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Expression of Murine IL-12 Is Regulated by Translational Control of the p35 Subunit

Jennifer M. Babik, Elizabeth Adams, Yukiko Tone, Paul J. Fairchild, Masahide Tone, and Herman Waldmann

IL-12 is a heterodimer of two subunits, p35 and p40, encoded by separate genes that are regulated independently. To investigate the mechanisms underlying the regulation of the p35 gene, we characterized murine p35 expression in the B cell lymphoma line A20 and in bone marrow-derived dendritic cells. Multiple transcription start sites were identified in both cell types, resulting in four p35 mRNA isoforms (types I–IV) that differ in the number and position of upstream ATGs in their 5′ untranslated regions. In nonstimulated cells, the predominant forms of p35 message (types II and IV) contained an additional upstream ATG, whose presence was shown to inhibit the downstream translation of the p35 subunit. After LPS stimulation, however, transcription initiated from alternate positions, so that the proportion of transcripts not containing this upstream ATG (types I and III) was significantly increased in the population of p35 mRNA. These type I and type III transcripts readily supported translation of the p35 subunit and its incorporation into bioactive IL-12. Furthermore, p35 mRNA levels were substantially up-regulated after LPS stimulation in both cell types. Thus, our results show that p35 gene expression is highly regulated by both transcriptional and translational mechanisms. The Journal of Immunology, 1999, 162: 4069–4078.

Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

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2 Address correspondence and reprint requests to Dr. Masahide Tone, Sir William Dunn School of Pathology, South Parks Rd., Oxford, United Kingdom OX1 3RE. E-mail address: mtone@molbio1.ox.ac.uk

3 Abbreviations used in this paper: UTR, untranslated region; bmDC, bone marrow-derived dendritic cells; RACE, rapid amplification of complementary deoxyribonucleic acid ends; EF-1α, elongation factor-1α; ORF, open reading frame; aa, amino acids; HPRT, hypoxanthine phosphoribosyl transferase.
Materials and Methods

Cell culture
A20 and RAW 264 cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 5% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. The bmdC were prepared from CBA/Ca mice based on the method of Inaba et al. (27) with minor modifications. Isolated bmdC were 90–95% pure, as determined by expression of the dendritic cell marker CD11c. When required, A20, RAW 264, and bmdC were stimulated with LPS (20 μg/ml) for 42 h (luciferase assay), 24 h (primer extension assay, Northern blot hybridization, IL-12 bioassay), or 18 h (RACE, RT-PCR).

Primer extension assay

Primer extension was performed on poly(A)+ RNA (20 μg) from A20 cells using the following 5'-labeled antisense primers: primer 1, GCC CAA CTG GAG GAG GAG GAG; primer 2, GAC TTG GAG CAC TTG GAG; or primer 3, GAC GG CAG GAC CAT. These primers correspond to sequences in exon 3; primer 2, GAC CT TCC GAG ACT GTG TCG TGC; or primer 3, GAC GGT TTT GAC TAT GAT ATG. Corresponding fragments were amplified on a genomic template. All amplified products were purified primer extension products were separated on a 6-M urea/6% polyacrylamide gel in parallel with sequencing ladders prepared using the same primers.

Construction of promoter and p35 5′-UTR-containing luciferase reporter plasmids

The Pro1 and Pro3 promoter fragments were made by amplification of a 0.81-kb fragment (Pro1, −458 to +354 in Fig. 2A) or a 0.72-kb fragment (Pro3, −458 to +265 in Fig. 2A) by PCR. The Pro2 promoter fragment was made by mutation of the ATG codon (+288 to +290 in Fig. 2A) to an ATC in the Pro1 fragment by PCR. Amplified fragments were introduced into the pG3-Enhancer Vector (Promega, Madison, WI) upstream of the luciferase gene.

The p35 5′ UTR fragments were made by PCR amplification using the antisense primer P33 (CGG TGC CAG CCA GTG TGT GC GAC ACC GAC ACT GAG GAG GAG) for fragments pII, pIIa, pIV, pIIa, pIII, or pIIIC or the antisense primer P33F (CGG TGC CAG CCA GTG TGT GC GAC ACC GAC ACT GAG GAG) for fragments pIII, pIIIb, pIIIc, or pIVa. These primers correspond to +340 to +375 in Fig. 2A and contain a NcoI site (underlined). Primer P33F contains a −1 frame shift at +356. The sense primers used were as follows: pII, GACAAACCTTAAAATGTTGTCTCAGG AGT GAC; or pIIa, GACAAACCTTAAAATGTTGTCTCAGG AGT GAC; or pIII, GACAAACCTTAAAATGTTGTCTCAGG AGT GAC; or pIIIb, GACAAACCTTAAAATGTTGTCTCAGG AGT GAC; or pIIIc, GACAAACCTTAAAATGTTGTCTCAGG AGT GAC; or pIVa, GACAAACCTTAAAATGTTGTCTCAGG AGT GAC. Corresponding fragments were introduced downstream of the p35 I-IV, p35 II/IV, and p35 III fragments.

