

The antagonistic regulation of human MUC4 and ErbB-2 genes by the Ets protein PEA3 in pancreatic cancer cells: implications for the proliferation/differentiation balance in the cells

Valérie Fauquette, Michael Perrais, Sylvain Cerulis, Nicolas Jonckheere, Marie-Paule Ducourouble, Jean-Pierre Aubert, Isabelle Van, Pascal Pigny, Isabelle van Seuningen

▶ To cite this version:

Valérie Fauquette, Michael Perrais, Sylvain Cerulis, Nicolas Jonckheere, Marie-Paule Ducourouble, et al.. The antagonistic regulation of human MUC4 and ErbB-2 genes by the Ets protein PEA3 in pancreatic cancer cells: implications for the proliferation/differentiation balance in the cells. Biochemical Journal, 2005, 386, pp.35 - 45. hal-02905757

HAL Id: hal-02905757 https://hal.science/hal-02905757

Submitted on 29 Sep 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

The antagonistic regulation of human MUC4 and ErbB-2 genes by the Ets protein PEA3 in pancreatic cancer cells. Implications for the proliferation/differentiation balance in the cells.

REVISED

Valérie Fauquette^{*}, Michael Perrais^{*}, Sylvain Cerulis^{*}, Nicolas Jonckheere^{*}, Marie-Paule Ducourouble^{*}, Jean-Pierre Aubert^{*}, Pascal Pigny^{*,†, 1} and Isabelle Van Seuningen^{*}.

* Unité INSERM 560, Place de Verdun, 59045 Lille cedex, FRANCE.

[†] Université de Lille 2, Faculté de médecine, 59045 Lille cedex, FRANCE

¹ to whom correspondence should be sent:
Pascal Pigny, Ph.D.
Unité INSERM 560, place de Verdun, 59045 Lille cedex, France.
Phone: 33 (0)3 20 29 88 64; Fax: 33 (0)3 20 53 85 62; Email: pigny@lille.inserm.fr.

<u>Page heading title:</u> MUC4 and ErbB-2 transcriptional regulation by the Ets factor PEA3 <u>Abbreviations used:</u> DAC, ductal adenocarcinoma; WD, well-differentiated; PD, poorly differentiated; ECM, extra cellular matrix; RT-PCR, Reverse Transcriptase PCR; WT, wild type; MT, mutated type; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; TBE, Tris-Borate-EDTA.

Keywords: mucin, transcription, carcinogenesis, MUC4, PEA3, ErbB-2.

ABSTRACT

The human transmembrane mucin MUC4 is aberrantly expressed in 75% of pancreatic ductal adenocarcinomas (DAC) whereas no expression is found in normal pancreas. Therefore MUC4 appears as a useful biological marker for the diagnosis of DAC. Since rat Muc4, was shown to interact with ErbB-2 tyrosine kinase receptor and to either promote cell survival and differentiation or cell proliferation, it is postulated that MUC4 may also participate in pancreatic carcinogenesis. Our aim was to investigate in parallel the role of the Ets factor PEA3 in MUC4 and ErbB-2 transcriptional regulation in pancreatic cancer cells. Two MUC4 expressing well-differentiated (WD) (CAPAN-1 & CAPAN-2) and one MUC4 non expressing poorly differentiated (PANC-1) cell lines were used. The three cell lines express ErbB-2 at different levels. By co-transfection and site-directed mutagenesis, we show that PEA3 is a transactivator of MUC4 promoter and that the -216 and -2368 PEA3 binding sites of MUC4 promoter are essential. We also demonstrate that PEA3 acts in synergy with c-Jun and Sp1 to transactivate the proximal region of MUC4 promoter and increase MUC4 mRNA levels in WD cells. These results suggest that MUC4 is a new target gene of the Ets factor PEA3 in pancreatic cancer cells. Conversely, PEA3 represses the transcriptional activity of two fragments of ErbB-2 promoter in a dose-dependent manner and decreases the endogenous ErbB-2 mRNA levels in WD cell lines. Thus, PEA3, by its capacity to up-regulate the epithelial marker MUC4 and to down-regulate the ErbB-2 oncogene, appears as a key regulator of the differentiation/proliferation balance in pancreatic cancer cells.

INTRODUCTION

Pancreatic cancer is an especially devastating form of cancer, with only 3% of patients still alive five years after diagnosis. It constitutes the fifth leading cause of cancer-related death in Western countries. The poor prognosis of this disease is linked to the fact that it affects a rather unaccessible organ and remains asymptomatic until well advanced. Its diagnosis is therefore made at a late stage where peritoneal dissemination and liver metastasis have already occurred. Moreover pancreatic cancer is difficult to treat because of its resistance to chemotherapy and radiotherapy. A better understanding of the molecular mechanisms of pancreatic carcinogenesis may lead to the definition of new therapeutic targets and efficient chemotherapeutic interventions. Pathologically, most pancreatic cancers correspond to ductal adenocarcinoma (DAC) that develop from ductal epithelium. Hruban *et al.* [1] recently proposed a progression model for pancreatic cancer from intraepithelial neoplasia to invasive DAC similar to the adenoma-carcinoma sequence described in the colon. In the pancreatic carcinogenetic sequence, *K-ras* activation and *ErbB-2* overexpression are the earliest genetic events [2].

Mucins are high molecular weight O-glycoproteins encoded by at least ten genes in humans [3], that exhibit an aberrant pattern of expression in various human malignancies. The common structural feature of all mucins is the presence of tandemly repeated amino acid regions that serve as docking sites for O-glycans. Two groups of mucins are now distinguished [3]: (*i*) secreted mucins including gel-forming (MUC2, MUC5AC, MUC5B and MUC6) and non-gel-forming (MUC7) mucins; (*ii*) membrane-bound mucins that are thought to participate in cell-cell and cell-extracellular matrix (ECM) interactions or in cell signaling [4,5]. Human *MUC4* belongs to this last group. It encodes a large transmembrane mucin characterized by the presence of a repetitive domain rich in serine and threonine in its N-terminal region, two EGF-like domains, a transmembrane domain and a short cytoplasmic tail in its C-terminal part [6]. MUC4 is the human homologue of rat SMC/Muc4, a heterodimeric glycoprotein made of a mucin subunit ASGP-1 tighly bound to a transmembrane subunit ASGP-2. ASGP-2 contains two EGF-like domains in its extracellular part, one of which, EGF1, acts as a ligand for the ErbB-2/Neu oncogene [4]. By analogy, MUC4 is thought to interact with the HER2/ErbB-2 tyrosine kinase receptor.

Biochemical Journal Immediate Publication. Published on 5 Oct 2004 as manuscript BJ20040706

Fauquette et al. 2004

Several studies have shown that mucin genes are dysregulated in pancreatic cancer. While *MUC4* is not expressed in normal pancreas [7], up to 75% of pancreatic DAC and of pancreatic cancer cell lines show *de novo* expression of *MUC4* at the mRNA level [8]. Recently, Swartz *et al* [9] demonstrated a gradual expression of MUC4 at the protein level in pancreatic carcinogenesis with 17% of pancreatic intraepithelial neoplasia type 1 positive for MUC4 whereas in DAC the percentage rises to 89%. The rare expression of *MUC4* in non neoplastic lesions of the pancreas was also confirmed. According to its high specificity for pancreatic cancer, MUC4 could be used as a biological marker for the histopathological diagnosis of pancreatic adenocarcinoma and for its early detection in the peripheral blood [10]. Moreover *MUC4* is one of the few genes identified as an attractive target for novel therapeutic strategies in pancreatic cancer [11]. However at this time, the molecular mechanisms leading to *MUC4* dysregulation in pancreatic carcinoma are still poorly understood.

