Abstract. Com-1, candidate of metastasis-1, also known as p8, has been shown to regulate the growth and apoptosis of cancer cells and is associated with the disease progression in human cancers including prostate cancer. In the current study, we investigated a potential mechanism underlying the anti-cancer action of Com-1/p8 in human prostate cancer. Human prostate cancer cells were used. Full-length Com-1 cDNA was isolated from normal mammary tissues. Ribozyme transgenes that specifically target human Com-1 were constructed using the pEF6/V5-His vector. Com-1 interacting proteins were determined using immunoprecipitation method. Cell growth and invasiveness were investigated using in vitro methods. Using immunoprecipitation and Western blotting, Com-1 was found to be cross-reprecipitated with PGC-1, a coactivator of peroxisome proliferator activated receptor (PPAR)-α, but not PPAR-α itself. Elimination of Com-1 from prostate cancer cells resulted in a reduced response of the cells to ciglitizone, a PPAR-α agonist, whereas forced expression of Com-1 rendered cells more responsive to ciglitizone. We further demonstrated that the overexpression of Com-1/p8 resulted in changes in the expression of the PGC-1 responsive gene, fatty acid synthase (FAS). Com-1 may act as a tumour suppressor in human prostate cancer cells. The potential tumour suppressive effect of Com-1 is at least partly via its interaction with PGC-1, the PPAR-α coactivator.

Introduction

Com-1, candidate of metastasis-1, otherwise known as p8, which was discovered from metastatic breast tumours of nude rats, has been under active investigation in recent years.

Although initially thought to play a role in promoting metastasis and to have some degree of mitogenic activity (1,2), subsequent investigations have shown that the molecule appears to play a more diverse role in cancer, depending on the cell function and tumour type. In thyroid cancer (3), in fibroblasts (4), and in pancreatic cancer (5,6), Com-1 appears to be associated with the aggressiveness of the tumour cells. However, in other tumour types, Com-1 has a different expression pattern. In breast cancer cells, Com-1 is associated with 1,25-Dihydroxyvitamin D3 induced growth inhibition which coincides with a rise of Com-1 (7). In clinical breast cancer, low levels of Com-1 transcript are associated with a poor clinical outcome of the patients (8). In pancreatic cancer, the overexpression of Com-1/p8 by transfection resulted in growth inhibition (9), and p8-silenced cells by way of an antisense approach grew more rapidly than control cells transfected with the empty retrovirus. It has also been shown that inhibitors to MEK1/2 and JNK can upregulate p8 expression, and that in fibroblasts, p8 is a cell growth inhibitor that facilitates apoptosis induced by DNA damage (10) which may involve p53.

The p8 polypeptide is a phosphoprotein subject to constitutive degradation by the ubiquitin/proteasome system mediated by phosphatidylinositol 3-kinase and protein kinase B/Akt. By contrast, stabilization of the p8 protein requires glycogen synthase kinase-3 (11). García-Montero et al (12) have shown that TGF-β-1 can activate Com-1/p8 expression, which in turn enhances the Smas-transactivation in the TGF-β pathway. Com-1/p8 is primarily a nuclear protein and has been suggested to possibly be involved in the p27kip1 and p21cip1 pathway. We have recently shown that Com-1 interacts with ER-β and counteracts oestrogen-mediated effects on breast cancer cells (13).

