

**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**

**Ravinder Singh, Sreedhar Subramanian, Jonathan M. Rhodes and Barry J. Campbell.**

Division of Gastroenterology, School of Clinical Science, Nuffield Building, Crown Street, University of Liverpool, Liverpool, L69 3BX, UK.

**Running title:** PNA binds TF antigen on CD44v6 activating c-Met and MAPK

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

Correspondence should be addressed to:

Dr Barry J Campbell, Division of Gastroenterology, School of Clinical Science, Nuffield Building, Crown Street, University of Liverpool, Liverpool, L69 3BX, UK.

Tel: +44(0)151 794 6829 Fax: +44(0)151 794 6825 e-mail: [bjcampbl@liv.ac.uk](mailto:bjcampbl@liv.ac.uk)

## **Abstract**

Peanut lectin (PNA) binds the Thomsen-Friedenreich (TF) oncofetal carbohydrate antigen (galactose $\beta$ 1-3N-acetylgalactosamine $\alpha$ -) 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 signalling mechanism underlying the proliferative response to PNA. This was investigated in HT29, T84 and Caco2 colon cancer cells.

Parallel lectin and immuno-blotting 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 downstream activation of p44/p42 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, which express CD44v6 but not in Caco2 cells, which 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 pre-cancerous colon.

## Introduction

Glycoconjugate abnormalities are commonly seen in epithelial malignancies and in cancer pre-cursors such as colonic polyps and inflammatory bowel disease (Campbell *et al.*, 2001). These alterations correlate with the invasive and metastatic potential of tumour cells (Hakamori, 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 antigen (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 sulphation (Martinez-Menarguez *et al.*, 1992; Lance and Lev, 1991). Increased expression of TF antigen has been demonstrated in hyperplastic and adenomatous colonic polyps, in inflammatory bowel disease, and in colon cancer (Campbell *et al.*, 1995; Rhodes *et al.*, 1988) 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; 1994a). PNA also stimulates proliferation in colonic mucosae from patients with polyps and inflammatory bowel disease (Ryder *et al.*, 1994b) 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 faeces 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 (Thorne *et al.*, 2004; Ponta *et al.*, 2003) some of which are over-expressed during colorectal tumorigenesis in man (Wielenga *et al.*, 1998; 2000a). Over-expression 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; 2000a). CD44v6 associates with the proto-oncogene product c-Met, a receptor tyrosine kinase that is itself over-expressed 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 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 1C). This confirmed that CD44v6 and c-Met co-immunoprecipitate and therefore suggests they may be physically associated.

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

Immunoblot analysis of HT29 cells treated with PNA 25 µg/ml, using a phospho-specific Met antibody that recognises 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 ( $p < 0.001$  ANOVA versus control;  $n=3$ ), returning to near baseline levels after 30 min (Figure 2B). PNA (25 µg/ml; 5 min) failed to activate c-Met in the presence of 100 µg/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 mins in Caco2 cells and to a lesser degree in HT29 cells (Figure 2D).

***PNA activates MAPK and inhibition of the MEK1/2-MAPK signalling pathway blocks PNA-induced cell proliferation.***

Immunoblotting of PNA-treated HT29, using specific anti-phospho p44/42 MAPK antibodies, demonstrated activation of both p42 and p44 MAPK (Figure 3A). After 5 min, 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-fold and 1.8 fold higher than 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 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- $^3\text{H}$ ]-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 PNA for 5 min. It is worth noting that although lower levels of CD44v6 were detected in T84 cells (Figure 4 A), stimulation of T84 cells with PNA resulted in a significant increase in phosphorylation 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 each were compared with their respective untreated controls (Figure 4B and 4C). PNA failed to significantly activate MAPK or c-Met in the CD44v6 negative Caco2 colon cancer cell-line (see Figure 4A, B and C).

