

## Peanut lectin stimulates proliferation of colon cancer cells by interaction with glycosylated CD44v6 isoforms and consequential activation of c-Met and MAPK: functional implications for disease-associated glycosylation changes

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**Peanut agglutinin lectin (PNA) binds the Thomsen–Friedenreich (TF) oncofetal carbohydrate antigen (galactose $\beta$ 1-3N-acetyl-galactosamine $\alpha$ ) that shows increased expression in colon cancer, adenomas, and inflammatory bowel disease. PNA is mitogenic, both *in vitro* and *in vivo*, for colon epithelial cells. In these cells, PNA binds predominantly to cell-surface TF antigen expressed by high molecular weight isoforms of the transmembrane glycoprotein CD44 that are generated in inflamed and neoplastic colonic epithelia by altered RNA splicing. Our aim was to identify the signaling mechanism underlying the proliferative response to PNA. This was investigated in HT29, T84, and Caco2 colon cancer cells. Parallel lectin and immunoblotting of PNA affinity-purified HT29 cell membrane extracts showed PNA binding to high molecular weight CD44v6 isoforms. Within 5 min, PNA (25  $\mu$ g/mL) caused a 6-fold increase in phosphorylation of hepatocyte growth factor receptor c-Met, known to co-associate with CD44v6. This was followed by the downstream activation of p44/p42 mitogen-activated protein kinase (MAPK) over 15–20 min. The presence of 100  $\mu$ g/mL asialofetuin, a TF antigen-expressing glycoprotein, blocked both PNA-induced c-Met and MAPK activation. A similar PNA-induced c-Met and MAPK phosphorylation was also seen in T84 cells that express CD44v6 but not in Caco2 cells that lack CD44v6. PNA-induced cell proliferation was completely blocked by 1  $\mu$ M PD98059, an inhibitor of MAPK activation ( $p < 0.0001$ ). The expression of TF antigen by CD44 isoforms in colonic epithelial cells allows lectin-induced mitogenesis that is mediated by phosphorylation of c-Met and MAPK. It provides a mechanism by which dietary, microbial, or endogenous galactose-binding lectins could affect epithelial proliferation in the cancerous and precancerous colon.**

**Key words:** CD44v6/c-Met/MAPK/peanut lectin/proliferation

### Introduction

Glycoconjugate abnormalities are commonly seen in epithelial malignancies and in cancer precursors such as colonic polyps and inflammatory bowel disease (Campbell *et al.*, 2001). These alterations correlate with the invasive and metastatic potential of tumor cells (Hakomori, 2001), but the mechanisms of their effects on cellular function are poorly understood.

Alterations in carbohydrate expression in colon cancer include neo-expression of O-linked oncofetal carbohydrate antigens. Probably, the commonest alteration is increased expression of the Thomsen–Friedenreich (TF) oncofetal carbohydrate antigen (galactose $\beta$ 1-3N-acetylgalactosamine $\alpha$ ) (Campbell *et al.*, 1995), which is a core carbohydrate structure of O-linked oligosaccharide chains on glycoproteins. It is expressed in neonatal colon, but in adult colon, the TF antigen is normally masked by further glycosylation or sulfation (Lance and Lev, 1991; Martinez-Menarguez *et al.*, 1992). Increased expression of TF antigen has been demonstrated in hyperplastic and adenomatous colonic polyps, in inflammatory bowel disease, and in colon cancer (Rhodes *et al.*, 1988; Campbell *et al.*, 1995) but is undetectable in normal colonic mucosa (Boland and Roberts, 1988) unless sensitivity is enhanced by avidin–biotin amplification (Cooper and Reuter, 1983).

Peanut agglutinin lectin (PNA) binds to TF antigen and is mitogenic for colorectal cancer cell lines and increases crypt cell proliferation rates in normal colonic epithelium *in vitro* (Ryder *et al.*, 1992; Ryder, Smith *et al.*, 1994). PNA also stimulates proliferation in colonic mucosae from patients with polyps and inflammatory bowel disease (Ryder, Parker *et al.*, 1994) but has a relatively minor trophic effect in the mammalian small intestine (Jordinson *et al.*, 1999). PNA is highly resistant to digestion, can be recovered in active form from feces, and even enters the circulation as an intact protein (Wang *et al.*, 1998). Peanut ingestion causes increased proliferation of rectal epithelia in individuals with mucosal expression of TF antigen (Ryder *et al.*, 1998), but the mechanism of this proliferative effect was unknown.

We have previously shown that, in colon cancer, cell-surface TF antigen is predominantly expressed on high molecular weight splice variants of CD44 (Singh *et al.*, 2001). CD44 is a widely distributed type I transmembrane glycoprotein with importance in mediating cell–cell and cell–matrix interactions (Thorne *et al.*, 2004). Alternative splicing of CD44 can produce a large number of different isoforms (Ponta *et al.*, 2003; Thorne *et al.*, 2004) some of which are overexpressed during colorectal tumorigenesis in man

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(Wielenga *et al.*, 1998; Wielenga, van der Neut, Offerhaus *et al.*, 2000). Overexpression of CD44v6, the high molecular weight variant isoform of CD44 carrying amino acid sequences coded by exon 11 (Screaton *et al.*, 1992), is associated with poor prognosis (Wielenga *et al.*, 1998; Wielenga, van der Neut, Offerhaus *et al.*, 2000). CD44v6 associates with the proto-oncogene product c-Met, a receptor tyrosine kinase that is itself overexpressed in many cancers and whose activation affects growth, invasion, and metastasis in cancer cells (Birchmeier *et al.*, 2003). The association between CD44v6 and c-Met is essential for the activation of c-Met by its natural ligand, hepatocyte growth factor/scatter factor (HGF/SF), and subsequent mitogen-activated protein kinase (MAPK) activation (Orian-Rousseau *et al.*, 2002).