Here we report the potential interaction between Com-1 and the peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1). The human transcriptional coactivator PPAR-γ coactivator-1α (PGC-1α), indentified from a human cDNA library in a steroid-responsive yeast strain (14,15), has been characterized as a tissue-specific coactivator that enhances the activity of many nuclear receptors, coordinates transcriptional programs important for energy metabolism and
acts as a broad regulator of cellular energy metabolism. The orphan nuclear receptor estrogen-related receptor α (ERR-α) was identified in a yeast two-hybrid screen of a cardiac cDNA library as a novel PGC-1α-binding protein. It has been reported that PGC1-α and ERR-α interact with each other and this interaction is distinct from that of other nuclear receptor PGC-1α partners, including PPAR-α, hepatocyte nuclear factor-4α and estrogen receptor α. Thus, ERR-α and ERR-γ are novel PGC-1α interacting proteins. It would also appear that PGC-1α selectively binds transcription factor partners in the regulation of energy metabolism (16). Few responsive genes are known to PGC-1, including Farnenoid X receptor (Fxr), nucleus receptors (Nrf1) and fatty acid synthase (Fas) (17,18).

Following our recent study which revealed a reduction of Com-1 in prostate tumours and that Com-1 may act as a tumour suppressor using in vitro and in vivo models with prostate cancer (19), we examine the potential role of the PPAR-γ and PGC-1 pathway in the action of Com-1 in prostate cancer cells and report that in prostate cancer cells interaction between Com-1/p8 and PGC-1 represents a novel mechanism for the action of Com-1.

Materials and methods

Materials. Human prostate cancer cell lines PC-3, DU-145, CA-HPV-10 and LN-Cap were obtained from ATCC (American Type Cell Culture, Rockville, MD). RNA extraction and RT kits were obtained from AbGene Ltd. (Surrey, UK). PCR primers were designed using Beacon Designer (Palo Alto, CA) and synthesised by Invitrogen Ltd. (Pasley, UK). Materials and methods

RNA extraction, cDNA synthesis and preparation of Com-1/p8 knockdown and human Com-1 overexpressing cells. RNA was extracted from cells and the concentration of RNA was determined using a UV spectrophotometer. Reverse transcription was performed using a RT kit with an anchored oligo-dt primer (AbGene), using 1 μg of total RNA. The quality of the cDNA was verified using β-actin primers.

The hammerhead ribozyme transgenes and Com-1 expression cassette were prepared as in previous studies (13,19,21). The following stably transfected cells from PC-3, DU145 and CA-HPV-10; Com-1 knockout cells, PC3Δcom1, DU145Δcom1 and CA-HPVΔcom1; Com-1 overexpressing cells, PC-3Δcom1Exp, DU145Δcom1Exp, and CA-HPVΔcom1Exp; and plasmid only control cells, PC3ΔΔ, DU145ΔΔ and CA-HPVΔΔ, were used together with the respective wild-types.

Immunoprecipitation and Western blotting. Cells were extracted using a lysis buffer, containing 2.4 mg/ml Tris, 4.4 mg/ml NaCl, 5 mg/ml sodium deoxycholate, 20 μg/ml sodium azide, 1.5% Triton, 100 μg/ml PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin, for 30 min. Protein concentrations were measured using fluorescamine and dye.
quantified by using a multi-fluoroscanner (Denly, Sussex, UK). The samples were then boiled at 100˚C for 5 min before clarification at 13,000 g for 10 min. Equal amounts of protein from each cell sample (controls and treated) were added to an 8% poly-acrylamide gel. For immunoprecipitation, prostate cancer cells were pelleted and lysed in the same buffer with 1 mM sodium orthovandate. Anti-Com-1/p8 (80 μg) or anti-PGC-1 antibody was added to equal amounts of protein for 1 h with constant agitation, followed by the addition of protein A/G agarose for an additional 1 h and washing with the same lysis buffer. The immunoprecipitate was solubilised with a sample buffer that contained 5% 2-mercaptoethanol. The samples were then separated on 10% SDS-PAGE or 12.5% Tris-Tricine PAGE (for Com-1 only). Protein blots were probed with a panel of antibodies (n=64) from a pool of antibodies in our laboratories, followed by washing and subsequent probing with appropriate peroxidase-conjugated secondary antibodies. Protein bands were visualised with a chemiluminescence (ECL) kit.