Primer extension analysis in the murine B cell line A20 has identified two promoters, one upstream of exon 1 and one upstream of exon 2. Promoter activity has been detected in a 1.8-kb fragment containing both putative promoters (26). To further characterize this promoter region, we analyzed the 5′ flanking region of exon 2 (29). Amplified products were analyzed by Southern blot hybridization using 3′-labelled cDNA probes. Transcript levels were analyzed by phosphorimaging and autoradiography.

Results

Transcriptional regulation is involved in the control of IL-12 p35 expression

We previously mapped the p35 transcription start sites in the murine macrophage cell line WEHI-3D by 5′ RACE (25). Start sites were located in both exon 1, a 5′ noncoding exon, and in exon 2, which contains the ATG that encodes the first methionine of p35. Primer extension analysis in the murine B cell line A20 has identified four major transcription start sites in the intron region directly upstream of exon 2 (26). Taken together, these mapping studies suggest that p35 transcription might be driven by two promoters, one upstream of exon 1 and one upstream of exon 2. Promoter activity has been detected in a 1.8-kb fragment containing both putative promoters (26). To further characterize this promoter region, we analyzed the 5′ flanking region of exon 2 (29). Amplified products were analyzed by Southern blot hybridization using 3′-labelled cDNA probes. Transcript levels were analyzed by phosphorimaging and autoradiography.

Northern blot hybridization

Total RNA (10 μg) from the indicated cells was separated on a 1% formaldehyde/agarose gel, transferred to a nylon membrane, and subjected to hybridization using 3′-labelled p35 or hypoxanthine phosphoribosyltransferase (HPRT) cDNA probes. Transcript levels were analyzed by phosphorimaging (Molecular Dynamics, Sunnyvale, CA) and autoradiography.

IL-12 bioassay

The bioassay for IL-12-induced IFN-γ production was performed as previously described (26), using splenocytes from CBA/Ca mice and the mAbs C15.6.7 and C15.1.2 for capture of IL-12 and, when required, the mAb C17.8 for neutralization. IFN-γ production was measured by ELISA. IL-12 mAbs were a gift from Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA).

Rapid amplification of cDNA ends (RACE)

RACE was performed as previously described (25) but with the following modifications. First-strand cDNA was synthesized using the SMART cDNA Library Construction Kit (Clontech, Palo Alto, CA) according to the manufacturer’s specifications. The sense primer used in semi-nested PCR was a modified SMART primer containing an XbaI site (underlined): CAGTCTA GACGGCTGAGGAGGAAGCGACAAA. The semi-nest antisense primers both correspond to sequences located in exon 3 and have been previously described (25).

Semiquantitative RT-PCR

PCR amplification was performed for 18 cycles (p40, HPRT, p35 II/IV), 22 cycles (p35 I-IV), or 34 cycles (p35 III). These cycle numbers were experimentally determined to be the linear range of amplification and detection for all cDNAs tested. The p35 I-IV, p35 II/IV, and p35 III fragments were amplified using the sense primers P40:GGACCT CACGACACCTATCGTACAG, corresponding to +328 to +352 in Fig. 2A); P35II/IV (GGAAAGCTCGTACGGCTACTCCGAG, corresponding to +253 to +277 in Fig. 2A); or P35III (GGAAAGCTCGTACGGCTACTCCGAG, corresponding to +247 to +450 in Fig. 2A). Amplified products were analyzed by Southern blot hybridization using 3′-labelled cDNA probes. Transcript levels were analyzed by phosphorimaging and autoradiography.
Before the first ATG of p35 was introduced upstream of the luciferase reporter gene in the pGL3-Basic Vector and assayed for luciferase activity in A20 cells under nonstimulated and LPS-stimulated conditions. No promoter activity was detected using this reporter construct, even after LPS stimulation (data not shown). Since p35 transcripts are usually expressed at low levels (1), it is likely that the p35 promoter is relatively weak. Therefore, we inserted the same 0.81-kb fragment into the pGL3-Enhancer Vector, which contains an SV40 enhancer downstream of the luciferase gene. Promoter activity was assayed using this construct (Pro1) and compared with levels produced using pGL3-Enhancer Vector alone. Surprisingly, again no promoter activity was detected in this fragment, even after LPS stimulation (Fig. 1).

