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Mechanism of IL-4-Mediated Up-Regulation of the Polymeric Ig Receptor: Role of STAT6 in Cell Type-Specific Delayed Transcriptional Response

Hilde Schjerven, Per Brandtzaeg, and Finn-Eirik Johansen

The polymeric IgR (pIgR), also known as the transmembrane secretory component (SC), mediates the generation of secretory Abs (SIgA and SIgM) in exocrine secretions. Locally produced pIgs (mainly dimeric IgA, but also larger polymers of IgA and pentameric IgM) bind to the pIgR basolaterally on secretory epithelial cells, and are subsequently translocated across the epithelium. At the apical surface, the receptor is cleaved, giving rise to SIgA and SIgM Abs with bound SC as well as free SC derived from unoccupied pIgR (1, 2). Several clinical and experimental studies have provided evidence for the importance of SIgA Abs in the protection of mucosal surfaces (3, 4). Recently, pIgR knockout mice have confirmed the essential role of this receptor in the transport of pIgs to secretions (5, 6). Further analysis of such mice demonstrated the importance of secretory Abs in maintaining the barrier function of mucosal linings (5).

pIgR is expressed at mucosal surfaces in human tissues with the highest expression in the small and large intestine (1). In epithelial cell lines, its expression has been shown to be regulated by several factors, including cytokines, hormones, vitamin A, and butyrate (1). In situ studies have shown up-regulation of pIgR in several chronic inflammatory mucosal diseases such as celiac disease, Helicobacter pylori gastritis, and Sjögren’s syndrome afflicting salivary glands; this is suggested to be a secondary effect of cytokines produced locally in these disorders (7).

Several immunoregulatory and proinflammatory cytokines, including IL-4, IFN-γ, IL-1, and TNF-α, enhance the expression of pIgR in cell culture systems (1). Such cytokine-mediated up-regulation of pIgR is known to depend on de novo protein synthesis (8–11), and has recently been shown to take place at the level of transcription in which IL-4 had the greatest effect on the transcription rate of the tested cytokines (10). This effect of IL-4 has been observed both in a human lung carcinoma cell line, Calu-3 (11, 12), and in a human colonic carcinoma cell line, HT-29 (13, 14). Furthermore, IL-4-mediated up-regulation of pIgR is known to be sensitive to inhibitors of tyrosine phosphorylation, indicating a signaling pathway dependent on protein tyrosine phosphorylation (11, 15).

DNA elements that mediate regulation of pIgR expression have been extensively studied. We and others have demonstrated that an E-box in the proximal promoter is essential for the constitutive expression of the human and murine pIgR genes (16, 17). Hormone-regulated transcriptional elements have been identified both in exon 1 and in the far upstream region of the human pIgR gene (18, 19), as well as in the promoter of the murine pIgR gene (20). For IFN-γ-mediated transcriptional up-regulation of pIgR, an IFN-stimulated response element (ISRE) located in exon 1, and two other ISREs in the proximal promoter have been implicated as necessary (21). One report indicated that TNF-α mediates its transcriptional up-regulation of pIgR (at least partly) through the exon 1 ISRE (22). In support of this possibility, we found that TNF-α-induced binding of NFs from HT-29 cells to the exon 1 ISRE, although weaker than after IFN-γ stimulation (10). TNF-α also induced NF-κB binding to a putative target site in the pIgR promoter (10, 23), and inhibitors of this transcription factor partially blocked induction of pIgR (23). However, no transcriptional mechanisms for IL-4-mediated up-regulation of pIgR have been reported, perhaps because the previously published sequence of the human pIgR gene contains no obvious IL-4-responsive elements.
either in the proximal promoter or in the 5′ regulatory sequences (21, 24). Although the entire gene of mouse pIgR (29 kb) has been sequenced (25), only 5′ sequences have been studied in the mouse and the rat pIgR genes in the search of putative binding sites for regulatory transcription factors (17, 25–27).

IL-4 is a pleiotropic cytokine, known to be important for development of type 2 Th cell responses, thus supporting humoral immunity (28). IL-4 is involved in the differentiation and maturation of B cells; although it is most commonly thought of as a switch factor for the IgE and IgG (mouse IgG1 and human IgG4) isotypes, it may also be involved in switching to IgA (29). Studies of IL-4-deficient mice demonstrated an important role for IL-4 in the induction of intestinal Ab responses (30). It has furthermore been shown that IL-4 (and the related cytokine IL-13) plays an important role in protection against intestinal parasites (31) and references therein). Thus, IL-4 might exert effects on both B cells and mucosal epithelial cells to enhance the production of Sgs generated through the cooperation between these two cell types.

