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Insulin-like growth factor 1 activates methionine adenosyltransferase 2A transcription by multiple pathways in human colon cancer cells

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Abstract

We have previously reported that the expression of *MAT2A* (methionine adenosyltransferase 2A) is increased in human colon cancer and in colon cancer cells treated with IGF-1 (insulin-like growth factor-1), which was required for its mitogenic effect. The aim of the present study was to elucidate the molecular mechanisms of IGF-1-mediated *MAT2A* induction. Nuclear run-on analysis confirmed that the increase in *MAT2A* expression lies at the transcriptional level. DNase I footprinting of the *MAT2A* promoter region revealed a similar protein-binding pattern in colon cancer and IGF-1-treated RKO cells. IGF-1 induced *MAT2A* promoter activity and increased nuclear protein binding to USF (upstream stimulatory factor)/c-Myb, YY1 (Yin and Yang 1), E2F, AP-1 (activator protein 1) and NF- κ B (nuclear factor κ B) consensus elements. IGF-1 increased the expression of c-Jun, FosB, MafG, p65, c-Myb, E2F-1 and YY1 at the pre-translational level. Knockdown of p65, MafG, c-Myb or E2F-1 lowered basal *MAT2A* expression and blunted the inductive effect of IGF-1 on *MAT2A*, whereas knockdown of YY1 increased basal *MAT2A* expression and had no effect on IGF-1-mediated *MAT2A* induction. Consistently, mutation of AP-1, NF- κ B, E2F and USF/c-Myb elements individually blunted the IGF-1-mediated increase in *MAT2A* promoter activity, and combined mutations completely prevented the increase. In conclusion, IGF-1 activates *MAT2A* transcription by both known and novel pathways. YY1 represses *MAT2A* expression.

Keywords

c-Myb; colon cancer; E2F; insulin-like growth factor-1 (IGF-1); methionine adenosyltransferase 2A; Yin and Yang 1 (YY1)

INTRODUCTION

Colorectal cancer is the second most common fatal malignancy in the Western world, with approximately 150 000 new cases and accounting for 57 000 deaths in the United States each year [1]. Currently, four signalling pathways are recognized in colorectal

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AUTHOR CONTRIBUTION

Shelly Lu and Heping Yang designed the experiment. Heping Yang, Tony Li and Jian Peng performed the experiments. Heping Yang and Shelly Lu analysed the results. Shelly Lu, Heping Yang and José Mato prepared the manuscript.

carcinogenesis: (i) Wnt, (ii) K-ras, (iii) TGF β (transforming growth factor β), and (iv) p53 [2]. Mutations of key players in these pathways lead to inactivation of tumour suppressor function or activation of proto-oncogenes that favour growth. In addition, increased signalling through IGF-1R [IGF-1 (insulin-like growth factor-1) receptor] and EGFR [EGF (epidermal growth factor) receptor] has been shown to be important in colon cancer growth and metastasis [3–6]. Both higher levels of IGF-1 and IGF-1R have been shown in patients with colon cancer [4,6], and IGF-1R signalling plays an important role in tumour growth, angiogenesis and metastasis [5].

We have recently reported that growth factors such as IGF-1, leptin and EGF transcriptionally activated *MAT2A* [MAT (methionine adenosyltransferase) 2A] in human colon cancer cells [7]. *MAT2A* encodes the catalytic subunit of the MATII isoenzyme that is expressed in all non-hepatic tissues and is responsible for the biosynthesis of the principal biological methyl donor *S*-adenosylmethionine [8]. We found that *MAT2A* expression is induced in human colon cancer and when colon cancer cells were treated with mitogens [7]. Importantly, blocking the induction in *MAT2A* expression prevented the ability of the growth factors to exert their mitogenic effect [7]. Thus *MAT2A* appears to be a key downstream effector of these mitogens in colon cancer cells. The aim of the present study was to elucidate the molecular mechanisms of how IGF-1 induces *MAT2A* at the transcriptional level. We found that several known IGF-1-activated signalling pathways, as well as pathways not previously reported to be activated by IGF-1, all participate in the transcriptional up-regulation of *MAT2A*. In addition, the human *MAT2A* promoter contains a functional YY1 (Yin and Yang 1) *cis*-acting element, and YY1 represses *MAT2A* expression.

EXPERIMENTAL

Materials

Cell culture medium and FBS (fetal bovine serum) were obtained from Gibco BRL Life Technologies. Primers were purchased from the Molecular Genomics Core of USC Norris Comprehensive Cancer Center. The luciferase assay system was obtained from Promega. Recombinant human IGF-1 was obtained from Sigma. All restriction endonucleases were obtained from New England Biolabs. [³²P]dCTP (3000 Ci/mmol) was purchased from New England Nuclear. All other reagents were of analytical grade and were obtained from commercial sources.

