Xplore® mRNA Assays for the Quantification of IL-1 β and TNF- α mRNA in Lipopolysaccharide-Induced Mouse Macrophages

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ABSTRACT

Because the accurate measurement of a number of cytokine mRNA transcripts provides valuable knowledge about cytokine gene regulation, we have developed the Xplore[®] assay for the quantification of cytokine mRNA. This microplate-based assay is rapid (under four hours), quantitative over three orders of magnitude and carries no risk of false-positive values from contamination with amplified target. Here, we describe the use of Xplore assays to measure the steady-state mRNA levels of TNF- α and IL-1 β produced by mouse WEHI and J774 macrophage-like cell lines.

INTRODUCTION

Tumor necrosis factor- α (TNF- α) and interleukin-1 beta (IL-1 β) are inflammatory cytokines that play important roles in the activation of the immune system following invasion of the host by endotoxin-producing Gram-negative bacteria. Macrophages, when exposed to lipopolysaccharide (LPS), produce large amounts of TNF- α and IL-1 β , which is released into the circulatory system. The expression of these cytokines is controlled by a variety of mechanisms, including the regulation of transcription, mRNA stability, translation, protein processing and secretion (1–10,13–17). Different studies have reported conflicting conclusions about the relative contribution of transcription and translation to TNF- α regulation. It has been suggested that these different conclusions may be due in part to the different experimental methods used to measure TNF- α transcription rates and mRNA levels (15). Furthermore, few of these studies have quantified cytokine mRNA levels at multiple time points after LPS stimulation.

Here, we report the use of a novel and quantitative method, the Xplore[®] (Third Wave Technologies, Madison, WI, USA) mRNA assay (11,12,18), to measure the steady-state mRNA levels of TNF- α and IL-1 β produced by the mouse WEHI and J774 cell lines. The Xplore assay is based on a novel signal-amplification system for the quantitative detection of nucleic acids (11,12). It uses the endonuclease activity of a Cleavase[®] enzyme (Third Wave Technologies) to recognize a specific nucleic acid structure composed of two oligonucleotides that are hybridized to the mRNA target (Figure 1). The probes are designed so that the 3'-end of the upstream Invader[™] probe (Third Wave Technologies) overlaps the 5'-end of the downstream signal probe. The endonuclease recognizes this complex and cuts the signal probe. The reaction is incubated near the melting temperature of the signal probe to permit the rapid exchange of hybridized signal probe. This turnover (hybridization, cleavage, dissociation of cleaved signal probe and replacement with an uncleaved signal probe) occurs rapidly so that multiple copies of the probe are cleaved for each copy of the target sequence without temperature cycling, and amplify the signal from the reaction.

The signal accumulates in a linear manner at a rate proportional to the amount of target in the original sample. These signal probe fragments are captured in a streptavidin-coated microwell (via biotin label on a capture probe with a sequence complementary to the signal probe). The captured signal probe fragments are then labeled with fluorescein-12-dUTP, which is added to the 3'-end of the cleaved signal probe by a DNA polymerase. The incorporated fluorescein-12-dUTP is detected by an anti-fluorescein antibody conjugated with alkaline phosphatase. The fluorescent detection signal is then produced by the reaction of the alkaline phosphatase with the substrate, which is converted to a fluorescent molecule.

MATERIALS AND METHODS

Growth and Stimulation of Cells

Both J774A.1 (ATCC TIB67) and WEHI-3 (ATCC TIB68) cells were grown in RPMI 1640 media supplemented with 10% FCS, 1% Pen/Strep (Life Technologies, Rockville, MD, USA), and 2 mM L-glutamic acid at 37°C and 5% CO₂. The cells were plated at 2 × 10⁶ cells/well in 6-well plates and stimulated with the addition of 5 µg/mL LPS. Cells were incubated overnight before the addition of 5 µg/mL LPS. At selected intervals, the media were removed and saved for ELISA analysis; cells in the plate were stored for RNA isolation by the addition of 1 mL TRIzol[®] reagent (Life Technologies) and stored at -70°C.