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

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

## Discussion

These studies show that peanut lectin (PNA) binds to Thomsen-Friedenreich (TF) oncofetal carbohydrate antigen (galactose $\beta$ 1-3N-acetylgalactosamine $\alpha$ ) which resides on v6 isoforms of the high molecular weight glycoprotein CD44. Furthermore, the interaction between PNA and the TF-expressing CD44v6 splice variants on the cell-surface of HT29 and T84 colon cancer cells results in phosphorylation of associated c-Met receptor and subsequent activation of the p44/p42 MAPK (ERK 1/2) cell signalling 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 due to 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 nor MAPK activation with PNA and are known to show no significant proliferative response to the lectin (Ryder *et al.*, 1994a). Activation of both c-Met and MAPK is PNA-TF antigen interaction dependant 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 dependant and almost completely abolished by O-Glycanase<sup>TM</sup> treatment of asialofetuin (Singh *et al.*, 2001). Thus, 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 activation of c-Met not only confirms the close functional 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 *et al.*, 2001a,b) 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 signalling in HepG2 cells which do not express CD44, and also induces Met signalling 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 posttranslational modification (Thorne *et al.*, 2004; Ponta *et al.*, 2003). 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 tumour growth and metastasis of colon cancer cells *in vivo* and reduce invasiveness of fibrosarcoma cells *in vitro* (Ponta *et al.*, 1998; Reeder *et al.*, 1998; Seiter *et al.*, 1993). 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

since little had been known about which macromolecules, besides mucins (Campbell *et al.*, 1995), might carry the TF oligosaccharide.

It is known that stimulation of c-Met via its natural ligand, hepatocyte growth factor/scatter factor (HGF/SF), results in wide-ranging biological and biochemical effects in the cell which can include scattering, proliferation, enhanced cell motility, invasion, and eventually metastasis (Ma *et al.*, 2003). Activation of c-Met results in the recruitment of scaffolding proteins like growth-factor-receptor-bound protein 2 (Grb2) and Grb2-associated binder 1 (Gab1), which activate Shp2 and the Ras-Raf-ERK signalling 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, activation of c-Met in colorectal carcinoma cells leads to constitutive association of tyrosine-phosphorylated beta-catenin (Herynk *et al.*, 2003). Over expression of c-Met protein correlates with poor prognosis in gastrointestinal, hepatocellular, breast, endometrial and nasopharyngeal carcinomas (Ma *et al.*, 2003; Danilkovitch-Miagkova and Zbar, 2002; 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 activation of c-Met tyrosine kinase activity by its natural ligand, hepatocyte growth factor/scatter factor (HGF/SF), and subsequent activation of MAPK signalling (Orian-Rousseau *et al.*, 2002). CD44 isoforms decorated with heparin sulphate chains can bind the c-Met ligand, HGF/SF, and this

interaction promotes signalling through c-Met (van der Voort *et al.*, 1999). CD44v3 isoforms, which contain a site for heparin sulphate attachment, and c-Met are co-expressed on colorectal tumours and cell lines (Wielenga *et al.*, 2000b). 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 (Levitzki, 2003; Dancey, 2003). c-Met is also an important target for cancer therapy and many efforts are directed towards 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 pre-cancerous 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 and Campbell, 2002; Rhodes, 1996).

## **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 DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/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 (BBA 10) and CD44v6 (BBA 13) 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. Peanut lectin (PNA), and PNA-agarose were purchased from Sigma, Poole, UK. The MEK-1 (MAPK kinase) inhibitor, PD98059 was obtained from Calbiochem, Nottingham, UK. Hepatocyte growth factor/scatter factor (HGF/SF) was a kind gift from Professor M Clague (The Physiological Laboratory, University of Liverpool, UK). [Methyl-<sup>3</sup>H]-thymidine was purchased from ICN Pharmaceuticals Ltd, 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 75cm<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 x 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 x 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 OD 280nm 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 lyophilised 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 5mM PMSF using three 20 s pulses. Cell extracts obtained as above were pre-cleared with 20µl Protein A-agarose beads to remove nonspecific binding material. The supernatants were then incubated for 3 h with CD44v6 antibody (BBA 13) at 4°C on a shaker. Twenty microlitres of Protein A-agarose beads were 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) 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-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) BSA for 24 h. For analysis of activation of MAPK and c-Met receptor, 25  $\mu$ g/ml PNA was added for 1-60 min periods of time 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  $\mu$ g/ml PNA in the presence or absence of 100  $\mu$ g/ml asialofetuin, a TF-antigen expressing glycoprotein. We have previously shown that binding of PNA to asialofetuin is TF antigen dependant and almost completely abolished by O-Glycanase<sup>TM</sup> 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 phosphoylated 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  $\mu$ g/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 was performed using the anti c-Met (sc-10) and anti-CD44 (BBA-10) antibodies.

