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Integrin-Mediated Signalling of Gelatinase B Secretion in Colon Cancer Cells

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The progression of colon cancer has been linked to both cell adhesion molecules called integrins and matrix-degrading enzymes called metalloproteinases. Herein we report that the $\alpha v\beta 6$ integrin expressed in colon cancer cells induces gelatinase B secretion through the C-terminal cytoplasmic extension unique to the $\beta 6$ integrin subunit, and that this ligand-independent event involves activation of the protein-kinase-C pathway.

Tumour growth and invasion are regulated, at least partly, by matrix-degrading enzymes and cell adhesion receptors. In colon cancer, the invasive potential of colon cancer cells has been linked to the expression of one member of the matrix metalloproteinase (MMP) family called gelatinase B (1). Although the mechanism involved is not understood, the family of cell adhesion receptors called integrins has been implicated in signalling MMP secretion in other cell types (2,3). These heterodimeric cell adhesion molecules are comprised of two transmembrane subunits (α and β) in non-covalent association (4), and we have recently reported that one member of the integrin family, $\alpha v\beta 6$, is a major fibronectin-binding receptor on colon cancer cells (5). Importantly, heterologous expression of $\alpha v\beta 6$ in a colon cancer cell line has been shown by us to lead to enhanced tumour growth *in vivo,* and deletion of only the 11 amino acid C-terminal extension unique to the $\beta 6$ subunit cytoplasmic domain abolishes the $\beta 6$-mediated growth effect (6). In the present report we describe the structure-function relationship between the $\beta 6$ subunit cytoplasmic domain and gelatinase B secretion and show that $\alpha v\beta 6$-mediated gelatinase B secretion occurs independently of integrin ligation with substrate and involves protein-kinase-C signalling.

MATERIALS AND METHODS

Antibodies and reagents. The monoclonal antibody E7P6 directed to the extracellular domain of human $\beta 6$ was prepared as previously described (7). Monoclonal antibody L230 against αv integrins was prepared from hybridoma cells obtained from the American Type Culture Collection (ATCC, Rockville, MD) (7). Phycoerythrin-conjugated goat-anti-mouse IgG was obtained from Chemicon International Co., CA. Collagen type I, fibronectin and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. Mouse laminin was obtained from Becton Dickinson (Bedford, MA) and vitronectin purified from human plasma as previously described by Yatohgo et al. 1988 (8).

Cell line and preparation of tumour-conditioned medium. The colon cancer cell line SW480 was obtained from the ATCC. Stable transfectants of SW480 colon cancer cells expressing pcDNA1neo constructs containing wildtype or mutant forms of $\beta 6$, or the expression plasmid only (pcDNA1neo) were prepared as reported previously (6). The cytoplasmic domain mutations comprised $\beta 6$ lacking only the 11 amino acid C-terminal extension of the cytoplasmic domain which is unique to the $\beta 6$ integrin subunit, and a $\beta 6$ receptor lacking the entire cytoplasmic domain. The transfected cell lines were maintained in standard medium comprising Dulbecco's Modified Eagle's Medium (DMEM; 4.5gm/litre of glucose) with 10% heat-inactivated fetal bovine serum supplemented with HEPES, penicillin, streptomycin and the neomycin analogue G418. Tumour-conditioned medium (TCM) was prepared by seeding 2×10^6 cells/75cm flasks in standard medium for 48 hours followed by removal of fetal bovine serum-containing medium and three washes of the adherent cells with phosphate-buffered saline (PBS), before addition of chemically-defined serum-free medium. Serum-free medium comprised DMEM (minus phenolphthalein) supplemented with ITS (insulin, selenous acid, and transferrin), HEPES and penicillin/streptomycin, and was harvested 48 hours later. The TCM was cleared of cells and debris by centrifugation at 4000RPM for 10 minutes, followed by protein estimation using the BCA protein assay reagent (Pierce, Rockford, IL).