Since p35 mapping studies indicate that there are major transcription start sites in intron 1 and exon 2, we would expect that promoter activity would be located in the 5′ flanking region of these start sites. Therefore, in light of the fact that no promoter activity could be detected in this region, we analyzed the DNA sequence of our promoter fragment for possible regulatory elements. Interestingly, we identified two additional ATGs upstream of the ATG that encodes the first methionine of p35 (designated ATG1) (Fig. 2A). One ATG (designated ATG2) is located 74 bp upstream of ATG1, and the other (designated ATG4) is located 148 bp upstream of ATG1. Since upstream ATGs located in the 5′ UTR often inhibit translation from a downstream ATG (30, 31), we predicted that, given transcription of the luciferase mRNA (containing the p35 5′ UTR) driven by the p35 promoter fragment, the presence of these additional ATGs might inhibit downstream translation from the first ATG of luciferase. Transcription initiation sites in A20 were mapped upstream of ATG4 by primer extension (26), indicating that ATG2 and ATG4 are present in the p35–5′ UTR/luciferase mRNA transcribed from the Pro1 reporter plasmid. To examine the effects of these upstream ATGs, we constructed two additional reporter plasmids using the pGL3-Enhancer Vector: Pro2 was made by mutating ATG2 in the Pro1 fragment to an ATC codon, and Pro3 was made by deleting a 90-bp region (containing ATG2) from the 3′ end of the Pro1 promoter fragment. In nonstimulated and LPS-stimulated A20 cells, both reporter plasmids exhibited fivefold greater luciferase activity than that observed with the pGL3-Enhancer Vector alone (Fig. 1). Mutating ATG4 in the Pro2 construct to an ATC codon did not result in a further increase in luciferase activity (data not shown). These results suggest that promoter activity is located upstream of exon 2, and that the presence of ATG2 in the p35–5′ UTR/luciferase mRNA inhibits downstream translation of the luciferase protein. Furthermore, in the 90-bp region deleted from Pro1 to Pro3, a typical TATA box sequence (TATAAAA) is present. Deletion of this 90-bp region in Pro3 did not result in a decrease in promoter activity, suggesting that p35 transcription from upstream of exon 2 is controlled by a TATA-less promoter.

Multiple transcription start sites result in the production of different mRNA isoforms

Given that ATG2 can inhibit translation from the first ATG in the luciferase gene, it seemed likely that ATG2 could also inhibit translation from the first ATG in the p35 gene. It was important, therefore, to further analyze the different isoforms of p35 for the presence of ATG2 and/or other upstream ATGs. From previous 5′ mRNA mapping studies (25, 26), four possible mRNA isoforms can be defined based on the position and number of ATGs upstream of ATG1 in the 5′ UTR (Fig. 2, A and B). We have previously defined three of these isoforms (25): type I transcripts initiate from exon 2 and contain no additional ATG sequences; type II transcripts initiate from intron 1 and contain ATG2; and type III transcripts initiate from exon 1 and contain an additional ATG, designated ATG3, located 63 bp upstream of ATG1. Based on the primer extension results reported for the A20 cell line (26), we now define a new type of mRNA isoform, the type IV transcript, which initiates further upstream in intron 1 and contains ATG2 and ATG4. The open reading frames (ORFs) initiated by each upstream ATG are shown in Fig. 2C. ATG1 encodes the first methionine of the p35 protein. ATG2 is out-of-frame relative to ATG1 and initiates an ORF of 40 amino acids (aa), which terminates downstream of ATG1. ATG3 is in-frame relative to ATG1 and so initiates an ORF containing an additional 21 aa at the N-terminus of the p35 protein. ATG4 is followed directly by a stop codon, and so initiates an ORF of only 1 aa.

In the primer extension assay used to identify the type IV transcripts in A20 cells (26), the primer binding region was located upstream of ATG2 in a position unsuitable for the detection of type I, II, and III transcripts. To determine whether these transcripts were synthesized in A20 cells, we performed primer extension analysis using three different primers on mRNA from nonstimulated and LPS-stimulated A20 cells (Fig. 3). The binding regions of these three primers are shown in Fig. 3C. The binding region of primer 1 was located in exon 3 for detection of all isoforms of p35 mRNA, the binding region of primer 2 was located upstream of ATG2 for detection of both type II and type IV transcripts, and the binding region of primer 3 spanned the exon 1/exon 2 splice junction for detection of only type III transcripts.

Using primer 3, no extension products were detected in A20 cells, suggesting that type III transcripts are not a significant fraction of the p35 mRNA population in these cells. Using primer 1, extension products in both nonstimulated and LPS-stimulated A20 cells were located 215, 234, and 331 bp from the 5′ end of the primer (Fig. 3A). Since no extension products could be detected in exon 1 using primer 3, it was assumed that these transcription start sites were located in intron 1. The 331-bp extension product was the major start site detected using this primer. Using primer 2, this major start site was again identified as well as an additional start site of 215 and 234 bp.
site (showing the same band intensity) located 94 bp upstream (Fig. 3B). These results suggest that the two more 5' start sites are the major sites of p35 transcription initiation in A20 cells. These are designated +1 and +95, respectively, with the more 3' start sites identified using primer 1 numbered accordingly (+192 and +211). The precise locations of these start sites are shown in Fig. 2A. Our primer extension results thus confirm the location of start sites identified previously (26) and, furthermore, indicate that in both nonstimulated and LPS-stimulated A20 cells, p35 transcription occurs mainly from upstream of ATG4. Therefore, the type IV transcript (containing ATG2 and ATG4) is the predominant form of p35 message in this cell line.