In *vitro* cell growth assay. This was modified from a previously described method (22). Cells were plated into a 96-well plate at 2,500 cells/well, with or without treatment with ciglitizone (0.1-100 μM final concentration). Cells were fixed in 10% formaldehyde at the day of plating and daily for 6 subsequent days after plating, and then stained with 0.5% (w/v) crystal violet. Following washing, the stained crystal was extracted with 10% (v/v) acetic acid and the absorbance was determined using a multplate reader. The growth of cells is shown in this study as percentage of the respective untreated cells at a given day.

**Results**

*Interaction of Com-1 with PGC-1, a PPAR-γ coactivator.* To identify the potential binding partners of Com-1, we first used immunoprecipitated proteins that interacted with Com-1, using the anti-Com-1 antibody for immunoprecipitation. The precipitates were subsequently separated on multiple gels and blots probed with a panel of 64 antibodies that recognise potential nuclear as well as other proteins. From the panel, PGC-1, but not PPAR-γ, was one of the few proteins that co-precipitated with Com-1 (Fig. 1). Using reverse precipitation, precipitation with anti-PGC-1 antibody and probing with anti-Com-1 antibody, the interaction between the two proteins was verified (Fig. 1).

*Modification of Com-1 expression results in differential growth response to PPAR-γ.* To test if Com-1 is involved in PPAR-γ/PGC-1 mediated cell growth inhibition in response to the PPAR-γ agonist, we first generated a panel of genetically modified (GM) prostate cancer cell lines, whose expression of Com-1 was either knocked down by ribozymes or increased by forced expression (Fig. 2) (19). The effects of ciglitizone on the growth of these cells were evaluated. Ciglitizone inhibited cell growth over a range of concentrations (Fig. 3, open circles). However, Com-1 knockout prostate cancer cells had reduced sensitivity,
although not completely, to the growth inhibition of ciglitizone (Fig. 3, solid circles). Fig. 3 shows the cell response on day 5. Data for all the respective cells including wild-type, control plasmid-transfected, ribozyme-transgene transfected and expression vector-transfected cells are summarised in Table II.

We further treated the aforementioned GM cells with ciglitizone, an agonist to PPAR-γ. As shown in Fig. 4 (top panel), ciglitizone increased the Com-1 protein levels in both wild-type PC-3 and PC3pEFS as shown in Western blotting. However, the agonist had no marked effect on PGC-1 expression.

We further analysed the cellular and nuclear staining of Com-1, PGC-1 and PPAR-γ using immunocytochemistry (ICC) and Western blotting (WB). As shown in Fig. 4, treatment of wild-type PC-3 cells seems to only marginally increase the amount of Com-1 (Fig. 4: top, WB; bottom panel, ICC). This marginal increase appears to be in the nuclear fraction.

**Modification of Com-1 expression and results in changes of PGC-1 responsive genes.** To assess if the manipulation of Com-1 has an impact on the responsive genes of PGC-1, we evaluated the expression of FAS, FxR and NrF1, which are known PGC-1 responsive genes. We evaluated these gene products in Com-1 modified cells, PC3Δcom1, DU145Δcom1, CA-HPVΔcom1, Com-1 overexpressing cells, PC-3com1Exp, DU145com1Exp, and CA-HPVcom1Exp, plasmid only control cells, PC3pEFS, DU145pEFS and CA-HPVpEFS, used together with the respective wild-types. The overexpression of Com-1 rendered cells to express lower levels of FAS, but not FxR and NrF1, compared with wild-type and transfection control cells (Fig. 5).