Source of normal and cancerous colon tissue

Colon cancer and adjacent non-cancerous tissues were obtained from the Norris Cancer Center Tissue Repository based on availability of both normal and cancerous colon specimens from the same patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Keck School of Medicine University of Southern California's human research review committee.

Cell culture and IGF-1 treatment

RKO (a poorly differentiated human colon carcinoma cell line) and HT-29 (a human colon adenocarcinoma cell line) cells were obtained from the Cell Culture Core of the USC Liver Disease Research Center and grown according to instructions provided by the A.T.C.C. Prior to treatment with IGF-1, medium was changed to 0.1 % FBS overnight. Medium was then changed to without serum, and cells were treated with IGF-1 (10–100 ng/ml) or vehicle control from 15 min to 6 h for various assays described below.

Nuclear run-on transcription assay

Isolation of nuclei from colon tissues and nuclear run-on transcription assay were performed as described previously [9]. Identical amounts of labelled nuclear RNAs (1×10^8 c.p.m.) were hybridized overnight with *MAT2A* or β -actin cDNAs ($2 \mu\text{g}$ each) with a slot-blot apparatus (Bio-Dot[®] SF, Bio-Rad) as described previously [9]. After hybridization, filters were washed and subjected to autoradiography and densitometry as described above. Results of nuclear run-on assay were normalized to β -actin.

RNA isolation and gene expression analysis

Total RNA was isolated using TRIzol reagent (Invitrogen). Northern blot analysis, autoradiography and densitometry for *MAT2A*, c-Jun, p65, FosB, MafG, E2F-1, USF-1 (upstream stimulatory factor-1), YY1, c-Myb and β -actin were performed as described previously [10]. The human-specific complementary cDNA probes for Northern blot were: *MAT2A* [nucleotides 181–574 (GenBank[®] accession number NM_005911)], c-Jun (*JUN*) [nucleotides 1104–1601 (GenBank[®] accession number NM_002228)], p65 (*RELA*) [nucleotides 483–1308 (GenBank[®] accession number NM_021975)], FosB (*FOSB*) [nucleotides 723–1208 (GenBank[®] accession number NM_006732)], MafG (*MAFG*) [nucleotides 245–295 (GenBank[®] accession number NM_002359)], E2F-1 (*E2F1*) [nucleotides 491–960 (GenBank[®] accession number NM_005225)]; USF-1 (*USF1*) [nucleotides 241–966 (GenBank[®] accession number NM_007122)], YY1 [nucleotides 482–1320 (GenBank[®] accession number NM_003403)] and c-Myb (*CMYB*) [nucleotides 251–723 (GenBank[®] accession number NM_005375)]. Specific probes were labelled with [³²P]dCTP using a random-primed DNA labelling kit [10]. Results of Northern blot analysis were normalized to β -actin.

DNase I footprinting analysis

For DNase I footprinting analysis, two ³²P end-labelled fragments of the 5'-flanking region of the human *MAT2A* gene were generated by digestion with restriction endonucleases. DNase I footprinting analysis was performed using double-stranded fragments corresponding to nucleotides –571 to –165 and –164 to +60 of the human *MAT2A* gene, as described previously [10,11]. Singly end-labelled fragments were generated by filling 5'-protruding ends with [α -³²P]dCTP (3000 Ci/mmol) using the exo-Klenow enzyme. Labelled probes were purified by electrophoresis using a 2 % agarose gel. Approximately 5×10^4 c.p.m. of end-labelled DNA fragments were incubated with 0–30 μg of nuclear protein from normal human colon, colon cancer or RKO cells treated with or without IGF-1, EGF or leptin (all 100 ng/ml for 3 h). After 30 min incubation on ice, CaCl₂ and MgCl₂ were added to give a final concentration of 0.5 mM and 1 mM respectively. DNase I digestions were performed at 20 °C for 1 min. Upon phenol extraction and ethanol precipitation, DNA fragments were resolved by electrophoresis in a denaturing 8 % acrylamide sequencing gel.

MAT2A promoter constructs and transient transfection assays

The human *MAT2A* promoter constructs have been described previously [9,10], and were subcloned in the sense orientation upstream of the luciferase coding sequence of the pGL-3 basic vector (Promega). In addition, wild type *cis*-acting elements and corresponding mutants based on the human *MAT2A* promoter sequence was created using the GeneTailor site-directed mutagenesis system from Invitrogen. The primers used in creating the mutations to the *cis*-acting elements (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/436/bj4360507add.htm>) for YY1, USF/c-Myb (has binding for both), NF- κ B (nuclear factor κ B)/c-Myb (has binding for both), AP-1 (activator protein 1) and E2F are: YY1 forward, 5'-GCCGTGAGGAGCCCTCGCTTGACGGCCAAGATG-3' and reverse, 5'-AGCGAGGGCTCCTCACGGCCTTCCTTCGG-3'; USF/c-Myb forward, 5'-