The presence of ATG2, but not ATG3 or ATG4, in the 5' UTR of p35 transcripts inhibits translation from ATG1

Our results to date suggest that the presence of ATG2 in a transcript can inhibit translation from a downstream ATG, and that ATG2 is present in the 5' UTR of a high proportion of p35 transcripts. To further define the role of ATG2 and the other upstream ATGs in p35 expression, we used a luciferase reporter system similar to that used in our promoter assay to measure the effect of these multiple ATGs on translation from ATG1. The p35 cDNA fragments containing ATG1 and upstream ATG sequences were introduced upstream of the luciferase reporter gene under control of the SV40 promoter in the pGL3-Control Vector. The structure of reporter plasmids is shown in Fig. 4. The first ATG of luciferase was "replaced" by the ATG1 of p35 in all reporter plasmids. To determine the effect of ATG2 and ATG4 on translation from ATG1, these upstream ATGs were, in some constructs, mutated to ATC codons either independently or in combination. Because ATG3 is in-frame with ATG1, translation from this upstream ATG would introduce an additional 21 aa at the N-terminus of the luciferase protein. Luciferase proteins with these additional 21 aa might retain activity, and so a detectable difference in luciferase activity might not be observed as a result of translation from ATG3. Thus, to assay the efficiency of translation from ATG3 we introduced a 21 frame shift upstream of ATG1 so that, as in constructs containing ATG2, translation from ATG3 is out-of-frame with ATG1. Reporter plasmids were transiently transfected into both A20 cells and the murine macrophage cell line RAW 264 and
assayed for luciferase activity under nonstimulated and LPS-stimulated conditions (Fig. 4). Luciferase levels were measured relative to the level of luciferase produced by the pGL3-Control Vector, which contained the SV40 promoter but no p35 5' UTR insert.Reporter plasmids containing ATG2 (Fig. 4; pII, pIV, and pIVa) produced little or no luciferase activity in all cell types analyzed. When ATG2 was mutated to an ATC codon in these plasmids (Fig. 4; pIIa, pIVb, and pIVc, respectively), luciferase activity was restored to the level in the pGL3-Control. Thus, the presence of ATG2 inhibits translation from ATG1, and luciferase production is rescued by the mutation of this ATG to an ATC. Mutating ATG4 to an ATC codon had no effect on luciferase activity in any plasmid tested (Fig. 4; pIV-pIVc). Similarly, the presence or the absence of ATG3 had no effect on luciferase activity (Fig. 4; pIII-pIIIc), even when in combination with a −1 frame shift (Fig. 4; pIIIa and pIIIb). Taken together, these results indicate that in both nonstimulated and LPS-stimulated cells, the presence of ATG2, but not ATG3 or ATG4, inhibits translation from ATG1 in p35–5' UTR/luciferase mRNAs. This strongly suggests that only the type

FIGURE 3. Mapping of p35 transcription start sites by primer extension. Twenty micrograms of poly(A) RNA from nonstimulated and LPS-stimulated A20 cells or 20 μg of yeast transfer RNA as a negative control were analyzed by primer extension using primer 1 (A), located in exon 3, or primer 2 (B), located in intron 1 between ATG2 and ATG4. Sequencing ladders were prepared using the same primers and were run in parallel with primer extension products. The positions of transcription start sites are denoted by arrows. The most 5' major start site is defined as +1. C. The relative locations of primers, transcription start sites, and upstream ATGs are shown.

FIGURE 4. Luciferase assay measuring the effect of upstream ATGs on translation from ATG1 in the p35 gene. Luciferase reporter plasmids were constructed by the insertion of p35 5' UTR fragments into the pGL3-Control Vector upstream of the luciferase gene. A20 and RAW 264 cells were transiently transfected with 20 μg of reporter plasmid and, when required, stimulated with LPS after transfection. Firefly luciferase levels were normalized to internal control Renilla luciferase values, and compared with the levels of luciferase produced using pGL3-Control Vector alone. Background luciferase levels were measured using pGL3-Basic Vector (no promoter) alone. Results are presented as the mean value of at least three experiments.
in the RAW 264 cell line, which after LPS stimulation produces constitutive EF-1 α mammalian expression vector pOXCD59neo under control of the ATG1 to GCG mutation (Fig. 5; 35.IIa) were introduced into the (Fig. 5; 35.I), ATG1 and ATG3 (Fig. 5; 35.III), or ATG3 and an S5 mutant type III p35 transcript. The entire p35 coding region and 5' UTR containing ATG1 alone (Fig. 5, 35.I), ATG1 and ATG2 (Fig. 5; 35.II), ATG1 and ATG3 (Fig. 5; 35.III), or ATG3 and an ATG1 to GCG mutation (Fig. 5; 35.IIIa) were introduced into the mammalian expression vector p0XCD59neo under control of the constitutive EF-1α promoter. Stable transfectants were generated in the RAW 264 cell line, which after LPS stimulation produces high levels of p40 mRNA but little or no detectable p35 mRNA. Expression levels of the p35 transgene were similar in all transfectants and undetectable in untransfected RAW 264 cells, as measured by Northern blot hybridization (Fig. 5A) and phosphorimager analysis (data not shown). Supernatants from LPS-stimulated transfectants and untransfected RAW cells were tested in an IL-12 bioassay for the ability to induce IFN-γ production from spleen cells (Fig. 5B). Untransfected RAW cells did not induce any IFN-γ production, consistent with the fact that endogenous p35 levels in this cell line are too low to contribute to IL-12 production. Only the supernatants from transfectants 35.I and 35.III were capable of inducing high levels of IFN-γ. This IFN-γ induction was a result of the presence of bioactive IL-12 in supernatants, since IFN-γ production was completely abrogated by the addition of a neutralizing anti-IL-12 mAb. This confirmed our previous findings that the presence of ATG2 in p35 mRNA almost completely inhibits translation from ATG1. In contrast, the presence of ATG3 in transfectant 35.III had no effect on the production of p35, since IL-12 levels in this transfectant were similar to those secreted by transfectant 35.I. This was consistent with our luciferase assay result, which indicated that no translation occurred from ATG3. There was a small possibility that in transfectant 35.III, translation initiated from ATG3 but that the additional 21 aa at the N-terminus of the p35 protein had no effect on p35 secretion/function. To exclude this possibility, ATG1 was mutated to an alanine codon (GCG) in construct 35.I to make construct 35.IIa. No IL-12 activity was detected in the supernatant from this transfectant, confirming our previous observation that initiation of translation does not occur from ATG3.