Engagement of the IL-4R is known to activate several intracellular signaling pathways, one of which is the Janus kinases (JAK)/STAT signaling pathway (28, 32, 33). STAT6 is latent in the cytoplasm, and upon engagement of the IL-4R, the receptor-associated JAKs become activated and phosphorylate STAT6. Once phosphorylated, STAT6 dimerizes and translocates to the nucleus, where it binds to its DNA elements and activates transcription (33). Thus, this is a direct mechanism of transcriptional activation not requiring synthesis of novel factors. STAT6 is known to be directly involved in the transcriptional up-regulation of some genes important in the immune system such as CD23 (FceRII) (34, 35) and the germline Cε transcription before class switching (29). For these genes, it has been demonstrated that STAT6 requires cooperation with other transcription factors, for example, C/EBP for germline Cε transcription (36, 37) and NF-κB (34) or IRF-4 (38) for induction of CD23 expression (33). Thus, this is a direct mechanism of transcriptional activation (36, 37) and NF-κB (34) or IRF-4 (38) for induction of CD23 expression. The genomic DNA of the human pIgR gene has been cloned, spanning approximately 2.7 kb of upstream sequences and including all coding exons with intervening introns (39). In this study, we identify an IL-4-responsive enhancer in the 5.7-kb intron 1, located approximately 4.1 kb downstream of the transcriptional start site. We further characterize this IL-4-dependent enhancer and demonstrate that STAT6 is involved, both directly and indirectly, in the late de novo protein synthesis-dependent IL-4-mediated transcriptional up-regulation of human pIgR.

Materials and Methods

Plasmid construction

All primers used are shown in Table I. Nucleotide numbering is given relative to the transcriptional start site (16). Plasmids denoted pS1 through pS23 contained different lengths of regulatory sequences (including the promoter) from the human plgR/SC gene (36) subcloned into XhoI/NcoI-digested pGL3 enhancer vector (Promega, Madison, WI). pS1 was extended from −2684 to an NcoI site (indicated by PCR) to the ATG start codon in exon 2 of the human plgR gene. pS2 was made by substituting the genomic fragment from the BstEII site in exon 1 to the start codon in exon 2, with the equivalent fragment from pIgR cDNA. In pS3 through pS9, internal deletions were made by digesting with different restriction enzymes (indicated in Fig. 1, blunting with Klenow fragment where necessary, and religating. The plasmids pS16 through pS22 were made by digesting pS12 with NcoI (partial digestion) and HindIII, and then inserting either a HindIII linker (pS16), or different lengths of PCR-amplified NcoI/HindIII-digested DNA fragments, from pIgR intron 1.

Plasmids denoted p1 through p51 contained different fragments of DNA sequence from intron 1 of the human plgR gene, subcloned upstream of the SV40 promoter of the pGL3 promoter vector (designated p0) (Promega). p1 and p2 contained a 1.3-kb SacI-HindIII fragment from intron 1 in either orientation. For p12, p14, p35, and p48, DNA fragments (indicated in Fig. 3A) were amplified by PCR and subcloned into p0. The 4-bp mutations in p36-p41, p50, and p51 were introduced with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Point mutations were designed that changed the nucleotide from a purine to the noncomplementary pyrimidine and vice versa (i.e., A→C, T→G). The 4-bp deletion in p18 and pS23 was introduced into p12 and pS12, respectively, by digesting with PstI, blunting with T4-DNA polymerase, and religating. The Renilla luciferase control vector, pRL-PGK, was constructed by substituting the CMV promoter from the pRL-CMV vector (Promega) with the promoter of the housekeeping gene phosphoglycerokinase (PGK). pCDNA3−STAT6:ER* was made by subcloning a 3.6-kb EcoRI-Sali fragment from pMXH−STAT6:ER* (40) into the EcoRI-Xhol site of the pCDNA3 vector (Invitrogen, NV Leek, The Netherlands). The pCMV-GFP, a green fluorescent protein (GFP) expression plasmid, was constructed by inserting the CMV promoter from pCDNA3 into the multiple cloning site of the pEGFP-1 plasmid (Clontech, Palo Alto, CA).

DNA sequencing

Sequencing of the human pIgR intron 1 was performed at the Biotechnolology Center of Oslo (Oslo, Norway). The integrity of the vector-insert boundary of all subcloned DNA fragments, as well as mutations, was confirmed by sequencing with the cycle sequencing kit (Amersham International PLC, Slough, U.K.).