In order to decipher these mechanisms, we have previously characterized MUC4 promoter and showed that it contains a GC-rich and TATA-less proximal regulatory region and a distal regulatory region flanked by a TATA box [12]. Several consensus binding sites for the Ets transcription factors (5'-GGAA/T-3') were found both in the proximal and distal regions of MUC4 promoter. Among those, the proximal putative cis-element (P216) is conserved in the rat Muc4 promoter [13]. The Ets transcription factors have in common a 85-amino-acid helix-turn-helix DNA binding domain called the ETS domain. Interestingly, the Ets factors have been implicated in a wide range of cellular processes such as differentiation, proliferation and transformation [14]. Recently, Perez et al. [15] have demonstrated that PEA3, a founding member of an Ets protein subfamily, was capable of transactivating rMuc4 promoter in the mouse mammary epithelial cell line HC11. This effect relies in part on a direct binding of PEA3 to its proximal cis-element P₃₃₉ (5'-AGGAA-3') that is not perfectly conserved in the human MUC4 promoter (5'-AGGAG-3'). In light of earlier data on the regulation of ErbB-2 by PEA3 in COS cells [16] and in ovarian cancer cell lines [17], we undertook to investigate in parallel the effects of PEA3 on MUC4 and ErbB-2 regulation in human pancreatic cancer cell lines. As an experimental model, we used two welldifferentiated (WD) cell lines, CAPAN-1 and CAPAN-2, that express high levels of MUC4 mRNA

and a poorly differentiated (PD) cell line that does not express *MUC4*, PANC-1 [12]. The 3 cell lines express ErbB-2 protein at different levels. The aim of our study was to demonstrate whether in pancreatic cancer cell lines, PEA3 co-regulates *MUC4* and *ErbB-2* that encode 2 membrane-bound protein partners.

Copyright 2004 Biochemical Society

MATERIALS AND METHODS

<u>Cell cultures</u>. Pancreatic cancer cell lines CAPAN-1 and CAPAN-2 were purchased from ATCC (American Type Culture Collection), and PANC-1 from ECACC (European Collection of Animal Cell Cultures). The cells were cultured as previously described [12].

Transient transfections. Cells were passed at 0.5×10^6 cells/well the day before being transfected by pGL3 basic vector (Promega, France) containing a deletion mutant of MUC4 promoter upstream of the luciferase gene [12] using Effectene reagent as previously described [18], or by pXP2 vector containing a deletion fragment of ErbB-2 promoter [19] upstream of the luciferase gene (kind gift of Dr R. Winkler, Université de Liège, Belgium). Total cell extracts were prepared after a 48h incubation at 37°C using 1X RLB buffer according to the manufacturer's instructions (Promega, France). The luciferase activity and the protein content of the cell extract were measured as previously described [12]. Results were first expressed as luciferase activity/µg of protein. In order to take into account transfection efficiency, corrected results were obtained by dividing the luciferase activity/µg protein by those of the positive control plasmid (pGL3-control vector). Final results are expressed as fold of induction of the pGL3-control vector. In co-transfection studies, cells were transfected with pGL3 MUC4 promoter deletion mutants in the presence of an expression vector encoding either mPEA3 (pSV-HA-mPEA3, kind gift of Dr J.L. Baert, Institut Pasteur de Lille, France), ER81 (pSV-ER81), ERM (pSV-ERM, both kind gifts of Pr Y. de Launoit, Université Libre de Bruxelles, Belgium), Sp1 (pCMV-Sp1, kind gift of Dr J.M. Horowitz, Raleigh, USA), or c-Jun (pRSV-c-Jun). Results were expressed as fold induction relative to the cotransfection performed in the presence of the corresponding empty expression vectors. Each combination of plasmids was assayed in triplicate in three separate experiments. Data were analyzed by Student's t test with differences less than 0.05 considered significant.

<u>Site-directed mutagenesis</u>. The P_{216} PEA3 binding site was mutated from AGGAA to AAAAA in the -219/-1 deletion mutant using the Quick Site Directed Mutagenesis Kit (Stratagene, France) according to the manufacturer's instructions. The mutated plasmid DNA was purified and sequenced on both strands before being used in cell transfection experiments.

Biochemical Journal Immediate Publication. Published on 5 Oct 2004 as manuscript BJ20040706

Fauquette et al. 2004

Nuclear extract preparation and Western-blotting. Nuclear and cytoplasmic extracts from cell lines of interest were prepared as previously described [20] and kept at -80°C before use. Protein content was determined using the BCA method. Nuclear or cytoplasmic proteins (25µg) were separated by 10% SDS-PAGE, followed by an electrotransfer onto a 0.2 µm nitrocellulose membrane (Schleicher & Schuell, Germany). The membranes were first incubated with specific primary antibodies [anti-PEA3 mouse monoclonal (sc-113), anti-c-Jun rabbit polyclonal (sc-44) and anti-Sp1 goat polyclonal antibody (sc-59) all from Santa Cruz Biotechnology (Tebu, France); anti-ErbB-2 mouse monoclonal antibody (NCL-CB11) from Novo Castra (Tebu, France)] before a second incubation with a peroxydase-conjugated secondary antibody (PEA3, c-Jun, erbB-2) or alkaline phosphatase conjugated IgG (Sp1). Immunoreactive bands were visualized using the chemiluminescence Super Signal system (PEA3, c-Jun, ErbB-2) and Hyperfilm ECL (Amershampharmaciabiotech, UK) or (Sp1) Nitrotetrazolium Blue Chloride and 5-bromo-4-chloro-3-indolyl phosphate substrate (Life Technologies, France). The autoradiograms were scanned and a densitometry analysis was performed using the Clara Vision Gel Smart-Gel Analysis software. Values were expressed as arbitrary units.

Electrophoretic Mobility Shift Assays (EMSA). Binding studies were performed with double stranded oligonucleotides containing the P₂₁₆ PEA3 binding site of the *MUC4* proximal promoter (sense oligonucleotide: 5' CGGTGTTTTCT<u>TTCCT</u>GGTGG 3'). The oligonucleotides were annealed before being phosphorylated at the 5'-end with $[\gamma^{-32}P]$ ATP (Amersham Biosciences, France) and T4 polynucleotide kinase. The binding reaction was performed by preincubating 9 µg of nuclear extract in 10 mM HEPES pH 7.5, 50 mM KCl, 5 mM MgCl2, 0.5 mM EDTA, 1 mM DTT, 12.5% glycerol and 2 µg polyd(I,C) in a final volume of 20 µl for 10 min at room temperature. Then the radiolabelled probe (90,000 c.p.m.) was added to the reaction mixture and incubated for an additional 30 min at room temperature as previously described [21]. Competition experiments using a 100-fold molar excess of wild type or mutated (sense oligonucleotide: 5'CGGTGTTTTCT<u>TTGGT</u>GGTGG 3') cold probe were performed as described before [21]. Supershift experiments were performed by adding 2 µl of an anti-PEA3 or anti-ErbB-2 antibody to the reaction mixture together with the radiolabelled probe [21]. The reaction was stopped by adding

 $2 \mu l$ of 10X loading buffer. Samples were run on a 6% non denaturating polyacrylamide gel (200V for 3 hours at 4°C) with 0.5X TBE (Tris Borate EDTA) buffer. Gels were dried and autoradiographed. The PEA3 fusion protein was prepared using the pSV-HA-mPEA3 expression vector and the TNT T7 Quick Coupled Transcription/Translation System (Promega, France) according to the manufacturer's instructions. Binding reaction was performed by using 5 μ l of the PEA3 fusion protein preparation and the experimental protocol reported by Perez et al [15].