I and type III transcripts (those not containing ATG2) are capable of supporting translation of the p35 subunit. To confirm these results, we examined the production of bioactive IL-12 from transfectants containing type I, type II, type III, or a mutant type III p35 transcript. The entire p35 coding region and 5' UTR containing ATG1 alone (Fig. 5, 35.I), ATG1 and ATG2 (Fig. 5; 35.II), ATG1 and ATG3 (Fig. 5; 35.III), or ATG3 and an ATG1 to GCG mutation (Fig. 5; 35.IIIa) were introduced into the mammalian expression vector p0XCD59neo under control of the constitutive EF-1α promoter. Stable transfectants were generated in the RAW 264 cell line, which, after LPS stimulation produces high levels of p40 mRNA but little or no detectable p35 mRNA. Expression levels of the p35 transgene were similar in all transfectants and undetectable in untransfected RAW 264 cells, as measured by Northern blot hybridization (Fig. 5A) and phosphorimager analysis (data not shown). Supernatants from LPS-stimulated transfectants and untransfected RAW cells were tested in an IL-12 bioassay for the ability to induce IFN-γ production from spleen cells (Fig. 5B). Untransfected RAW cells did not induce any IFN-γ production, consistent with the fact that endogenous p35 levels in this cell line are too low to contribute to IL-12 production. Only the supernatants from transfectants 35.I and 35.III were capable of inducing high levels of IFN-γ. This IFN-γ induction was a result of the presence of bioactive IL-12 in supernatants, since IFN-γ production was completely abrogated by the addition of a neutralizing anti-IL-12 mAb. This confirmed our previous findings that the presence of ATG2 in p35 mRNA almost completely inhibits translation from ATG1. In contrast, the presence of ATG3 in transfectant 35.III had no effect on the production of p35, since IL-12 levels in this transfectant were similar to those secreted by transfectant 35.I. This was consistent with our luciferase assay result, which indicated that no translation occurred from ATG3. There was a small possibility that in transfectant 35.III, translation initiated from ATG3 but that the additional 21 aa at the N-terminus of the p35 protein had no effect on p35 secretion/function. To exclude this possibility, ATG1 was mutated to an alanine codon (GCG) in construct 35.I to make construct 35.IIa. No IL-12 activity was detected in the supernatant from this transfectant, confirming our previous observation that initiation of translation does not occur from ATG3.

The proportion of type I transcripts in the p35 mRNA population is up-regulated after LPS stimulation

Our bioassay results suggest that biologically significant amounts of the p35 subunit and IL-12 heterodimer can only be produced when type I and type III transcripts are present. By primer extension assay, however, we have shown that in both nonstimulated and LPS-stimulated A20 cells, the major type of p35 mRNA synthesized is the type IV transcript. This apparent paradox can be explained if we consider the possibility that minor start sites that represent transcripts not containing ATG2 may have been missed when type I and type III transcripts (those not containing ATG2) are capable of supporting translation of the p35 subunit. To confirm these results, we examined the production of bioactive IL-12 from transfectants containing type I, type II, type III, or a mutant type III p35 transcript. The entire p35 coding region and 5' UTR containing ATG1 alone (Fig. 5, 35.I), ATG1 and ATG2 (Fig. 5; 35.II), ATG1 and ATG3 (Fig. 5; 35.III), or ATG3 and an ATG1 to GCG mutation (Fig. 5; 35.IIIa) were introduced into the mammalian expression vector p0XCD59neo under control of the constitutive EF-1α promoter. Stable transfectants were generated in the RAW 264 cell line, which, after LPS stimulation produces high levels of p40 mRNA but little or no detectable p35 mRNA. Expression levels of the p35 transgene were similar in all transfectants and undetectable in untransfected RAW 264 cells, as measured by Northern blot hybridization (Fig. 5A) and phosphorimager analysis (data not shown). Supernatants from LPS-stimulated transfectants and untransfected RAW cells were tested in an IL-12 bioassay for the ability to induce IFN-γ production from spleen cells (Fig. 5B). Untransfected RAW cells did not induce any IFN-γ production, consistent with the fact that endogenous p35 levels in this cell line are too low to contribute to IL-12 production. Only the supernatants from transfectants 35.I and 35.III were capable of inducing high levels of IFN-γ. This IFN-γ induction was a result of the presence of bioactive IL-12 in supernatants, since IFN-γ production was completely abrogated by the addition of a neutralizing anti-IL-12 mAb. This confirmed our previous findings that the presence of ATG2 in p35 mRNA almost completely inhibits translation from ATG1. In contrast, the presence of ATG3 in transfectant 35.III had no effect on the production of p35, since IL-12 levels in this transfectant were similar to those secreted by transfectant 35.I. This was consistent with our luciferase assay result, which indicated that no translation occurred from ATG3. There was a small possibility that in transfectant 35.III, translation initiated from ATG3 but that the additional 21 aa at the N-terminus of the p35 protein had no effect on p35 secretion/function. To exclude this possibility, ATG1 was mutated to an alanine codon (GCG) in construct 35.I to make construct 35.IIa. No IL-12 activity was detected in the supernatant from this transfectant, confirming our previous observation that initiation of translation does not occur from ATG3.
The schematic drawing of the p35 5’ region (numbering as in Fig. 2A).