Studies were therefore performed to assess the signal transduction processes that might be involved in PNA-induced mitogenesis with particular attention to the consequences of its interaction with CD44 isoforms.

## Results

### *PNA binds CD44v6, which is associated with the receptor tyrosine kinase c-Met, in colon cancer cells*

Immunoblot analysis of PNA-agarose affinity-purified cell-surface membrane proteins from HT29 cells using anti-CD44v6 antibodies revealed two PNA-reactive CD44v6 isoforms (Figure 1A). CD44v6 immunoprecipitated from HT29 cell lysate was subjected to immunoblot analysis using either anti-CD44v6 or anti-c-Met antibodies (Figure 1B

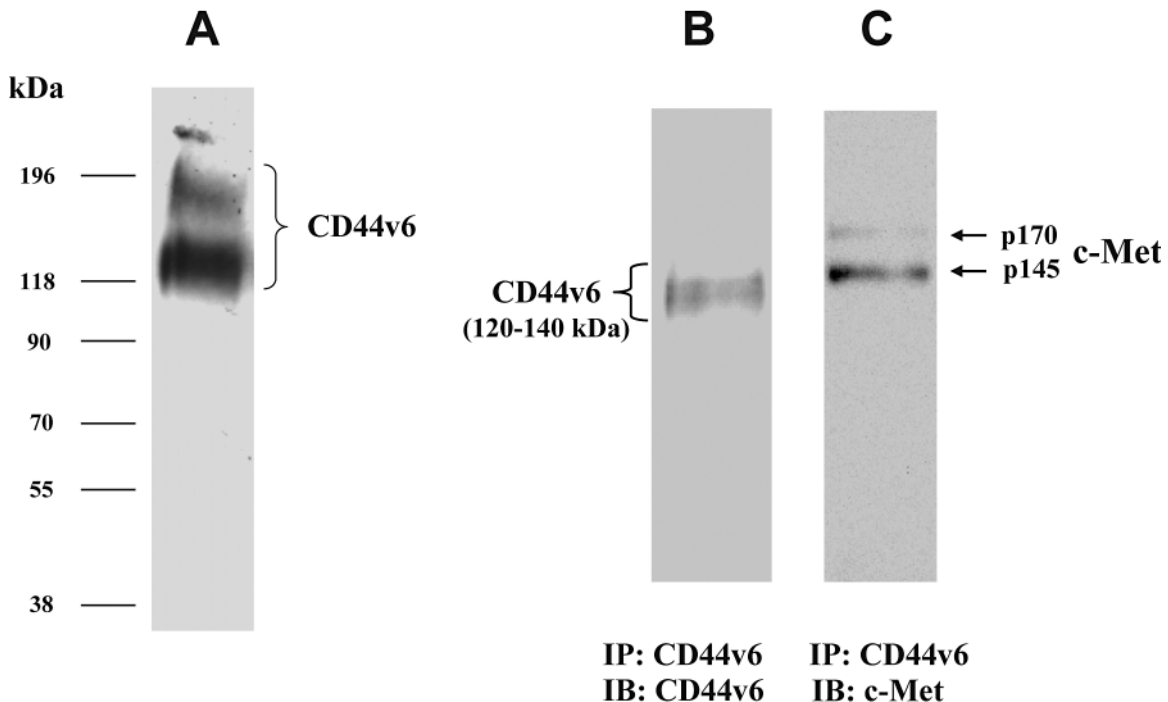
and C). This confirmed that CD44v6 and c-Met co-immunoprecipitate and therefore suggests that they may be physically associated.