Reverse Transcriptase(RT)-PCR analysis. Total RNAs from pancreatic cancer cells were prepared using the RNeasy mini-kit (Qiagen SA, France) as already described [12]. cDNA was synthesized from 1.5 µg RNA using the Advantage-RT for PCR kit (BD Bioscience, France). To evaluate MUC4 expression, PCR was performed on 2 µl of cDNA using previously described primers [12]. To evaluate PEA3 expression, a specific pair of primers was designed (E1Af: 5' ACCATGGCGAGCAGTGCCTT 3', E1Ar: 5' TCCGACAGCTGGTGTTGGTA 3') that amplifies a 288 bp fragment outside of the ETS domain, that starts in exon 6 and ends in exon 8 of the human gene. PCR was carried out in a final reaction volume of 50 µl containing 5 µl of cDNA, 2µl of AmpliTaq (Roche, France), 10 pmoles of each primer and 1.5 mM MgCl₂ using the following program: denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 1 min; primer annealing at 57°C for 1 min, and elongation at 72°C for 1 min. Final elongation was conducted at 72°C for 5 min. To check the specificity of the primers, the 300 bp band was gelpurified before being sequenced using the ABI PRISM 377 XL automatic sequencer and the ABI PRISM Ampli Taq FS Big Dye Terminator cycle sequencing ready reactions Kit (Applied Biosystems, France). The nucleotide sequence was analyzed by the EMBL FASTA Server [22] showing a 100% identity with the sequence of PEA3/E1AF mRNA (GenBank Accession Number D12762). To evaluate *ErbB-2* expression we used the following pair of primers (NAU2126: 5' CGCGAGCTCGGAGGACGAGTGTGTGGG NAU2127: 5' CGCGGTACC-3' and CAGAATGCCAACCACCGC 3') that amplifies a 485 bp fragment that showed a 100% identity with the human c-erb-B2 mRNA (GenBank Accession Number X03363). The PCR program was as followed: denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30s;

primer annealing at 60°C for 30s, and elongation at 72°C for 30s. Final elongation was conducted at 72°C for 5 min. 28S or β actin were used as internal controls.

Immunohistochemistry. Cells were passed at 1.5×10^6 cells / 75 cm² flask 48h before being transfected with 4 µg of vector as described before. Forty-eight hours later, the cells were trypsinized, centrifuged and washed once with 1X PBS. The pellet was fixed in 4% formaldehyde, embedded in paraffin and 3 µm sections were prepared. Immunohistochemistry was performed on serial sections of the same blocks using an automatic immunostainer (ES, Ventana Medical System, Strasbourg, France) as previously described [23]. The monoclonal anti-MUC4 antibody, that recognized the tandem repeat region of the protein [9], was used at a 1/10000 dilution.

RESULTS

Expression of MUC4, ErbB-2 and PEA3 in pancreatic cancer cells. Expression of the different partners studied in this report was evaluated by RT-PCR or Western-blotting. As already shown [12], MUC4 mRNA were found only in the WD cell lines CAPAN-1 and CAPAN-2 (Fig. 1A). PEA3 expression was first evaluated by RT-PCR using a specific pair of primers designed to amplify a 5' region of human PEA3 cDNA outside of the conserved ETS domain. The results showed that the 3 cell lines express PEA3 mRNA (Fig 1A), at an apparently lower level in PANC-1 cells. To look for statistically significant differences, a *PEA3/\beta actin* ratio was calculated on 2 independent experiments and a t test performed, showing that CAPAN-1 (mean ratio (SD): 0.865 (0.007)), CAPAN-2 (0.945 (0.106)) and PANC-1 cells (0.616 (0.220)) express similar levels of PEA3. We also examined PEA3 expression at the protein level by Western-blotting using nuclear (lanes 1-3) and cytosolic (lanes 4-6) extracts prepared from the three cell lines (Fig. 1B). PEA3 is found at higher levels in CAPAN-1 (lane 1) and CAPAN-2 (lane 2) nuclear extracts when compared with PANC-1 (lane 3). On the contrary, PANC-1 cytosolic extracts (lane 6) contain higher amounts of PEA3 than CAPAN-1 (lane 4) or CAPAN-2 (lane 5). Finally, we controlled the levels of ErbB-2 protein expressed by the 3 cell lines by Western blotting (Fig. 1B). The densitometry analysis of the bands showed that CAPAN-1 cells expressed the lowest levels of ErbB-2 (p<0.05 vs CAPAN-2, n=2) whereas CAPAN-2 and PANC-1 expressed similar levels (mean ErbB-2/actin ratio (SD): 0.89 (0.014) for CAPAN-1; 1.50 (0.009) for CAPAN-2 and 1.11 (0.24) for PANC-1).

PEA3 selectively transactivates the promoter of MUC4

The promoter of *MUC4* contains several canonical Ets binding sites (5'-AGGAA-3') located in the proximal transcriptional unit ($P_{216} \& P_{349}$) upstream of the transcription start site (Fig. 2, upper panel) and in the distal transcriptional unit upstream (P_{2950} , P_{2872} , P_{2647}) or downstream (P_{2368}) of the transcription start site (Fig. 2, lower panel). To study the effects of the Ets protein PEA3 on *MUC4* promoter, we co-transfected a panel of deletion mutants covering both promoters of *MUC4* [12] in the presence of an expression vector encoding full-length PEA3. In CAPAN-1 cells (Fig. 3A, white

bars), PEA3 strongly transactivates the proximal promoter especially the -219/-1 region containing the PEA3 site P_{216} . A stronger effect is not seen with longer fragments outlining the importance of this site. Mutagenesis of P_{216} (5'-AGGAA-3' to 5'-AAAAA-3') led to a 60% reduction of the PEA3 transactivating effect on the -219/-1 region (Figure 3A, MT -219/-1). When the luciferase activities of wild type (WT -219/-1) and mutant (MT -219/-1) constructs were compared in the absence of PEA3, we observed a 70% reduction of activity for the mutated form (Fig. 3B). This indicates that the PEA3 P_{216} site acts as an important positive regulatory element required for *MUC4* promoter activity in CAPAN-1 cells.

In CAPAN-2 cells (Fig. 3A, black bars), which express lower levels of *MUC4* mRNA (see Fig. 1A), the transactivating effect of PEA3 on the -144/-1 and -219/-1 regions was 50% less intense than in CAPAN-1 cells. Interestingly, the response to PEA3 in these cells increased as longer fragments of *MUC4* promoter were transfected. These results suggest that positive elements for PEA3 regulation are present in the -461/-219 region of the promoter. Accordingly, the mutation of the conserved PEA3 site P_{216} did not significantly affect the transcriptional activity of the -219/-1 region in CAPAN-2 cells (Fig. 3B). PEA3 also induces the luciferase activity driven by the -3135/-2307 region of *MUC4* promoter in CAPAN-1 (4-fold) and CAPAN-2 (2-fold) cells (Fig. 3A) whereas it does not modify the activity of the -3135/-2572 region. The -3135/-2307 region contains one additional Ets binding site (P_{2368}) and two closely-linked AP-1 *cis*-elements compared to the -3135/-2572 region. Thus these sites are probably crucial for PEA3 transactivating effect in those two cell lines.

Finally, the weakest effects of PEA3 were observed in the *MUC4* non-expressing cell line PANC-1 (Fig. 3A, hatched bars) in which the transactivating effect of PEA3 remained less than 2 fold except for the -386/-1 region of the proximal promoter where it reached 2.7 (2.7 ± 0.8). Nevertheless, this value was significantly lower than those obtained in CAPAN-2 cells (3.8 ± 0.57 , p<0.05, Student's t test).

To check whether PEA3 transactivating effect on *MUC4* promoter is shared by other members of its sub-family, co-transfection studies were carried out using expression vectors encoding ER81 or ERM both in CAPAN-1 and CAPAN-2 cells. Three fragments of *MUC4* promoter corresponding

to either the -3135/-2307, -219/-1 or -461/-1 region were chosen. In CAPAN-1 cells, ER81 and ERM did not transactivate the proximal nor the distal regions of *MUC4* promoter thus demonstrating an effect restricted to PEA3. On the contrary, in CAPAN-2 cells ER81 and ERM are at least as potent transactivators as PEA3 on the 3 regions of *MUC4* promoter (data not shown).

