Control of expression of the lectin-like protein Reg-1 by gastrin: role of the Rho family GTPase RhoA and a C-rich promoter element

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The expression of members of the Reg family of secreted lectin-like proteins is increased in response to stress, inflammation and damage in many tissues. In the stomach, Reg is located in enterochromaffin-like cells, where its expression is stimulated by the gastric hormone gastrin. We have examined the mechanisms by which gastrin stimulates expression of Reg-1. Deletional mutations of 2.1 to 0.1 kb of the rat Reg-1 promoter in a luciferase reporter vector were transiently transfected into gastric cancer AGS-G_R cells. All promoter fragments tested showed similar relative increases in luciferase expression in response to gastrin (1 nM). The response to gastrin of the smallest (104 bp) construct was 4.2 ± 0.4 -fold over basal. These responses were reduced by Ro-32-0432, a protein kinase C inhibitor, by C3-transferase, a *Clostridium botulinum* toxin and a selective inhibitor of the Rho family GTPase RhoA, and by co-transfection with a dominant negative form of RhoA. Co-transfection with a constitutively active form of RhoA stimulated expression 11.6 ± 1.7 -fold over

basal. Mutations through the 104 bp construct identified a C-rich element (C⁻⁷⁹CCCTCCC⁻⁷²) required for responses to gastrin, PKC (protein kinase C) and L63RhoA (the constitutively active form of human RhoA protein containing a glutamine-to-leucine substitution at position 63). EMSAs (electrophoretic-mobility-shift assays) using nuclear extracts of control and gastrin-stimulated AGS-G_R cells and a probe spanning –86 to –64 bp revealed multiple binding proteins. There was no effect of gastrin on the pattern of binding. Supershift assays indicated that transcription factors Sp1 and Sp3 bound the C-rich sequence. We conclude that gastrin stimulates Reg expression via activation of PKC and RhoA, that a C-rich region (–79 to –72) is critical for the response and that Sp-family transcription factors bind to this region of the promoter.

Key words: gastric epithelium, growth factor, Reg-1, RhoA, Sp1/3, transcription.

INTRODUCTION

The epithelium of the gastrointestinal tract exhibits a range of adaptive responses to infection, inflammation and damage, as well as to delivery of nutrients [1,2]. Several independent studies have identified the Reg family of peptides as novel growth factors that exhibit markedly increased expression in response to damaging or stressful stimuli. These peptides are typically about 140 amino acid residues in length and resemble the C-type lectins [3]. Members of the family have variously been named ‘pancreatic thread protein’, ‘pancreatic stone protein’, ‘lithostathine’ and ‘Reg’, and are closely related to the pancreatitis-associated proteins [4–6]. A cluster of Reg-related genes is found in tandem at chromosome 4q33–34 in the rat, 6c in mouse and 2p12 in human [7–9]. A putative Reg receptor, EXTL-3 (exostoses-like-3), has been identified which belongs to the multiple-exostoses family of tumour suppressors [10]. In general, however, little is known of the control of Reg expression or its mechanisms of action.

The name ‘Reg’ reflects the discovery of a transcript that was markedly up-regulated in a model of regenerating pancreas (partial pancreatectomy and nicotinamide treatment). In this model, Reg is expressed in islet β -cells and is associated with stimulation of islet growth [5,11]. It is now clear that Reg is also expressed in many other tissues. Recent microarray studies in inflammatory bowel disease identified members of the Reg family as exhibiting the greatest increase in expression compared with controls [12,13]. Moreover, these proteins are not limited to epithelia, since members of the Reg family have been found to be substantially increased in motor neurons after axotomy [14] and may be overexpressed in Alzheimer’s disease [15]. In the

human stomach, Reg is located in pepsinogen-secreting chief and histamine-secreting ECL (enterochromaffin-like) cells [16], and there is also expression of Reg in rat ECL cells [17]. In both man and rat, there is evidence that Reg expression is increased by the gastric hormone gastrin [16,18]. In addition, gastric Reg expression is increased by stress and by mucosal damage possibly via the chemokine CINC-2 β (cytokine-induced neutrophil chemoattractant-2 β) acting at CXCR-2 (CXC chemokine receptor 2) receptors [17,19].