### *PNA activates c-Met in HT29 colon cancer cells*

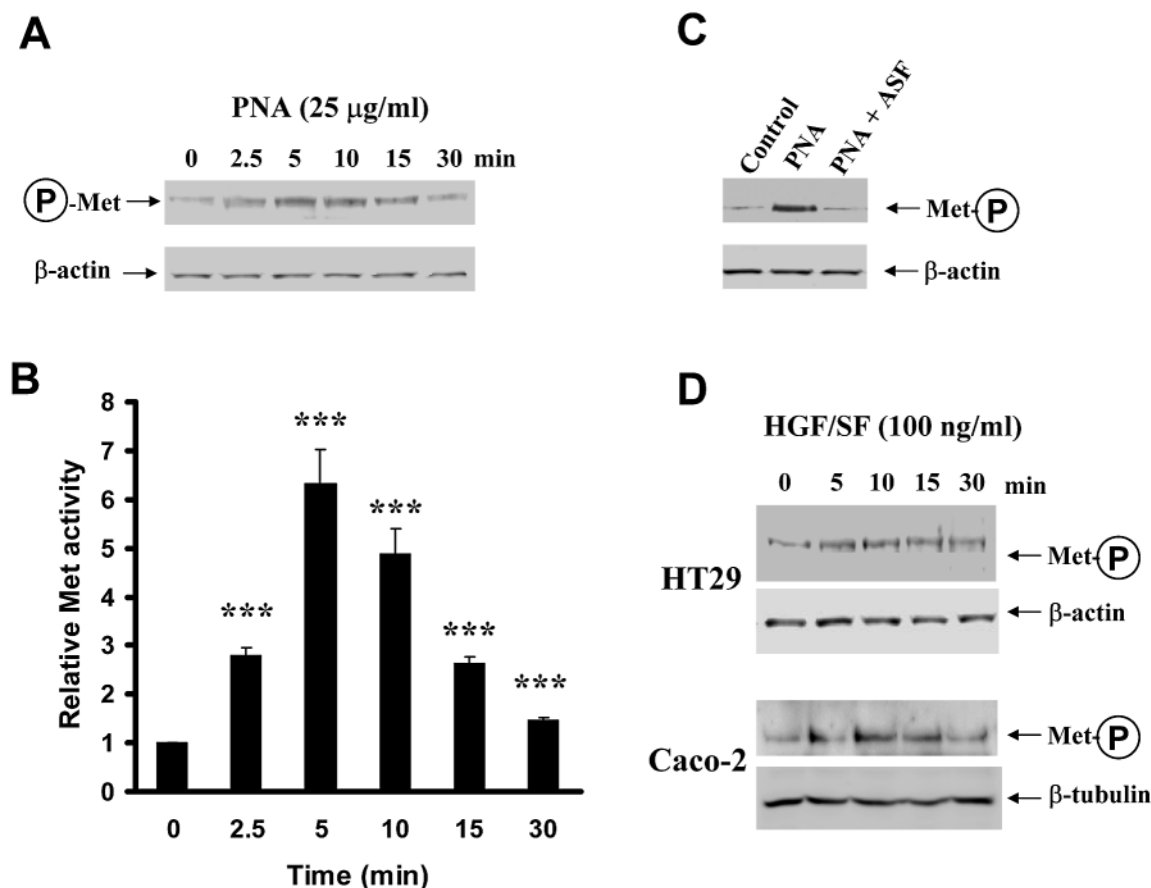
Immunoblot analysis of HT29 cells treated with 25  $\mu\text{g}/\text{mL}$  PNA, using a phospho-specific Met antibody that recognizes a peptide containing phospho-Tyr (1349) that provides a docking site on activated Met for downstream factors, revealed rapid phosphorylation of c-Met in response to PNA (Figure 2A). Densitometric analysis revealed significant increases in c-Met phosphorylation of  $2.8 \pm 0.17$  (mean  $\pm$  SD) and  $6.3 \pm 0.68$ -fold at 2.5 and 5 min, respectively ( $p < 0.001$  analysis of variance [ANOVA] versus control;  $n = 3$ ), returning to near baseline levels after 30 min (Figure 2B). PNA (25  $\mu\text{g}/\text{mL}$ ; 5 min) failed to activate c-Met in the presence of 100  $\mu\text{g}/\text{mL}$  asialofetuin (Figure 2C). In response to treatment with 100 ng/mL HGF/SF, used as a positive control for Met receptor activation, significant phosphorylation of c-Met was seen, from 5 to 15 min, in both HT29 cells and Caco2 cells. This response started to fall at 30 min in Caco2 cells and to a lesser degree in HT29 cells (Figure 2D).

### *PNA activates MAPK, and inhibition of the MEK1/2-MAPK signaling pathway blocks PNA-induced cell proliferation*

Immunoblotting of PNA-treated HT29, using specific anti-phospho p44/42 MAPK antibodies, demonstrated the activation of both p42 and p44 MAP (Figure 3A). After 5 min,



**Fig. 1.** CD44v6 binds PNA and associates with c-Met. (A) Immunoblot of PNA-agarose affinity-purified cell-surface glycoproteins from HT29 human colon cancer cells using anti-CD44v6 antibody (BBA13) demonstrates two PNA-reactive CD44v6-expressing splice variants of CD44. (B and C) Immunoprecipitation (IP) of CD44v6 from crude HT29 cell lysates followed by immunoblotting (IB) of SDS-PAGE resolved proteins, using either primary antibody to (B) CD44v6 (BBA13) or (C) antibody to c-Met (sc-10). Results suggest that CD44v6 and c-Met may be physically associated within the cell.



**Fig. 2.** PNA activates c-Met and induces tyrosine phosphorylation in HT29 cells. (A) Immunoblot of HT29 cell lysates following treatment with 25 µg/mL PNA (2.5–30 min) using antibodies against Tyr 1349 phosphorylated c-Met, illustrating the activation of c-Met by PNA. (B) Densitometric analysis of the immunoblots expressed relative to control ( $n = 3$  experiments; mean  $\pm$  SD, all \*\*\* $p < 0.001$  ANOVA). (C) PNA (25 µg/mL PNA, at 5 min) failed to activate c-Met in presence of 100 µg/mL asialofetuin (ASF), a TF antigen-expressing glycoprotein. (D) Immunoblot with antibody to phosphorylated c-Met for following treatment of HT29 and Caco-2 cells with 100 ng/mL HGF/SF, as a positive control for c-Met activation.

addition of 25 µg/mL PNA to HT29 cells initiated significant p44 MAPK phosphorylation (2.2-fold) reaching a peak at 20 min ( $3.1 \pm 0.07$ -fold; mean  $\pm$  SD,  $p < 0.001$  ANOVA;  $n = 3$ ). Phosphorylation of p44 MAPK remained 2.2- and 1.8-fold higher than that of control at 45 and 60 min, respectively. Phosphorylation of p42 MAPK showed a similar pattern, again initiated within 5 min (1.9-fold), reaching a peak at 15 min (2.5-fold) and remaining 1.4-fold higher than that of control even at 60 min,  $p < 0.01$  ANOVA (Figure 3B). Again, the presence of 100 µg/mL asialofetuin blocked MAPK activation induced by PNA in HT29 cells (Figure 3C). In addition, PNA (25 µg/mL) produced a  $38 \pm 1.5\%$  (mean  $\pm$  SD,  $n = 3$ ) increase in [methyl- $^3$ H]-thymidine incorporation into HT29 cells compared with untreated control (expressed as  $100 \pm 2\%$ ;  $n = 3$ ). PD98059, an inhibitor of MAPK activation, at a concentration of 1 µM, completely blocked PNA-induced proliferation in HT29 cells ( $p < 0.0001$  ANOVA) (Figure 3D).