FACScan analyses. Monolayer cultures of SW480 transfectants were harvested with 20mM EDTA and then blocked with goat serum at 4°C for 10mins. Cells were washed once with PBS and incubated with primary antibody (E7P6) for 20 mins at 4°C and then washed twice with PBS. Cells were then stained with secondary antibody conjugated with phycoerythrin for 20mins at 4°C, washed twice with PBS and re-suspended in 0.5ml PBS prior to FACScan analysis (Becton Dickinson, Rutherford, NJ).

Zymography. The gelatinases (A and B) were analysed using SDS-substrate gels (9). Gelatin was added to the 10% acrylamide

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separating gel at a final concentration of 0.1mg/ml. Tumour-conditioned medium collected under serum-free conditions was mixed with substrate gel sample buffer (10% SDS, 50% glycerol, 25mM Tris-HCl, pH 6.8 and 0.1% bromophenol blue) and 70μl loaded on to the gel without prior boiling. Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30mins at room temperature to remove the SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50mM Tris HCl and 5mM $CaCl_2$ at pH 8.0. The gels were stained with 0.15% coomassie blue R250 in 50% methanol, 10% glacial acetic acid for 20mins at room temperature and de-stained in the same solution without coomassie blue. The gelatin-degrading enzymes were identified as clear bands against the blue background of stained gel.

RESULTS

Effect of β6 Subunit Cytoplasmic Domain Deletions on Secretion of Gelatinase B

The β6 subunit cytoplasmic domain contains two NPXY motifs shared by other β subunit cytoplasmic domains and an 11 amino acid C-terminal cytoplasmic extension found only in the β6 integrin subunit (10) as shown diagrammatically in Figure 1A. To investigate whether the cytoplasmic extension unique to β6 is required for induction of gelatinase B secretion, we examined the ability of SW480 cells stably transfected with truncated β6 gene constructs to promote gelatinase B secretion. One truncated variant lacked the entire β6 cytoplasmic domain and the other only the C-terminal 11 amino acids (738t and 777t, respectively, as shown in Figure 1A). Gelatin zymography of non-concentrated TCM obtained from the SW480 transfectants revealed that cells expressing either of the truncated variants of β6 secreted similar amounts of gelatinase B to that seen for SW480 wild-type and mock transfectants (Figure 1B). In contrast, markedly enhanced secretion of gelatinase B was observed for TCM from cells expressing full-length β6. SW480 cells secrete much less gelatinase A than gelatinase B and the slight increase in gelatinase A (72kD) seen for full-length β6 transfectants in Figure 1B was not a consistent finding between mock and β6 transfected cell lines.

To exclude the possibility that the enhanced gelatinase B secretion observed for cells expressing full-length β6 compared with truncated β6 transfectants might be associated with more αvβ6 on the cell surface, FACScan analyses were performed as shown in Figure 1C. Surface expression of β6 in the full-length β6 transfectants was similar to that observed for the truncated receptors and we have previously reported that heterologous expression of these mutant versions, like expression of the full-length receptor, does not produce systematic alterations in expression of other integrins (6).

αvβ6-Mediated Gelatinase B Secretion Occurs Independently of Integrin Ligation

To examine the effect of αvβ6 ligation with ligand on gelatinase B secretion, cells were cultured as adherent monolayers on irrelevant and relevant β6-substrates for 24 hours and the serum-free, non-concentrated TCM exposed to gelatin zymography. In addition, gelatinase activity was examined in TCM obtained from cells seeded on plastic pre-coated with bovine serum albumin (BSA). As shown in Figure 2, the enhanced gelatinase B secretion from SW480 β6-expressing cells compared with mock transfectants was maintained under all culture conditions (non-adherent cells seeded on BSA-treated plastic and adherent cell monolayers on irrelevant substrates such as laminin and collagen). Exposure of β6 transfectants to blocking anti-αv antibody at a concentration known to abolish β6-mediated adhesion to fibronectin (the major ligand for αvβ6) also had no effect on gelatinase B secretion compared with cells seeded on fibronectin in the absence of antibody (Figure 2).