### ***SDS-PAGE and immunoblotting***

Solubilised proteins from HT29, T84 and Caco2 colon cancer cells were separated by SDS-PAGE either on 7.5% or 12% resolving polyacrylamide gels and electrotransferred to nitrocellulose membrane for 1 h, at 100V, 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-labelled 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 [<sup>3</sup>H]-thymidine incorporation***

Cell proliferation was determined using [methyl-<sup>3</sup>H]-thymidine incorporation under similar conditions to those described (Singh *et al.*, 2001; Yu *et al.*, 1997). 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  $\mu$ g/ml) in the presence or absence of 1  $\mu$ M PD98059 for 24 h, followed by a 3 h pulse with 0.8  $\mu$ Ci/well [methyl-<sup>3</sup>H]-thymidine. After two washes in PBS, the cells were treated with 5% trichloroacetic acid (TCA) for 1h 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 solubilisation with 0.2 M NaOH. Five millilitre of EcoLite scintillation cocktail (ICN Pharmaceuticals Ltd, Basingstoke, UK) was added to the dissolved

precipitates, and the radioactivity incorporated was determined using a Wallac 1219 Rackbeta counter (Milton Keynes, UK).

### **Statistical analysis**

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

### **Acknowledgements**

RS was funded was by a North West Cancer Research Fund UK award (CR565). SS was funded by an award from the National Association for Colitis and Crohn's Disease UK (M/03/2). Further support was provided by a Medical Research Council Co-operative Grant (GR990432).

## References

Bell, M.V., Cowper, A.E., Lefranc, M.P., Bell, J.I. and Sreaton, G.R. (1998) Influence of intron length on alternative splicing of CD44. *Mol. Cell Biol.*, **18**, 5930-41.

Birchmeier, C., Birchmeier, W., Gherardi, E. and Vande Woude, G.F. (2003) Met, metastasis, motility and more. *Nat. Rev. Mol. Cell Biol.*, **4**, 915-25.

Boland, C.R. and Roberts, J.A. (1988) Quantitation of lectin binding sites in human colon mucins by use of peanut and wheat germ agglutinins. *J. Histochem. Cytochem.*, **36**, 1305-7.

Campbell, B.J., Finnie, I.A., Hounsell, E.F and Rhodes, J.M. (1995) Direct demonstration of increased expression of Thomsen-Friedenreich (TF) antigen in colonic adenocarcinoma and ulcerative colitis mucin and its concealment in normal mucin. *J. Clin. Invest.*, **95**, 571-6.

Campbell, B.J., Yu, L.G. and Rhodes, J.M. (2001) Altered glycosylation in inflammatory bowel disease: a possible role in cancer development. *Glycoconjugate J.*, **18**, 851-858

Cooper, H.S. and Reuter, V.E. (1983) Peanut lectin-binding sites in polyps of the colon and rectum. Adenomas, hyperplastic polyps, and adenomas with in situ carcinoma. *Lab. Invest.*, **49**, 655-61.

Dancey, J.E. (2003) Recent advances of molecular targeted agents: opportunities for imaging. *Cancer Biol. Ther.*, **2**, 601-9.

Danguy, A., Camby, I., and Kiss, R. (2002) Galectins and cancer. *Biochim. Biophys. Acta*, **1572**, 285-93.

Danilkovitch-Miagkova, A. and Zbar, B. (2002) Dysregulation of Met receptor tyrosine kinase activity in invasive tumors. *J. Clin. Invest.*, **109**, 863-7.

Gunthert, U., Hofmann, M., Rudy, W., Reber, S., Zoller, M., Haussmann, I., Matzku, S., Wenzel, A. *et al.* (1991) A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell*, **65**, 13-24.

Hakomori, S. (2001) Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. *Adv. Exp. Med. Biol.*, **491**, 369-402.

Hammond, D.E., Carter, S., McCullough, J., Urbe, S., Vande Woude, G. and Clague, M.J. (2003) Endosomal dynamics of Met determine signaling output. *Mol. Biol. Cell.*, **14**:1346-54.