Cell culture and transfections

The human colonic adenocarcinoma cell line, HT-29.m3, previously selected for high expression of pIgR (41), was maintained in RPMI 1640 medium supplemented with 2 mM l-glutamine, and 10% FCS. For transient transfections, approximately 1.5 × 10^6 cells per 9.6-cm^2 well were plated out on day 1. On day 2, the cells were transfected with 3 μg/ml human rIL-4, 2.5 μg/ml cycloheximide (CHX), or 1 μg/ml gentamicin, 2 mM L-glutamine, and 10 μg/ml human rIL-4. 2.5 μg/ml cycloheximide (CHX), or 1 μg/ml 4-hydroxynonenoxen (4-HNE; Sigma, St. Louis, MO). Unless otherwise stated, cells were harvested after 24 h of stimulation, and the luciferase activity of both the reporter gene (Firefly luciferase) and the internal control plasmid pRL-PGK (Renilla luciferase) was measured in a luminometer (Victor; Victor, Wallace, Turku, Finland) with the Dual Luciferase Reporter Assay System (Promega). Transfection efficiency of HT-29.m3 cells was approximately 0.5–2%, as determined by FACS analysis of pCMV-GFP-transfected cells.

<table>
<thead>
<tr>
<th>Name of Construct</th>
<th>Name of Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pS1 and pS2</td>
<td>nco-primer.rev</td>
<td>AGCAATGGCTTGGAGTTGCT</td>
</tr>
<tr>
<td>pS16</td>
<td>HindIII linker</td>
<td>CAAAGCTTGG</td>
</tr>
<tr>
<td>pS17</td>
<td>nco2407i</td>
<td>GAATATTCATGGACCTGAAAGA</td>
</tr>
<tr>
<td>pS18</td>
<td>nco2407i</td>
<td>CTATTGCCATGGCTCAG</td>
</tr>
<tr>
<td>pS19</td>
<td>nco2407i</td>
<td>AGAGCCATGGACCTC</td>
</tr>
<tr>
<td>pS20</td>
<td>hind2407i</td>
<td>TGGCCCAAGCTTCTACAG</td>
</tr>
<tr>
<td>pS21, p14</td>
<td>hind2407i</td>
<td>CTCCTCACAGTTGAGC</td>
</tr>
<tr>
<td>pS22, p12</td>
<td>hind2407i</td>
<td>AGCCCAAGCTTAGAGGAGG</td>
</tr>
<tr>
<td>p35</td>
<td>Bgl2.rev</td>
<td>TGTGGAATATAGAACTTTATGTCAG</td>
</tr>
<tr>
<td>p48</td>
<td>Kpn5.fwd</td>
<td>CCCATATGGTACACTCC</td>
</tr>
</tbody>
</table>

Mutations are underlined.
(data not shown). Cos-1 monkey fibroblasts were grown in DMEM medium supplemented with gentamicin, l-glutamine, and FCS, and transfected essentially as described for HT-29.m3 cells, except that 102 cells were plated per well. Data in Figs. 1, 2, 3A, and 6 show the mean ± SEM of three or more independent experiments.

**Quantitative RT-PCR**

Cytoplasmic RNA was isolated from untreated and treated HT-29.m3 cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. RNA (1 μg), primed with oligo(dT), and reverse transcribed with Superscript II, was used for a 20 μl cDNA reaction. Specific mRNA was quantified by real-time PCR with the Light Cycler (Roche Diagnostics) and SYBR Green I reagents, according to the manufacturer’s protocol (Table II). The amplification coefficient was calculated by determining the crossing point (number of cycles required to reach a set threshold) for a series of 2-fold dilutions of cDNA template for each gene analyzed. The calculated crossing point at 1 μl cDNA template was used as a measure of gene-specific RNA quantity, and fold induction was calculated by the following equation: 

\[ K_{\text{trans}} = \frac{\Delta C_p \text{~gene~test}}{\Delta C_p \text{~gene~control}} \]

where \( K_{\text{trans}} \) is the amplification coefficient, and \( \Delta C_p \) is (the crossing point for the RT-PCR from unstimulated cells) − (the crossing point for the RT-PCR from IL-4-stimulated cells) for that gene.

**Nuclear extract preparation and EMSA**

Preparation of nuclear extracts from HT-29.m3 cells was performed essentially as described (42), except for the use of 0.6% Nonidet P-40 for cell lysis instead of mechanical lysis. Also, freshly added 1 mM Na3VO4 (Sigma) was included in buffer A, and buffer C was supplemented with fresh 1 mM Na3VO4, 1 μg/ml peptatin A, 1 μg/ml leupeptin, and 1 μg/ml antipain. Nuclear extracts were aliquoted, frozen in liquid nitrogen, and stored at −70°C until used, and protein concentrations were determined with Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Approximately 5 μg of nuclear proteins was incubated with 32P end-labeled double-stranded oligonucleotide probe (0.5 pmol/reaction) in buffer containing 0.1 mM EDTA, 50 mM KCl, 1 mM DTT, 0.1 μg/ml dl/EC, 0.05% Nonidet P-40, 10 mM Tris, 1 mM MgCl2, 6% glycerol, and 20 mM HEPES (pH 7.9) for 30 min at room temperature. The reaction was separated by electrophoresis on a 5% polyacrylamide gel (0.25 m Tris/borate/EDTA) at 150 V for approximately 1.5 h at room temperature, dried, and visualized on x-ray film overnight. Cold competitors were added in 10- or 100- fold excess before addition of the labeled probe when indicated. For supershift experiments, 4 μg of the anti-STAT6 polyclonal Ab (M-200; Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture before addition of the labeled oligonucleotide, and incubated overnight at 4°C. The labeled probe was added to the reactions and incubated for another 30 min at 4°C, before electrophoresis on a 5% polyacrylamide gel for 2 h at 4°C. The top strands of the oligonucleotide probes used were: wild-type, 5'-TTTATTCGTGACACGAGGAGCTGCCAGA-3'; mutated, 5'-TTTATTCGGCGAAAGAACAAGCTGAGGAGCTGCCAGA-3'; and Cε, 5'-GATGACCCCTCTTTCACAAGAATCTAC-3' (as used in Ref. 40).