FIGURE 6. Determination of the proportion of type I and type II/IV transcripts in the p35 mRNA population by 5’ RACE. mRNAs from non-stimulated and LPS-stimulated A20 and bmDC were reverse transcribed using the cap-dependent SMART cDNA synthesis method. The p35 5’ end was then amplified from first-strand cDNA by semi-nested PCR using p35-specific primers. To reduce the likelihood that the results were biased by PCR amplification, two independent experiments were performed on the same mRNA sample. Multiple transcription start sites were identified in all cell types and were classified based on their positions relative to ATG2. The number of clones starting upstream of ATG2 (type II/IV transcripts, nonfunctional mRNA for p35 translation) and downstream of ATG2 (type I transcripts, functional mRNA for p35 translation) in each cell type are shown. The relative number of type I transcripts is shown in parentheses. The sequence range spanned by each cluster of start sites is indicated above the schematic drawing of the p35 5’ region (numbering as in Fig. 2A).

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FIGURE 7. Semiquantitative RT-PCR analysis of p35 mRNA populations. To characterize the p35 mRNA populations from nonstimulated and LPS-stimulated A20 and bmDC, sense primers were chosen whose binding regions were located upstream of ATG1 to identify all forms of p35 transcripts (p35 I–IV), upstream of ATG2 to identify only type II or type IV transcripts (p35 II/IV), or upstream of ATG3 to identify only type III transcripts (p35 III). mRNA levels of p40 and HPRT were also determined. Thermal cycling was performed for 18 cycles (p40, HPRT, p35 II/IV), 22 cycles (p35 I–IV), or 34 cycles (p35 III). Amplified products were analyzed by Southern blot hybridization using 32P-labeled cDNA probes. The results shown are representative of at least three experiments.

or type IV transcripts). After LPS stimulation, however, the proportion of type I transcripts was up-regulated to 7.0% of the p35 mRNA population. In nonstimulated bmDC, 1.7% of transcripts initiated from downstream of ATG2, and this was up-regulated to 16.4% after LPS stimulation. Some of these transcripts, particularly type I transcripts, were not previously detected in A20 cells because 5’ mapping in this cell line was performed using the less sensitive primer extension method. These results indicate that in both A20 and bmDC, LPS stimulation up-regulates the proportion of type I transcripts (functional mRNA for p35 translation) within the p35 mRNA population.

Three start sites downstream of ATG2 were detected, and these were located at +311, +330, and +347 (open arrowheads in Fig. 2A). These three start sites correlate with several bases of the three exon 2 start sites identified in the WEHI-3D cell line in our previous study (25).

p35 transcription can initiate from exon 1 after LPS stimulation

We had previously identified the presence of type III transcripts in WEHI-3D cells (25), yet by RACE 5’ mapping in A20 and bmDC, no transcripts of this type were detected. We considered the possibility that in these cells, type III transcripts might represent an even smaller fraction of the mRNA population than do type I transcripts. To explore this possibility, we performed semiquantitative RT-PCR on mRNA from nonstimulated and LPS-stimulated A20 and bmDC (Fig. 7). To characterize the p35 mRNA populations in these cells, p35 sense primers were chosen whose binding sites were located either upstream of ATG1 to identify all p35 isoforms (Fig. 7, p35 I–IV), upstream of ATG2 to identify only the type II or type IV isoforms (Fig. 7, p35 II/IV), or upstream of ATG3 to identify only type III isoforms (Fig. 7, p35 III). Type II and type IV transcripts cannot be differentiated using these primers. mRNA levels of p40 and the housekeeping gene HPRT were also determined. Transcript levels were determined by phosphorimager analysis and normalized to HPRT levels.