We then checked whether PEA3 could bind to the P_{216} binding site by gel retardation analysis. As shown in figure 4A one retarded complex (lanes 1 and 6, arrow #1) was obtained when incubating CAPAN-1 or CAPAN-2 nuclear extracts with the T₇₆ radiolabelled probe containing the P₂₁₆ site (5'-AGGAA-3'). Competition experiments carried out with a 100-fold excess of cold T₇₆ oligonucleotide led to a substantial decrease of the shifted band (lanes 2 and 7) whereas an oligonucleotide with a mutated PEA3 binding site (T_{76MT} : 5'-ACCAA-3') did not modify the binding (lanes 3 and 8). Therefore the data indicate that an Ets protein is involved in the formation of the retarded complex. Surprinsingly, no supershift was observed when an anti-PEA3 antibody was added in the binding reaction (lanes 4 and 9) instead a strong increase of the retarded band was noticed, being more pronounced with CAPAN-2 nuclear extracts (compare lane 9 to lane 6, and lane 4 to lane 1). The addition of an irrelevant antibody (anti-ErbB-2) to the medium did not modify the retarded band formation (lanes 5 and 10). To show that PEA3 binds directly to the P_{216} site, we produced a fusion protein encoding a HA-mPEA3 fusion protein and checked its ability to bind to the T₇₆ probe. As shown in figure 4B, the high mobility band (arrow #2, lane 2) represents a specific DNA-protein complex. The upper band is not specific as it was also found in the control reaction containing no added DNA plasmid (lane 1, n.s.). The specificity of complex #2 was confirmed by performing cold competition with a 100-fold excess of T_{76} that led to a total loss of the shifted band (lane 3). On the contrary, when the T_{76MT} oligonucleotide with a mutated PEA3 binding site was used, the intensity of the shift band decreased very slightly (lane 4). This shows that PEA3 can bind specifically to the P_{216} site in *MUC4* promoter.

c-Jun and Sp1 transactivating effects on the promoter of MUC4

PEA3 is often considered as a weak transcription factor that needs to cooperate with partners such as Sp1, c-Jun to efficiently transactivate its target genes [14]. Morevover, both the proximal and

distal promoters of MUC4 have several consensus putative cis-elements for AP-1 and Sp1 transcription factors (see Fig. 2). To show whether Sp1 and/or AP-1 cooperate with PEA3 to activate MUC4 transcription, we cotransfected the -461/-1 and -219/-1 regions of the proximal promoter and the -3135/-2307 region of the distal promoter with expression vectors encoding PEA3, Sp1 or c-Jun, respectively (Fig. 5). The -461/-1 region that is the most responsive to PEA3 in CAPAN-2 cells (see Fig. 3A) is also stimulated by Sp1 (3-fold induction; Fig. 5A, black bars) or c-Jun (idem). Interestingly, coexpression of Sp1 greatly increases the transactivating effect of PEA3 on the -461/-1 region in CAPAN-2 cells (12-fold; p<0.001 versus PEA3 alone). This is not observed on the shorter -219/-1 region (Fig. 5B) suggesting that the upstream Sp1 and the P₃₄₉ PEA3 cis-elements (Fig. 2) are required for this synergistic effect. An additive effect is seen between c-jun and PEA3 on the -461/-1 region (p<0.05) becoming synergistic on the shorter -219/-1 in CAPAN-2 cells (10-fold induction, p<0.01). Since this last region does not contain a canonical AP-1 cis-element, these data suggest that c-Jun acts indirectly probably by increasing PEA3 binding to the P₂₁₆ binding site as previously proposed [24]. On the contrary, Sp1 does not increase the transactivating effect of PEA3 on the -461/-1 or the -219/-1 regions in CAPAN-1 cells (Figs. 5A & 5B, white bars). A weak synergistic effect is noticed between c-Jun and PEA3 on the -219/-1 region in these cells (9-fold induction). In PANC-1 cells (hatched bars), the weak transactivating effect of PEA3 on the 2 proximal regions of MUC4 promoter is not modified by the coexpression of either Sp1 or c-Jun. Taken together, these results indicate that strong synergistic effects between PEA3/Sp1 or PEA3/c-jun occur on MUC4 proximal promoter in CAPAN-2 cells.

Concerning the distal promoter of *MUC4*, a 4-fold activation by c-Jun is obtained on the -3135/ -2307 region in CAPAN-1 cells (Fig. 5C) making this transcription factor as potent as PEA3. Interestingly, c-Jun and PEA3 act in synergy to transactivate this region both in CAPAN-1 (17-fold induction, p<0.001) and CAPAN-2 cells (10-fold, p<0.05). This effect is not seen on the -3135/-2572 region (data not shown). Sp1, alone or with PEA3, does not modify the luciferase activity driven by the -3135/-2307 region in either cell line (Fig. 5C). Taken together, these results outline the important role of the P₂₃₆₈ PEA3 site and of the two adjacent AP-1 *cis*-elements (see Fig. 2) to convey synergistic activation of *MUC4* distal promoter in both WD cell lines. c-Jun could either act

Biochemical Journal Immediate Publication. Published on 5 Oct 2004 as manuscript BJ20040706

Fauquette et al. 2004

directly by binding to its *cis*-elements and/or indirectly by increasing the binding of PEA3 to its cognate element.

PEA3 and its partners regulate MUC4 mRNA levels in pancreatic cancer cells

The response of the -3135/-2307 region to PEA3 and c-Jun in CAPAN-1 and CAPAN-2 cells, and those of the -461/-1 and -219/-1 regions to PEA3 and Sp1 and PEA3 and c-Jun in CAPAN-2 cells respectively, prompted us to evaluate whether these two combinations of transcription factors also affected the level of endogenous MUC4 mRNA in WD pancreatic cancer cells. To this end, we transiently cotransfected these cells as described before and isolated total RNA 48h later. RT-PCR (Fig. 6, upper panel) and the densitometry analysis of the bands (Fig. 6, lower panel) show that (i) PEA3, Sp1 and c-jun alone did not modify MUC4 mRNA levels in both WD cell lines; (ii) PEA3 + Sp1 strongly increased MUC4 expression in CAPAN-1 cells by 221%; (iv) PEA3 + c-Jun increased MUC4 expression by 160% both in CAPAN-1 and CAPAN-2 cells. None of these combinations was able to induce the expression of MUC4 in the PD PANC-1 cells (data not shown). The absence of induction of endogenous MUC4 expression with 0.2 µg of PEA3 expression vector was unexpected, and thus thought to be dose-dependent. Therefore higher amounts of the expression vector (0.5, 1 or 2 µg) were assayed and the level of MUC4 mRNA was measured as previously described. The results confirmed that hypothesis as in CAPAN-1 cells a high activation (5.7 fold) was obtained with 1 µg of PEA3 expression vector. In CAPAN-2 cells, an induction was also observed but remained weaker with a maximal value of 2.1 fold with 1 µg of expression vector. In conclusion, our results demonstrate that PEA3 alone, in a dose-dependent manner, or in association with c-Jun and/or Sp1, is a potent activator of MUC4 expression in the WD CAPAN-1 and CAPAN-2 cells.

PEA3 regulates MUC4 apomucin expression in pancreatic cancer cells.

Having shown that PEA3 transactivates *MUC4* promoter in CAPAN-1 and CAPAN-2 cells and induces *MUC4* mRNA levels in these cell lines, we undertook to evaluate its effects at the protein level. To this end, cells were transfected with either an empty expression vector or with pSV-HA-

mPEA3, and then processed for immunostaining with a specific anti-MUC4 antibody. In CAPAN-1 mock cells, 38 % of the cells were positively labeled with anti-MUC4 antibody (Fig. 7A). The staining was restricted to the cytoplasm. Transfection with PEA3 expression vector led to an increase of the percentage of positive cells to 60% accompanied by an increase of the staining intensity (Fig. 7C). In CAPAN-2 mock cells, 53 % of the cells were positively labeled with the anti-MUC4 antibody (Fig. 7A), a percentage that was not significantly modified to 56 % in the PEA3-transfected cells (Fig. 7D). The immunostaining intensity was weaker in CAPAN-2 cells than in CAPAN-1 cells. In conclusion, our results showed that PEA3 is also an activator of MUC4 apomucin expression in CAPAN-1 cells.