CACCTTCACATGGCCGACCACTACCGGCTCCCT-3' and reverse, 5'-GGTCGGCCATGTGAAGGTGGCCATCTTGGC-3'; NF- κ B/c-Myb forward, 5'-GAAAGCTATCCCGGCCAAAGTTTCGGAAGGGAGG-3' and reverse, 5'-TTGGCCGGGATAGCTTTCCCGGAGGTCGC-3'; AP-1 forward, 5'-GTCCGAAGGGAGGTGCCATGGCCCCGCACAGGG-3' and reverse, 5'-CATGGCACCTCCCTTCCGACCGTTGGCCG-3'; and E2F forward, 5'-CCCCGCGCAAGTGGTGCATTGACTCGCGGCGCCGA-3' and reverse, 5'-CATGGCACCTCCCTTCCGACCGTTGGCCG-3'. The mutated residues are shown underlined.

The -571/+60 *MAT2A* promoter construct was used as the template for creating the various site-directed mutations. Each mutagenesis within the -571/+60 *MAT2A* promoter construct was confirmed by DNA sequencing and shown in Supplementary Figure S1. The -571/+60 *MAT2A* promoter constructs containing mutations to a single *cis*-acting element include YY1, USF/c-Myb, NF- κ B/c-Myb, AP-1 and E2F. The -571/+60 *MAT2A* promoter constructs containing mutations to multiple *cis*-acting elements include: (i) NF- κ B/c-Myb + AP-1, (ii) NF- κ B/c-Myb + AP-1 + USF/c-Myb, (iii) NF- κ B/c-Myb + AP-1 + USF/c-Myb + YY1, and (iv) NF- κ B/c-Myb + AP-1 + USF/c-Myb + YY1 + E2F.

Transient transfection analysis of *MAT2A* promoter activity and activities of the *cis*-acting elements were performed as described previously [10]. The effect of IGF-1 was examined by measuring luciferase activity driven by these *MAT2A* promoter constructs (wild-type or mutated) in transfected RKO and HT-29 cells treated with IGF-1 (100 ng/ml) for 3 h. Each experiment was performed in triplicate and results are reported as the fold change over the vector control.

EMSA (electrophoretic mobility-shift assay) and supershift assay

EMSAs for the putative binding sites were performed as described previously [10,12]. Nuclear protein (10 or 15 μ g) from RKO cells treated with or without IGF-1 (10–100 ng/ml, from 15 min to 6 h) were pre-incubated with 2 μ g of poly(dI-dC) in a buffer containing 10 mM Hepes (pH 7.6), 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM MgCl₂ and 10 % glycerol for 10 min on ice. ³²P-End-labelled double-stranded DNA fragments corresponding to different *cis*-acting elements (see Supplementary Figure S1) were then added with or without a 50–100-fold excess of unlabelled specific probe as a competitor. Mixtures were incubated for 20 min on ice, loaded on to a 4 % non-denaturing polyacrylamide gel and subjected to electrophoresis in 50 mM Tris/HCl, 45 mM borate and 0.5 mM EDTA (pH 8.0). Further confirmation of the identity of the binding proteins was done by antibody supershift assays for JunB, c-Fos, c-Jun, FosB, MafG, c-Myb and USF-1 (Cell Signaling, Novus and Abcam). A 1.5 μ l amount of these antibodies was added to respective samples after 20 min of incubation with the labelled probe, and all samples were further incubated for a further 45 min on ice before electrophoresis. Gels were dried and subjected to autoradiography.

ChIP (chromatin immunoprecipitation) assay

To verify changes in protein binding to the human *MAT2A* promoter following IGF-1 treatment in an endogenous chromatin configuration, a ChIP assay was carried out following the ChIP assay kit protocol provided by Millipore. RKO cells were treated with IGF-1 (100 ng/ml for 3 h) or vehicle control, and processed for the ChIP assay as described previously [13]. Antibodies used for immunoprecipitation were anti-E2F-1, anti-YY1, anti-MafG, anti-c-Jun, anti-USF-1, anti-p65, anti-FosB and anti-c-Myb antibodies (Cell Signaling, Novus and Abcam). PCRs of the human *MAT2A* promoter region from -539 to -280 used the forward primer 5'-GCCGAAGGAAGCCGTGAG-3' and the reverse primer 5'-

AAGCGACTGGGGCTTGCTCCAC-3', and the region from -270 to +54 used the forward primer 5'-ATCAAACAAGGAAGAGCAATCCCCC-3' and the reverse primer 5'-GACAGCGTTCTACTCGTAGCAGGC-3'. All PCR products were run on 8 % acrylamide gels and stained with ethidium bromide for 15–30 min.