The cellular mechanisms regulating Reg expression are poorly understood. In the pancreas, it has been reported that the combination of IL-6 (interleukin-6), dexamethasone and nicotinamide stimulates transcription via a mechanism involving poly(ADP-ribose) polymerase (PARP) acting at a GC-rich sequence (–81 to –70) [20]. Because there appears to be robust regulation of Reg *in vivo* by gastrin, in the present study we examined the cellular mechanisms responsible. Our studies identify the C-rich region in the Reg promoter as required for stimulation by gastrin, and indicate that transcription from this site is mediated by PKC (protein kinase C) and by RhoA.

EXPERIMENTAL

Plasmids

The luciferase-reporter vector pXP2 [21] was a kindly provided by Professor Timothy C. Wang (University of Massachusetts Medical Center, Worcester, MA, U.S.A.). Dominant-negative (N19RhoA $\equiv$ [¹⁹Asn]RhoA) and constitutively active (L63RhoA) mutants of RhoA were gifts from Professor Alan Hall (MRC

Abbreviation used: CCK, cholecystokinin; CgA, chromogranin A; ECL-cell, enterochromaffin-like cell; EMSA, electrophoretic-mobility-shift assay; G17, heptadecapeptide gastrin; IL, interleukin; PAI-2, plasminogen activator inhibitor type 2; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C; TFF1, trefoil factor 1; TGF, transforming growth factor; TNF, tumour necrosis factor; VMAT2, vesicular monoamine transporter type 2.

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Laboratory for Molecular Cell Biology and Cell Biology Unit, University College London, London, U.K.). The pGEM[®]-T Easy Vector System for cloning was obtained from Promega (Southampton, U.K.), and the TA cloning vector pCRII was purchased from Invitrogen (Leek, The Netherlands).

Cell culture

The gastric cancer cell line AGS-G_R, which expresses the gastrin-cholecystokinin (CCK)_B (or CCK-2) receptor, was cultured as previously described in Ham's F12 medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin [22]. For studies with luciferase reporter vectors, cells were plated at 2×10^5 cells/well in six-well plates, transfected 24 h later, and the effect of various treatments studied 16 h after that. In different experiments, cells were treated with combinations of G17 (hepta-decapeptide gastrin; Peninsula, St Helen's, Merseyside, U.K.), PMA (100 nM), mithramycin (90 nM; Sigma, Poole, Dorset, U.K.), C3-transferase (417 ng/ml; Oncogene, Nottingham, U.K.) or Ro-32-0432 (1 μ M; Calbiochem, Nottingham, U.K.). IL-1 β , IL-6, IL-8, TNF (tumour necrosis factor)- α and TGF (transforming growth factor)- β (Calbiochem) were used at the concentrations described.

Cloning of the rat Reg-1 promoter and mutants

An upstream sequence corresponding to 2111 bp of the wild-type rat Reg-1 promoter, together with exon 1, intron 1 and 16 bp of exon 2, was amplified by PCR from rat genomic DNA and cloned into PCRII. The promoter fragment was excised from PCRII and cloned into pXP2 between *Bam*HI and *Xho*I sites of the multiple cloning region to give Reg-1-luc. All PCR reactions were with Bio-X-act DNA polymerase (Biolone, Dollis Hill, London, U.K.) in a PerkinElmer Genamp 2400 thermal cycler. Deletional mutants containing 601, 408, 338, 236 and 104 bp of promoter were generated by PCR.