Total RNA Isolation

Total RNA was isolated from cells using the TRIzol reagent according to the manufacturer's specifications. RNA pellets were dissolved in 30 µL RNase-free water, and the concentration was determined by measuring the absorbance at 260 nm. (Any potential impact of errors in RNA concentration determination on Xplore mRNA assay results was corrected by normalizing results to GAPDH mRNA concentrations.)

Cytokine Concentration Determination

The concentration of mouse TNF-α and IL-1β in culture media was measured using ELISA kits according to the manufacturer's instructions (Third Wave Technologies).

Quantification of mRNA by Xplore Assays

Mouse TNF-α, IL-1β and GAPDH mRNA concentrations were measured using Xplore mRNA assays following the manufacturer's instructions. The Cleavase reactions were assembled in RNase-free 200-µL polypropylene tubes. Each 15-µL reaction contained 500 ng total RNA sample. The tubes were capped and then incubated in an oven at 54°C for 4 h. After the Cleavase incubation, the reaction mixture was transferred to the wells of a streptavidin-coated 96-well plate for capture of the 5' fragments of the cleaved signal probes in the plate using

a biotinylated capture oligonucleotide (which is complementary to the cleaved 5'-end of the signal probe). The cleaved probes were labeled with fluorescein-dUTP using a DNA polymerase that extended the 3'-end of the cleaved fragment on the capture probe template in a 15-min incubation at 37°C.

The plate was washed three times to remove any nonhybridized components. The fluorescein label was detected by adding 100 µL anti-fluorescein-alkaline phosphatase antibody conjugate and incubating for 15 min at 37°C. The excess antibody was removed by washing the plate three times. Bound antibody was detected by adding 100 µL AttoPhos[™] reagent (JBL Scientific, San Luis Obispo, CA, USA), and the plate was incubated for 30 min at 37°C. The fluorescence was measured on a Cytofluor[®] plate reader (PE Biosystems, Foster City, CA, USA) (excitation = 450/50, emission = 580/50, gain = 50, reads/well = 10 and temperature = 37°C).

Standard curves for the assays were run with known amounts of TNF-α, IL-1β or GAPDH in vitro-synthesized mRNA transcripts that were provided in the Xplore mRNA quantification kit. The signal from these standards was used to calculate the concentrations of the mRNA targets in the samples. The values shown are the averages of duplicate samples (Figure 3). The reported concentrations of IL-1β and TNF-α mRNA were normalized to the concentration of GAPDH mRNA present in those samples (determined by an Xplore assay for mouse GAPDH mRNA; data not shown). This was done by multiplying the uncorrected cytokine mRNA concentration by the time zero GAPDH mRNA concentration and then dividing that value by the time "X" GAPDH mRNA concentration.

Northern Blot Analysis of mRNA

Northern blot analysis of TNF-α and IL-1β mRNA was performed on 5 µg samples of total RNA using Northern Max-Gly[™], Strip-Ez RNA[™], BrightStar Psoralen-Biotin[™] and BrightStar BioDetect[™] kits according to the manufacturer's instructions (Ambion, Austin, TX, USA).

RESULTS

As predicted from the results of previous studies (1–10, 13–17), the macrophage-like cell lines WEHI and J774 responded to stimulation with LPS by producing TNF-α and IL-1β mRNA and protein (Figures 2 and 4). The Xplore assays provided accurate quantitative detection of cytokine mRNA, and this revealed new information pertaining to the regulation of these cytokines in these cell types.

TNF-α Regulation

WEHI cells responded to LPS by increasing the level of TNF-α from a baseline level of 1 amol (in 500 ng total RNA) to 3.6 amol within 30 min. The steady-state level of TNF-α mRNA continued to increase, reaching a peak of 52 amol at 4 h after stimulation (Figure 2, B and 3). The quantitative values obtained using the Xplore mRNA assay correlated well with the results from the Northern blot analysis of TNF-α mRNA in the same samples (Figure 2C). Although the time course of TNF-α mRNA production was similar for both WEHI and J774 cells (detectable increases by 30 min and peaking at 4 h), J774 cells produced over four times more

Figure 1. Probe design for the mIL-1β Xplore mRNA assay. The 3' base of the Invader probe (bold) invades the region where a base of the signal probe hybridizes to the target sequence. The Cleavase enzyme recognizes this complex and cuts the signal probe [the 5' arm of the signal probe (underlined) is not complementary to the target].