Western blot analysis

Western blot analysis was done on nuclear or cytosolic proteins for levels of c-Jun, p65, FosB, MafG, USF-1, E2F-1, c-Myb and YY1 as described previously [12]. Sources of antibodies for transcription factors were the same as for the supershift assays.

siRNA (small interfering RNA) treatments

Double-stranded siRNA against p65 (sc-41228), E2F-1 (sc-29297), c-Myb (sc-29855), USF-1 (sc-36783), YY1 (sc-36863), MafG (sc-38099), c-Jun (sc-29223), and negative scrambled control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology. RKO cells were transfected with these siRNAs or scrambled siRNA (10 nM per 1×10^5 cells) using LipofectamineTM RNAiMAX Transfection Reagent (Invitrogen) in six-well plates at 30 % confluency for 24 h, followed by IGF-1 (100 ng/ml) or vehicle control treatment for 3 h, and Northern blot analysis was performed as described above for *MAT2A*.

Statistical analysis

Results are given as means $\pm$ S.E.M. Statistical analysis was performed using Student's *t* test for comparison of paired samples and ANOVA followed by Fisher's test for multiple comparisons. Significance was defined by $P < 0.05$.

RESULTS

MAT2A is transcriptionally activated in human colon cancer

Using the nuclear run-on assay, Figure 1(A) shows that the *MAT2A* gene is transcriptionally activated in human colon cancer (CA) as compared with normal (NL) colon tissues from the same patients (CA is 188 ± 12 % of NL, $P < 0.05$). To see whether increased transcription can be due to differential protein binding to the *MAT2A* promoter region, we compared the protein-binding pattern using the DNase I footprinting assay. Figure 1(B) shows multiple DNase I-protected regions in the colon cancer specimen only that contains PEA3 (polyoma enhancer activator 3), YY1, USF, c-Myb, NF- κ B, AP1 and E2F consensus elements (see Supplementary Figure S1). Interestingly, this footprinting pattern in colon cancer differs from HCC (hepatocellular carcinoma) [12], which suggests that different molecular mechanism(s) are likely to be involved in increased *MAT2A* expression in these two cancers.

We previously reported that IGF-1, leptin and EGF all induced the promoter activity of *MAT2A* [7]. The DNase I footprinting assay revealed that this also involves increased binding of transcription factors to the *MAT2A* promoter region (Figure 2). The pattern of protein binding after IGF-1 and EGF treatments resembled that from the human colon cancer specimen. For the rest of the present study we chose IGF-1 as a representative mitogen and examined how it increases *MAT2A* transcriptional activity.

Transcription factors and *cis*-acting elements involved in IGF-1-mediated *MAT2A* induction

To see which part of the promoter contained important elements responsible for IGF-1-mediated induction of *MAT2A*, we used serial deletion constructs of the *MAT2A* promoter. Figure 3 shows that IGF-1 treatment of RKO cells for 3 h increased the promoter activity driven by *MAT2A* -270/+60-LUC and -571/+60-LUC (but not -47/+60-LUC) in a dose-dependent manner, with a maximum effect at an IGF-1 concentration of 50 ng/ml. Higher

activity was observed with the longer construct, suggesting that important enhancer element(s) lies in the region between -571 and -270. Enhancer element(s) are also expected between -270 and -47.

We next used EMSAs and supershift assays to determine whether IGF-1 treatment increased protein binding to consensus elements present in the regions implicated from the results of transient transfection analysis. Figures 4 and 5 show the effect of IGF-1 treatment on protein binding to consensus elements PEA3, YY1, USF/c-Myb, NF- κ B/c-Myb, AP-1 and E2F (see Supplementary Figure S1). IGF-1 treatment increased binding to all of these elements, except for PEA3 (Figure 5, lower left-hand gel). A maximum effect was observed at 3 h and at a dose of 100 ng/ml (Figures 4A and 4B). Following IGF-1 treatment, dominant proteins bound to the AP-1 site at -332 were c-Jun, FosB and MafG (Figure 4C). Dominant protein bound to the USF/c-Myb site at -496 was c-Myb (Figure 5, upper right-hand gel).

We used the ChIP assay to verify that there was increased binding of these putative transcription factors to the *MAT2A* promoter following IGF-1 treatment in the native chromatin structure. Figure 6 shows that following IGF-1 treatment, there is increased E2F-1, YY1, MafG, c-Jun, p65, FosB and c-Myb binding to the region between -539 and -281, and E2F-1 binding to the region between -270 and +54. There is no change in USF-1 binding following IGF-1 treatment (Figure 6A).

Effect of IGF-1 on the expression of transcription factors

To understand how IGF-1 treatment leads to an increase in binding of the transcription factors described above to the *MAT2A* promoter region, we examined whether the expression of these transcription factors is altered by IGF-1 treatment. Figure 7 shows that IGF-1 treatment resulted in a time-dependent increase in the expression of c-Jun (*JUN*), p65 (*REL*A), FosB (*FOSB*), c-Myb (*MYB*), E2F-1 (*E2F1*), USF-1 (*USF1*), YY1 and MafG (*MAFG*). The mechanism lies at the pre-translational level as the mRNA levels (Figure 7A) increased to a comparable degree as the protein levels (Figure 7B).

