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Possible role of the transcription factor interferon regulatory factor 1 (IRF-1) in the regulation of ornithine decarboxylase (ODC) gene expression during IFNγ macrophage activation

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Abstract

In this report we discuss the role of interferon regulatory factor 1 (IRF-1) in the regulation of ornithine decarboxylase (ODC) transcription during IFNγ human macrophage activation. We show that a binding sequence for the transcription factor IRF-1 is contained in the first intron of the human ODC gene (from nt +2711 to nt +2722) and we demonstrate that the level of expression of IRF-1 increases in human macrophages and in the human promonocytic cell line, U937, previously differentiated in monocytes/macrophages by phorbol myristate acetate (PMA), after 2 h of IFNγ stimulation. We also show that the hamster tk-ts13 cell line, stably transfected with the IRF-1 cDNA, over-expresses ODC. In addition, a specific complex was detected, by gel-shift assay after incubating a 20 bp double-stranded oligomer containing the binding sequence for IRF-1 with nuclear proteins extracted from human macrophages and from (PMA-differentiated) U937 cells stimulated with IFNγ for 2 h.

Key words: Interferon-γ, Interferon regulatory factor 1; Ornithine decarboxylase; Gene expression; Transcription regulation; Macrophage activation

1. Introduction

The enzyme ornithine decarboxylase (ODC) is the key regulator of the synthesis of polyamines that are essential for cell growth and proliferation. Expression of this gene, which is sustained in rapidly proliferating cells, transiently increases upon stimulation by growth factors [1–3], but becomes constitutively activated during cell transformation induced by carcinogens [4,5], viruses [6–8], or oncogenes [9–12]. Moreover, ODC behaves like a protooncogene [13]. The role of polyamines does not seem to be restricted only to cell growth and proliferation; in fact, it has been shown that cell differentiation and the function of terminally differentiated cells also involve the activation of ODC and the accumulation of polyamines [14]. In previous reports we have demonstrated that the stimulation of human monocytes and mouse macrophages with lipopolysaccharide (LPS), interferon-γ (IFNγ), and tumor necrosis factor (TNF) leads to ODC accumulation [15,16], and that the induction of ODC gene expression followed by polyamine accumulation has a functional significance for human macrophage activation [16,17]. In 1992, by the differential screening of a cDNA library prepared from lymphokine-treated mouse macrophage-like Raw 264.7 cells, Farber [18] identified 11 genes induced early in the response to macrophage-activating factors. One of these genes encodes for a transcription factor, interferon regulatory factor 1 (IRF-1), which regulates the expression of IFN-β and some IFN-inducible genes [19–21]. IRF-1 is expressed upon IFNγ macrophage activation [18]. Since we also found that the activation of human macrophages and U937 cells with IFNγ leads to the expression of the IRF-1 gene, and that a consensus binding sequence for IRF-1 is present in the first intron (non-coding region) of the ODC gene (from nt +2711 to nt+2722) (Fig. 1), we hypothesized that IRF-1 binds to this sequence in the ODC gene and regulates ODC expression during IFNγ macrophage activation.

2. Materials and methods

2.1. Cells and culture conditions

Fresh human buffy coats were obtained from a blood bank and, after 2-fold dilution with PBS supplemented with 2.5 mM EDTA, were layered on a Ficoll-Hypaque (Pharmacia) gradient. After 40 min centrifugation at 400 × *g* the interphase of peripheral blood mononuclear cells was collected, washed twice with PBS, and placed in plastic tissue culture flasks at a concentration of 1–2 × 10^6 cells/cm surface area. The cells were cultured in Iscove medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg/ml of penicillin and streptomycin. After 3–4 h of incubation the non-adherent cells were washed out using PBS with Ca^{2+} and Mg^{2+}. The adherent cells (monocytes) were next cultured overnight and then stimulated with human recombinant IFNγ (Amersham) (50 U/ml). After established periods of time the cells were harvested for RNA extraction or protein isolation. The U937 cell line was grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, and 1% penicillin/streptomycin. These cells were differentiated into monocytes/macrophages by PMA (20 ng/ml) for 3 days and stimulated with IFNγ (100 U/ml) as described above for human monocytes.