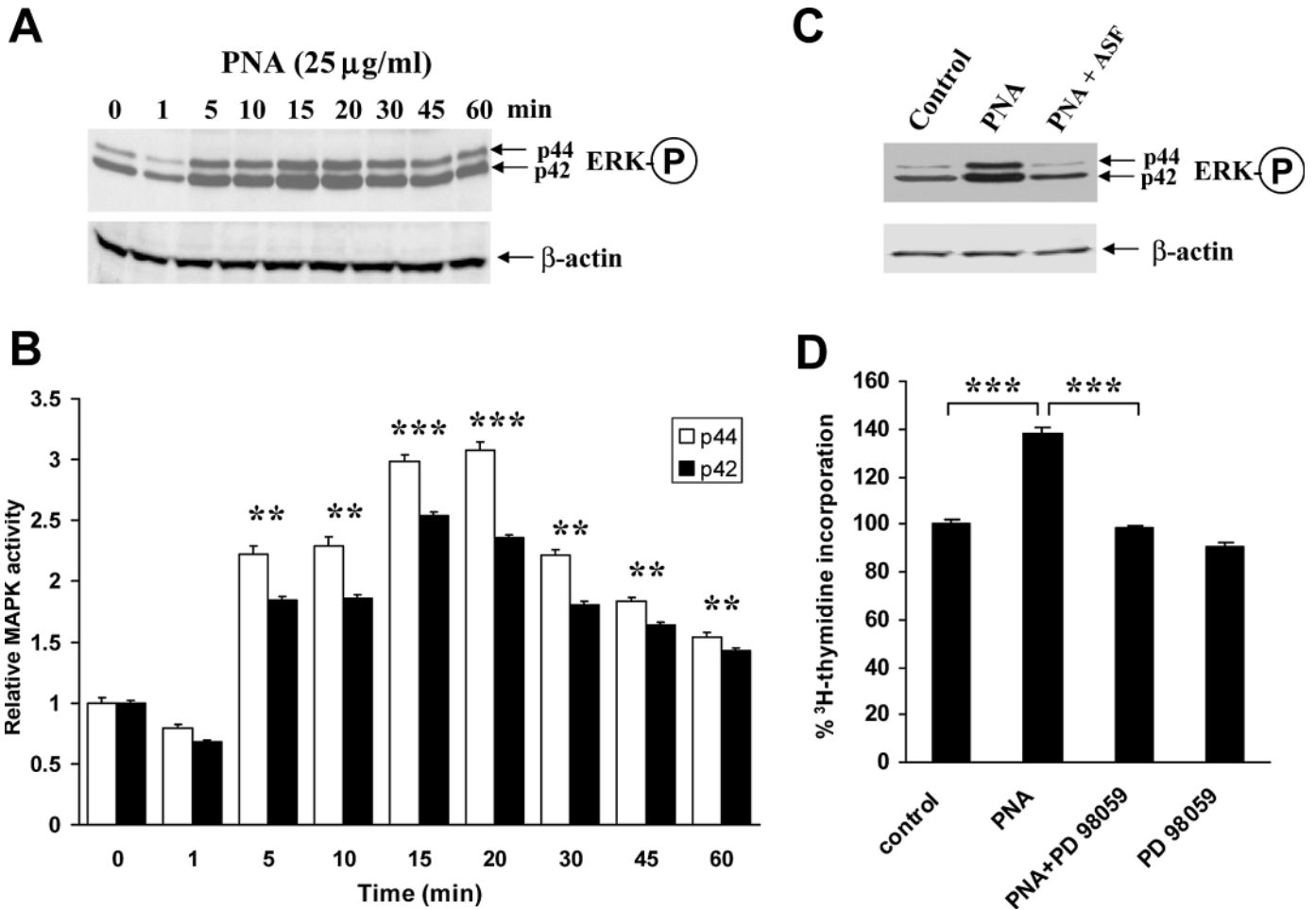
#### The PNA-induced c-Met and MAPK activation is CD44v6 isoform dependent

As seen for CD44v6-expressing HT29 cells, similar activation of c-Met and p42/p44 MAPK also occurred in

CD44v6-positive T84 colon cancer cells following treatment with 25 µg/mL of PNA for 5 min. It is worth noting that although lower levels of CD44v6 were detected in T84 cells (Figure 4A), stimulation of T84 cells with PNA resulted in a significant increase in phosphorylation of c-Met ( $2.6 \pm 0.16$ -fold increase;  $n = 3$ ;  $p < 0.001$  unpaired  $t$ -test), similar to the PNA-induced response observed in HT29 cells ( $2.8 \pm 0.21$ -fold increase;  $p < 0.001$ ) when they each were compared with their respective untreated controls (Figure 4B). PNA failed to significantly activate MAPK or c-Met in the CD44v6-negative Caco2 colon cancer cell line (Figure 4A and B).