αvβ6-Mediated Gelatinase B Secretion Involves Signalling via the Protein-Kinase-C Pathway

To determine whether the enhanced secretion of gelatinase B observed for SW480 β6-expressing cells might be regulated by protein-kinase-C, TCM obtained from cells cultured in the absence/presence of calphostin-C was subjected to gelatin zymography. Calphostin-C is a specific inhibitor of protein-kinase-C and, as shown in Figure 3, the enhanced gelatinase B secretion observed following treatment of β6-expressing cells with 100nM calphostin-C reduced levels of gelatinase B to those seen for TCM obtained from mock transfectants.

DISCUSSION

In the present report we show that αvβ6-mediated gelatinase B secretion requires the presence of the entire β6 cytoplasmic domain including the 11 amino acid C-terminal extension unique to the β6 integrin subunit. αvβ6 is the major fibronectin-binding integrin expressed on colon cancer cells (5) and we have previously reported that the 11 amino acid C-terminal extension unique to the β6 cytoplasmic domain is not essential for β6-mediated binding to fibronectin (6). To explore the possibility that the unligated receptor is capable of inducing gelatinase B secretion, cells were seeded on a range of matrix substrates. As shown, β6-induced gelatinase B secretion was maintained in cultures of non-adherent cells as well as in cultures of adherent cell monolayers when cells were seeded on irrelevant matrix substrates such as laminin and collagen. However, since SW480 cells themselves secrete large amounts of fibronectin (6), it was not possible to study the effect of β6 expression on gelatinase B secretion in the absence of the ligand for αvβ6. Nevertheless, inhibition of αvβ6-ligand binding by anti-αv antibody