PEA3 and its partners are expressed in pancreatic cancer cells

Having demonstrated a strong correlation between the transactivating effect of PEA3 on *MUC4* promoter activity (Fig. 3A) and the level of *MUC4* mRNA expressed by the three pancreatic cancer cell lines (Fig. 1), we undertook to determine the level of expression of PEA3 and its partners in these cells. As previously shown, PEA3 is found at higher levels in the nuclear extracts of the WD cell lines than in those of the PD PANC-1 cells. These results suggest a cytosolic sequestration of PEA3 in PANC-1 cells that may prevent it to exert its transcriptional effects. Moreover, since the different levels of *MUC4* expression in the three cell lines may be due to a different pattern of expression of c-Jun, and/or Sp1 that cooperate with PEA3, we determined their levels of expression in the same nuclear extracts by Western-blotting (Fig. 8). c-Jun is mainly found under the dimeric form (band b) in the three cell lines. Interestingly, the intensity of the band corresponding to the dimeric form of c-Jun is much stronger in CAPAN-1 (lane 3) and CAPAN-2 (lane 2) cells than in PANC-1 (lane 1) suggesting that c-Jun is engaged in active transcriptional complexes in *MUC4* positive cell lines. The amount of Sp1 (band c) is similar in the three cell lines.

PEA3 down regulates the expression of ErbB-2 in pancreatic cancer cells

Since MUC4 is the ligand of ErbB-2 we wanted to check whether they were both positively coregulated by PEA3. To this aim, co-transfection experiments were performed using two proximal

fragments of the human *ErbB-2* promoter i.e. the p255 and the p537 fragments [19]. The p255 contains a conserved consensus PEA3 binding site (5'-AGGAA-3') near the TATA box and several core elements (5'-GGAA-3') for Ets proteins; the p537 contains one more PEA3 putative binding site nearby a GATA-1 and an AP-1 binding sites [19]. The transfection experiments showed that in CAPAN-1, CAPAN-2 and PANC-1 cells, PEA3 repressed the transcriptional activity of the two fragments of *ErbB-2* promoter in a similar way (Fig. 9A). This suggests that the proximal PEA3 consensus binding site in the p255 fragment is sufficient to mediate PEA3 inhibitory effect on *ErbB-2* promoter. Moreover, the down-regulation of *ErbB-2* promoter by PEA3 is dose-dependent, as increasing amounts of PEA3 repress the promoter activity of the p255 region up to 90% in the three cell lines. In conclusion, these results indicate that in contrast to what was observed on *MUC4* promoter, PEA3 is a potent transrepressor of the human *ErbB-2* promoter in pancreatic cancer cell lines.

The inhibition of *ErbB-2* expression by PEA3 was confirmed at the mRNA level. In CAPAN-1 cells and, to a lower extent in CAPAN-2 cells, endogenous *ErbB-2* mRNA levels substantially decreased when PEA3 was overexpressed in the cells (Fig. 9B). The maximun decrease was 75-80% in CAPAN-1 cells (for 1 µg expression vector) and 50% in CAPAN-2 cells (for 2 µg expression vector, Fig. 9C). Thus expression level in cells correlated well with regulatory data at the promoter level. When one compared the levels of expression of PEA3, MUC4 and ErbB-2 (Fig. 1) in the 3 cell lines, it came to our attention that the WD CAPAN-1 cells expressed both high levels of PEA3 and MUC4 and the lowest levels of ErbB-2. On the contrary, the PD PANC-1 cells that contained low levels of active PEA3, express ErbB-2 but no MUC4.

DISCUSSION

The aim of this study was to better understand the mechanisms leading to MUC4 aberrant expression in pancreatic cancer cell lines, and especially to further characterize the transcription factors and the *cis*-regulatory elements involved in the control of MUC4 expression. In this study we focused our attention on the Ets transcription factor PEA3 for two reasons. First, both the distal and proximal promoters of MUC4 contain numerous binding sites for Ets transcription factors, among those the P₂₁₆ is conserved in human [12] and rat [13] promoters. Second, PEA3 is a key transcriptional regulator of the proto-oncogene *ErbB-2* [16,17]. Moreover, the fact that ratMuc4 was shown to be a ligand of the rat homologue of ErbB-2 (p185neu) [4] suggests a link between MUC4, PEA3 and ErbB-2.

Our data demonstrate that PEA3 acts as a potent transactivator of both regions of MUC4 promoter in the MUC4-expressing cell lines and that its transactivating effect is correlated to the level of MUC4 mRNA expressed by these cells. We also showed by EMSA experiments that PEA3 binds to the P₂₁₆ PEA3 element of MUC4 promoter and consequently acts directly. The fact that the interaction between PEA3 and its *cis*-element, is stronger in the presence of an anti-PEA3 antibody could rely on a relief of PEA3 DNA binding autoinhibition by preincubation with the antibody as previously described [25]. These data are in agreement with a recent work showing that PEA3 transactivates the proximal promoter of rat Muc4 in mammary cancer cells [15]. Thus PEA3 appears as an important factor that could upregulate MUC4 expression in tumor cells.

Chotteau-Lelièvre *et al.* [26] previously showed using *in situ* hybridization that in adult mice PEA3 expression is restricted to the brain, skeletal muscles and colon while during development PEA3 is widely expressed. An important point was therefore to check PEA3 expression in pancreatic cancer cells in order to document its biological role in the control of *MUC4* expression. Here we show for the first time that PEA3 is expressed in pancreatic cancer cells, as recently shown in human breast [16] and colon cancer cell lines [27]. Moreover, PEA3 is present at high levels in the nuclear compartment of *MUC4* positive WD cell lines allowing it to exert its transactivating effect. In the *MUC4*-negative PD PANC-1 cells, the level of PEA3 mRNA is similar to those observed in the *MUC4* positive cells. However, in PANC-1 cells, PEA3 protein

is mostly found in the cytosol compartment and did not transactivate MUC4 promoter. Thus it may be hypothesized that in PANC-1 cells the transduction pathway(s) involved in PEA3 activation, translocation and binding to DNA is/are altered, preventing it from exerting its transactivating effect or that MUC4 locus is inactivated by epigenetic mechanisms [28]. Bojovic and Hassell [25] have previously suggested that PEA3 is found in mammalian cells in a dynamic equilibrium between 2 states: one able and one unable to bind to DNA. The transition between the active and inactive states depends on post-translational modifications such as phosphorylation [29] and/or on interactions with protein partners. Altogether, the differences seen in the transactivating effect and in the intracellular distribution of PEA3 between MUC4-expressing and non expressing cell lines suggest the involvement of PEA3, and possibly of other Ets proteins, in the regulation of MUC4 transcription in pancreatic tumors. The PEA3-mediated increase of MUC4 endogenous expression in CAPAN-1 and CAPAN-2 cells was dose-dependent and became efficient at high doses of PEA3. This is in agreement with the fact that PEA3 is considered as a weak transactivator that often cooperates with other transcription factors to regulate the expression of target genes [30]. Among the already known partners of PEA3, we focused our attention on Sp1 and c-Jun transcription factors. C-Jun is a major member of the AP-1 family, which regulates a variety of cellular processes such as proliferation, differentiation and apoptosis [31]. Our results indicate that PEA3 and c-Jun strongly transactivate the distal region -3135/-2307 of MUC4 promoter in CAPAN-1 and CAPAN-2 cells and the proximal region -219/-1 in CAPAN-2 cells. The activation of the promoter was associated with an increase of endogenous MUC4 expression in these cells, especially in CAPAN-1 cells. Moreover, the fact that the amounts of c-Jun engaged in active transcriptional complexes are higher in CAPAN-1 and CAPAN-2 than in PANC-1 cells further document the key regulatory role of this factor in MUC4 expression. The underlying mechanisms resulting in different levels of c-Jun activity are currently unknown. Differences in the activity of c-Jun Nterminal kinase (JNK) pathway, responsible for the activation of c-Jun by phosphorylation [31], seem unlikely since the MEKK1-JNK pathway is active in PANC-1 cells [32]. An involvement of the extracellular signal-regulated kinase (ERK) pathway that is known to play an important role in PANC-1 proliferation [33] could not be currently ruled out. Interestingly, both PEA3 and its co-

factor c-Jun transcriptional activities are regulated by the MAPK pathway. JNK can induce AP-1 activity by phosphorylation of c-Jun [31]. Similarly, PEA3 activity is positively regulated by the ERK and JNK pathways [29]. Further studies are required to determine the nature of the signaling pathway(s) that regulate PEA3 activity in pancreatic cancer cells and whether PEA3 is a downstream target gene of ErbB-2.