#### PNA increases CD44 and p170 c-Met expression in HT29 colon cancer cells

Following 24-h treatment of HT29 colon cancer cells with 15–60 µg/mL PNA, immunoblot analysis demonstrated no significant increase in the expression of c-Met reactivity, although a modest increase was observed in the p170 precursor protein, and not active p145 c-Met, at 60 µg/mL PNA. A similar modest increase in the expression of CD44 was also seen with 60 µg/mL PNA when compared with untreated controls (data not shown).



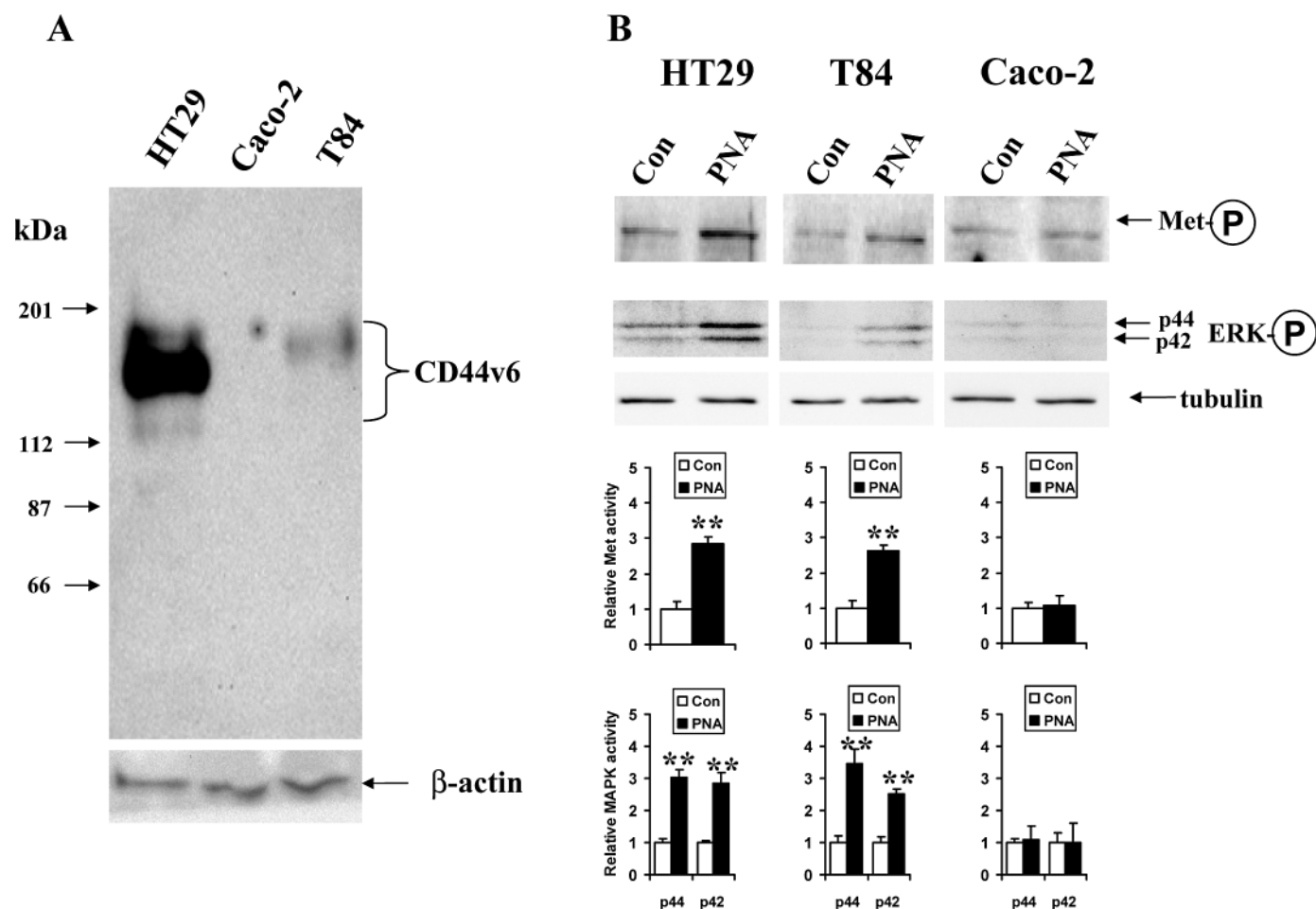
**Fig. 3.** PNA activates p44/p42 MAPK, and inhibition of MAPK activation abrogates PNA-induced cell proliferation. (A) Immunoblot of HT29 cell lysates following treatment with 25 µg/mL PNA (1–60 min) using antibodies against phospho-ERK 1/2. PNA activates p44/p42 MAPK within 5 min, with peak activation at 15 min. Explanation for the transient decrease seen at 1 min is unknown, but this might reflect recent manipulation of the serum-starved cells. (B) Densitometric analysis of the immunoblots expressed relative to control ( $n = 3$  experiments; mean  $\pm$  SD). Significant differences are indicated for both p44 and p42,  $**p < 0.01$  and  $***p < 0.001$  ANOVA. (C) PNA (25 µg/mL; 15 min) failed to activate MAPK in presence of 100 µg/mL asialofetuin (ASF). (D) Effect of PNA 25µg/mL, 24 h with or without 1 µM PD98059 on HT29 [<sup>3</sup>H]-thymidine incorporation into HT29 cells. PNA significantly stimulates HT29 cell proliferation ( $***p < 0.001$  ANOVA) and inhibition of MAPK activation by PD98059 abrogates PNA-induced proliferation ( $***p < 0.001$  ANOVA). The results represent means  $\pm$  SD of triplicate determinations.