Herynk, M.H., Tsan, R., Radinsky, R. and Gallick, G.E. (2003) Activation of c-Met in colorectal carcinoma cells leads to constitutive association of tyrosine-phosphorylated beta-catenin. *Clin. Exp. Metastasis*, **20**, 291-300.

Itzkowitz, S.H. (1997) Galectins: multipurpose carbohydrate-binding proteins implicated in tumor biology. *Gastroenterology*, **113**, 2003-5.

Jordinson, M., Goodlad, R.A., Brynes, A., Bliss, P., Ghatel, M.A., Bloom, S.R., Fitzgerald, A., Grant, G., Bardocz, S., *et al.* (1999) Gastrointestinal responses to a panel of lectins in rats maintained on total parenteral nutrition. *Am. J. Physiol.*, **276**, G1235-42.

Kermorgant, S., Aparicio, T., Dessirier, V., Lewin, M.J.M. and Lehy, T. (2001) Hepatocyte growth factor induces colonic cancer cell invasiveness via enhanced motility and protease overproduction. Evidence for PI3 kinase and PKC involvement. *Carcinogenesis*, **22**, 1035-1042.

Kermorgant, S., Dessirier, V., Lewin, M.J.M. and Lehy, T. (2001) HGF upregulates and modifies subcellular distribution of proteins in colon cancer cell enterocytic differentiation. *Am. J. Physiol.*, **281**:1068-1080.

Lance, P. and Lev, R. (1991) Colonic oligosaccharide structures deduced from lectin-binding studies before and after desialylation. *Hum. Pathol.*, **22**, 307-12.

Levitzki, A. (2003) Protein kinase inhibitors as a therapeutic modality. *Acc. Chem. Res.*, **36**, 462-9.

Ma, P.C., Maulik, G., Christensen, J. and Salgia, R. (2003) c-Met: structure, functions and potential for therapeutic inhibition. *Cancer Metastasis Rev.*, **22**, 309-25.

Macdonald, D.C., Leir, S.H., Brooks, C., Sanders, E., Lackie, P. and Rosenberg, W. (2003) CD44 isoform expression on colonic epithelium mediates lamina propria lymphocyte adhesion and is controlled by Th1 and Th2 cytokines. *Eur. J. Gastroenterol. Hepatol.*, **15**, 1101-10.

Martinez-Menarguez, J.A., Ballesta, J., Aviles, M., Madrid, J.F. and Castells M.T. (1992) Influence of sulphate groups in the binding of peanut agglutinin. Histochemical demonstration with light- and electron-microscopy. *Histochem. J.*, **24**, 207-16.

Orian-Rousseau, V., Chen, L., Sleeman, J.P., Herrlich, P. and Ponta, H. (2002) CD44 is required for two consecutive steps in HGF/c-Met signaling. *Genes Dev.*, **16**, 3074-86.

Ponta, H., Wainwright, D. and Herrlich, P. (1998) The CD44 protein family. *Int. J. Biochem. Cell Biol.*, **30**, 299-305.

Ponta, H., Sherman, L. and Herrlich, P.A. (2003) CD44: from adhesion molecules to signalling regulators. *Nat. Rev. Mol. Cell Biol.*, **4**, 33-45.

Reeder, J.A., Gotley, D.C., Walsh, M.D., Fawcett, J. and Antalis, T.M. (1998) Expression of antisense CD44 variant 6 inhibits colorectal tumor metastasis and tumor growth in a wound environment. *Cancer Res.*, **58**, 3719-26.

Rhodes, J.M., Black, R.R., and Savage, A. (1988) Altered lectin binding by colonic epithelial glycoconjugates in ulcerative colitis and Crohn's disease. *Dig. Dis. Sci.*, **33**, 1359-63.

Rhodes, J.M. (1996) Unifying hypothesis for inflammatory bowel disease and associated colon cancer: sticking the pieces together with sugar. *Lancet*, **347**, 40-4.

Rhodes, J.M. and Campbell, B.J. (2002) Inflammation and colorectal cancer: IBD-associated and sporadic cancer compared. *Trends Mol Med.*, **8**, 10-6.