The p35 transcripts (p35 I–IV and p35 II/IV) were detected in nonstimulated A20 and bmDC. After stimulation, these messages were up-regulated by 3.6- and 4.4-fold, respectively, in A20 cells, and by 7.1- and 6.2-fold, respectively, in bmDC. In both cell types, type III transcripts were detected only after LPS stimulation. To ensure that the p35 type III band was not an artifact, this band was cloned, and the type III sequence was confirmed. The substantial up-regulation of all p35 isoforms observed after LPS stimulation suggests that transcriptional mechanisms also play a role in p35 gene regulation.

By RACE and RT-PCR, we have shown that LPS-stimulated bmDC can produce functional mRNA for translation of p35 (type I and type III transcripts). These cells also synthesize p40 transcripts after LPS stimulation (Fig. 7) and thus can produce IL-12 heterodimer. A20 cells differ from bmDC in their ability to produce IL-12 because they do not synthesize p40 mRNA, even after LPS stimulation (Fig. 7). However, because A20 and bmDC have a similar pattern of p35 expression, and because it has been shown that p35 and p40 are regulated independently (11–14), A20 is a good and useful model for the study of p35 gene regulation.
Since the type I-IV, type II/IV, and type III mRNA isoforms were detected using different primer pairs, we cannot quantify the relative proportions of these transcripts in the p35 mRNA population from these RT-PCR results. However, our RACE data indicate that type II/IV transcripts are a significant portion of the p35 mRNA population before and after LPS stimulation in both cell types. Furthermore, the absence of type III transcripts in our RACE pool of 5’ clones suggests that type III transcripts are a rare population of mRNAs in LPS-stimulated A20 and bmDC. Taken together with our RACE and primer extension data, these RT-PCR results clearly demonstrate that nonstimulated cells synthesize transcripts that initiate almost entirely from upstream of ATG2 (type II and type IV transcripts, nonfunctional for p35 translation), and upon LPS stimulation there is a shift in transcription initiation so that cells synthesize a higher proportion of transcripts that do not contain ATG2 (type I and type III transcripts, functional mRNA for p35 translation). These type I and type III mRNA isoforms, according to our luciferase assay and IL-12 bioassay results, can support translation of the p35 subunit and its incorporation into the IL-12 heterodimer.

Discussion

In the present study, we demonstrate that p35 expression is controlled 1) by transcriptional regulation, in terms of both the expression level and the type of p35 mRNA isoform transcribed; and 2) by translational control, in which the presence of an upstream ATG in the 5’ UTR of p35 mRNA inhibits translation of the p35 subunit. The translational and transcriptional mechanisms employed in p35 regulation are intimately linked, such that the negative translational controls exerted under nonstimulated conditions can be bypassed, after LPS stimulation, by a switch in the location of transcription initiation. This type of regulation has also been observed in other genes, including the murine complement factor B gene (36) and the IL-15 gene (37).

IL-12 gene regulation occurs at many levels. Coexpression of both subunits in the same cell is required to produce bioactive IL-12 heterodimer (10), yet these subunits are controlled independently (11–14). We have shown here that the p35 gene is highly regulated at the level of translation by the presence or the absence of an upstream ATG in the 5’ UTR. The majority of genes containing upstream ATGs are cytokines, cytokine receptors, growth factors, transcription factors, signal transduction molecules, and proto-oncogenes (31). These molecules, when overproduced, might constitute a potential danger to the host. Therefore, it seems likely that the reason why IL-12 expression is so tightly controlled is to ensure that enough safeguards are in place to prevent overproduction of this potentially dangerous cytokine. Furthermore, the independent regulation of the IL-12 subunits allows, in theory, for safe expression of only one subunit at a time (2, 16, 17, 38, 39). Thus, it is possible that during development, the IL-12 subunits are expressed one at a time to generate self tolerance without putting the host in the position of having to achieve immunological tolerance to a cytokine that itself counteracts tolerance (40).

We have demonstrated p35 promoter activity upstream of exon 2. This promoter activity is quite weak compared with that of the p40 gene (18, 19, 23) (M. Tone, unpublished observations). This may reflect an additional mechanism to prevent overproduction of IL-12. However, this would seem a disadvantage for a cell when it needs to rapidly induce expression of the IL-12 protein. The translational control of the p35 subunit may play a role here, whereby the continued synthesis of untranslatable p35 message would maintain the transcriptional locus in an open and active state, with the basal transcriptional machinery and most or all transcription factors already recruited and in place. The cell would only need to shift its site of transcriptional initiation so as to synthesize type I and/or type III transcripts (functional mRNA for p35 translation). Maintaining the transcription locus in an open form has also been suggested as a mechanism for the rapid induction of transcription in the IL-2 gene (41, 42). In resting T cells, the RNA polymerase is “paused” but not active at the start site of transcription, and only becomes active upon assembly of other enhancer/promoter elements upon T cell activation.