2.2. RNA extraction and hybridization

Following culture, the adherent cells were washed twice with PBS

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and than guanidium-lysed directly in the culture flasks for RNA extraction using the procedure of Chomczynsky and Sacchi [22]. 15 μg of total cellular RNA of each sample (as measured by A_{260} absorption) were next electrophoresed in 1.2% agarose with 2.2 M formaldehyde in the presence of 0.5 μg of ethidium bromide and added directly to the sample. After electrophoresis the gel was capillary-blotted on to nitrocellulose. Prehybridization and hybridization were carried out at 42°C in a mixture containing 50% formamide, 5 × SSC, 5 × Denhardt's solution, 20 mM sodium phosphate buffer, pH 6.5, and 100 μg of denatured, sheared salmon sperm DNA per ml. In the case of hybridization, the mixture was supplemented with 10% dextran sulfate and denatured probe. The cDNA probes; human ODC and IRF-1 (a 369 bp fragment obtained by RT-PCR from HL60 cells) were labeled by the random primer method to a mean specific activity of 0.5–1.3 × 10^6 cpm/μg DNA. Membranes were hybrydized for 18–24 h and then washed twice with a mixture containing 2 × SSC and 0.2% sodium dodecyl sulfate (SDS) for 5 min at room temperature and 3 times with a mixture of 0.1 × SSC and 0.1% SDS for 30 min at 52°C. Membranes were exposed for 24–72 h at −80°C using intesifying screens.

2.3. Protein extracts and gel-shift assay

The nuclear protein extracts and gel shift assay were prepared by a modification of the protocol described by Schreiber et al. [23]. After the established times of culture the cells, typically 2 × 10^6, were washed twice with PBS and than harvested with a rubber policeman in 500 μl cold buffer containing: 10 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM EDTA, 1 mM dithiotreitol, 0.5 mM PMSF, and 10 μg/ml aprotinin.

The homogenates were centrifuged for 30 s in a microcentrifuge at 4°C. The nuclear pellets were resuspended in 50 μl of ice-cold buffer and the tubes were vigorously rocked at 4°C for 15 min. The buffer contained: 20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.2 mM EDTA, 1 mM dithiotreitol, 0.5 mM PMSF, and 10 μg/ml aprotinin. The nuclear extracts were centrifuged for 5 min at 4°C and the supernatants were frozen in aliquots at −80°C. The protein content was estimated with a kit for protein microdetermination (Sigma Diagnostics). The 22 bp double-stranded oligonucleotide containing the binding sequence for the IRF-1 protein found in the non-coding region of the ODC gene, was labeled with terminal transferase and purified on a spin column according to the method described by Maniatis et al. [24]. Binding reactions were carried out by incubating the probe (0.3 ng; 30,000-40,000 cpm) with 10 μl of incubation buffer containing: 4% Ficoll, 20 mM HEPES, 50 mM KCl, 0.2 mM EDTA, 1 mM dithiotreitol, 0.25 mg/ml BSA, 8 μg/ml poly(dI-dC), and 5 μg of nuclear extract. In the gel-shift assay an unlabeled oligonucleotide containing a high affinity IRF-1 binding sequence four times repeated (AAGTGA) and an unlabeled non-specific oligonucleotide containing the binding sequence for the AP-1 complex were added in molar excess (500–100 ×) for competition. After 30 min of incubation at room temperature, samples were electrophoresed through a 4% polyacrylamide gel for 1.5 h at 120 V. Prior to electrophoresis 0.01% Bromophenol blue was added to the samples. Gels were exposed to X-ray film with intensifying screens for 24–48 h.

3. Results and discussion

Monocytes/macrophages play a central role in immune and inflammatory responses. Macrophages can be activated by endogenous cytokines such as interferons, granulocyte-macrophage colony stimulating factor, and tumor necrosis factor, as well as exogenous factors such as lipopolysaccharide. When macrophages are activated,

(a) 5' GGAACTGAAACT 3'

(b) 5' G(A)AAA(G/C)(T/C)GAAA(G/C)(T/C) 3'

Fig. 1. (a) IRF-1 binding sequence in the first intron of the ODC gene. (b) IRF-1 consensus binding sequence.