Directed mutagenesis within the 104 bp promoter region was achieved by standard and recombinant PCR techniques using the wild-type 104 bp deletional mutant (104 bp Reg-1-luc) as a template [23]. PCR products were purified on agarose gels, recovered using a gel extraction kit (Qiagen, Crawley, Sussex, U.K.), and ligated into the pGEM[®]-T Easy vector. Inserts were excised and directionally cloned between *Bam*HI and *Xho*I sites of pXP2. The integrity of all constructs was confirmed by automated dideoxy sequencing.

Luciferase assays

AGS-G_R cells were transiently transfected using TransFast[™] Transfection Reagent (6 μ l/ μ g of DNA; Promega) in serum-free medium (840 μ l/well; 0.5–1.0 μ g of Reg-1-luc plasmid and 0.1 μ g of *Renilla* luciferase vector). Cells were harvested using 1 $\times$ Glo Lysis Buffer (Promega; 0.5 ml/well); in initial experiments a dual injection luminometer (Lumat LB9507; Berthold, Redbourne, Herts., U.K.) was used, and, subsequently, luciferase assays performed using Promega Dual-Glo[™] Luciferase assay kits and a Packard (Pangbourne, Berks., U.K.) luminometer plate reader according to the manufacturers' instructions. Protein was determined as appropriate to monitor plating efficiency.

EMSAs

Nuclear extracts of AGS-G_R cells were prepared using the Pierce NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents Kit,

Figure 1 Deletional mutation of the Reg-1 promoter sequence

(A) In AGS cells transiently transfected with luciferase reporter vectors there was a decrease in basal activity between –408 and –338 bp relative to the transcriptional start site. The activity of the promoterless vector was barely discernable, and is not shown. Abbreviation: RLU, relative light units. (B) The responses to G17 (1 nM) of the same range of mutants expressed as the relative increase over basal expression were similar. Representative results (means $\pm$ S.E.M.) from three independent experiments, obtained using a dual injection luminometer, are shown.

the manufacturer's guidelines being followed. Complementary oligonucleotides spanning the region –86 to –64 of the rat Reg-1 promoter and containing 3 bp 5' overhangs were annealed, labelled with [³²P]dATP using Klenow enzyme and 0.1 mM dNTP (minus dATP; 30 μ l, 22 $^{\circ}$ C, 20 min) and terminated by heat inactivation (75 $^{\circ}$ C, 5 min). The resultant probe was adjusted to 100 μ l with 0.05 $\times$ TBE buffer (89 mM Tris/borate/2 mM EDTA, pH 8.3) and diluted 1 in 10 with 1 $\times$ EMSA buffer (10 mM Tris/HCl at pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA and 1 mM dithiothreitol). Binding reactions (20 μ l, 20 min) contained 50% (v/v) glycerol (4 μ l), 10 $\times$ EMSA buffer (2 μ l), nuclear extract (5 μ g), poly(dA $\cdot$ dT) (1 μ g/ml, 1 μ l), probe (2 μ l) and water. For competition assays, double-stranded competitor oligonucleotide was preincubated (20 min) with nuclear extract in 100-fold excess (unless otherwise stated) prior to the addition of probe. For supershifts, nuclear extracts were preincubated (20 min) on ice with antibodies to Sp1, Sp3 and Sp4 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Samples were separated on non-denaturing 6% (w/v) polyacrylamide gels in 0.025 $\times$ TBE (20 mA, 3 h), the gels dried under vacuum, exposed overnight to a phosphor screen, visualized on a Phosphorimager (Bio-Rad, Hemel Hempstead, Herts., U.K.) and analysed using Quantity One (Bio-Rad) image-analysis software.

Statistics

Results are presented as means $\pm$ S.E.M. Comparisons were made by Student's *t* test, two-tailed where appropriate, and assuming unequal variance.