Role of p65, MafG, c-Myb, E2F-1, USF-1, and YY1 in basal *MAT2A* expression and IGF-1-mediated induction

To confirm an important role of the transcription factors identified above in IGF-1-mediated *MAT2A* induction, we employed siRNA to acutely reduce the expression of each transcription factor in RKO cells before treatment with IGF-1. Knockdown efficiency of these siRNAs was comparable and resulted in a significant reduction of their expression after 24 h (see Supplementary Figure S2 at <http://www.BiochemJ.org/bj/436/bj4360507add.htm>). Knockdown of p65, MafG, c-Myb and E2F-1 resulted in lower baseline *MAT2A* mRNA levels, and significantly reduced the inductive effect of IGF-1 on *MAT2A* (Figure 8). Although USF-1 knockdown did not affect the baseline *MAT2A* mRNA level, it blunted the inductive effect of IGF-1 on *MAT2A* (Figure 8D). In contrast, knockdown of YY1 resulted in a higher *MAT2A* expression at baseline, and little to no effect on IGF-1-mediated *MAT2A* induction (Figure 8D).

Role of *cis*-acting elements in IGF-1-mediated induction of *MAT2A* promoter activity

To prove that the *cis*-acting elements identified from EMSAs are functional, we examined the effect of mutating these sites on baseline and the IGF-1-mediated increase in *MAT2A* promoter activity in two different human colon cancer cell lines. In RKO cells, mutating the NF- κ B/c-Myb, AP-1, USF/c-Myb and E2F sites individually all resulted in reduced baseline *MAT2A* promoter activity and a blunted response to IGF-1 treatment (Figure 9A). In contrast, mutating the YY1 site resulted in a higher baseline *MAT2A* promoter activity and a heightened IGF-1 response. Combining mutations in NF- κ B/c-Myb, AP-1, USF/c-Myb and

E2F sites resulted in the lowest baseline activity and IGF-1 no longer had an inductive effect (Figure 9A). Very similar results were obtained using HT-29 cells, with combined mutations in NF- κ B/c-Myb, AP-1, USF/c-Myb and E2F sites having the lowest basal activity and IGF-1 was no longer inductive, whereas a YY1 mutation resulted in the highest basal promoter activity and IGF-1 induced this further (Figure 9B).

DISCUSSION

MAT is an essential enzyme as it is responsible for the biosynthesis of S-adenosylmethionine, the principal methyl donor for all *trans*-methylation reactions and precursor of polyamines that are required for growth [8]. Two genes encode the MAT catalytic subunit, *MAT1A* and *MAT2A* [8]. While *MAT1A* is largely expressed in normal differentiated liver, *MAT2A* is widely expressed in all non-hepatic tissues [8]. Increased expression of *MAT2A* was found in liver cancer [14] and, more recently, colon cancer [7] (Figure 1A). Increased expression of *MAT2A* is essential for mitogen-induced growth in both liver [15] and colon cancer cells [7]. The mechanisms for *MAT2A* up-regulation in liver cancer have been previously reported [9,12]. Increased c-Myb and Sp1 (specificity protein 1) expression, and binding to the *MAT2A* promoter occurred in human liver cancer and contributed to the *MAT2A* up-regulation [12]. The molecular mechanism(s) responsible for *MAT2A* up-regulation in colon cancer is unknown and is the subject of the present study.

In human colon cancer, the mechanism for *MAT2A* up-regulation lies at the transcriptional level and is, at least in part, due to increased binding of transcription factors to enhancer elements. Since IGF-1 treatment of RKO cells resulted in similar DNase I-protected regions as compared with colon cancer, we employed IGF-1 treatment in RKO cells as a convenient model to dissect the molecular mechanisms responsible. This is reasonable as the IGF-1 signalling pathway has been demonstrated to play an important role in colon cancer by activating pro-survival, growth and proliferation mechanisms [3–6,16]. IGF-1R and IGF-1 are commonly expressed at high levels in colon cancer cells to help promote tumour progression, angiogenesis and metastasis [2,16]. Given that the mitogenic effect of IGF-1 in colon cancer cells requires *MAT2A* induction [7], it is important to elucidate how IGF-1 induces *MAT2A* expression.