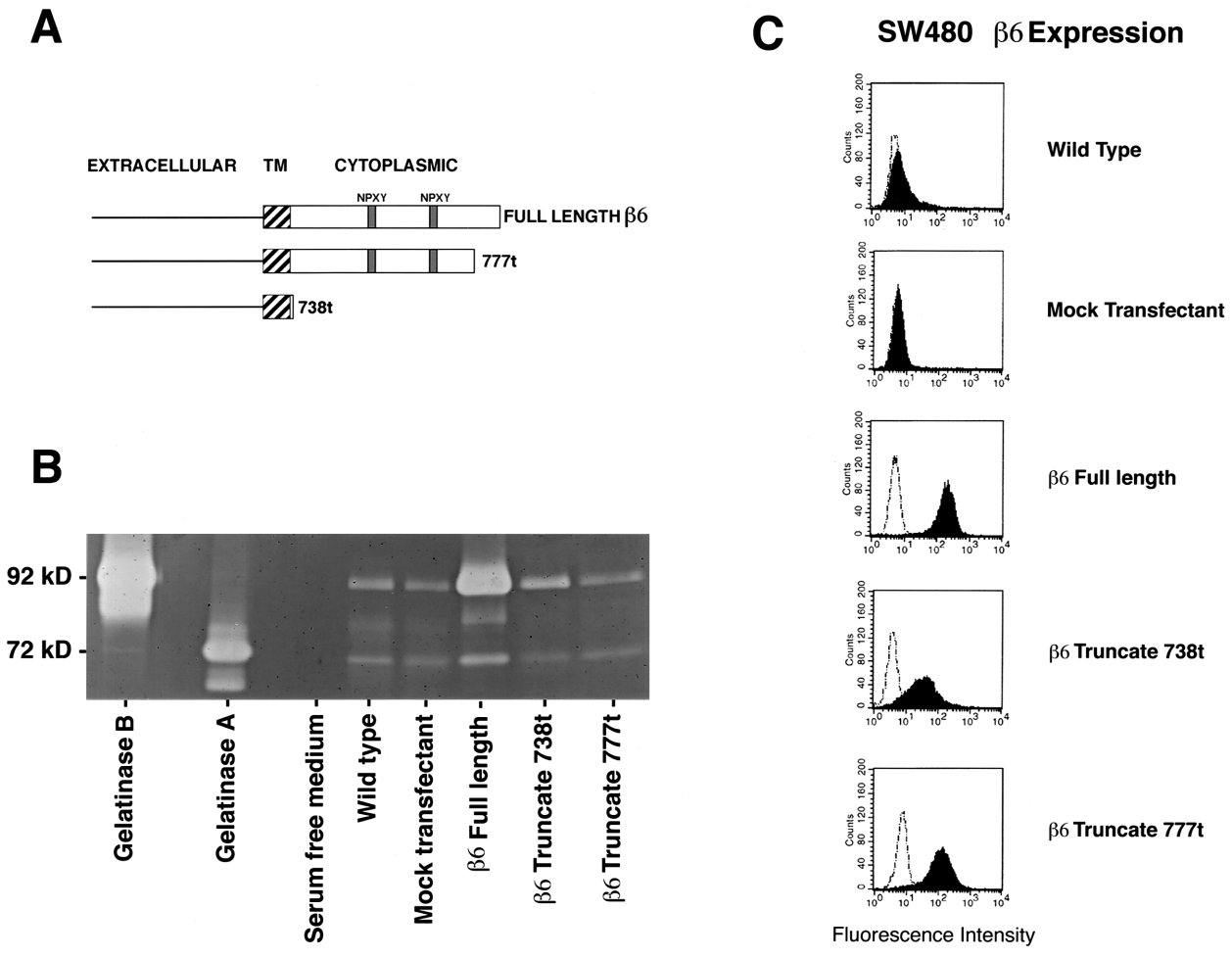

FIG. 1. Effect of β6 subunit cytoplasmic domain deletions on secretion of gelatinase B. (A) Schematic representation of the extent of the truncation in the β6 cytoplasmic domain: 777t lacking the 11 amino acid C-terminal extension unique to the β6 subunit, and 738t lacking the entire β6 cytoplasmic domain. (B) Gelatin zymogram showing amount of gelatinase B present in TCM (non-concentrated) obtained from SW480 wild-type cells, and cell populations stably transfected with vector alone (mock), the full-length β6 construct or truncated β6 cytoplasmic domain variants (777t and 738t). No gelatinolytic activity was detected in serum-free medium used to generate TCM, and the positions of purified gelatinases (A and B) are shown on the left. (C) Flow cytometric analyses of binding of mAb E7P6 (anti-β6) to SW480 wild-type cells and parent cell populations stably transfected with either vector alone (mock), the full-length β6 construct or truncated β6 cytoplasmic domain variants (777t and 738t). The cells were incubated with either no primary antibody (white histograms) or with mAb E7P6 (black histograms) and with goat anti-mouse IgG conjugated with phycoerythrin.

had no effect on gelatinase B secretion when β6-expressing cells were cultured on a fibronectin substrate. Taken together, these findings suggest that β6-induced gelatinase B expression occurs independently of integrin ligation.

Given that phorbol ester response elements are present in the gelatinase B promoter (11-13) we sought to determine whether $\alpha v\beta$6-mediated gelatinase B secretion involved signalling via the protein-kinase-C (PKC) pathway. Gelatinase B secretion by SW480 β6 transfectants was reduced in the presence of calphostin-C to levels seen for SW480 mock transfectants, indicating that β6-induced expression of this enzyme involves activation of the PKC pathway. It was unlikely that the attenuating effect of calphostin-C on gelatinase B levels in TCM from β6-expressing cells was due to toxic effects of the agent because neither cell proliferation nor cell viability were affected in the presence of the inhibitor (data not shown). The results of the present study do not allow us to differentiate between PKC signalling directly via the 11 amino acid C-terminal extension unique to the β6 subunit, or indirectly through induced secretion of second messengers such as inflammatory cytokines. Depending on the cell type, secretion of gelatinase B has been shown to be augmented by various cytokines, such as tumor necrosis factor α, transforming growth factor β, interleukin-1β and epidermal growth factor (14-17). Given that endog-