Ryder, S.D., Smith, J.A. and Rhodes, J.M. (1992) Peanut lectin: a mitogen for normal human colonic epithelium and human HT29 colorectal cancer cells. *J. Natl. Cancer Inst.*, **84**, 1410-16.

Ryder, S.D., Smith, J.A., Rhodes, E.G., Parker, N. and Rhodes, J.M. (1994a) Proliferative responses of HT29 and Caco2 human colorectal cancer cells to a panel of lectins. *Gastroenterology*; **106**, 85-93.

Ryder, S.D., Parker, N., Parker, N., Ecclestone, D., Haqqani, M.T. and Rhodes, J.M. (1994b) Peanut lectin stimulates proliferation in colonic explants from patients with inflammatory bowel disease and colon polyps. *Gastroenterology*, **106**, 117-24.

Ryder, S.D., Jacyna, M.R., Levi, A.J., Rizzi, P.M. and Rhodes, J.M. (1998) Peanut ingestion increases rectal proliferation in individuals with mucosal expression of peanut lectin receptor. *Gastroenterology*, **114**, 44-49.

Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI. (1992) Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc. Natl. Acad. Sci. USA*, **89**, 12160-4.

Seiter, S., Arch, R., Reber, S., Komitowski, D., Hofmann, M., Ponta, H., Herrlich, P., Matzku, S. and Zoller, M. (1993) Prevention of tumor metastasis formation by anti-variant CD44. *J. Exp. Med.*, **177**, 443-55.

Singh, R., Campbell, B.J., Yu, L.G., Fernig, D.G., Milton, J.D., Goodlad, R.A., FitzGerald, A.J. and Rhodes, J.M. (2001) Cell surface-expressed Thomsen-Friedenreich antigen in colon cancer is predominantly carried on high molecular weight splice variants of CD44. *Glycobiology*, **11**, 587-92.

Takeuchi, H., Bilchik, A., Saha, S., Turner, R., Wiese, D., Tanaka, M., Kuo, C., Wang, H.J. and Hoon, D.S. (2003) c-MET expression level in primary colon cancer: a predictor of tumor invasion and lymph node metastases. *Clin. Cancer Res.*, **9**, 1480-8.

Thorne, R.F., Legg, J.W. and Isacke, C.M. (2004) The role of the CD44 transmembrane and cytoplasmic domains in co-ordinating adhesive and signalling events. *J. Cell Sci.*, **117**, 373-80.

van der Voort, R., Taher, T.E., Wielenga, V.J., Spaargaren, M., Prevo, R., Smit, L., David, G., Hartmann, G., Gherardi, E., *et al.* (1999) Heparan sulfate-modified CD44 promotes hepatocyte growth factor/scatter factor-induced signal transduction through the receptor tyrosine kinase c-Met. *J. Biol. Chem.*, **274**, 6499-506.

Wang, Q., Yu, L.G., Campbell, B.J., Milton, J.D. and Rhodes, J.M. (1998) Identification of intact peanut lectin in peripheral venous blood. *Lancet*, **352**, 1831-2.

Wielenga, V.J., van der Voort, R., Mulder, J.W., Kruijt, P.M., Weidema, W.F., Oosting, J., Seldenrijk, C.A., van Krimpen, C., Offerhaus, G.J., *et al.* (1998) CD44 splice variants as prognostic markers in colorectal cancer. *Scand. J. Gastroenterol.*, **33**, 82-7.

Wielenga, V.J., van der Neut, R., Offerhaus, G.J. and Pals, S.T. (2000a) CD44 glycoproteins in colorectal cancer: expression, function, and prognostic value. *Adv. Cancer Res.*, **77**, 169-87.

Wielenga, V.J., van der Voort, R., Taher, T.E., Smit, L., Beuling, E.A., van Krimpen, C., Spaargaren, M. and Pals, S.T. (2000b) Expression of c-Met and heparan-sulfate proteoglycan forms of CD44 in colorectal cancer. *Am. J. Pathol.*, **157**, 1563-73.

Yu, L.G., Jansson, B., Fernig, D.G., Milton, J.D., Smith, J.A., Gerasimenko, O.V., Jones, M. and Rhodes, J.M. (1997) Stimulation of proliferation in human colon cancer cells by human monoclonal antibodies against the TF antigen (galactose beta1-3 N-acetyl-galactosamine). *Int. J. Cancer*, **73**, 424-31.