RESULTS

Mutational analysis of the rat Reg-1 promoter

In initial studies we examined the expression of rat Reg-1 promoter–luciferase-reporter constructs in transiently transfected AGS-G_R cells. Approx. 2 kb of Reg-1 promoter induced luciferase expression that was increased 4–7-fold by gastrin (1 nM) (Figures 1A and 1B). There was a substantial decrease in unstimulated

Figure 2 Block mutations within the proximal promoter region indicate that gastrin acts via a C-rich sequence

(A) The block mutants studied included deletion of a putative GATA site (–91 to –86) and a C-rich sequence (–79 to –76). Abbreviation: R.L.U., relative light units. (B) Basal expression of promoter-reporter sequences. (C) Responses to G17 (1 nM) normalized to basal activity of the relevant mutant. Note marked reduction in response of M3 corresponding to the loss of the C-rich sequence. Results obtained using luminometer plate reader and Dual-Glo™ Luciferase assay kits. Results are means ± S.E.M.; **P* < 0.05 compared with wild-type (WT).

luciferase expression when a 408 bp fragment of promoter was deleted to 338 bp, corresponding to loss of a putative Sp1 site; however, approx. 20% of basal activity was retained by a sequence extending to –104 bp relative to the transcriptional start site (Figure 1A). Gastrin (1 nM) stimulated expression of the deletional mutants to a similar extent (Figure 1B).

We then examined the effects of block mutations within the proximal –104 bp of promoter (Figure 2A). There was up to 50% reduction in basal activity with these mutations (Figure 2B), including those that deleted a putative GATA site (–91 to –86) and a C-rich sequence (–79 to –76). Mutation of a putative GATA site had little effect on the response to gastrin (Figure 2C). In contrast, mutating the sequence C^{–79}CCC^{–76} substantially inhibited the response to gastrin (Figure 2C). We then examined the effects of a series of mutations through the C-rich region. Mutations of 2 bp within the sequence G^{–80}CCCCCTCCA^{–71} (Figure 3A) reduced basal expression and inhibited the response to gastrin by 50–80% (Figures 3B and 3C).

It has previously been reported that mithramycin A binds GC-rich sequences and inhibits transcriptional activity dependent on these sites [24,25]. In view of the data already described, we therefore questioned whether mithramycin influenced the response to gastrin. Pretreatment of cells with mithramycin inhibited the response to gastrin by 66%, but had little effect on the basal expression of 104 bp Reg-1-luc (Figure 3D).

Activation of PKC mediates the effect of gastrin

Previous studies have established that gastrin stimulates transcription via activation of PKC [22,26–28]. We found that PMA (100 nM) increased expression of the –104 bp construct, but the response was consistently about 60% of that caused by gastrin (Figure 4A). The PKC inhibitor Ro-32-0432 fully inhibited the

Figure 3 Mutations within the C-rich sequence (–80 to –71) confirm their importance for gastrin responses

(A) Mutations (2 bp) introduced through the C-rich sequence. (B) Basal expression of promoter-reporter mutants. Abbreviation: R.L.U., relative light units. (C) Responses to G17 (1 nM) normalized to basal activity of the relevant mutant. (D) Effect of mithramycin (90 nM) on the response to G17 of the wild-type 104 bp Reg-1-luc construct. Results are means ± S.E.M.; **P* < 0.05 (B and C), compared with corresponding wild-type (WT) expression.

response to PMA and reduced the response to gastrin by about 60% (Figure 4A).

We then examined the effect of mutations, including the sequence CCCC described above, on responses to PMA. Mutations within the latter substantially reduced responses to PMA and, overall, the pattern of responses was qualitatively similar to that evoked by gastrin (Figure 4B). The data indicate that gastrin-induced expression of Reg-1 from the C-rich sequence includes both a PKC-dependent and a PKC-independent pathway.

RhoA mediates the effects of gastrin

Because gastrin is known to activate RhoA [29], and this has been shown to be involved in gastrin-dependent transcriptional responses [22,27], we then examined the role of RhoA. Initially cells were incubated with the *Clostridium botulinum* toxin C3-transferase, which is a selective RhoA inhibitor. This significantly reduced the response to gastrin (Figure 5A). Consistent with the notion that RhoA mediates the effect of gastrin, we found that co-transfection of cells with 104 bp Reg-1-luc and a plasmid encoding a dominant-negative RhoA (N19RhoA) also significantly inhibited the luciferase responses to gastrin (Figure 5A). In addition, N19RhoA inhibited the response to PMA, placing PKC upstream of RhoA activation in this system.

