expression via Sp1 (14). Overall, Bcl-w seems to promote gastric cancer cell invasion by inducing the PI3K-Akt-Sp1-MMP-2 pathway.

Role of Bcl-w in other gastric cancer cell types. To investigate the cell type specificity of Bcl-w action, Bcl-w was overexpressed in SNU-638 cells and, as in SNU-484 cells, this resulted in an increase in MMP-2 activity, its protein and mRNA levels, and in cell invasion (Fig. 4*A*). Both SNU-484 and SNU-638 cells grow as adherents. Therefore, we next examined the role of Bcl-w in floating types of gastric cancer cells (i.e., SNU-16 and SNU-620). It has been reported that SNU-620 cells constitutively hyperexpress Bcl-w (4). Bcl-w levels in SNU-620 cells were similar to those in Bcl-w-overexpressing SNU-484 cells. Nevertheless, SNU-620 cells neither expressed MMP-2 nor displayed an invading activity through Matrigels (data not shown). Similar results were obtained using untransfected or Bcl-w–overexpressing SNU-16 cells. Therefore, Bcl-w seems to promote gastric cancer cell invasion only in adherent cell types.

Role of Bcl-2 in gastric cancer cell invasion. Given that Bcl-2 is also up-regulated in gastric cancer cells (15, 16), we overexpressed Bcl-2 in SNU-484 cells. This treatment protected these cells from γ-irradiation (2.5-10 Gy; data not shown), which confirmed the function of exogenous Bcl-2 in SNU-484 cells. Nevertheless, Bcl-2–overexpression did not increase MMP-2 expression and cell invasion (Fig. 4*B*). Thus, Bcl-2 and Bcl-w probably function differently in terms of the regulation of gastric cancer cell invasion.

Discussion

The present study shows for the first time that Bcl-w can enhance the invasiveness of cancer cells. This novel function of
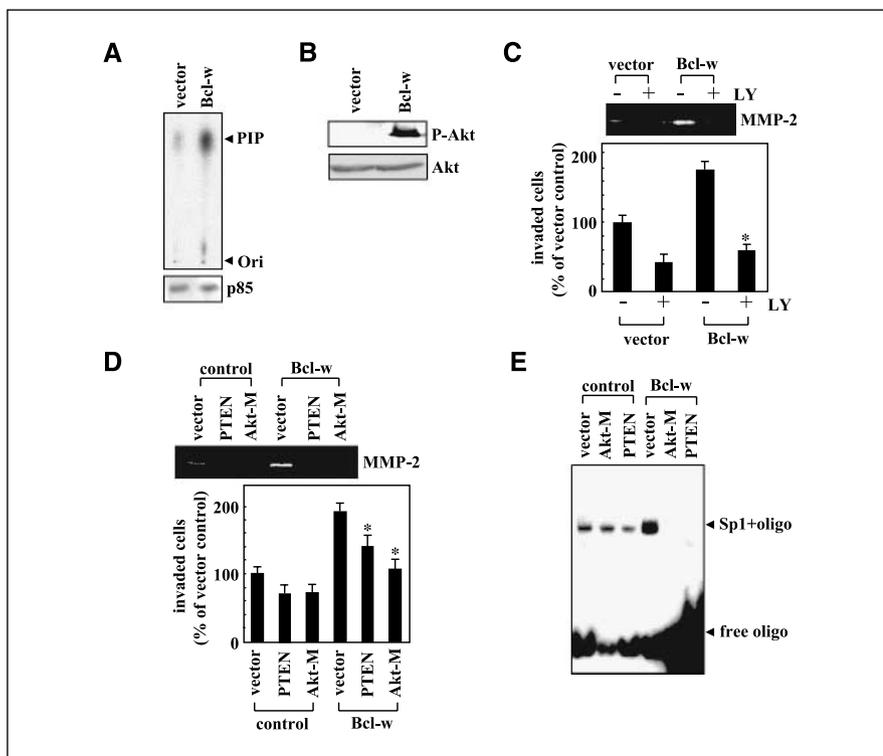
Figure 3. PI3K and Akt act upstream of Sp1. *A,* the indicated SNU-484 transfectants were lysed and PI3K activities in lysates were analyzed by *in vitro* kinase assays using L-α-phosphatidylinositol as a substrate. Levels of the p85 subunit of PI3K were compared by Western blotting. *B,* levels of phosphorylated and unphosphorylated Akt in samples were compared by Western blotting. *C,* transfectants were treated with serum-free media with or without LY294002 (*LY*; 10 μmol/L). Gelatin zymography and Matrigel invasion assays were done 24 hours after treatment. *, $P < 0.001$, versus untreated Bcl-w control ($n = 3$). *D,* SNU-484 cells were cotransfected as indicated and subjected to gelatin zymography and Matrigel invasion assays. *, $P < 0.05$, versus untreated Bcl-w control ($n = 5$). *E,* cotransfected cells were analyzed for Sp1 activity by EMSA.