**Discussion**

**Com-1 interacts with PGC-1, but not PPAR-γ.** We conducted a co-precipitation test using a panel of antibodies, and found that the peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1, PGC-1, is one of the few proteins that co-precipitated with Com-1. The human transcriptional coactivator PPAR-γ coactivator-1α (PGC-1α), identified from a human cDNA library in a steroid-responsive yeast strain (16,17), has been characterized as a tissue-specific coactivator that enhances the activity of many nuclear

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**Table II. Com-1 expression and ciglitizone-regulated cell growth (72 h).**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Variant</th>
<th>1 μM</th>
<th>5 μM</th>
<th>20 μM</th>
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<tr>
<td>PC-3</td>
<td>Wild-type</td>
<td>93.3</td>
<td>93.3</td>
<td>56.4</td>
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<tr>
<td></td>
<td>pEFa control</td>
<td>91.7</td>
<td>89.9</td>
<td>60.2</td>
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<td></td>
<td>Com1Rib</td>
<td>138.2</td>
<td>101.6</td>
<td>63.9</td>
</tr>
<tr>
<td></td>
<td>Com1Exp</td>
<td>108.8</td>
<td>80.7</td>
<td>40.0</td>
</tr>
<tr>
<td>DU-145</td>
<td>Wild-type</td>
<td>92.0</td>
<td>96.9</td>
<td>85.9</td>
</tr>
<tr>
<td></td>
<td>pEFa control</td>
<td>94.1</td>
<td>98.4</td>
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<tr>
<td></td>
<td>Com1Rib</td>
<td>107.0</td>
<td>97.9</td>
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<tr>
<td></td>
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<td>92.7</td>
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<tr>
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<td>Wild-type</td>
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<td>105.7</td>
<td>72.79</td>
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<td></td>
<td>pEFa control</td>
<td>108.3</td>
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</table>
receptors, coordinates transcriptional programs important for energy metabolism and is a broad regulator of cellular energy metabolism. PGC-1 promotes transcription through the assembly of a complex that includes the histone acetyltransferases steroid receptor coactivator-1 (SRC-1) and CREB binding protein (CBP)/p300 (23). PGC-1 acts to coordinate the process of metabolic adaptation in the liver, by interacting with HNF4α and cAMP-response element-binding protein (CREB) (24,25). PGC-1 is a coactivator of MEF2C, a muscle-selective transcription factor, and can control the level of endogenous GLUT4 gene expression in muscle (26). Few PGC-1 responsive genes are known. Farnecoid X receptor (FxR), nuclear receptor family group H members and fatty acid synthase are amongst the known genes (17,18). The over-expression of PGC-1 can decrease the expression of fatty acid synthase. The current study has shown that the overexpression of Com-1 in prostate cancer cells also results in the reduction of fatty acid synthase expression. However, manipulating Com-1 expression has not affected the expression of NrF1 and FxR. Taken together, Com-1 may operate with PGC-1 in regulating PGC-1 responsive genes, including fatty acid synthase.

The impact of Com-1 and PGC-1 requires further investigation. However, PGC-1 is involved in the transcriptional regulation of the UCPI gene in humans and mediates the effects of PPAR-α and PPAR-γ agonists and retinoic acid (27) and is a coactivator for RXRα (28). PGC-1 has also been implicated in the cachectic response and oxidative metabolism in skeletal muscle (28-31). These collectively suggest an important role for Com-1 in the metabolic response in malignancies.

A potential role for Com-1 in the PGC-1/ER connection. PGC-1 is a bona fide ERα coactivator, which may serve as a convergence point between PPAR-γ and ERα signalling (32). A PGC-1 related coactivator, PERC (PGC-1 related estrogen receptor coactivator), is known to interact with the AF2 domain of ERα in an estrogen-dependent manner. PERC and PGC-1 show distinct preferences for enhancing ERα in different promoter contexts. PERC enhances the ERα-mediated response to the partial agonist tamoxifen, while PGC-1 modestly represses it. The two coactivators are likely to mediate distinct, tissue-specific responses to estrogens (33). Akt can mediate the downregulation of peroxisome proliferator-activated receptor (PPAR)-α coactivator-1 (PGC-1) and PPAR-γ (34). Recently, we reported that Com-1 forms a complex with ER-β in human breast cancer cells (8,13). In breast cancer cells, the loss of Com-1 resulted in cells responding more vigorously to oestrogen with a rapid increase in cell growth. Vice versa, the overexpression of Com-1 in breast cancer cells resulted in the cells being less responsive to oestrogen and less invasive. We suggested that Com-1, by forming a complex with ER-β and PGC-1, acts as a tumour suppressor in human solid tumours including prostate and breast cancers (8).