In order to further define the role of RhoA on Reg expression, we then examined responses to the co-expression of 104 bp Reg-1-luc and a plasmid encoding constitutively active RhoA (L63RhoA). The latter induced an increase in luciferase activity of 11.6 ± 1.7 -fold (mean ± S.E.M., *n* = 17 independent experiments). To determine whether the response to L63RhoA depended on the *cis* element in 104 bp Reg-1-luc that is required for responses to gastrin, we examined induction of luciferase after co-transfection of cells with Reg-1-luc mutants and constitutively

Figure 4 PKC in part mediates the effect of gastrin

(A) There was stimulation of Reg-1-luc by PMA (100 nM) which was completely inhibited by Ro-32-0432 (1 μ M). The response to G17 (1 nM) was also significantly reduced by Ro-32-0432 (1 μ M). (B) The reduced effect of PMA (100 nM) on the mutants shown in Figure 2, indicates an important role for the C-rich sequence in mediating the effects of PKC. * $P < 0.05$ (B) compared with WT).

active L63RhoA (Figures 5B and 5C). Responses to the latter were significantly attenuated by mutation of the sequence CCCCTCCCC described above (Figures 5B and 5C).

Since mithramycin inhibited the effect of gastrin, we then examined its effects on L63RhoA-induced Reg-1-luc expression. There was a substantial inhibition of Reg-1-luc response when co-transfected with L63RhoA, providing further evidence that activation of RhoA increases expression via the C-rich sequence (Figure 5D).

Cytokines

Reg expression in pancreatic β -cells is increased by IL-6 and dexamethasone and by inhibition of PARP [20]. We found that gastrin-stimulated Reg-1-luc expression was unaffected by addition of the PARP inhibitor 3-aminobenzimide up to 3 mM (results not shown). We also found that IL-6 (5, 50 and 500 pg/ml) had no effect on 104 bp Reg-1-luc and 2.1 kb Reg-1-luc expression in AGS-G_R cells. In addition, other inflammatory mediators, i.e. TGF- β (0.1, 1, 10 and 100 ng/ml), IL-8 (3, 30 and 300 ng/ml), TNF- α (5, 50 and 500 pg/ml) and IL-1 β (1, 10 and 100 pg/ml) had no effect on expression of either the 2.1 kb Reg-1-luc and 104 bp Reg-1-luc constructs (results not shown). Moreover, IL-1 β at 1, 10 and 100 pg/ml did not change the response of 104 bp Reg-1-luc and 2.1 kb Reg-1-luc to gastrin at 0.1 and 1 nM (results not shown).

Figure 5 RhoA mediates the effects of gastrin

(A) The action of G17 (1 nM) on Reg-1-luc was inhibited by treatment of cells with C3-transferase (C3T; 417 ng/ml) and by co-transfection with dominant-negative RhoA (N19RhoA; 0.25 μ g/well). The latter also inhibited responses to PMA (100 nM), indicating that PKC is upstream of RhoA. (B and C) Co-transfection of a constitutively active mutant of RhoA (L63RhoA; 0.25 μ g/well) stimulated Reg-1-luc activity, and the response was markedly decreased by mutation of the C-rich sequence (see Figure 2). (D) In a separate experiment, mithramycin (Mith; 90 nM) inhibited the response to L63RhoA. * $P < 0.05$ [B and C compared with corresponding wild-type (WT) expression].