Our results demonstrate that PEA3 transactivates the MUC4 promoter in cooperation with c-Jun in WD pancreatic tumor cells. These results are consistent with previous data showing that c-Jun synergizes with PEA3 to transactivate the matrilysin promoter in kidney epithelial cells [27]. Moreover the activity of several promoters including those of metalloproteinase-13 [25], and cyclooxygenase-2 (COX-2) [34] requires closely spaced cis-elements for PEA3 and AP-1 suggesting that these transcription factors function synergistically. PEA3 alone induces the expression of several metalloproteinase genes such as collagenase-IV by MCF-7 cells, thus promoting the degradation of the ECM [35]. Therefore, the Ets transcription factors and especially PEA3 are involved in the invasiveness of tumor cells [36]. However the function of PEA3 may not be limited to this step of tumorigenesis, since it also regulates the expression of other genes such as COX-2 [34], ErbB-2 [16-17] and MUC4 (this report), which are known to modulate cell proliferation and/or apoptosis [37,38]. Our working hypothesis was that PEA3 may induce the expression of both MUC4 and ErbB-2 simultaneously thereby promoting the formation of a stable complex between these two proteins at the membrane level and consequently the phosphorylation of ErbB-2, followed by activation of intracellular signaling pathways. These pathways are either linked to epithelial cell proliferation and cell transformation [38] or to epithelial cell differentiation, depending on the presence of neuregulin, an ErbB-3 ligand [39].

In normal epithelial cells, rat Muc4 is apically localized [39,40] whereas the ErbB-2 tyrosine kinase receptor is present at the basolateral membrane together with the other ErbB receptors. This localization depends on a specific interaction between the C-terminal end of ErbB-2 and the PDZ domain of ERBIN [41], and occurs only when ErbB-2 is not phosphorylated. Recently Ramsauer *et al.* [40] demonstrated that rat Muc4 was able to trigger ErbB-2 translocation from the basolateral to the apical membrane of polarized human colon carcinoma Caco-2 cells. This

relocalization prevents the receptor to interact with partners (other ErbB receptors) to form an array of homo- and heterodimers and promotes the differentiated state of the cell. Interestingly, our results indicate that PEA3 could act as both an activator of MUC4 expression and a repressor of ErbB-2 expression in WD pancreatic cancer cells. Such a repression of ErbB-2 expression by PEA3 has already been observed in breast and ovarian cancer cell lines [17]. This inhibitory function seems to be restricted to PEA3 since ESX (Epithelial restricted with Serine boX)/Elf-3, an epithelial-specific Ets protein, transactivates the ErbB-2 promoter in mammary cells [42], and is capable of conferring aggressive and metastatic properties to the non tumorigenic human mammary epithelial MCF-12A cells [43]. Moreover, Brembeck et al. [44] have previously demonstrated that ESX suppresses the promoter activity of keratin 4, that plays a key role in the early differentiation of the esophageal squamous epithelium. These data suggest a link between up-regulation of ErbB-2, promotion of cell transformation and inhibition of differentiation. By contrast, the stable expression of PEA3, that represses ErbB-2 promoter activity in breast cancer cells [17], did not induce a cell transformation in MCF-12A cells [43]. Therefore, several lines of evidence suggest that PEA3 down-regulates ErbB-2 oncogene expression in tumor cells. The fact that concomittantly, PEA3 up-regulates the expression of MUC4, an epithelial marker merely expressed in differentiated cells [39,40] suggests that PEA3 promotes the differentiation of pancreatic cancer cells. Our results thus favor the hypothesis that PEA3 could be one of the key regulators of the differentiation/proliferation balance in pancreatic cancer cells (Fig. 10).

ACKNOWLEDGEMENTS

We are indebted to Danièle Petitprez, Dominique Demeyer, Christine Mouton, Annette Leclercq and Brigitte Hémon for their excellent technical help. We also thank Drs. S. Aubert and M.C. Copin for their help in interpretating the immunohistochemical data. This work was supported by grants from l'Association de Recherche contre le Cancer (n°5785 to IVS) and le Comité du Pas-de-Calais de la Ligue Nationale contre le Cancer (to IVS). N. Jonckheere is a recipient of a Centre Hospitalier Régional Universitaire de Lille – Région Nord Pas-de-Calais Ph.D. fellowship and V. Fauquette of a Ministère de la Recherche et de la Technologie Ph.D. fellowship.

REFERENCES

Hruban, R.H., Wilentz, R.E., and Kern, S.E. (2000) Genetic progression in the pancreatic ducts. Am.
 J. Pathol. 156, 1821-1825

2 Hilgers, W., and Kern, S.E. (1999) Molecular genetic basis of pancreatic adenocarcinoma. Genes Chromosomes Cancer **26**, 1-12

3 Dekker, J., Rossen, J.W., Buller, H.A., and Einerhand, A.W. (2002) The MUC family: an obituary. Trends Biochem. Sci. **27**, 126-131

4 Carraway, K.L., Ramsauer, V.P., Haq, B., and Carothers Carraway, C.A. (2003) Cell signaling through membrane mucins. BioEssays **25**, 66-71

5 Hollingsworth, M.A., and Swanson B.J. (2004) Mucins in cancer: protection and control of the cell surface. Nat. Rev. Cancer **4**, 45-60

6 Moniaux, N., Nollet, S., Porchet, N., Degand, P., Laine, A., and Aubert, J.P. (1999) Complete sequence of the human mucin MUC4 : a putative cell membrane-associated mucin. Biochem. J. **338**, 325-333

7 Balagué, C., Audié, J.P., Porchet, N., and Real, F.X. (1995) In situ hybridization shows distinct patterns of mucin gene expression in normal, benign, and malignant pancreas tissues. Gastroenterology **109**, 953-964

8 Andrianafahanana, M., Moniaux, N., Schmied, B.M., Ringel, J., Friess, H., Hollingsworth, M.A., Buchler, M.W., Aubert, J.P., and Batra, S.K. (2001) Mucin gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumor marker of diagnostic significance. Clin. Cancer Res. **7**, 4033-4040

9 Swartz, M.J., Batra, S.K., Varshney, G.C., Hollingsworth, M.A., Yeo, C.J., Cameron, J.L., Wilentz, R.E., Hruban, R.H., and Argani, P. (2002) MUC4 expression increases progressively in pancreatic intraepithelial neoplasia. Am. J. Clin. Pathol. **117**, 791-796

10 Ringel, J. and Lohr, M. (2003) The MUC gene family: their role in diagnosis and early detection of pancreatic cancer. Mol. Cancer, **2**, 9

11 Iacobuzio-Donahue, C.A., Ashfaq, R., Maitra, A., Volkan Asay, N., Shen-Ong, G.L., Berg, K., Hollingsworth, M.A., Cameron, J.L., Yeo, C.J., Kern, S.E., Goggins, M., and Hruban, R.H. (2003) Highly expressed genes in pancreatic ductal adenocarcinomas: a comprehensive characterization and comparison of the transcription profiles obtained from three major technologies. Cancer Res. **63**, 8614-8622

12 Perrais, M., Pigny, P., Ducourouble, M.P., Petitprez, D., Porchet, N., Aubert, J.P., and Van Seuningen, I. (2001) Characterization of human mucin gene MUC4 promoter. J. Biol. Chem. **276**, 30923-30933

13 Price-Schiavi, S.A., Perez, A., Barco, R., and Carraway, K.L. (2000) Cloning and characterization of the 5' flanking region of the sialomucin complex/rat Muc4 gene: promoter activity in cultured cells. Biochem. J. **349**, 641-649

14 Oikawa, T., and Yamada, T. (2003) Molecular biology of the Ets family of transcription factors. Gene **303**, 11-34

15 Perez, A., Barco, R., Fernandez, I., Price-Schiavi, S.A., and Carraway, K.L. (2003) PEA3 transactivates the Muc4/Sialomucin complex promoter in mammary epithelial and tumor cells. J. Biol. Chem. 278, 36942-36952

Copyright 2004 Biochemical Society

Biochemical Journal Immediate Publication. Published on 5 Oct 2004 as manuscript BJ20040706

