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Posttranscriptional Regulation of IL-10 Gene Expression Through Sequences in the 3’-Untranslated Region

Mark J. Powell, Sara A. J. Thompson, Yukiko Tone, Herman Waldmann, and Masahide Tone

Interleukin-10 is a potent antiinflammatory and immunosuppressive cytokine, initially described as “cytokine synthesis inhibitory factor” because of its ability to suppress production of IFN-γ, IL-2, and proinflammatory cytokines (1, 2).

IL-10 can be expressed by a wide range of cell types including T cells, B cells, monocytes, macrophages, and keratinocytes, and many tumor cells (1, 3–8). Recently, we have shown that IL-10 transcription is regulated by the translation factors Sp1 and Sp3 which are known to be constitutively and ubiquitously expressed (9). We have also shown that the low levels of IL-10 mRNA in resting T cell and macrophage cell lines as well as bone marrow dendritic cells could be up-regulated by activation (9). Weak PMA response elements were identified between −802 and −305 of the IL-10 promoter (9). However, the weak activity of the response elements could not account for the large increase in IL-10 mRNA accumulation upon PMA stimulation. We wondered whether IL-10 production is regulated by other posttranscriptional regulatory elements such as enhancers and/or posttranscriptional control mechanisms. The latter possibility had been implied by the finding that a T cell clone that was actively transcribing IL-10 did not yield detectable mature IL-10 mRNA (10). Consistent with this, we observed multiple copies of potential mRNA-destabilizing motifs AUUUUA and AU-rich elements (ARE)1 (11) in the 3’-untranslated region (UTR) of mouse IL-10 mRNA (12).

Our own observations on posttranscriptional control of this gene began with the finding that although comparable promoter activity could be measured in resting and stimulated EL4 (T cell) and RAW 264 (macrophage) cell lines, the levels of IL-10 mRNA varied between the lines after activation (9). In this article, we have been able to show directly that the 3’-UTR of IL-10 mRNA is indeed destabilizing for IL-10 mRNA. We have identified the sequences concerned using transient and stable transfectants. The results of this and the IL-10 promoter analysis described in the accompanying paper (9) led us to conclude that many cell types might be constitutively transcribing IL-10 mRNA but that much of the regulation is determined posttranscriptionally. This might ensure that the immune system can react rapidly in moderating an inflammatory stimulus.

Materials and Methods

Plasmids

Luciferase reporter plasmids were constructed using the pG3L-Control Vector (Promega, Madison, WI) carrying a SV40 promoter/luciferase expression unit. Four fragments, AU1 (+727 to +818), AU2 (+807 to +936), AU3 (+1157 to +1293), and AU4 (+727 to +1239), containing the 3’-UTR sequences of IL-10 cDNA were amplified by PCR and cloned into an XbaI site (+1934) located between the luciferase gene and the poly(A) signal in the pG3L-Control Vector. The pG3L-Basic Vector was used as a negative control plasmid for a luciferase assay.

A fusion gene of mouse IL-10 (+11 to +609) (12) and the human Ig (Ig) heavy chain constant region (+427 to +1132) (13) was amplified and assembled by PCR. This fragment was cloned downstream of the EF-1α promoter in the pMTF expression vector carrying a neomycin resistance gene. The resulting plasmid (IL-10/Ig “no-UTR”) was used to construct plasmid IL-10/Ig “plus-UTR.” A fragment containing a 3’-UTR sequence (+610 to +1295) of IL-10 cDNA was amplified and cloned downstream of the IL-10/Ig fragment in plasmid IL-10/Ig no-UTR.

Cell culture and transfection

EL-4 and RAW 264 cells were cultured in IMDM with 5% FCS. For the luciferase reporter assay, 2 × 10⁵ cells were electroporated with 10 μg luciferase reporter plasmids and 1 μg pRl-TK (Promega) as an internal control plasmid. Transfected cells were cultured in 10 ml IMDM-10% FCS. If required, PMA (50 ng/ml) or LPS (20 μg/ml) was added 6 h postelectroporation. Cells were harvested 48 h postelectroporation, and luciferase activities were analyzed by the Dual-Luciferase Reporter Assay System (Promega). These assays were repeated more than three times, and the activities were normalized to Renilla luciferase activities.