**Results**

**DNA elements in intron 1 mediate IL-4-induced up-regulation of plgR**

To identify DNA elements that mediate IL-4-induced transcriptional up-regulation of plgR, we made luciferase reporter constructs containing putative regulatory sequences of the human plgR gene. Thus, the construct pSC1 contained approximately 2.7 kb of upstream sequences, exon 1, the 5.7-kb large intron 1, and was fused in frame with the luciferase gene at the ATG start codon in exon 2. A second reporter construct, pSC2, contained the same 2.7-kb upstream sequences, exon 1, and exon 2, but lacked the entire intron 1. Transient transfections of these constructs were performed into HT-29.m3 cells that were either left untreated or treated with IL-4 for 24 h. The activity of the intron-containing reporter gene was enhanced more than 7-fold after IL-4 treatment (Fig. 1, upper panel; pSC1), whereas that of the intron-less construct was only marginally enhanced by such treatment (Fig. 1, upper panel; pSC2). Up-regulation of the plgR luciferase reporter construct by IL-4 was hence mediated through DNA elements in intron 1.

**IL-4-mediated up-regulation of plgR and pSC1-luciferase mRNA is blocked by CHX**

The IL-4-mediated up-regulation of endogenous plgR in HT-29.m3 cells has been shown to depend on de novo protein synthesis (10, 11). We therefore tested whether this was true also for IL-4-mediated up-regulation of the reporter construct pSC1 by performing quantitative RT-PCR with mRNA from transfected HT-29.m3 cells. Cells were left untreated or stimulated with IL-4 for 24 h, either in the presence or absence of CHX. Cytoplasmic RNA was isolated from these HT-29.m3 cells, and the levels of different specific mRNAs were quantified by real-time RT-PCR. For each sample, we analyzed message for the endogenous plgR gene, the transfected pSC1 gene, and the housekeeping gene GAPDH as an internal control for mRNA integrity and yield. We found that the mRNA level for endogenous plgR was increased approximately 16-fold after IL-4 treatment, while simultaneous treatment with CHX abolished this up-regulation (Table III). The level of mRNA for pSC1 luciferase was increased approximately 5-fold after 24 h with IL-4 stimulation (Table III). This relatively small induction compared with the endogenous gene could be explained by a higher basal level of transcription of the plasmid-encoded reporter gene, or it could be due to the absence of other important regulatory DNA elements. However, CHX blocked this IL-4-mediated effect, documenting that the pSC1 luciferase reporter construct mimicked the protein synthesis-dependent mechanism of IL-4-mediated up-regulation of the endogenous plgR gene. By contrast, GAPDH expression was only increased approximately 1.4-fold after IL-4 treatment and 1.2-fold after addition of CHX together with IL-4.

**Table II. Primers and cycle parameters for real time RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Product Size (bp)</th>
<th>Annealing Temperature (°C)</th>
<th>Measuring Temperature (°C)</th>
<th>Amplification Coefficient (Ktrans)</th>
</tr>
</thead>
<tbody>
<tr>
<td>plgR</td>
<td>AGAGCAGGGGTTACCAACT</td>
<td>267</td>
<td>62</td>
<td>86</td>
<td>1.92</td>
</tr>
<tr>
<td>pSC1-luc</td>
<td>AGAGCAGGGGTTACCAACT</td>
<td>192</td>
<td>61</td>
<td>82</td>
<td>1.86</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AAATCCCCATCAATGATCCCTCCGATAC</td>
<td>313</td>
<td>60</td>
<td>72</td>
<td>1.68</td>
</tr>
</tbody>
</table>

* The forward and reverse primers, respectively, are given for the genes investigated, together with the product size and annealing temperature of the RT-PCR. The temperature at which the signal was measured and the calculated amplification coefficients (fold increase per cycle) for each gene are given as well. The amplification coefficients are calculated as described in Materials and Methods.