Fauquette et al. 2004

Benz, C.C., O'Hagan, R.C., Richter, B., Scott, G.K., Chang, C.H., Xiong, X., Chew, K., Ljung,
B.M., Edgerton, S., Thor, A., and Hassell, J.A. (1997) HER2/Neu and the Ets transcription activator PEA3 are coordinately upregulated in human breast cancer. Oncogene 15, 1513-1525

Xing, X., Wang, S.C., Xia, W., Zou, Y., Shao, R., Kwong, K.Y., Yu, Z., Zhang, S., Miller, S.,
Huang, L., and Hung, M.C. (2000) The Ets protein PEA3 suppresses HER2/neu overexpression and inhibits
tumorigenesis. Nature Med. 6, 189-195

18 Perrais, M., Pigny, P., Copin, M.C., Aubert, J.P., and Van Seuningen, I. (2002) Induction of MUC2 and MUC5AC mucins by factors of the EGF family is mediated by EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. J. Biol. Chem. **277**, 32258-32267

19 Grooteclaes, M., Pasleau, F., Dijkmans, H., Berzi, P., Albert, A., and Winkler Gol, R. (1994) The 6 kb c-erbB2 promoter contains positive and negative regulatory elements functional in human mammary cell lines. Cancer Res. **54**, 4193-4199

20 Van Seuningen, I., Ostrowski, J., Bustelo, X.R., Sleath, P., and Bomsztyk, K. (1995) The K protein domain that recruits the IL1-responsive K protein kinase lies adjacent to a cluster of c-Src and Vav SH3binding sites. Implications that K protein acts as a docking platform. J. Biol. Chem. **270**, 26976-26985

21 Su, Z., Shi, Y., Friedman, R., Qiao, L., McKinstry, R., Hinman, D., Dent, P., and Fisher, P.B. (2001) PEA3 sites within the progression elevated gene-3 (PEG-3) promoter and mitogen-activated protein kinase contribute to differential PEG-3 expression in Ha-ras and v-raf oncogene transformed rat embryo cells. Nucl. Acid Res. **29**, 1661-1671

22 Pearson, W.R., and Lipman, D.J. (1988) Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85**, 2444-2448

23 Mariette C., Perrais M., Leteurtre E., Jonckheere N., Hemon B., Pigny P., Batra S., Aubert J.P., Triboulet J.P., and Van Seuningen, I. (2004) Transcriptional regulation of human mucin MUC4 by bile acids in oesophageal cancer cells is promoter-dependent and involves activation of the PI3-K signalling pathway. Biochem. J. **377**, 701-708

24 Tardif, G., Reboul, P., Dupuis, M., Geng, C., Duval, N., Pelletier, J.P., and Martel-Pelletier, J.

(2001) TGF- β induced collagenase-3 production in human osteoarthritic chondrocytes is triggered by Smad proteins: cooperation between activator protein-1 and PEA-3 binding sites. J. Rheumatol. **28**, 1631-1639

25 Bojovic, B.B., and Hassell, J.A. (2001) The PEA3 Ets transcription factor comprises multiple domains that regulate transactivation and DNA binding. J. Biol. Chem. **276**, 4509-4521

26 Chotteau-Lelievre, A., Desbiens, X., Pelczar, H., Defossez, P.A., and de Launoit, Y. (1997) Differential expression patterns of the PEA3 group transcription factors through murine embryonic development. Oncogene **15**, 937-952

27 Crawford, H.C., Fingleton, B., Gustavson, M.D., Kurpios, N., Wagenaar, R.A., Hassell, J.A., and Matrisian, L.M. (2001) The PEA3 subfamily of Ets transcription factors synergizes with beta-catenin-LEF-1 to activate matrilysin transcription in intestinal tumors. Mol. Cell. Biol. **21**, 1370-1383

Jonckheere, N., Perrais, M., Mariette, C., Batra, S.K., Aubert, J.P., Pigny, P., and Van Seuningen, I. (2004) A role for human MUC4 mucin gene, the ErbB2 ligand, as a target of TGF- β in pancreatic carcinogenesis. Oncogene **23**, 5729-5738

29 O'Hagan, R.C., Tozer, R.G., Symond, M., McCormick, F., and Hassell, J.A. (1996) The activity of the Ets transcription factor PEA3 is regulated by two distinct MAPK cascades. Oncogene **13**, 1323-1333

Biochemical Journal Immediate Publication. Published on 5 Oct 2004 as manuscript BJ20040706

Fauquette et al. 2004

30 Sharrocks, A.D. (2001) The ETS-domain transcription factor family. Nat. Rev. Mol. Cell. Biol. 2, 827-837

31 Shaulian, E., and Karin, M. (2002) AP-1 as a regulator of cell life and death. Nat. Cell. Biol. **4**, E131-E136

Hirano, T., Shino, Y., Saito, T., Komoda, F., Okutomi, Y., Takeda, A., Ishihara, T., Yamaguchi, T.,
 Saisho, H., and Shirasawa, H. (2002) Dominant negative MEKK1 inhibits survival of pancreatic cancer cells.
 Oncogene 21, 5923-5928

33 Ding, X.Z., and Adrian, T.P. (2001) MEK/ERK-mediated proliferation is negatively regulated by p38 MAP Kinase in the human pancreatic cancer cell line, PANC-1. Biochem. Biophys. Res. Com. 282, 447-453

34 Subbaramaiah, K., Norton, L., Gerald, W., and Dannenberg, A.J. (2002) Cyclooxygenase-2 is overexpressed in HER-2/neu-positive breast cancer. J. Biol. Chem. **277**, 18649-18657

Higashino, F., Yoshida, K., Noumi, T., Seiki, M., and Fujinaga, K. (1995) Ets-related protein E1A-F can activate three different matrix metalloproteinase gene promoters. Oncogene **10**, 1461-1463

Trojanowska, M. (2000) Ets factors and regulation of the extracellular matrix. Oncogene 19, 6464-

37 Komatsu, M., Jepson, S., Arango, M.E., Carothers Carraway, C.A., and Carraway, K.L. (2001) Muc4/sialomucin complex, an intramembrane modulator of ErbB2/HER2/Neu, potentiates primary tumor growth and suppresses apoptosis in a xenotransplanted tumor. Oncogene **20**, 461-470

38 Singh, A.P., Moniaux, N., Chauhan, S.C., Meza, J.L., Batra, S.K. (2004) Inhibition of MUC4 expression suppresses pancreatic tumor cell growth and metastasis. Cancer. Res. **64**, 622-630

Jepson, S., Komatsu, M., Haq, B., Arango, M.E., Huang, D., Carothers Carraway, C.A., and Carraway, K.L. (2002) Muc4/sialomucin complex, the intramembrane ErbB2 ligand, induces specific phosphorylation of ErbB2 and enhances expression of p27^{kip}, but does not activate MAPK or PKB/Akt pathways. Oncogene **21**, 7524-7532

40 Ramsauer, V.P., Carothers Carraway, C.A., Salas, P.J.I., and Carraway, K.L. (2003) Muc4/sialomucin complex, the intramembrane ErbB2 ligand, translocates ErbB2 to the apical surface in polarized epithelial cells. J. Biol. Chem. **278**, 30142-30147

41 Borg, J.P., Marchetto, S., Le Bivic, A., Ollendorff, V., Jaulin-Bastard, F., Saito, H., Fournier, E., Adélaide, J., Margolis, B., and Birnbaum, D. (2000) ERBIN: a basolateral PDZ protein that interacts with the mammalian ERBB2/HER2 receptor. Nat. Cell. Biol. 2, 407-414

42 Eckel, K.L., Tentler, J.J, Cappetta, G.J., Diamond, S.E., and Gutierrez-Hartmann, A. (2003) The epithelial-specific ETS transcription factor ESX/ESE-1/Elf-3 modulates breast cancer-associated gene expression. DNA Cell. Biol. **22**, 79-94

43 Schedin, P.J., Eckel-Mahan, K.L., McDaniel, S.M., Prescott, J.D., Brodsky, K.S., Tentler, J.J., and Gutierrez-Hartmann, A. (2004) ESX induces transformation and functional epithelial to mesenchymal transition in MCF-12A mammary epithelial cells. Oncogene **23**, 1766-1779