Bcl-w was shown using adherent, but not floating, types of gastric cancer cell lines. Cell lines that grow in suspension may have lost the ability to interact with and invade extracellular materials. The invasion-promoting activity of Bcl-w seems to be important *in vivo*, given our previous analysis of patient samples, which showed that the majority of Bcl-w-expressing gastric tumors display an infiltrative (diffuse) morphology (4). Our findings suggest that the survival and invasion of cancer cells can be linked and that Bcl-w provides such a link at least in gastric tumors. In contrast, Bcl-2 failed to facilitate the invasion of gastric cancer cells, a finding that is consistent with the previous observation that Bcl-2 is predominantly expressed in noninfiltrative (intestinal) gastric tumor types (15, 16). Nevertheless, Bcl-2 was found to be cytoprotective in gastric cancer cells, which suggests that the prosurvival activities of Bcl-2 family members do not always guarantee their invasion-promoting activities. Given that Bcl-2 overexpression can increase the invasive potential of other tumors, such as glioma (17) and non–small-cell lung cancer cells (18), the ability of Bcl-2 family members to support cell invasion seems to depend on tissue type.

We also found that the invasion-promoting action of Bcl-w in gastric cancer cells is mediated by a cellular pathway that sequentially involves PI3K, Akt, Sp1, and MMP-2. It has been reported that Bcl-2 can also up-regulate MMP-2 in glioma (17) and non–small-cell lung cancer cells (18). In this latter case, AP-1 has been proposed to be a transcription factor involved in Bcl-2–induced MMP-2 expression. Given that AP-1 inhibitors failed to attenuate the action of Bcl-w in the present system, Bcl-2 and Bcl-w seem to promote cell invasion by different mechanisms. This speculation is also supported by the present observation that Bcl-w, but not Bcl-2, promotes cell invasion in SNU-484 cells.

In conclusion, this study shows the invasion-promoting activity of Bcl-w and its underlying mechanism. These findings significantly advance our understanding not only of the actions of Bcl-w but also of the pathogenesis of gastric cancer.
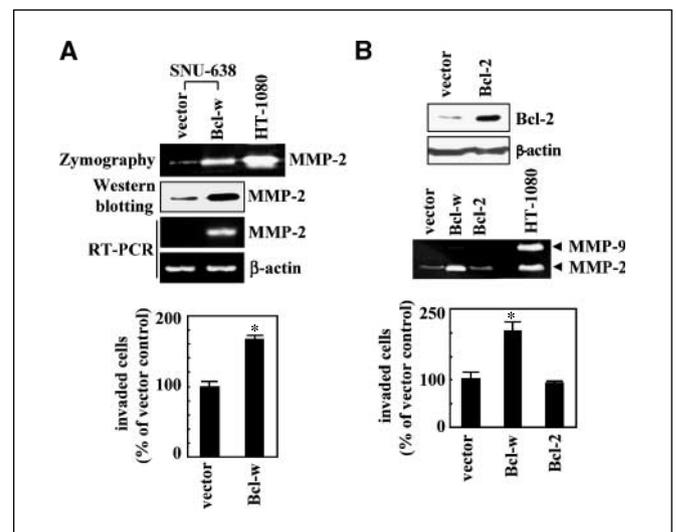

Figure 4. Cell type specificity of Bcl-w action and the role of Bcl-2 in gastric cancer cell invasion. *A,* SNU-638 cells, rather than SNU-484, were transfected with Bcl-w. Conditioned media were prepared using control and Bcl-w–transfected cells. Samples were then analyzed by gelatin zymography and Western blotting to compare MMP-2 activity and its protein levels, respectively. Alternatively, the cellular levels of MMP-2 mRNA were analyzed by RT-PCR using β-actin as a control. Transfectants were also subjected to Matrigel invasion assays. *, $P < 0.01$, versus vector control ($n = 3$). *B,* SNU-484 cells were transfected with Bcl-2 instead of Bcl-w. The indicated transfectants were subjected to gelatin zymography and Matrigel invasion assays. *, $P < 0.005$, versus vector control ($n = 3$).

Acknowledgments

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