* From Jaehnsen et al. (50).
Multiple elements within a 554-bp region cooperate for maximal IL-4-mediated pIgR induction

To map the localization of the IL-4-responsive element(s) more closely, we sequenced intron 1 of the human pIgR gene (sequence data available from European Molecular Biology Laboratory (EMBL), accession number AJ276452), and made several constructs with sequential internal deletions in this intron. When transiently transfected into HT-29.m3 cells, we found that deletion of bases 847–1855, 1855–4118, or 4866–5163 in the reporter constructs pSC3, pSC4, and pSC9, respectively, did not affect their IL-4-mediated induction (Fig. 1, top panel). However, deletion from position 3464 to 4866 in intron 1 (pSC8) abolished IL-4 responsiveness (Fig. 1, top panel). Therefore, the IL-4-responsive element(s) was localized to a 748-bp fragment between position 4118 and 4866, present in pSC4 but absent in pSC8 (Fig. 1, top panel). For more precise mapping, we made smaller stepwise deletions within this 748-bp region. Reporter constructs pSC16, pSC18, pSC19, and pSC20 lost their ability to be induced by IL-4; all of them lacked a 198-bp fragment indicated in Fig. 1 (lower panel). pSC17 and pSC21, which contained this fragment (bases 4250–4447) with some additional flanking sequences, both showed some IL-4 responsiveness, although reduced compared with the full-length pSC1 (Fig. 1). In contrast, pSC22, which contained 554 bp of the indicated 748-bp fragment, retained almost full IL-4 responsiveness.

The IL-4-late-responsive region of the pIgR gene behaves like a general IL-4-responsive enhancer

To determine whether the identified IL-4-responsive region behaved like a general enhancer, we subcloned a 1.3-kb fragment corresponding to the deleted region in pSC8 (Fig. 1, top panel) upstream of the minimal SV40 promoter in both orientations, and tested the luciferase activity of these reporter constructs (designated p1 and p2, respectively) as above (Fig. 2). The basal viral promoter (designated p0) was unaffected by IL-4 treatment, while both p1 and p2 showed approximately 6-fold induction after IL-4 treatment (Fig. 2). Furthermore, the 554-bp fragment identified in pSC22 (Fig. 1, lower panel) also retained full IL-4 responsiveness when transfected into HT-29.m3 cells (Fig. 3A; p12). Therefore, further studies were based on this plasmid rather than on the longer p1 reporter construct.

Internally in the 554-bp intronic fragment of p12, there was a unique PstI restriction enzyme recognition site; this was used to make a 4-bp deletion that had a dramatic effect on the IL-4-mediated gene induction in transfected HT-29.m3 cells, reducing it to less than 2-fold (Fig. 3A; p18). The same negative effect was seen when this small deletion was introduced into the full-length pSC1 background (pSC23; data not shown). Interestingly, the 4-bp deletion was located immediately downstream of a putative STAT6 site (37, 43, 44).

A consensus STAT6 element is required for IL-4 responsiveness of the pIgR gene

To more precisely identify required DNA element(s) in the proximity of the PstI restriction enzyme recognition site, we made sequential 4-bp mutations in p12 that covered 32 bp of the sequence spanning the putative STAT6 site. Two sets of four point mutations, upstream of the consensus STAT6 site, did not reduce IL-4 responsiveness, while the three sets of point mutations that abolished the STAT6 binding site also abolished IL-4 responsiveness (Fig. 3A). Furthermore, the 4-bp mutation just downstream of the STAT6 site, corresponding to the 4-bp PstI deletion, reduced induction to approximately 2-fold (Fig. 3A). Two mutations further downstream that both mutated a putative C/EBP site (45) had little or no effect on IL-4-mediated induction of the reporter genes (Fig. 3A).

The STAT6 element exerts its effect on the pIgR gene in cooperation with surrounding DNA sequences

To test whether the STAT6 element and the partially overlapping putative C/EBP site were sufficient to confer IL-4 responsiveness, as seen in the germline e promoter (36, 37), we subcloned single or up to four (2-mer or 4-mer) copies of an oligonucleotide containing these two binding sites (indicated in Fig. 3B) upstream of the basal promoter in p0. Neither single nor multimerized copies were sufficient to confer IL-4 responsiveness when transfected into HT-29 cells (data not shown). We also made sequential deletions from either end of the p12 reporter construct and found that a region significantly larger than the STAT6 site was required for IL-4 responsiveness. A 340-bp fragment that contained 214 bp 5′ and 116 bp 3′ of the STAT6 site retained full IL-4 responsiveness (Fig. 3A; p14). However, deleting 102 bp from the 3′ end of this
reporter gene abolished IL-4 responsiveness, despite the fact that this deletion ended 14 bp before the identified STAT6 site (Fig. 3A; p35). Deletion of 195 bp from the 5′ end of p14 reduced IL-4 responsiveness from 5.5-fold to approximately 2.5-fold (Fig. 3A, lower panel, compare p48 with p14).