## Figure legends

**Figure 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.

**Figure 2. PNA activates c-Met and induces tyrosine phosphorylation in HT29 cells.** *A*, Immunoblot of HT29 cell lysates following treatment with 25  $\mu\text{g/ml}$  PNA (2.5 to 30 min) using antibodies against Tyr 1349 phosphorylated c-Met illustrating 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  $\mu\text{g/ml}$  PNA, at 5 min) failed to activate c-Met in presence of 100  $\mu\text{g/ml}$  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.

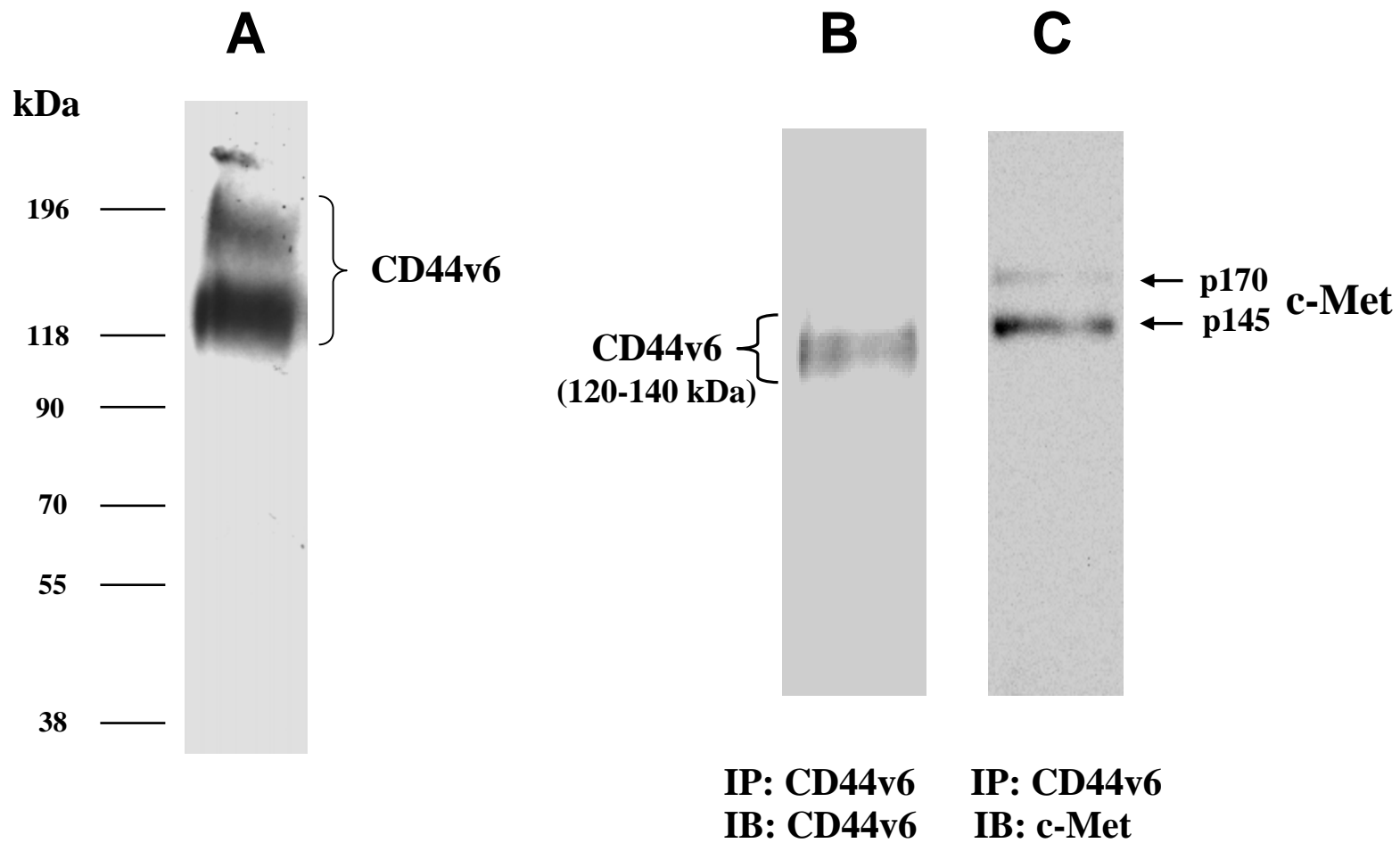
**Figure 3. PNA activates p44/p42 MAPK and inhibition of MAPK activation abrogates PNA-induced cell proliferation.** *A*, Immunoblot of HT29 cell lysates following treatment with 25  $\mu\text{g/ml}$  PNA (1 to 60 min) using antibodies against phospho-ERK 1/2. PNA activates p44/p42 MAPK within 5 min, 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  $\mu$ g/ml; 15 min) failed to activate MAPK in presence of 100  $\mu$ g/ml asialofetuin (ASF). *D*, Effect of PNA (25 $\mu$ g/ml, 24h) with or without 1  $\mu$ M 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.