44 Brembeck, F.H., Opitz, O.G., Libermann, T.A., and Rustgi, A.K. (2000) Dual function of the epithelial specific ets transcription factor ELF3 in modulating differentiation. Oncogene **19**, 1941-1943

LEGENDS TO FIGURES

- Figure 1
- (A) Analysis of the expression of *MUC4* and *PEA3* mRNAs in pancreatic tumor cells by **RT-PCR.** Cells were cultured in 75 cm² flasks to 80% confluence. RT-PCR was performed using specific primer pairs as described in the Materials and Methods section. 28S or β actin were used as internal controls. PCR products were separated on 1.5% agarose gel containing ethidium bromide in 1X TBE buffer. The size of the PCR products were as follows: MUC4: 596 bp; 28S: 231 bp; β actin: 661 bp.
- (B) Expression of ErbB-2 and PEA3 proteins by Western-blotting in pancreatic cancer cells. Cells were cultured in 75 cm² plates at 100% confluence before being scrapped in PBS buffer. Cytosolic and nuclear extracts were prepared as described in Material and Methods and then separated on 10% SDS-PAGE (25 μg). Proteins were electrotransferred onto nitrocellulose and the blot was probed with a specific anti-human PEA3 antibody. For ErbB-2 analysis total cellular extracts were used (see Materials and Methods) and separated by 8% SDS-PAGE (20 μg).

• Figure 2

Schematic representation of the proximal and distal regulatory regions of *MUC4* promoters. The numbering refers to the A of the first ATG, and the main *cis*-elements for Sp1, AP-1, AP-2 and PEA3 (Pxxx) are shown. The broken arrows represent the transcriptional start site locations. The nucleotide sequence and the orientation of the PEA3 *cis*-elements are boxed.

• Figure 3

PEA3 transactivates *MUC4* **promoter in a cell-specific manner in pancreatic tumor cell lines.** (A) The MUC4-pGL3 deletion mutants were transiently transfected in CAPAN-1, CAPAN-2 and PANC-1 cells in the presence of 0.2 μ g of pSV-mPEA3 expression vector or empty pSV. The MT -219/-1 fragment represents the -219/-1 region in which the PEA3 *cis*-element P₂₁₆ was mutated. Results are expressed as fold induction of luciferase activity relative to the empty expression vector. Values are the mean of 3 independent experiments where cotransfections were run in triplicate. The error bar represents the standard deviation (SD) of the mean. \blacktriangle , p<0.05; *, p<0.01. (B) Effect of mutation of the P₂₁₆ site on the luciferase activity driven by the -219/-1 region in CAPAN-1 and CAPAN-2 cells. The luciferase activity of the wild type region (WT -219/-1) was arbitrarily set at 100%. Results are expressed as the mean and SD of 3 independent experiments.

• Figure 4

DNA binding of PEA3 to the P₂₁₆ cis-element of MUC4 proximal promoter.

(A) Nuclear extracts from CAPAN-1 and CAPAN-2 cells were used in EMSA experiments as described in the Material and Methods. A specific retarded complex (arrow #1) was seen both in CAPAN-1 (lane 1) and CAPAN-2 cells (lane 6). Specificity was assessed by addition of a 100-fold excess of a unlabelled wild type probe (lanes 2 and 6), or mutated probe (lanes 3 and 7). Supershift experiments were carried out by adding 2 μ l of an anti-PEA3 antibody (lanes 4 and 9) or an anti-ErbB-2 antibody (lanes 5 and 10).

(B) The DNA binding activity of a full-length mouse PEA3 protein was evaluated by EMSA. 5 μ l of the transcription-translation reaction performed without plasmid DNA (control reaction, lane 1) or 5 μ l of mPEA3 protein (lanes 2 and 3) were incubated with a radiolabelled T76 probe. A non specific DNA-protein complex (# n.s.) is observed both in lanes 1 and 2. The specific complex (#2) corresponds to the high mobility band that disappeared upon addition of an excess of cold probe (lane 3) but decreased only weakly upon addition of an excess of the mutated probe (lane 4).

• Figure 5

PEA3 acts in synergy with c-Jun or Sp1 to transactivate different regions of *MUC4* **promoter.** PEA3, Sp1, and c-Jun were transiently cotransfected alone or in combination in pancreatic tumor cells with fragments encoding the -461/-1 (A), -219/-1 (B) or the -3135/-2307 (C) regions of *MUC4* promoters. Results are expressed as fold induction relative to the empty expression vector. Values are the mean of 3 independent experiments where cotransfections were run in triplicate. \blacktriangle , p<0.05; **, p<0.001 versus PEA3 alone.

• Figure 6

(A) Effects of c-Jun, Sp1 or PEA3 alone or in combination on the expression of endogenous *MUC4*. Cells were cultured in 40 mm dishes at 40 % confluence before being transfected with 0.2 μ g of expression vectors encoding PEA3, Sp1 and/or c-Jun or the empty expression vector (C: control). Analysis of *MUC4* mRNA was assessed by RT-PCR as described in Materials and Methods. *28S* was used as an internal control.

(B) **Representation of the level of** *MUC4* **mRNA expressed by the cell lines.** Results are expressed as the *MUC4/28S* ratio after densitometry analysis of the bands. The ratio obtained with the empty expression vector was set at 1. The data shown are the mean \pm range of 2 independent experiments.

• Figure 7

Effect of PEA3 on MUC4 apomucin expression in CAPAN-1 and CAPAN-2 cells. Immunohistochemistry was performed as described in the Materials and methods section. CAPAN-1 cells transfected by 4 μ g of a control empty vector (A) or PEA3 expression vector (C). CAPAN-2 cells transfected by 4 μ g of a control empty vector (B) or PEA3 expression vector vector (D). Magnification x 200.

• Figure 8

Analysis of the expression of c-Jun and Sp1 in nuclear extracts of PANC-1 (lane 1), CAPAN-2 (lane 2) and CAPAN-1 (lane 3) cells by Western Blot. Nuclear proteins (20 μ g) were separated on 10% SDS-PAGE before being transferred onto nitrocellulose. The blot was stained using specific anti c-Jun or anti Sp1 antibody. Migration of the molecular weight (MW) standard is shown on the right hand side. The arrows point at (a) the monomeric c-Jun (around 40 kDa); (b) dimeric c-Jun; (c) Sp1 protein (\approx 105 kDa).

• Figure 9

- (A) Effects of increasing amounts of PEA3 expression vector on the promoter activity and the expression of human *ErbB-2* promoter. Expression vector (0.2 to 2 µg) was transiently cotransfected with deletion fragments of *ErbB-2* promoter in CAPAN-1, CAPAN-2 and PANC-1 cells. Results are expressed as in Figure 3A. ▲, p<0.05; *, p<0.01 and **, p<0.001 vs controls.</p>
- (B) Effects of increasing amounts of PEA3 expression vector on the expression of endogenous *ErbB-2*. Cells were cultured in 40 mm dishes at 40 % confluence before being transfected with 0.2 to 2 μg of expression vectors encoding PEA3 or with empty expression vector. Analysis of *ErbB-2* mRNA was assessed by RT-PCR as described in Materials and Methods. 28S was used as an internal control.
- (C) **Representation of the level of** *ErbB-2* **mRNA expressed.** Results are expressed as described in figure 7B and are the mean \pm range of the 2 independent experiments.
- Figure 10

Model for the roles of PEA3 both on the regulation of *MUC4* and *ErbB-2* expression in relationship with the proliferation-differentiation balance in pancreatic cancer cells. (+: stimulating effect, -: inhibiting effect, ?: not determined).



Figure 1



PROXIMAL PROMOTER



Figure 2

Copyright 2004 Biochemical Society







Figure 4

Copyright 2004 Biochemical Society



CAPAN-1



CAPAN-2

 C
 c-Jun
 Sp1
 C
 PEA3
 PEA3
 C
 PEA3
 PEA3
 C
 Sp1
 c-Jun

 + c-Jun
 + Sp1
 + c-Jun
 + c-Jun
 + c-Jun
 + c-Jun
 + c-Jun



A.







Figure 8