Identification of Sp1 and Sp3 binding to the Reg promoter

In order to identify putative DNA-binding proteins, we used EMSAs employing nuclear extracts from AGS-G_R cells and probes spanning the sequence CCCCTCCCC (Figures 6A–6C). In nuclear extracts of unstimulated cells there were three bands that exhibited specific binding (identified as 1–3 in Figure 6C). In competition experiments the wild-type sequence in 100-fold excess competed fully with the probe for binding to these proteins. Oligonucleotides containing mutations outside of the region CCCCTCCCC [M (mutant)13, M14, M15 and M20] also competed for binding, whereas oligonucleotides containing mutations within this sequence (M11, M12 and M16–19) did not. The same pattern of bands was identified in nuclear extracts from cells treated with gastrin (Figure 6D). In some lanes there was also a fourth band that ran just below band 2; this was fully competed for by most mutants (Figure 6C) and, because it could not be linked to a specific DNA sequence, it was not considered further.

Since the sequence CCCCTCCCC is a putative binding site for the Sp family of transcription factors, we examined the effects of incubation with antibodies to Sp-family members. A supershifted band was identified after incubation with antibodies to Sp1 together with a modest depletion of band 1, whereas antibodies to Sp3 produced a supershifted band and depletion of band 2 (Figure 6E). Antibodies to Sp4 did not change the pattern of binding.

DISCUSSION

The present study shows that, in gastric epithelial cells, increased expression of Reg-1 in response to gastrin is mediated by PKC

Figure 6 Sp1 and Sp3 bind the gastrin-response element of the Reg-1 promoter in AGS-G_R cells

(A) Sequences of mutants used for EMSA. (B) Nuclear extracts of AGS-G_R cells show retardation of an oligonucleotide probe (arrows indicate complexes 1 and 2, and 3; see below) corresponding to the C-rich region when gels were run under conditions in which unbound probe (P) can be seen at the bottom of the gel (PO, probe only; NC, non-competitor; WT, 100-fold excess of wild-type oligonucleotide). (C) At least three distinct proteins (1–3) binding the oligonucleotide probe were separated on gels run further to increase resolution (the bottom panel shows an enhanced image of band 3). Binding of the probe was inhibited by the wild-type sequence, and mutants outside, but not within, the sequence CCCCTCCC. A fourth faint band can be seen in some lanes (open arrowhead), although in this case the pattern of competition did not allow identification of a specific DNA-binding sequence. (D) Nuclear extracts of gastrin-treated cells (G17) exhibited similar patterns of binding to that of control (Con) cells. (E) In supershift assays, antibody to Sp1 produced a supershifted band (*) and modest depletion of band 1; antibody to Sp3 depleted band 2 compared with controls and also produced a supershifted band (*); antibody to Sp4 had no effect (NA, no antibody). The position of the wells is indicated by the broken arrow, and the open arrowhead indicates the band described in (C) above.

and activation of the small GTPase RhoA. It is now clear that, in many different systems, the expression of Reg-family members is highly regulated and, in particular, that there are marked increases in expression with tissue damage, inflammation and regeneration. Thus, for instance, there is increased Reg expression in pancreatic regeneration and acute pancreatitis [5,30], in stress-induced damage to the stomach [17], in the affected segment in inflammatory bowel disease [12] and in nerve fibres after axotomy [14]. However, the cellular signalling systems responsible for these changes are poorly understood. The present data implicate the activation of RhoA and a putative Sp1/Sp3 element in the stimulation of Reg-1 expression.

Recent studies have shown that Reg is increased in the gastric epithelium by gastrin and stress [16,18,19]. It also seems likely that stimulation by gastrin accounts for the increased Reg expression found in gastric ECL-cell carcinoid tumours [16]. In addition, there is reported to be increased expression in gastric cancer, where there is an association with decreased survival [31,32]. It is possible that gastrin plays a role in regulating Reg expression in some gastric carcinomas, although generally the gastrin–CCK_B (or CCK-2) receptor is only expressed in a subset of these tumours [33]. Activation of this receptor is, however, well recognized to play a role in control of gastric epithelial proliferation, and this is now thought to be secondary to release of growth factors [34]. Stimulation of Reg expression is therefore a potential component of the proliferative response to gastrin.