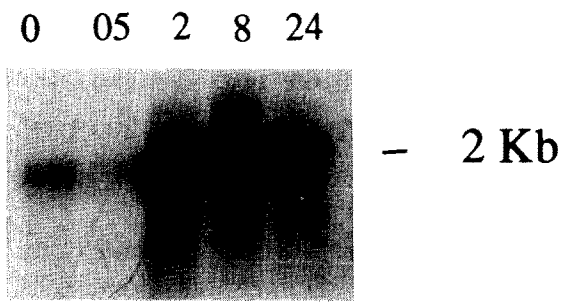

Fig. 2. Effect of IFNγ on accumulation of IRF-1 mRNA in human macrophages.

they carry out a number of important immune functions, including the presentation of antigens, phagocytosis, and the killing of microbial and tumor cells. IFNγ is a key lymphokine in macrophage activation. IFNγ-mediated activation causes the induction of specific genes [25–28]; for instance, there are many data available indicating the role of ODC [29–31]. Rapid accumulation of ODC mRNA has been associated with a stimulaton of a plethora of physiological responses, suggesting that the ODC gene may be involved in the phenomenon of cell activation; in fact, activation of ODC expression has been repeatedly linked to the stimulation of cell proliferation and induction of differentiation. The intracellular availability of ODC is controlled at multiple levels, including both transcription-dependent and -independent mechanisms, translation, protein stability, and endogenous inhibition of its enzymatic activity [32,33]. In this report we studied the gene expression of IRF-1 in human macrophage cells upon IFNγ activation and we addressed the question of whether it regulates ODC gene expression. In fact, we found a binding sequence for IRF-1 in the first intron (non-coding region) of the ODC gene. To demonstrate that the transcription factor IRF-1 is involved in the monocyte/macrophage IFNγ activation signal transduction pathway we first checked the expression of IRF-1 in IFNγ-activated human macrophages and monocytes/macrophages of differentiated U937 cells for 0, 30 min, 2 h, 8 , and 24 h. After RNA extraction Northern blot assays were performed and the nylon filters were hybridized with IRF-1 cDNA probe (Figs. 2,3). These figures show that IFNγ causes an accumulation of IRF-1 mRNA after 2 h of stimulation. To demonstrate the functional role of IRF-1 on ODC gene ex-

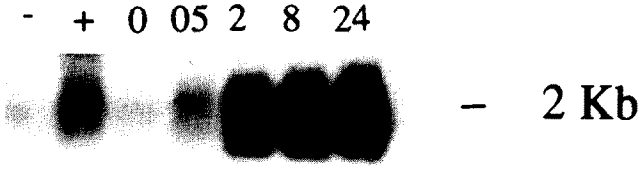

Fig. 3. Effect of IFNγ on accumulation of mRNA in human U937 cell line after PMA differentiation.

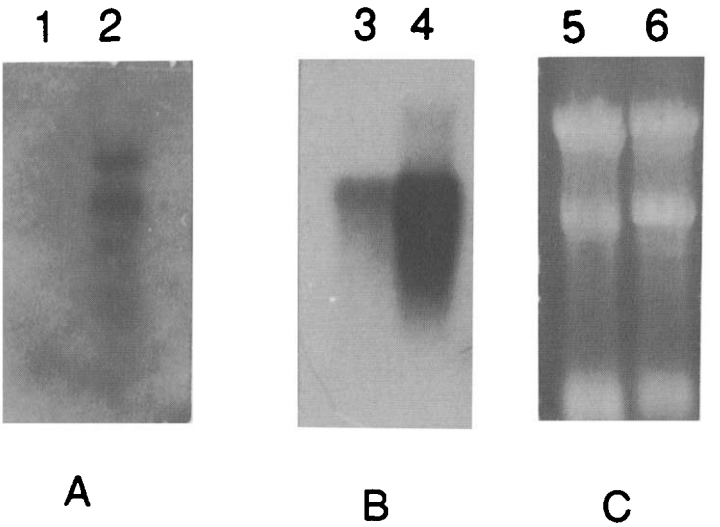

Fig. 4 (A) IRF-1 mRNA levels in tk-ts13 cells transfected with SV-IRF-1 plasmid (lane 2) and with the empty SV40 control plasmid (lane 1); (B) ODC mRNA levels in tk-ts13 cells transfected with SV-IRF-1 plasmid (lane 4) and with the empty SV40 control plasmid (lane 3); (C) control rRNA ethidium bromide bands.