To generate stable transfectants, 2 × 10⁶ EL-4 cells were electroporated with 20 μg of plasmids IL-10/Ig plus-UTR or no-UTR. Stable transfectants were selected by G418 (1 mg/ml).
Enzyme-linked immunosorbent assay

EL-4 transfectants were cultured with or without PMA (50 ng/ml) for 24 h. Culture supernatants were harvested and diluted (1/1 to 1/152). IL-10/Ig fusion protein in culture supernatants was analyzed by ELISA using anti-human IgG (The Jackson Laboratory, Bar Harbor, ME) as the capture Ab and biotin conjugated anti-mouse IL-10 (SXC10, PharMingen, San Diego, CA) as the detection Ab.

Analysis of RNA half-life

EL-4 transfectants generated using plasmids IL-10/Ig no-UTR or plus-UTR were cultured for 6 h with or without PMA (50 ng/ml). Actinomycin D was added (10 µg/ml), and cells were harvested at different time points (0, 0.5, 1, 1.5, 2, 3, and 4 h). RNA were isolated, and IL-10/Ig RNA was analyzed by Northern blot hybridization using a human Ig constant-region cDNA as a probe. As a control, HPRT mRNA was also analyzed. To measure the half-life of IL-10/Ig RNA, the intensity of IL-10/Ig and HPRT bands was analyzed by phosphorimaging (Molecular Dynamics, Sunnyvale, CA).

Results

Identification and characterization of mRNA destabilizing activities in the 3′-UTR of IL-10 mRNA

Potential mRNA destabilizing motifs AUUUUA and related sequences (AUUUUA, AUUUUUA) are located in the 3′-UTR of IL-10 mRNA. The relative positions of these sequences are indicated in Fig. 1A. We have classified these AUUUAs and related sequences into three potential regulatory regions by both location and surrounding sequences. The first consists of three nonclustered AUUUAs located between +741 and +806, and with surrounding sequences that are not AU-rich (Fig. 1, A and B, fragment AU1). The second consists of clustered AUUUAs located between +827 and +895. Here, the surrounding sequences are of the typical AU-rich type (Fig. 1, A and B, fragment AU2) which are similar to those previously identified as mRNA-destabilizing elements (11). The third is located near the 3′-end of IL-10 mRNA (between +1183 and +1225). It contains nonclustered AUUUAs and AUUUUUA sequences with surrounding sequences that are not AU-rich (Fig. 1, A and B, fragment AU3). To investigate whether these sequences regulate stability of IL-10 mRNA, a luciferase reporter assay was performed. Four fragments containing AUUUAs and related sequences (AU1, +727 to +818; AU2, +807 to +936; AU3, +1157 to +1239; AU4, +727 to +1239) from the 3′-UTR of IL-10 cDNA (Fig. 1, A and B) were cloned between the luciferase cDNA and the poly(A) additional signal in the pGL3-Control Vector carrying a SV40 promoter/luciferase expression unit. Transcripts from the resulting plasmids would therefore contain the luciferase-coding region plus a part of the 3′-UTR of IL-10 mRNA. Luciferase activities in transfected cells using these plasmids were compared with that using a control plasmid (no insert), pGL3-Control Vector. If the inserted fragments from the IL-10 3′-UTR contain RNA-destabilizing activity, the luciferase activity generated will be less than that of the control plasmid. In nonstimulated EL-4 cells, reporter activity fell by ~50% when any of the AU1, AU2, or AU3 sequences in the 3′-UTR were used (Fig. 1C) and by 80% with plasmid AU4, which carries the longest 3′-UTR sequence (containing AU1, AU2, and AU3 sequences) (Fig. 1C). However, the results in PMA-stimulated EL-4 cells were different. We observed a reduction of reporter activity with plasmids AU1, AU3, and AU4, but not with plasmid AU2. This suggests that some part of mRNA destabilizing activity may have been overridden by PMA stimulation, whereas some still remained functional. Similar results were obtained using macrophage cell line RAW 264 cells except that the strong mRNA-destabilizing activity of fragment AU3 was sustained in LPS-stimulated RAW 264 cells, implicating the region between +1157 to +1239 in this process. These results suggest that the stability of IL-10 mRNA is regulated by heterogeneous mRNA-destabilizing elements that may be dependent on cell type and nature of activation signals located in the 3′-UTR of IL-10 mRNA.