In vivo, gastrin increases the expression in ECL cells of a number of genes, including those coding for histidine decarboxylase, VMAT2 (vesicular monoamine transporter type 2) and CgA (chromogranin A) as well as for Reg [35–37]. Each of these genes can be linked to well-known actions of gastrin in regulating gastric function (histamine synthesis, storage and secretion, or

proliferation) [34]. Quite recently a number of other genes expressed in gastric mucosa have also been shown to be regulated by gastrin including TFF1 (trefoil factor 1), PAI-2 (plasminogen activator inhibitor type 2) and matrix metalloproteinase 9 (gelatinase B) [22,38,39]. These seem to be part of the system by which gastrin produces relatively long-term effects on mucosal remodelling [40]. Transcriptional activation of many of these gastrin-regulated genes depends on the integrity of GC-rich promoter elements. Putative transcription factors thought to be implicated in these responses include Sp family members (CgA, TFF1), MAZ (TFF1), CREB (cAMP-response-element-binding protein) (VMAT2, CgA, PAI-2) as well as a number of still uncharacterized binding proteins [22,28,38,41]. Several of the relevant transcriptional responses to gastrin have been shown to be mediated by PKC and activation of RhoA [22,27], but, in general, little has been done to link RhoA activation in this system to specific promoter elements.

The sequence C^{−79}CCCTCCC^{−72} was found to be critical for the response to gastrin, PMA and RhoA in gastric epithelial cells. Interestingly, the same region of the Reg promoter has previously been implicated in the increased expression of Reg in the pancreas in response to nicotinamide and stimulation by IL-6 and dexamethasone, and has been shown to involve binding of PARP [20]. We found no evidence that PARP inhibition or cytokines stimulated Reg expression in AGS cells, suggesting that the increased expression of Reg that occurs in different cell types is mediated by distinct signalling mechanisms. Sequences similar to that identified in the rat Reg-1 promoter were found in the promoter of the proto-oncogene *c-myc* many years ago, and were shown to bind the transcription factors Sp1 and MAZ [42]. They are now recognized to be widely distributed and, for example, mediate gastrin-stimulated transcription of TFF1 in

AGS-G_R cells [38]. However, the possible role of RhoA in regulating transcription from these sites has not previously attracted attention.

Transcriptional responses to activation of RhoA have been intensively studied in many systems, particularly activation of serum response factor and ternary complex factor [43]. Although RhoA has been reported to regulate p21^{waf/cip} (a cell-cycle inhibitor) via a GC-rich Sp1 sequence similar to that found in the present study [44], the response was inhibitory, unlike the present excitatory effect. We found that mithramycin, which is known to inhibit transcription from Sp1/Sp3 sites, significantly reduced the effects of gastrin, PMA and L63 RhoA. However, in gel-shift assays, gastrin did not change the pattern of nuclear protein binding, so that there are presumably other mechanisms involved in mediating the effects downstream of RhoA. Conceivably these may include induction of transcriptional co-activators or post-translational modification to the complex binding to the Reg-1 promoter.

A variety of functions have been ascribed to Reg-family members. These include stimulation of cell proliferation and, at higher concentrations, apoptosis [10]. In addition, Reg forms fibrils on mild tryptic digestion and so may be a component of the extracellular matrix [45]. In spite of the overwhelming evidence that Reg expression is strongly regulated, the relevant control mechanisms remain unclear. While previous studies in the pancreas identified PARP as a putative regulator of Reg transcription [20], it remains unclear whether this accounts for changes in expression in other tissues. Our data now show that activation of a G-protein-coupled receptor, the gastrin-CCK_B or CCK-2 receptor, is able to regulate Reg-1 expression by activation of PKC and RhoA. The link between RhoA and a C-rich region of the Reg-1 promoter was unexpected. Given the potential importance of Reg expression, the present observations suggest it will be fruitful in the future to determine the precise mechanisms by which RhoA regulates transcription from this sequence.

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