## Discussion

These studies show that PNA binds to TF oncofetal carbohydrate antigen (galactose $\beta$ 1-3*N*-acetylgalactosamine $\alpha$ ) that resides on v6 isoforms of the high molecular weight glycoprotein CD44. Furthermore, the interaction between the PNA and the TF-expressing CD44v6 splice variants on the cell surface of HT29 and T84 colon cancer cells results in the phosphorylation of associated c-Met receptor and subsequent activation of the p44/p42 MAPK (ERK 1/2) cell signaling pathway. Continuous exposure to PNA results in a reduction in the amount of phosphorylated c-Met following the initial increase, a phenomenon likely to be because of endocytosis of the c-Met receptor rather than receptor saturation (Hammond *et al.*, 2003). Caco2 cells, that do not express CD44v6, do not show c-Met or MAPK activation with PNA and are known to show no significant proliferative response to the lectin (Ryder, Smith *et al.*, 1994). The activation of both c-Met and MAPK is PNA–TF antigen

interaction dependent as shown by abrogation of response to PNA in the presence of the TF antigen-expressing glycoprotein, asialofetuin. Binding of PNA to asialofetuin is TF antigen dependent and almost completely abolished by *O*-glycanase treatment of asialofetuin (Singh *et al.*, 2001). Thus, the TF antigen expression on CD44v6 has functional significance for the lectin-induced proliferation of colon cancer cells. The kinetics of MAPK activation and the inhibition of PNA-induced proliferation by PD98059, a specific inhibitor of MAPK kinase (MEK-1), which phosphorylates and activates p44/42 MAPKs, strongly suggest that this is the mechanism for the proliferative effect of PNA on colonocytes.

It is known that CD44v6 is required for c-Met activation by HGF/SF and subsequent MAPK activation in several cell lines, including HT29 cells (Orlan-Rousseau *et al.*, 2002). The demonstration that exposure to PNA results in the activation of c-Met not only confirms the close functional



**Fig. 4.** PNA-induced c-Met and MAPK activation is CD44v6 isoform dependent. (A) Immunoblot analysis, following 7.5% SDS-PAGE, reveals CD44v6 isoforms expressed in HT29 and T84 cells but not in the Caco-2 colon cancer cell line. (B) Upper panel: as seen for HT29 cells, immunoblot analysis demonstrated that similar activation of c-Met and p42/p44 MAPK also occurred in CD44v6-positive T84 colon cancer cells following treatment with PNA (25  $\mu$ g/mL; 5 min) but failed to occur in the CD44v6-negative Caco-2 colon cancer cell line. Lower panel: densitometry of three separate experiments (mean  $\pm$  SD). Significant changes are indicated as \*\* $p < 0.001$  ANOVA;  $n = 3$ .

relationship between c-Met and CD44v6 but also suggests an important function for the TF glycan that is selectively expressed by high molecular weight CD44 splice variants (Singh *et al.*, 2001). However, in Caco2 cells, known to express a functioning HGF receptor (Kermorgant, Aparicio *et al.*, 2001; Kermorgant, Dessirier *et al.*, 2001), we demonstrated that HGF/SF activation of c-Met can occur despite the lack of CD44v6. It is known that HGF/SF is able to induce Met signaling in HepG2 cells that do not express CD44 and also induces Met signaling in fibroblasts derived from CD44 null mice. In these HGF/SF-responsive but CD44-negative cells, it is thought that substitute molecules may compensate for the lack of CD44 and allow Met activation (Orian-Rousseau *et al.*, 2002).

CD44 isoforms are generated by extensive alternative splicing, and additional variability is introduced by post-translational modification (Ponta *et al.*, 2003; Thorne *et al.*, 2004). The expression of isoforms bearing sequences encoded by exons v4–v7 or v6 and v7 have been shown to be sufficient to confer metastatic potential to non-metastatic cells (Gunthert *et al.*, 1991). Antibodies directed against

CD44v6, or CD44v6 antisense, inhibit tumor growth and metastasis of colon cancer cells *in vivo* and reduce invasiveness of fibrosarcoma cells *in vitro* (Seiter *et al.*, 1993; Ponta *et al.*, 1998; Reeder *et al.*, 1998). The mechanism for variant splicing of CD44 in cancerous and inflamed epithelia is not well understood. It may be affected either by intron length (Bell *et al.*, 1998) or by the effect of pro-inflammatory cytokines (Macdonald *et al.*, 2003). The fact that TF expression is specific for some of the high molecular weight CD44 splice variants suggests that these may contain amino acid sequences that are particularly susceptible to *O*-glycosylation with this disaccharide (Singh *et al.*, 2001). This is also an important observation, because little had been known about which macromolecules, besides mucins (Campbell *et al.*, 1995), might carry the TF oligosaccharide.