**IL-4 stimulation induces binding of STAT6 to the intronic STAT6 site of the plgR gene**

To determine whether IL-4 stimulation affected protein-DNA interactions, we isolated nuclear extracts from HT-29.m3 cells treated with IL-4 for various time periods and performed in vitro EMSA experiments with a probe spanning the identified STAT6 site (Fig. 4A). We found that IL-4 induced the formation of two protein-DNA complexes within 10 min that still remained after 24 h, the longest time point investigated (Fig. 4; complex I and II, see arrows). The higher mobility complex (I) peaked after 10 min, then decreased in intensity after 2 h, while the relatively weak lower mobility complex (II) remained stable in intensity over time (Fig. 4A). Competition experiments demonstrated that both complexes were abolished by an excess of wild-type oligonucleotide (Fig. 4, A and B), while an oligonucleotide with a mutated STAT6 site had no effect (Fig. 4B). Furthermore, a consensus STAT6 binding site from the human germline promoter (40) competed to the same extent as the wild-type oligonucleotide (Fig. 4B). Finally, incubation with a polyclonal Ab against STAT6 resulted in a shift for the higher mobility complex (Fig. 4B; complex III), unequivocally documenting that IL-4 induced activation of STAT6 and its binding to this DNA element from intron 1.

**IL-4-mediated up-regulation of the plgR-derived reporter constructs displays delayed kinetics**

IL-4 treatment of HT-29.m3 cells induced STAT6 binding to the plgR intronic enhancer within 10 min, much faster than the time delay for transcriptional activation previously reported (10). Therefore, to further investigate the kinetics of plgR up-regulation, we compared the time-dependent response to IL-4 of two plgR-based reporter constructs, with a previously described synthetic IL-4-responsive promoter, p(1e-IL4RE)4-Luc (46). pSC1 (which contained plgR sequence from −2684 to the start codon at position 5928), p12 (which contained the IL-4-responsive enhancer upstream of an unrelated promoter), or p(1e-IL4RE)4-Luc was transiently transfected into HT-29.m3 cells, which were stabilized with IL-4 for different time periods. The p(1e-IL4RE)4-Luc reporter construct showed no activity above background in unstimulated cells, but was induced rapidly by IL-4, reaching maximal induction after 6 h (Fig. 5). By contrast, the two plgR-derived reporter constructs, pSC1 and p12, demonstrated significantly slower kinetics. Only modest induction was seen after 6 h; maximal induction was not reached until after 24 h of IL-4 treatment (Fig. 5). Thus, the kinetics of the identified IL-4-responsive region from the plgR intron 1 differed substantially from the synthetic multimerized IL-4-responsive enhancer derived from the germline promoter, p(1e-IL4RE)4-Luc.

**STAT6 activation is sufficient to up-regulate plgR transcription in HT-29.m3 cells, but not in Cos-1 cells**

To test whether activation of the STAT6 signaling pathway was sufficient to activate transcription from the plgR IL-4-responsive enhancer region, we cotransfected a conditionally active form of STAT6, the STAT6:ER* fusion protein (40), together with the p12 reporter construct into HT-29.m3 cells. The cells were then either left untreated or treated with 4-HT for 24 h to induce dimerization and activation of the STAT6:ER* fusion protein. The reporter construct p12 was induced by the activated STAT6:ER* fusion protein (Fig. 6A). Thus, activation of the STAT6 signaling pathway was sufficient to provide up-regulation of the p12 reporter construct in HT-29.m3 cells.

To test whether cell type-specific factors were needed for this STAT6-mediated up-regulation of p12, we cotransfected the STAT6:ER* fusion plasmid with either p(1e-IL4RE)4-Luc or p12 into monkey fibroblast Cos-1 cells, and incubated the cells with or without 4-HT for 24 h to activate the STAT6:ER* fusion protein. Its activation was sufficient to up-regulate transcription of the p(1e-IL4RE)4-Luc reporter gene approximately 20-fold (Fig. 6B), but did not provide enhanced expression of p12 in Cos-1 cells (Fig. 6B).

**Discussion**

In this study, we have identified a 554-bp DNA fragment within intron 1 of the human plgR gene that is necessary and sufficient for its IL-4-mediated induction. The resulting slow transcriptional response was mimicked by STAT6 activation alone and shown to depend on complex cooperation of several transcription factors: activated STAT6, a de novo synthesized protein(s), and the presence (or induction) of a cell type-specific factor(s). Thus, STAT6 mediates cell type-specific up-regulation of the plgR gene both directly (by binding to its DNA element in intron 1) and indirectly (by inducing the de novo expression of one or more possibly cell type-specific required factors).