pression, we cotransfected a hamster fibroblast cell line, tk-ts13 (timidine kinase-lacking gene), with the SV-IRF-1 plasmid containing the full-length IRF-1 cDNA under the control of the SV40 promoter and with the p11tk plasmid containing the timidine kinase gene. Positive colonies were selected in medium containing gHAt. After the stable integration of IRF-1 in the cellular genome we tested the IRF-1 expression in a mixed population of colonies transfected with IRF-1 (Fig. 4A, lane 2) compared with colonies transfected with the empty SV40 plasmid as a control (Fig. 4A, lane 1). We than checked the expression of ODC and found that the cells that constitutively express IRF-1 over-express the ODC gene (Fig. 4B, lane 4). To demonstrate the direct interaction

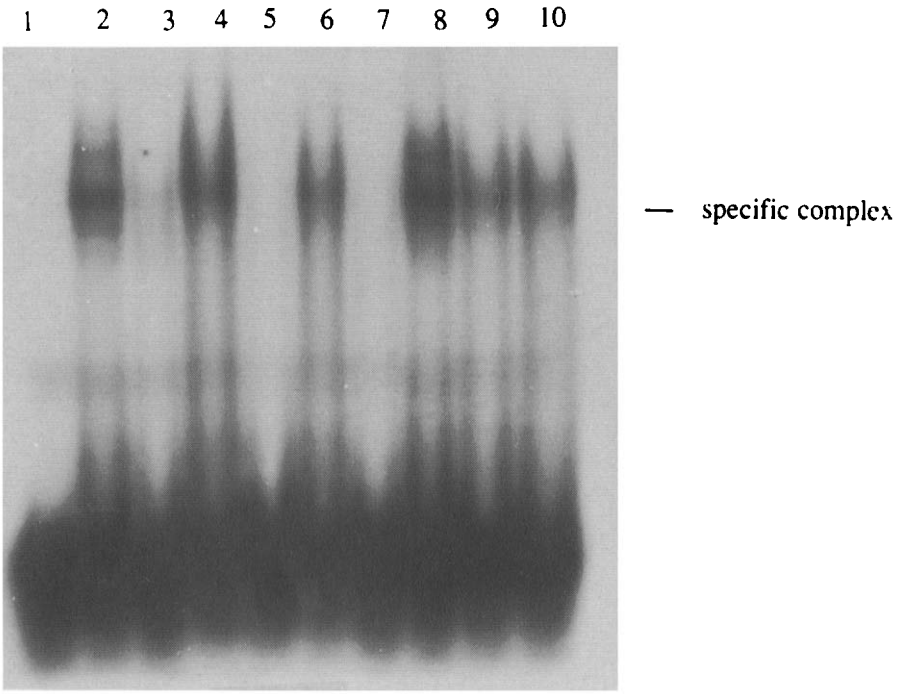

Fig. 5. Gel retardation analysis of the ^{32}P-labeled 20 bp oligonucleotide containing the binding sequence for IRF-1 incubated with nuclear extracts from human macrophages and U937 cells (differentiated in macrophages by PMA) stimulated with IFNγ for 2 h, and from K562 cells activated with IFNγ for 2 h as a positive control. Lanes: (1) free probe. Probe incubated with: (2) nuclear extracts from K562 cells; (3) K562 nuclear extracts and the specific competitor; (4) U937 nuclear extracts; (5) U937 nuclear extracts and the specific competitor; (6) macrophage nuclear extracts; (7) macrophage nuclear extracts and the specific competitor; (8) K562 nuclear extracts and the aspecific competitor; (9) U937 nuclear extracts and the aspecific competitor; (10) macrophage nuclear extracts and the aspecific competitor.

between IRF-1 and the ODC gene, a 20 bp double-stranded ^{32}P-labeled oligonucleotide containing the consensus binding sequence for IRF-1 found in the ODC gene was reacted with nuclear proteins extracted from human macrophages and from U937 cells treated with PMA activated by IFNγ for 2 h, the time at which there is a high expression of IRF-1 (Fig. 5). A specific complex was detected (Fig. 5, lanes 2,4,6); this complex disappeared when a 24 bp double-stranded unlabeled oligomer containing a high affinity IRF-1 binding examer repeated four times $(AAGTGA)_4$, was added in molar excess as a competitor (Fig. 5, lanes 3,5,7). Moreover, the complex was still present after competition with a double-stranded aspecific competitor (Fig. 5, lanes 8,9,10). From our results it is clear that the transcription factor IRF-1 binds to the first intron of the ODC gene and transactivates the transcription of this gene. In order to better establish the functional role of IRF-1 in the regulation of ODC gene expression in human macrophages activated by IFNγ other studies are being carried out.

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