It is known that the stimulation of c-Met via its natural ligand, HGF/SF, results in wide-ranging biological and biochemical effects in the cell that can include scattering, proliferation, enhanced cell motility, invasion, and eventually metastasis (Ma *et al.*, 2003). The activation of c-Met results in the recruitment of scaffolding proteins such as HGF

receptor-bound protein 2 (Grb2) and Grb2-associated binder 1 (Gab1), which activate Shp2 and the Ras-Raf-ERK signaling pathway. This causes changes in gene expression of cell-cycle regulators, (such as retinoblastoma protein, Cdk6 and p27), extracellular matrix proteinases (such as matrix metalloproteinases and urokinase plasminogen activator), and in alterations of cytoskeletal functions that control migration, invasion, and proliferation (Birchmeier *et al.*, 2003). In addition, the activation of c-Met in colorectal carcinoma cells leads to constitutive association of tyrosine-phosphorylated  $\beta$ -catenin (Herynk *et al.*, 2003). Overexpression of c-Met protein correlates with poor prognosis in gastrointestinal, hepatocellular, breast, endometrial, and nasopharyngeal carcinomas (Danilkovitch-Miagkova and Zbar, 2002; Ma *et al.*, 2003; Takeuchi *et al.*, 2003).

Recent studies have provided good evidence for functional collaboration between CD44 isoforms and c-Met. Association between CD44v6 and c-Met receptor is essential for the activation of c-Met tyrosine kinase activity by its natural ligand, HGF/SF, and subsequent activation of MAPK signaling (Orian-Rousseau *et al.*, 2002). CD44 isoforms decorated with heparin sulfate chains can bind the c-Met ligand, HGF/SF, and this interaction promotes signaling through c-Met (van der Voort *et al.*, 1999). CD44v3 isoforms, which contain a site for heparin sulfate attachment, and c-Met are co-expressed on colorectal tumors and cell lines (Wielenga, van der Voort, Taher *et al.*, 2000). These studies further support a possible therapeutic role for MEK1/2-MAPK inhibition by tyrosine kinase inhibitors such as imatinib mesylate and gefitinib in cancer (Dancey, 2003; Levitzki, 2003). c-Met is also an important target for cancer therapy, and many efforts are directed toward the identification of inhibitors that are active *in vivo* (Birchmeier *et al.*, 2003).

This study raises the possibility that other galactose-binding lectins may have similar interactions with cancerous or precancerous colonic epithelial cells via TF-expressing CD44v6 and its association with c-Met. Such lectins might include members of the galectin family of endogenous galactose-binding lectins whose expression is markedly altered in cancer (Itzkowitz, 1997; Danguy *et al.*, 2002) or lectins expressed by bacteria in the colonic lumen (Rhodes, 1996; Rhodes and Campbell, 2002).

## Materials and methods

### Cell culture

The human colon cancer cell lines HT29, T84, and Caco2 (ECACC 91072201, 88021101, and 86010202, respectively) were obtained from the European Collection of Animal Cell Culture at the Public Health Laboratory Service, Porton Down, Wiltshire, UK. All cells were grown as monolayers in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 4 mM glutamine and maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>, 95% air.

### Materials

Rabbit polyclonal antibodies against phosphorylated p44/p42 MAPK (ERK1/2) were purchased from Promega,

Southampton, UK. Monoclonal anti-phosphotyrosine antibody PY20, conjugated to horse-radish peroxidase (HRP), was obtained from Becton and Dickinson, Oxford, UK. Antibodies against standard CD44 (BBA10) and CD44v6 (BBA13) were obtained from R&D Systems, Abingdon, UK. Anti-c-Met (sc-10) and anti-phospho-Met (Tyr1349) antibodies were purchased from Autogen Bioclear (Calne, UK) and New England Biolabs (Hitchin, UK), respectively. PNA and PNA-agarose were purchased from Sigma, Poole, UK. The MEK-1 (MAPK kinase) inhibitor, PD98059, was obtained from Calbiochem, Nottingham, UK. HGF/SF was a kind gift from Prof. M. Clague (The Physiological Laboratory, University of Liverpool, Liverpool, UK). [Methyl-<sup>3</sup>H]-thymidine was purchased from ICN Pharmaceuticals, Basingstoke, UK. All other reagents were of analytical grade.

### PNA-agarose affinity purification of TF-expressing cell-surface glycoproteins from HT29 colon cancer cells

Confluent cells in 75-cm<sup>2</sup> flasks were washed three times in phosphate-buffered saline (PBS), pH 7.4. The cells were then scraped into PBS containing 5 mM phenylmethyl sulfonyl fluoride (PMSF), sonicated using three 20-s pulses and centrifuged at 100,000  $\times$  g for 1 h. The membrane-rich pellet was solubilized by sonication in PBS containing 1% (v/v) Nonidet P40 (NP40) and 5 mM PMSF using five 20-s pulses, left on ice for 2 h, and then centrifuged at 100,000  $\times$  g for 1 h. The supernatant was collected and loaded on to a buffer equilibrated 2 mL PNA-agarose column, which was then washed with PBS until the absorbance of the wash-through at optical density (OD) 280 nm returned to the baseline. Bound proteins were then eluted with PBS containing 0.2 M galactose. The eluate was desalted using a PD-10 Sephadex GM25 column (Amersham Biosciences, Little Chalfont, UK), and the void volume fractions were lyophilized and stored at -80°C for subsequent immunoblot analysis using anti-CD44v6 antibody (BBA13).