TNF- α mRNA than WEHI cells (227 vs. 52 amol in 500 ng total RNA) (Figure 2, A and B). If the efficiency of translational or post-translational processes was the same in J774 and WEHI cells, we would predict that the amount of TNF- α protein secreted by the J774 cells should be at least four times higher than that of the WEHI cells. However, the peak level of TNF- α protein secreted by the J774 cells was only twice that of the WEHI cells (3300 pg/mL vs. 1600 pg/mL).

IL-1 β Regulation

Xplore mRNA analysis of IL-1 β mRNA in the WEHI cell total RNA samples showed that IL-1 β mRNA levels did not increase until 1 h after induction, reached a peak level of 156

amol at 7 h and remained unchanged at 10 h (159 amol) (Figure 4B). These quantitative values correlated well with the results from Northern blot analysis of IL-1 β mRNA in the same samples (Figure 4C). The WEHI cells produced more IL-1 β mRNA than the J774 cells (132 vs. 85 amol in 500 ng total RNA at 4 h after induction) (Figure 4, A and B). Because of the greater abundance of IL-1 β mRNA in the WEHI cells, we predicted that the WEHI cells would produce more IL-1 β protein. However, the J774 cells secreted nearly twice as much IL- β protein as the WEHI cells (1255 vs. 621 pg/mL). This difference between mRNA and protein levels suggests that the translational or post-translational processes are regulated differently in J774 and WEHI cells.

DISCUSSION

The Xplore mRNA assays provided accurate quantification of the steady-state levels of IL-1 β and TNF- α mRNA. This quantitative measurement revealed information about the regulation of these cytokines that would be difficult to obtain with other mRNA analysis methods such as Northern analysis. The effective dynamic range of the Xplore assay was larger than that of a single X-ray film exposure of the Northern blot (Figures 2C and 3). An increase of TNF- α mRNA from 1 amol to 3.6 amol was observed 30 min after treatment of the WEHI cells with LPS, yet no band corresponding to TNF- α mRNA is seen in the lane of the Northern blot containing the 30-min sample, even when the 4-h peak sample band produced a strong exposure.

Both the J774 and WEHI cell lines are described as macrophage-like cells, and it might be predicted that these cell lines would regulate the production of inflammatory cy-

Figure 2. Quantification of TNF- α mRNA and protein produced by WEHI and J774 cells after stimulation with LPS over time. See Materials and Methods section for details. (A) Xplore assay analysis of TNF- α mRNA, and ELISA measurement of protein produced by LPS-stimulated J774 cells (■, mRNA; ○, protein). (B) Xplore assay analysis of TNF- α mRNA and ELISA measurement of protein produced by LPS-stimulated WEHI cells (■, mRNA; ○, protein). (C) Northern analysis TNF- α mRNA produced by LPS-stimulated WEHI cells.

Figure 3. Xplore assay analysis of TNF- α mRNA produced by LPS-stimulated WEHI cells over time (data shown in Figure 2B is replotted with an expanded Y-axis). The average value of replicate samples is plotted (error bars represent the range of values of the duplicates). The amount of TNF- α mRNA (in amol) detected in 500 ng total RNA is indicated next to each time point.

tokines in a similar manner. However, while both cell lines responded to LPS stimulation by producing IL-1 β and TNF- α , the J774 cells produced much more IL-1 β protein per IL-1 β message than the WEHI cells. This was not because of any general difference in mRNA translation efficiency between the two cell types, since the WEHI cells produced more TNF- α protein per TNF- α message than J774 cells. The measurement of steady-state mRNA and protein levels supports the conclusion that these cytokines were regulated differently in the two cell lines.

Figure 4. Quantification of IL-1 β mRNA and protein produced by WEHI and J774 cells after stimulation with LPS over time. See Materials and Methods section for details. (A) Xplore assay analysis of IL-1 β mRNA and ELISA measurement of protein produced by LPS-stimulated J774 cells (■, mRNA; O, protein). (B) Xplore assay analysis of IL-1 β mRNA and ELISA measurement of protein produced by LPS-stimulated WEHI cells (■, mRNA; O, protein). (C) Northern analysis IL-1 β mRNA produced by LPS-stimulated WEHI cells.

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