The role of Com-1 in prostate cancer cells. We and others have reported that PGC-1 expression, together with PPAR-γ, is reduced in human cancer (31,35). Furthermore, ER-β, which plays an opposite role to ER-α in cancer cells, has also been reported to be reduced in certain types of cancer such as breast cancer.
Peroxisome proliferator-activated receptor-γ (PPAR-γ) belongs to a nuclear hormone receptor super-family that regulates gene expression. PPAR-γ contains DNA, lipid and A/B binding domains that recognise DNA response elements in the promoters of their target genes and interact with lipid ligands and prostaglandins (PGI2). Chang et al has shown that ligands of PPAR-γ such as troglitazone, BRL49653, 15-hydroxyicosatetraenoic acid and 15-deoxye2ta12,14-prostaglandin J2 are capable of inducing apoptosis of cancer cells and inhibiting tumourigenesis (36,37). It has also been demonstrated that PPAR-γ forms a complex with its coactivators (PGC-1 and -2) and in association with other transcription factors such as SRC-1 and CREB binding proteins (38-40) regulates the expression of a number of target genes including the cMet protooncogene, E-cadherin and the cyclin regulator p27 Kip1. Studies by Hisitake (41) demonstrated that PPAR-γ agonists are capable of reducing the growth and progressive activity of prostate cancer cells as well as inhibiting the transcription of genes such as cMet and HGF both of which are associated with tumour progression.

Anomalies in PPAR group members have been shown to cause tumourigenesis in both human cancers and animal models. Previous studies have shown (42) that poly-unsaturated fatty acids activate PPAR-γ levels in cancer cells. PGC-1, as a key modulator of hepatic gluconeogenesis and as a central target of the insulin-cAMP axis in liver, regulates mitochondrial biogenesis and function (15,43). The transcriptional activity of PPARγ may not only be decreased by mutation but also by downregulation of the coactivator PGC-1 of PPAR-γ (44). Recently, we have also reported, for the first time, that the mRNA level of PGC-1 is also reduced in human breast cancer and the reduction is associated with an aggressive nature of the breast tumours (35). Taken together, it is suggested that in human tumours including prostate cancer, the loss and/or reduction of PGC-1 and Com-1 in cancer cells, particularly in the nuclear fraction, may act both on the control of transcription of tumour suppressor genes and cell growth. This would allow cancer cells to gain rapid growth and aggressiveness. A few other theories have been recently reported with regards to the mechanism of Com-1-regulated cell growth and death. Com-1/p8 has been shown to be one of the key modulators in the antitumour (pro-apoptotic) effects of the cannabinoids, and p8 mediates its apoptotic effect via upregulation of the endoplasmic reticulum stress-related genes ATF-4, CHOP, and TRB3 (45).

Furthermore, the interaction between Com-1/p8 and prothymosin α (ProTα) has been reported to be key to staurosporine-induced apoptotic response in HeLa cells (6). The interplay between Com-1, PGC-1 and ProTα warrants further investigation.

In conclusion, the current study has shown that Com-1/p8 is a potential tumour suppressor in human prostate cancer. One of the potential mechanisms underlying the action of Com-1 is its interaction with PGC-1. Levels of Com-1 influence cellular responses to PPAR-γ agonists. Com-1 may have important therapeutic implications as well as prognostic implications.

Acknowledgements

The authors thank Cancer Research Wales for supporting their work and Dr Gregory Harrison for his assistance in immunoprecipitation experiments.

References