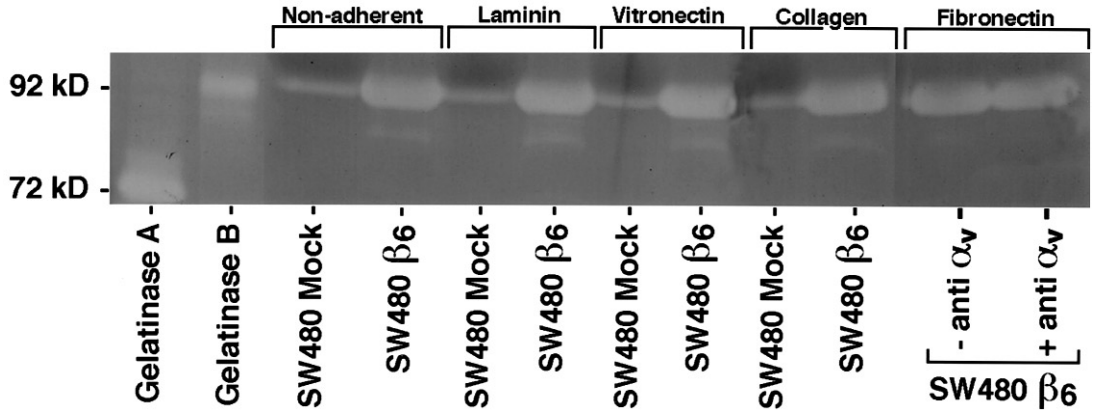

FIG. 2. $\alpha v\beta 6$-mediated gelatinase B secretion occurs independently of integrin ligation. Gelatin zymography (non-concentrated TCM): increased amounts of gelatinase B in TCM from SW480 $\beta 6$ transfectants compared with mock transfectants are shown for cells cultured for 24 hours on BSA-coated plastic (non-adherent cells) and as adherent monolayers on laminin (10μg/ml), vitronectin (10μg/ml) and collagen type I (10μg/ml). Non-specific binding was blocked by treatment of culture wells with 0.5% BSA in PBS prior to addition of cells. Gelatinase B secretion by SW480 $\beta 6$ transfectants seeded on fibronectin (3μg/ml) and exposed to anti-αv blocking antibody (L230; 20μg/ml) is shown in the far right-hand lane. Gelatinase B secretion by SW480 $\beta 6$ transfectants in the absence of anti-αv antibody is shown in the adjacent lane. The positions of purified gelatinases A and B are shown on the left.

enous activation of PKC signalling is elicited by several of these growth factors, further studies are underway to determine if $\beta 6$-mediated gelatinase B secretion reflects autocrine stimulation of colon cancer cells by secreted cytokines which act via the PKC signal transduction pathway.

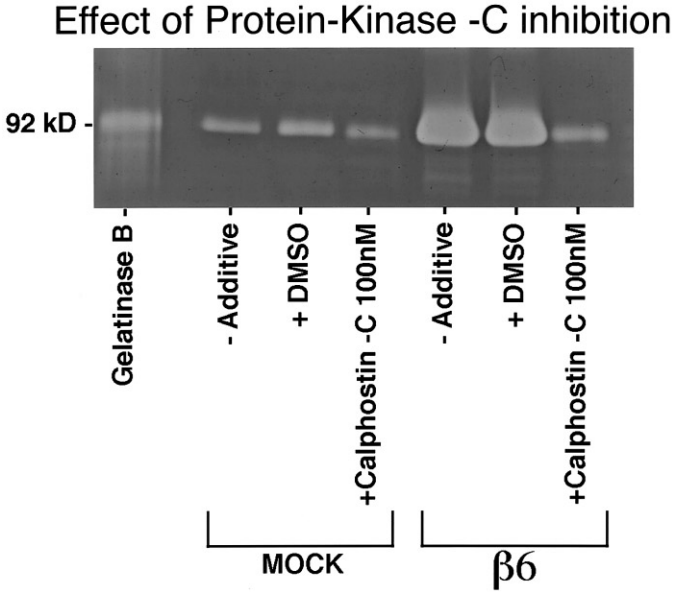

FIG. 3. Effect of calphostin-C on $\alpha v\beta 6$-mediated gelatinase B secretion. Gelatin zymogram: Gelatinolytic activity is shown for TCM collected from SW480 colon cancer cells (mock and $\beta 6$ transfectants) cultured for 48 hours either without additives or in the continuous presence of calphostin-C (100nM) or DMSO (vehicle control). The position of purified gelatinase B is shown on the left.

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