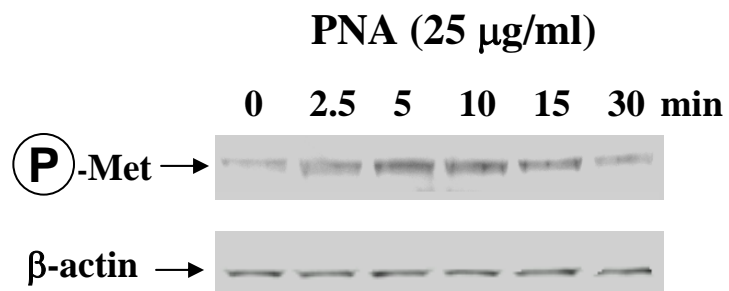
**Figure 4. PNA-induced c-Met and MAPK activation is CD44v6 isoform dependent.**

*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.

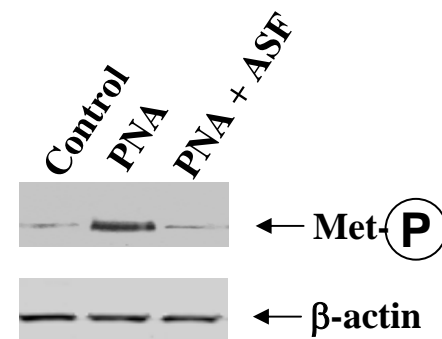
**Figure 1**  
Singh et al.



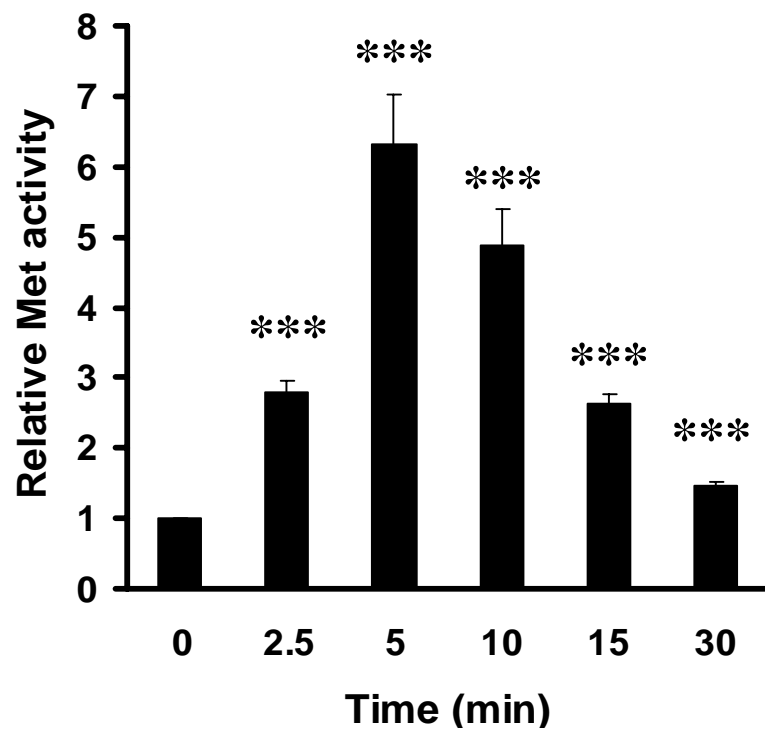
**A**



**C**



**B**



**D**