DNase I footprinting, transient transfection analysis and EMSAs identified increased binding, following IGF-1 treatment, to putative binding sites for YY1, USF, c-Myb, NF- κ B, AP-1 and E2F (Figures 2, 4 and 5, and Supplementary Figure S1). The PEA3 EMSA probe saw a significant amount of protein binding at baseline, but IGF-1 was unable to induce more binding (Figure 5). The strong baseline binding suggests that this region may be important for basal promoter activity. Supershift analysis of the AP-1 probe identified the increased binding of c-Jun, FosB and MafG, but not JunB or c-Fos after IGF-1 treatment (Figure 4C). MafG is a member of the AP-1 family of transcription factors that can homodimerize or heterodimerize with Nrf (nuclear factor-erythroid 2-related factor) 1, Nrf2, Nrf3, FosB and c-Fos, but not c-Jun, to bind to DNA [17,18]. ChIP analysis of the *MAT2A* promoter region encompassing all of the putative *cis*-acting elements confirmed the increase in binding of the transcription factors by IGF-1 treatment (Figure 6). Although the E2F consensus element is not present in the proximal region of the *MAT2A* promoter, there is increased E2F-1 binding detected on ChIP (Figure 6A). This may be due to known interactions of E2F-1 with YY1 [19] and p65 [20].

Some of the findings of the present study are expected, as IGF-1 has been shown to induce the activation of the PI3K (phosphoinositide 3-kinase) and MAPK (mitogen-activated protein kinase) signalling pathways in cancer cells [16]. AP1, NF- κ B, YY1 and E2F-1 nuclear-binding activities are known to be induced directly or indirectly by IGF-1 signalling

[21–24]. However, to the best of our knowledge, IGF-1 has not been reported to influence the nuclear-binding activity of USF-1 or c-Myb.

Increased transcription-factor-binding activity can either be the result of increase transcription factor expression or post-translational modifications to enhance its DNA-binding affinity. Previous reports have shown that IGF-1 signalling can directly induce the expression of E2F-1 and YY1 at the transcriptional level and activate NF- κ B post-translationally by phosphorylation [21,23,24]. We found an increase in both mRNA and protein levels of c-Jun, FosB, MafG, p65, USF-1, E2F-1 and YY1 after IGF-1 treatment (Figure 7). This suggests that, in colon cancer cells, IGF-1 induces enhanced binding to the putative *cis*-acting elements, at least in part, by increasing the expression of the respective transcripts. E2F-1 (*E2F1*) and YY1 induction by IGF-1 at the mRNA level agrees with previous reports [23,24]. However, IGF-1 induction of NF- κ B (*NFKB*), FosB (*FOSB*) and c-Jun (*JUN*) expression is contrary to previous published reports that showed IGF-1 inducing the activity of these factors via a post-transcriptional mechanism as opposed to its expression. For MafG (*MAFG*), this is the first time IGF-1 has been shown to induce its expression. Maf proteins can be divided into two groups, large and small Mafs, where both have a similar structure, except that the small Mafs lack a transactivation domain; MafG is a small Maf [18]. Large Mafs have been implicated in oncogenesis, whereas the small Mafs as homodimers have been shown to antagonize large Maf activity by competing for DNA binding [17,18]. However, MafG can act as either an activator or repressor, depending on its dimerization partner [18]. For c-Myb (*CMYB*), this is also the first time IGF-1 was shown to induce its expression at the mRNA level in any cell type.

We next established the functional importance of these transcription factors in regulating expression of *MAT2A* at baseline and in response to IGF-1 treatment. Using siRNA to acutely reduce the expression of these transcription factors, we found four transcription factors to be essential for basal expression in colon cancer cells, namely p65, MafG, c-Myb and E2F-1 (Figure 8). On the other hand, YY1 acts as a repressor for *MAT2A* expression. The inductive effect of IGF-1 on *MAT2A* expression requires normal levels of p65, MafG, c-Myb, USF-1 and E2F-1. Since IGF-1 also induces YY1, this probably serves to dampen the level of *MAT2A* induction.

Some of these transcription factors act at multiple *cis*-acting elements. Indeed, c-Myb can bind to both USF/c-Myb and NF- κ B/c-Myb sites. E2F-1 also interacts with YY1 and NF- κ B sites. To see which of these *cis*-acting elements are important for basal activity and the inductive effect of IGF-1 on *MAT2A* promoter activity, these sites were mutated singly or in combination. Using this strategy, we confirmed that mutations to the YY1 site increased the basal promoter activity and further enhanced the induction by IGF-1 of the *MAT2A* promoter activity (Figure 9). This confirms that YY1 works as a repressor for *MAT2A*. YY1 can act as an activator or repressor depending on its context with other transcription factors [24]. YY1 has been shown to target both oncogenes and tumour suppressor genes in malignant tissues [24]. With the exception of YY1, mutation of any single *cis*-acting element reduced basal promoter activity and partially reduced the inductive effect of IGF-1 on *MAT2A* promoter activity (Figure 9). Mutations of multiple sites further decreased basal promoter activity and the inductive effect of IGF-1, with the lowest activity observed when NF- κ B/c-Myb, AP-1, USF/c-Myb and E2F sites are all mutated, and IGF-1 was no longer able to induce *MAT2A* promoter activity (Figure 9). This proves the importance of these *cis*-acting elements in regulating *MAT2A* basal expression and in mediating the inductive effect of IGF-1 on *MAT2A* expression in colon cancer cells.