### Immunoprecipitation of CD44v6 and associated proteins from HT29 cells

Confluent HT29 cells were lysed by sonication, on ice, in PBS containing 1% (v/v) NP40 and 5 mM PMSF using three 20-s pulses. Cell extracts obtained as above were precleared with 20  $\mu$ l Protein A-agarose beads to remove non-specific binding material. The supernatants were then incubated for 3 h with CD44v6 antibody (BBA13) at 4°C on a shaker. Twenty microliters of Protein A-agarose beads was then added and the mixture incubated on an end-over-end mixer overnight at 4°C. Beads and the associated immunoprecipitate were collected by centrifugation and washed once in PBS containing 0.5% (v/v) Triton X-100, 0.1% (w/v) sodium dodecyl sulphate (SDS), once in PBS containing 0.5% (v/v) Triton X-100 and 0.5 M NaCl, and twice more in the former buffer. Pellets were resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (62.5 mM Tris [pH 6.8] containing 2% [w/v] SDS, 10% [v/v] glycerol, 5% [v/v]  $\beta$ -mercaptoethanol, and 0.001% [w/v] bromophenol blue) and boiled for 5 min. Immunoprecipitates were then separated on polyacrylamide (7.5 or 12%) gels and probed using antibodies against CD44v6 (BBA13) and c-Met (sc-10).

### PNA treatment of colon cancer cells and preparation of cell lysates

Cells were seeded at  $2 \times 10^5$  in 6-well plates in complete culture medium. After 24 h, the medium was replaced with serum-free medium containing 0.5% (w/v) bovine serum albumin (BSA) for 24 h. For the analysis of activation of MAPK and c-Met receptor, 25  $\mu\text{g}/\text{mL}$  PNA was added for 1–60 min, and HGF/SF (100 ng/mL; 5–30 min) was used as a positive control for c-Met activation. In addition, HT29 cells were incubated with 25  $\mu\text{g}/\text{mL}$  PNA in the presence or absence of 100  $\mu\text{g}/\text{mL}$  asialofetuin, a TF antigen-expressing glycoprotein. We have previously shown that binding of PNA to asialofetuin is TF antigen dependent and almost completely abolished by *O*-glycanase treatment of asialofetuin (Singh *et al.*, 2001). Cells were immediately lysed with equal volumes of boiling SDS–PAGE sample buffer. Immunoblot analysis was performed using antibodies against phosphorylated p44/p42 MAPK and phosphorylated c-Met.

In experiments to assess whether PNA up-regulated CD44 and c-Met expression, HT29 cells were treated with increasing concentrations of PNA (15–60  $\mu\text{g}/\text{mL}$ ) for 24 h. Following treatment, cells were then lysed, on ice, by ultrasonication in PBS containing 1% (v/v) NP40 and 5 mM PMSF using three 20-s pulses. Immunoblot analyses of cell lysates were performed using the anti-c-Met (sc-10) and anti-CD44 (BBA10) antibodies.

### SDS–PAGE and immunoblotting

Solubilized proteins from HT29, T84, and Caco2 colon cancer cells were separated by SDS–PAGE either on 7.5 or on 12% resolving polyacrylamide gels and electrotransferred to nitrocellulose membrane for 1 h, at 100 V, in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. After transfer, membranes were blocked with 1% (w/v) BSA in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 for 1 h and then probed with appropriate antibodies. Bound peroxidase-labeled secondary antibody was detected using enhanced chemiluminescence (ECL, Amersham Biosciences). Equal loading was confirmed using  $\beta$ -actin or tubulin as control. Densitometry of immunoblots was performed using the Quantity One analysis software (BioRad, Hemel Hempstead, UK). All immunoblots shown are representative of experiments performed in triplicate.

### Cell proliferation measured using [ $^3\text{H}$ ]-thymidine incorporation

Cell proliferation was determined using [methyl- $^3\text{H}$ ]-thymidine incorporation under similar conditions to those described (Yu *et al.*, 1997; Singh *et al.*, 2001). Briefly, HT29 cells were seeded at a density of  $2 \times 10^4$ /well in 0.5 mL of DMEM containing 5% (v/v) FCS in 24-well plates. After 24 h, the culture medium was replaced with DMEM containing 1% (v/v) FCS for 24 h. The cells were then incubated with or without PNA (25  $\mu\text{g}/\text{mL}$ ) in the presence or absence of 1  $\mu\text{M}$  PD98059 for 24 h, followed by a 3 h pulse with 0.8  $\mu\text{Ci}$ /well [methyl- $^3\text{H}$ ]-thymidine. After two washes in PBS, the cells were treated with 5% trichloroacetic acid (TCA) for 1 h at 4°C. The precipitates were washed once with 5% TCA

at 4°C and twice with 95% ethanol at 4°C followed by air drying at room temperature and solubilization with 0.2 M NaOH. Five milliliters of EcoLite scintillation cocktail (ICN Pharmaceuticals) was added to the dissolved precipitates, and the radioactivity incorporated was determined using a Wallac 1219 Rackbeta counter (PerkinElmer, Beaconsfield, UK).

### Statistical analysis

Statistical analysis was performed using unpaired *t*-test or one-way ANOVA followed by Newman and Keuls multiple pairwise comparisons of treatment means (StatsDirect v2.3.1; StatsDirect, Sale, UK). Differences were considered significant when  $p < 0.05$ .

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### Conflict of interest statement

None declared.

### Abbreviations

ANOVA, analysis of variance; FCS, fetal calf serum; HGF, hepatocyte growth factor; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethyl sulfonyl fluoride; PNA, peanut agglutinin lectin; SDS, sodium dodecyl sulfate; SF, scatter factor; TF, Thomsen–Friedenreich.

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