IL-10 production is negatively regulated by its 3′-UTR in nonstimulated EL-4 cells

To further investigate IL-10 mRNA stability, we generated stable EL-4 transfectants using IL-10 transgenes possessing or lacking
the 3′-UTR sequence. To distinguish the transgene product from endogenous IL-10, we used an IL-10/human Ig constant-region fusion protein gene (IL-10/Ig), which was cloned into an expression vector carrying the constitutive EF-1α promoter to ensure strong expression, and a neomycin resistance gene for selection (Fig. 2A). The resulting plasmids IL-10/Ig “no-UTR” (lacking the 3′-UTR of IL-10 cDNA) or IL-10/Ig “plus-UTR” (possessing the 3′-UTR) were transfected into EL-4 cells, and clones of stable transfectants were selected. The ELISA assay, which measured the Ig component of the IL-10/Ig construct, was performed using culture supernatants diluted appropriately so as to provide the most sensitive readout. We observed no difference in the amount of IL-10/Ig protein that accumulated in the culture supernatants of nonstimulated and PMA-stimulated “no-UTR” transfectants (Fig. 3). These results suggest that IL-10 production was negatively regulated by the 3′-UTR of IL-10 mRNA in nonstimulated EL-4 cells, although PMA stimulation is capable of partially overriding the effect.

The IL-10/Ig mRNA half-life increases significantly after PMA stimulation

We have shown that IL-10 production is regulated through post-transcriptional mechanisms. We estimated the extent of IL-10 mRNA stability in nonstimulated and PMA-stimulated EL-4 cells by measuring the half-life of IL-10/Ig mRNA. EL-4 transfectants were cultured with or without PMA for 6 h and then treated with actinomycin D to block transcription. RNAs were isolated at different time points and analyzed by Northern blot hybridization using human Ig and mouse HPRT cDNAs as probes (Fig. 4). The “no-UTR” RNA was quite stable in both nonstimulated and PMA-stimulated EL-4 cells (half-life, >12 h) (Fig. 4); in contrast, the half-life of IL-10/Ig “plus-UTR” RNA was 1 h in nonstimulated cells increasing to 3 h in the PMA-stimulated transfectants. Within the 6 h PMA-stimulated groups, the “no-UTR” RNA was more stable than “plus-UTR” RNA, suggesting that some mRNA destabilizing signals in the 3′-UTR of IL-10 mRNA were still active.

Discussion

In the accompanying paper (9), we have shown that the transcription of IL-10 gene is regulated by the factors Sp1 and Sp3 and that IL-10 mRNA could be up-regulated by PMA activation. We were also able to detect weak PMA response elements in the IL-10 promoter (9). However, because constitutively expressed Sp1 is a key transcription factor in IL-10 expression, it is difficult to explain the up-regulation of IL-10 mRNA by promoter activity alone. Here, we show that IL-10 expression is controlled by post-transcriptional regulation. Constitutively expressed IL-10 mRNA was unstable in nonstimulated cells, and therefore poorly translated. Activation by PMA was able to restabilize the mRNA, thus leading to protein production. We infer that there must be equivalent signals in normal cells that can also increase IL-10 mRNA stability.

We have classified AUUUA motifs and the related sequences into three potential regulatory regions by both location and surrounding sequences. These regions identified in the mouse IL-10 mRNA sequence (12) are also conserved in the human sequence (14). AUUUA motifs between +807 and +936 (Fig. 1, A and B, fragment AU2) of the 3′-UTR are present in AU-rich sequences.

**FIGURE 2.** Impact of the 3′-UTR of IL-10 mRNA on translation of the protein. A, Stable transfectants were generated using plasmids IL-10/Ig “no-UTR” and “plus-UTR”. The structure of the expression plasmids is shown. IL-10/Ig “plus-UTR” contains the 3′-UTR of IL-10 mRNA (+610 to +1295) indicated by a solid line. B, Culture supernatants of nonstimulated (open square) and PMA-stimulated (closed circle) transfectants IL-10/Ig “no-UTR” and “plus-UTR” were titrated, and IL-10/Ig protein was detected by ELISA using anti-human IgG as a capture Ab and anti-mouse IL-10 as a detection Ab.

**FIGURE 3.** Measurement of the impact of the 3′-UTR on the expression of an IL-10/Ig fusion protein in stable transfectants. EL-4 cells were transfected using plasmid IL-10/Ig “no-UTR” or “plus-UTR”. Twenty-four independent transfectants were randomly selected and cultured for 24 h without (Non) or with (PMA) PMA (50 ng/ml). IL-10/Ig protein in the culture supernatants was detected by ELISA.