**Table III. IL-4-mediated up-regulation of plgR and pSC1-luciferase mRNA is blocked by CHX**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Induction with IL-4</th>
<th>Fold Induction with IL-4 + CHX</th>
</tr>
</thead>
<tbody>
<tr>
<td>plgR</td>
<td>15.8 ± 1.3</td>
<td>1.7 ± 0.04</td>
</tr>
<tr>
<td>pSC1</td>
<td>5.1 ± 1.0</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1.4 ± 0.3</td>
<td>1.2 ± 0.04</td>
</tr>
</tbody>
</table>

* Calculation of fold induction was performed as described in Materials and Methods. Data are the average of three independent experiments ± SD.
Mapping of the IL-4-responsive region

We identified an IL-4-responsive enhancer within intron 1, and found this region necessary and sufficient to mediate gene induction by IL-4, both in the context of the plgR promoter and a heterologous promoter. Location of this IL-4-responsive region 4.1 kb downstream of the transcriptional start site opens for the possibility that the regulatory element(s) functions at the RNA level. This mode of up-regulation has been demonstrated for other genes, such as the TAT-mediated activation of HIV (long terminal repeat-directed) transcription (47, 48). However, because the enhancer region still conferred the same degree of IL-4 responsiveness when situated upstream of a heterologous promoter in either orientation, the role of RNA elements could be eliminated.

Further mapping of the IL-4-responsive region was performed in this heterologous context by the use of sequential deletions, or with specific point mutations. The IL-4 responsiveness was abolished by mutations affecting the consensus STAT6 site, because such mutations also reduced the IL-4 responsiveness significantly, indicating that this putative C/EBP site is not required for the IL-4-responsive enhancer in the plgR gene. A similar observation has been reported for an IL-4-responsive enhancer in the mouse CD23 (FcεRII) promoter, in which mutation of a STAT6 site abolished IL-4-mediated up-regulation, while an adjacent C/EBP site was not required for IL-4 responsiveness (34, 35). In addition, our observation that neither a single copy nor multimerized copies of an oligonucleotide containing the STAT6 site and the putative C/EBP site from the plgR gene were sufficient to confer IL-4 responsiveness to a basal promoter suggested that this was not a functional C/EBP site (as compared with the C/EBP site in the multimerized STAT6-C/EBP site from the germline ε promoter, p[IL-4RE]ε-Luc). In support of this, the putative C/EBP site was not conserved in the mouse, as judged from analysis of transcription factor databases (45). While the STAT6 site and two flanking bases on both sides were 100% conserved (Fig. 3B). However, participation of C/EBP in the observed IL-4-mediated up-regulation of plgR could not be completely ruled out because another putative C/EBP site is located approximately 70 bp upstream of the STAT6 site within the 554-bp intronic fragment required for complete responsiveness. However, it is beyond the scope of this article to analyze all putative transcription factor-binding sites identified by searches against database matrices.

We found that a 214-bp deletion of the 3’ end of the 554-bp fragment did not affect the IL-4 responsiveness significantly (Fig. 3A; comparing p14 with p12), while deleting the same bp in the context of the complete intron reduced induction slightly, possibly due to positional or context-dependent effects (Fig. 1; compare pSC21 and pSC22). However, we found that more than 14 bp 3’ of
the STAT6 site was required for IL-4 responsiveness and that more than 17 bp upstream was needed for maximal induction. Taken together, these findings suggested that the identified STAT6 site needs to cooperate with DNA elements located both upstream and downstream to confer full IL-4 responsiveness of the pIgR gene.

Role of STAT6 in pIgR gene induction

Using EMSA with nuclear extracts from HT-29 cells, we identified two IL-4-inducible complexes that bound the pIgR STAT6 site from pIgR intron 1, as used as a labeled probe. A, IL-4 treatment for different time periods (10 min-24 h) induced two complexes (I and II) indicated with arrows. A 100-fold molar excess of unlabeled wild-type (wt) oligonucleotide was added where indicated. B, Oligonucleotide competition experiment with 10- or 100-fold molar excess (Fxs) of the cold competitors (cc): wild-type (wt), mutated STAT6 site (m), or a consensus STAT6 site (37) (c). Lanes 8–14 were run at 4°C with nuclear extracts from unstimulated (lane 8) or IL-4-stimulated cells (lanes 9–14), with addition of cold competitors as indicated, and addition of polyclonal STAT6 Ab (lanes 12–14). The appearance of a supershifted complex III is indicated by arrow (lanes 12 and 14).