Synthesis of type II and type IV transcripts seems to be controlled by a TATA-less promoter. Promoter activity upstream of exon 2 is not dependent upon the presence of the TATA sequence (Fig. 1, Pro3), and in addition, this typical TATA sequence is located at position +281 to +287 in the p35 gene, far downstream of the type II and type IV transcription start sites. However, after LPS stimulation, transcription can initiate from three start sites in exon 2, and the major start site is located at position +311, 30 bp downstream of the TATA box. It is possible, therefore, that the TATA box functions only after LPS stimulation and may play a role in the switching of transcriptional initiation to downstream of ATG2. It is of interest that a structurally related cytokine, IL-6 (25, 43), contains a functional TATA-like sequence in a position similar to that of the TATA box in p35 (25). Thus, it is likely that the TATA box in the p35 gene was at one time functional and may still continue to play a well-defined role in the regulation of p35 gene expression.

The translational mechanism of p35 regulation defined here can explain why, in some cases, the coexpression of p35 and p40 messages is not sufficient to produce IL-12 heterodimer (16, 17, 38, 39). For example, when macrophages from BALB/c mice are infected by Salmonella dublin, there is a significant up-regulation of both p35 and p40 mRNA (15). However, while the p40 subunit was readily detected in cultures of infected macrophages, no IL-12 protein was observed (38). Pretreatment of macrophages with IFN-γ, however, led to an increase in IL-12 protein production after Salmonella infection. Thus, it is likely that in this system, IFN-γ can induce a shift in transcription initiation in p35 so that the type I and/or type III transcripts (functional mRNA for p35 translation) are synthesized. Similarly, it has been reported that in microglia (17), the resident macrophage of the brain, and astrocytes (39), the major glial cell type in the central nervous system, LPS stimulation induces the expression of both p35 and p40 mRNA but not that of IL-12 protein. Only after a combination of LPS/IFN-γ treatments can any IL-12 heterodimer be detected in these cell cultures.

We have shown previously that LPS stimulation was sufficient to up-regulate the transcription of p35 type I and type III mRNA isoforms from the macrophage cell line WEHI-3D (25), and here we report a similar finding in A20 cells and bmDC. However, in the case of Salmonella-infected macrophages and LPS-stimulated astrocytes and microglia, a single stimulus was not sufficient to drive the production of IL-12 from these cells. Only the combined treatment of LPS/IFN-γ could induce IL-12 secretion. Thus, the mechanism driving the switch in p35 transcription to make type I and/or type III transcripts is likely to depend on both the nature of the stimulus and the type of responding cell. In addition, the importance of CD40 ligation for the induction of IL-12 from APCs has been demonstrated (7), and its role, if any, in the switching of transcription initiation in p35 should be examined. We expect that CD40 ligation will induce a similar change in the type of p35 message transcribed by APCs.

Although translational control is a potent mechanism of gene regulation, we cannot rule out the possibility that the type II and type IV transcripts might have another function aside from serving...
as a buffer of untranslatable message to prevent the overproduction of the p35 subunit. For example, the 40-aa peptide encoded by the ATG2 ORF may have a specific function unrelated to p35 translational control. However, database searches have not revealed any striking similarities with known proteins (J. Babik, unpublished observation).

Interestingly, the presence of ATG2 in the 5′ UTR of p35 transcripts is conserved between human (44) and murine genes. The favorable context for translation initiation (30) around murine ATG2 is also exactly conserved in the human gene, so it is likely that translation occurs from this upstream ATG in human p35 mRNA. However, in the human gene, ATG2 is in-frame with the p35 protein, resulting in a potential additional 34-aa fragment at the N-terminus of the p35 signal peptide. In cells transfected with human p35 cDNA, translation of p35 occurs from constructs without the in-frame ATG2, and so the presence of this upstream ATG is not necessary for production of the p35 subunit (44). The effect of the in-frame ATG2 on the translational efficiency of human p35 transcripts has not been reported. It is possible that, given efficient translation from ATG2, the resulting modification of the signal peptide may affect the secretion and/or targeting of human p35. It has been suggested that this 34-aa sequence may be important for generating a membrane-bound form of human p35 (44), and indeed, human IL-12 has been shown to exist in a membrane-bound form on the surface of macrophages (45). Taken together, the regulatory mechanisms involved in human p35 expression remain poorly defined and demand further investigation.

Conventional methods of analyzing p35 expression levels (i.e., RT-PCR or Northern blot hybridization) cannot distinguish between the different isoforms of p35 and so do not accurately reflect the expression of mRNA. However, in the human gene, ATG2 is in-frame with the p35 protein, resulting in a potential additional 34-aa fragment at the N-terminus of the p35 signal peptide. In cells transfected with human p35 cDNA, translation of p35 occurs from constructs without the in-frame ATG2, and so the presence of this upstream ATG is not necessary for production of the p35 subunit (44). The effect of the in-frame ATG2 on the translational efficiency of human p35 transcripts has not been reported. It is possible that, given efficient translation from ATG2, the resulting modification of the signal peptide may affect the secretion and/or targeting of human p35. It has been suggested that this 34-aa sequence may be important for generating a membrane-bound form of human p35 (44), and indeed, human IL-12 has been shown to exist in a membrane-bound form on the surface of macrophages (45). Taken together, the regulatory mechanisms involved in human p35 expression remain poorly defined and demand further investigation.

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