We have previously shown the importance of NF- κ B, c-Myb and AP-1 in *MAT2A* activation in liver cancer cells [10,12]. In the present study we show that these factors are also

important in basal *MAT2A* expression and IGF-1 induction of *MAT2A* in colon cancer cells. In addition, we have identified functional YY1 and E2F-1 elements in the human *MAT2A* promoter, and while YY1 represses, E2F-1 activates *MAT2A* expression. The majority of the IGF-1 responsive factors in the present study are involved in growth, proliferation and anti-apoptosis. The new finding that IGF-1 induces c-Myb expression is of particular importance as c-Myb is a known proto-oncogene and found to be overexpressed in approximately 80 % of all colon cancers [25]. It can target the expression of survival genes such as Bcl2 and growth-promoting genes such as c-Myc, cyclins A1, B1, E1, and c-Kit [25]. In addition, c-Myb has been demonstrated to induce the expression of IGF-1 and IGF-1R, both of which are expressed at high levels in colon cancer [4,26]. Thus IGF-1 can induce c-Myb expression and in turn c-Myb can increase IGF-1 signalling in colon cancer. This might represent a feed-forward mechanism between c-Myb and IGF-1 signalling to help provide colon cancer cells with a mechanism of enhanced survivability and to provide a growth advantage to the cancer cells. The requirement for such a system may be due to the need to maintain high c-Myb levels in colon cancer cells since the half-life of c-Myb is approximately 30 min [25]. This would enable c-Myb to activate its target genes that are involved in growth and proliferation, such as *MAT2A*.

In summary, *MAT2A* expression is increased in human colon cancer and when colon cancer cells are treated with IGF-1 due to increased transcription factor binding to key enhancer elements present in the *MAT2A* promoter. IGF-1 increases the expression of these transcription factors, which in turn work in concert to activate *MAT2A* expression. Although IGF-1 also increases YY1 expression, YY1 serves to dampen the inductive effect of IGF-1 on *MAT2A* expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AP-1	activator protein 1
ChIP	chromatin immunoprecipitation
EGF	epidermal growth factor
EGFR	EGF receptor
EMSA	electrophoretic mobility-shift assay
FBS	fetal bovine serum
IGF-1	insulin-like growth factor-1
IGF-1R	IGF-1 receptor

MAT	methionine adenosyltransferase
MAT2A	gene encoding MAT 2A
NF-κB	nuclear factor κ B
Nrf	nuclear factor-erythroid 2-related factor
PEA3	polyoma enhancer activator 3
siRNA	small interfering RNA
USF-1	upstream stimulatory factor-1
YY1	Yin and Yang 1

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Figure 1. *MAT2A* gene transcription and protein binding to the *MAT2A* promoter region in human colon cancer

(A) *MAT2A* gene transcription in normal (NL) and cancerous colon (CA) from the same patients. Nuclei were isolated from normal and cancerous colon for the nuclear run-on transcription assay performed as described in the Experimental section. (B) DNase I footprinting in normal and cancerous colon. DNA fragments were end-labelled and digested with DNase I in the absence (0) or presence of 15–30 μ g of nuclear protein extracts from normal and cancerous colon. The position of the protected regions observed only in cancerous colon is indicated on the right-hand side of the Figures. Lanes G + A represent a Maxam–Gilbert sequencing reaction in the same fragments.

Figure 2. Effects of leptin, EGF and IGF-1 treatments on DNase I footprinting analysis of the human *MAT2A* promoter

The DNA fragment was end-labelled and digested with DNase I in the absence (0) or presence of 30 μ g of nuclear protein extracts from RKO cells treated with leptin (Lep), EGF or IGF-1 (all at 100 ng/ml for 3 h). The position of the protected region is indicated on the right-hand side of the Figure. Lane G + A represents a Maxam–Gilbert sequencing reaction in the same fragment. Representative DNase I footprinting is shown.

Figure 3. Effect of IGF-1 treatment on luciferase activity driven by the human *MAT2A* promoter
 RKO cells were transfected with human *MAT2A* promoter constructs of different lengths or pGL-3 vector alone and treated with IGF-1 (0–100 ng/ml for 3 h). Cells were co-transfected with *Renilla* luciferase for control of transfection efficiency. Results represent means $\pm$ S.E.M. from three independent experiments performed in triplicate. Results are expressed as relative luciferase activity to that of the pGL-3 vector control, which is assigned a value of 1.0. * $P < 0.05$ compared with the respective control, ** $P < 0.005$ compared with the respective control.