FIGURE 4. IL-4 treatment induces binding of STAT6 to the required DNA element in intron 1 of the human pIgR gene. EMSA experiments were performed with nuclear extracts from HT-29.m3 cells stimulated with IL-4 for the indicated time periods. A 24-bp fragment, spanning the STAT6 site from pIgR intron 1, was used as a labeled probe. A, IL-4 treatment for different time periods (10 min-24 h) induced two complexes (I and II) indicated with arrows. A 100-fold molar excess of unlabeled wild-type (wt) oligonucleotide was added where indicated. B, Oligonucleotide competition experiment with 10- or 100-fold molar excess (Fxs) of the cold competitors (cc): wild-type (wt), mutated STAT6 site (m), or a consensus STAT6 site (37) (c). Lanes 8–14 were run at 4°C with nuclear extracts from unstimulated (lane 8) or IL-4-stimulated cells (lanes 9–14), with addition of cold competitors as indicated, and addition of polyclonal STAT6 Ab (lanes 12–14). The appearance of a supershifted complex III is indicated by arrow (lanes 12 and 14).

oligonucleotide centered around the STAT6 site, spanning only 24 bp. Conversely, in the endogenous pIgR gene, as well as in the pIgR-derived reporter constructs, large flanking sequences would allow for other transcription factors to bind, thereby stabilizing the binding of the IL-4-induced complex(es). It has been shown in vitro that binding of C/EBP to a DNA element adjacent to a
STAT6 site (in the germline ε promoter) stabilized the binding of STAT6 by decreasing the dissociation rate (49). A similar mechanism of transcription factor cooperation is likely to occur also for other factors that bind in the proximity of STAT6 in other genes.

The JAK-STAT6 pathway is an immediate early response, whereas the IL-4-mediated transcriptional up-regulation of plgR depends on de novo protein synthesis. We found that two key plgR reporter constructs had similar slow-response kinetics, reaching a maximum luciferase level after approximately 24 h. This was significantly different from the early responsive enhancer derived from the germline ε promoter, which gave a more rapid response to IL-4, reaching a maximum approximately 6 h after stimulation. Thus, the plgR-derived reporter constructs required other cellular events in addition to STAT6 activation for their induction by IL-4.

The finding that activation of a conditionally active STAT6:ER* fusion protein was sufficient to up-regulate the plgR-derived enhancer construct (p12) to the same extent as IL-4 in HT-29 cells suggested that IL-4 mediated its effects on the plgR gene mainly through the STAT6 signaling pathway. However, when we quantified plgR and reporter gene mRNA in unstimulated cells and in IL-4-induced HT-29 cells, the IL-4 responsiveness of both the endogenous plgR gene and the reporter gene (pSCL1) was shown to be CHX sensitive. This agreed with previous studies, which demonstrates that IL-4 induction of this gene depends on de novo protein synthesis (10, 11). Most likely, therefore, STAT6 has a dual role in the IL-4-mediated up-regulation of plgR: directly by binding to its DNA element in the IL-4-responsive region in intron 1, and indirectly by inducing de novo synthesis of a required protein (Fig. 7). This protein could be a transcription factor or a coactivator that cooperates with STAT6 to enhance the transcription rate. Alternatively, it might be an enzyme or some other protein that modulates transcription through its effect on other DNA-bound factors.

We found that activation of STAT6 was sufficient to up-regulate the plgR-derived enhancer construct (p12) in HT-29 cells, but not in the monkey fibroblast Cos-1 cell line, suggesting that the plgR-derived IL-4-responsive enhancer is cell-type specific. The required lineage-specific transcription factor(s) might be a constitutively expressed protein, or it might be the de novo synthesized protein(s) failing to be induced by STAT6 in Cos-1 cells. The fact that activation of STAT6 was sufficient to up-regulate the p(1-IL4RE)2-Luc reporter gene in Cos-1 cells, but not the plgR-derived reporter gene, may in the future be exploited in a search for this required factor by complementation experiments.

In conclusion, we have shown that a consensus STAT6 binding site within intron 1 of the human plgR gene is absolutely necessary for its IL-4-mediated up-regulation. Nevertheless, this DNA element was not sufficient, but depended on cooperation with other DNA elements located both upstream and downstream, thus suggesting a complex transcription factor cooperation involving several different DNA elements. Furthermore, the requirement for cell type-specific factors and de novo protein synthesis suggested a mechanism of IL-4 induction similar in principle to that recently described for CD23 in lymphoid cells (38). The mechanism of IL-4-mediated up-regulation of CD23 in B cells, and of plgR in mucosal epithelial cells may hence constitute an emerging paradigm for how pleiotropic signaling substances exert differential transcriptional responses in distinct tissue compartments. Molecular characterization of the additional factor(s) required for plgR gene induction, as well as more detailed description of the target DNA elements involved, will be required to further understand how IL-4 mediates enhanced plgR expression in secretory epithelia. IL-4 may thus increase the external transport of secretory Abs during infections that provoke a type 2 Th cell response, and at the same time stimulate plgA production by mucosal B cells.

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References

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