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phosphoribosyltransferase (HPRT) RNA was also measured. In Fig. 2. IL-10/Ig RNA was analyzed by Northern blot hybridization using "no-UTR" and "plus-UTR". The same transfectants were previously used nonstimulated (Non) and PMA-stimulated (PMA) transfectants IL-10/Ig points (0, 0.5, 1, 1.5, 2, 3, and 4 h) post-actinomycin D treatment from also confirmed these data with stable transfectants.

mRNA may be dependent on cell type and/or stimuli. We have served in LPS-stimulated RAW 264 cells. Stabilization of IL-10 mRNA-destabilizing activity in the fragment AU3 was ob-

served in LPS-stimulated RAW 264 cells. However, mRNA-destabilizing activity remained in the fragment AU2 and partial destabilizing activity on AU1 binding to AU2, resulting in the outcome of no apparent destabi-

lization. This may be overridden by the destabilization activities of AU1 and AU3 in full length sequence. In nonstimulated RAW 264 cells, the luciferase activity produced by the AU2 plasmid was only slightly less than that of the control plasmid. Because this reduction of luciferase activity is not large, we cannot conclude the presence of mRNA-destabilizing activity. However, in LPS-stim-

ulated RAW 264 cells, we did not observe any reduction in lucifer-

erase activity with the AU2 plasmid. This suggests that destabili-

zing activity was lacking in stimulated cells. Because RAW 264 is a macrophage cell line, RAW 264 cells might have been partially stimulated (through "danger") by DNA transfection. Under these circumstances, RNA-destabilizing activity might have been partially inhibited.

Although regulatory region AU1 and AU3 contain AUUUA and AUUUUUA sequences, these sequences are not clustered, and the surrounding sequences are not AU rich. Therefore, these regions are not similar to typical AREs previously identified as mRNA-

destabilizing sequences. Lagnado et al. (21) also proposed that a minimal mRNA destabilizing sequence is not AUUUA but may be UUAUUUA(U/A)(U/A). The IL-10 3'-UTR contains similar se-

quences, AUAUUUAU and CUAUUUAAU in fragment AU1 and the identical sequence UUAUUUAU in fragment AU3. These sequences might function as mRNA-destabilizing se-

quences. A protein binding to the short AU-rich sequence UUAUUUAU has also been identified (19). Such a factor may regulate IL-10 mRNA stability through the region AU1 and AU3.

Comparison of the luciferase activities produced using AU1 and AU3 plasmids with that using control plasmid in PMA-stimulated EL-4 cells resulted in a small reduction of luciferase activity (Fig. 1, AU1 and AU3 in EL-4/PMA) (given the inherent variability of the luciferase assay, we could not conclude that these re-
ductions were significant). However, the same plasmids in non-

stimulated EL-4 cells produced large reductions in luciferase activity. We conclude that the putative RNA-destabilizing factors binding to AU1 and AU3 may express poorly in PMA-stimu-

lated cells but also that these factors may be more stable than those binding to AU2, resulting in the outcome of no apparent destabi-

lizing activity on AU2 and partial destabilizing activity on AU1 and AU3. We need additional experiments to resolve this issue (e.g., identification and characterization of factors binding to AU1, AU2, and AU3).

mRNA-destabilizing activity in the longest fragment (AU4) is stronger than the others. We speculate that this might be caused by gathering of destabilizing activity in each fragment. However, it is possible that these regions might cooperate with each other to regu-

late RNA stability.

In LPS-stimulated RAW 264 cells, similar RNA-destabilizing activities in fragments AU3 and AU4 (fragment AU4 contains the sequence in fragment AU3) were detected. IL-10 mRNA in LPS-

stimulated RAW 264 cells seems to be destabilized mainly through the sequence in fragment AU3. The likelihood is, then, that the different RNA destabilizing elements described here might depend on distinct destabilizing signals. The corollary is that cells capable of producing high levels of IL-10 (Tr1 cells, Th2 cells, and certain tumors) may fail to destabilize mRNA.

The results of this and previous (9) papers lead us to the "regu-

latory" model of IL-10 expression. Transcription of IL-10 is ubiqui-

tously and constitutively regulated by Sp1 and Sp3. In nonstimu-

lated cells, the constitutively expressed IL-10 mRNA is kept at low levels through mRNA-destabilizing signals. Upon activation,
IL-10 transcription is up-regulated through PMA response elements located in IL-10 promoter, and then IL-10 mRNA levels are further increased through RNA stabilization controlled by regulatory regions located in the 3'-UTR. Variable expression levels of IL-10 may be determined by the heterogeneity of mRNA-stabilizing signals.

This combination of ubiquitous transcriptional and posttranscriptional mechanisms for IL-10 expression might be critical to the role of this cytokine. Low concentrations of IL-10 might maintain the background tone of the immune system, thus controlling proinflammatory cytokine expression. When appropriate activation signals arise, IL-10 production could be rapidly up-regulated at sites of inflammation to limit proinflammatory cytokine cascades and restore immune homeostasis via negative feedback.

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References