Figure 4. Effect of IGF-1 treatment on EMSA and supershift assay of the AP-1 site
RKO cells were treated with IGF-1 (0–100 ng/ml) for 3 h or 100 ng/ml for 15 min to 6 h and processed for EMSA and supershift assay as described in the Experimental section. **(A)** Dose-dependent effect of IGF-1 on nuclear binding to the AP-1 site at –332. **(B)** Time-dependent effect of IGF-1 on AP-1 binding. **(C)** Supershift analysis after 3 h of IGF-1 (100 ng/ml) treatment shows that the dominant proteins bound to the AP-1 site are c-Jun, FosB and MafG. A representative EMSA with supershift is shown.

Figure 5. Effect of IGF-1 treatment on nuclear binding activity of YY1, USF, PEA3, NF-κB and E2F sites

RKO cells were treated with IGF-1 (100 ng/ml) for up to 6 h and processed for EMSA and supershift assay as described in the Experimental section. USF and NF-κB probes also contain a c-Myb-binding site. Location of the sites are indicated below each gel. IGF-1 treatment exerted a time-dependent increase in binding to all of these sites with the exception of the PEA3 site. The dominant protein bound to the USF site is c-Myb (upper right-hand gel). A representative EMSA with supershift is shown.

Figure 6. Effect of IGF-1 treatment on transcription factor binding to the *MAT2A* promoter using ChIP

RKO cells were treated with IGF-1 (100 ng/ml for 3 h) and processed for ChIP analysis using the antibodies indicated on the right-hand side. (A) The promoter region covered spans from −539 to −280 and IGF-1 treatment increased the binding of E2F-1, YY1, MafG, c-Jun, p65, FosB and c-Myb to this region. (B) The promoter region covered spans from −270 to +54 and IGF-1 treatment increased E2F-1 binding to this region. Input genomic DNA (Input DNA) was used as a positive control and immunoprecipitation with an antibody against the SV40T-ag (simian virus 40 T antigen) was used as a negative control. Numbers below the blots represent densitometric values expressed as mean percentage of the control

± S.E.M. from two independent experiments performed in duplicate. All of the changes are significantly different from control (Con) with $P \leq 0.05$, except for USF-1.

Figure 7. Effect of IGF-1 treatment of RKO cells on mRNA and protein levels of c-Jun, p65, FosB, c-Myb, E2F-1, USF-1, YY1 and MafG

RNA and protein were isolated from RKO cells treated with IGF-1 (100 ng/ml) for up to 6 h for Northern or Western blot analyses (15 μ g of RNA or protein/lane). Membranes were stripped and re-probed with β -actin for Northern blot analysis, or Histone H3 (for nuclear protein) and actin (cytosolic protein) for Western blot analysis to ensure equal loading. Representative blots from three separate experiments are shown, and numbers below the blots represent densitometric values expressed as the mean percentage of zero time control (Con) $\pm$ S.E.M. (A) and (B) are Northern blots, and (C) and (D) are Western blots. IGF-1 induced the expression of all of these transcription factors significantly in a time-dependent manner with $P < 0.05$.

Figure 8. Effect of acute knockdown of transcription factors on basal and IGF-1-mediated *MAT2A* induction

RKO cells were transfected with siRNA against p53 (A), MafG (B), c-Myb (C), E2F-1 (D), USF-1 (D) or YY1 (D) for 24 h followed by IGF-1 treatment (100 ng/ml) for 3 h. Northern blot analyses were performed to assess the effect of these treatments on *MAT2A* mRNA levels. Representative blots from three separate experiments are shown and numbers below the blots represent densitometric values expressed as the mean percentage of control (Con) $\pm$ S.E.M. Acute knockdown of p53, MafG, c-Myb and E2F-1 lowered, whereas YY1 knockdown raised *MAT2A* mRNA levels ($P < 0.05$ for all). Knockdown of all of these transcription factors with the exception of YY1 significantly blunted the inductive effect of IGF-1 on the *MAT2A* mRNA level ($P < 0.05$).

Figure 9. Effect of mutation of *cis*-acting elements alone or together on the *MAT2A* promoter activity at baseline and in response to IGF-1 treatment

RKO (A) and HT-29 (B) cells were transfected with wild-type or mutated *MAT2A* promoter constructs and treated with IGF-1 (100 ng/ml) for 3 h for measurement of luciferase activity as described in the Experimental section. See Supplementary Figure S1 (at <http://www.BiochemJ.org/bj/436/bj4360507add.htm>) for different elements and sites of mutation. Results represent means $\pm$ S.E.M. from three independent experiments performed in triplicate for RKO cells, and two independent experiments performed in triplicate for HT-29 cells. Results are expressed as relative luciferase activity to that of pGL-3 vector control, which is assigned a value of 1.0. * $P < 0.01$, ** $P < 0.05$ compared with the respective construct without IGF-1 treatment; † $P < 0.05$, †† $P < 0.01$ compared with the wild-type -571/+60 promoter construct.