Ipaf is upregulated by tumor necrosis factor-α in human leukemia cells

Olga Gutierrez, Carlos Pipaon, Jose L. Fernandez-Luna*

Unidad de Genetica Molecular, Hospital Universitario Marques de Valdecilla, Edificio Escuela Universitaria de Enfermeria, Av. Valdecilla s/n, 39008 Santander, Spain

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Abstract Ipaf has been associated with apoptosis, cytokine processing, and nuclear factor-B activation. Here, we describe that Ipaf is highly expressed in myelomonocytic cells and that the mRNA levels of Ipaf progressively increase during differentiation of CD34$^+$ progenitors to granulocytes and monocytes. Additionally, treatment with tumor necrosis factor-α and exposure to UV radiation induced the transcriptional activation of Ipaf in human leukemia HL-60 cells. Thus, Ipaf may contribute to modulate the response of myeloid cells to genotoxic and pro-inflammatory stimuli.

Keywords: Ipaf; c-Jun; TNFα; CD34$^+$ progenitor; HL-60

1. Introduction

Ipaf, also called CARD12 or CLAN, belongs to a family of proteins structurally characterized by a nucleotide-binding oligomerization domain (NOD) followed by multiple leucine-rich repeats (LRRs). In addition, Ipaf contains an N-terminal caspase recruitment domain (CARD) as already described for other members of the family such as Nod1, Nod2 and CIITA [1,2]. Recently, Nod proteins have been shown to recognize bacterial components including lipopolysaccharides (LPS) and/or bacterial muramyl dipeptide [3,4], and this interaction leads to the activation of nuclear factor κB (NFκB), a transcription factor that plays a central role in innate immunity and cell survival. Very little is known about the transcriptional regulation of the NOD family of proteins. Recently, it has been described that Nod2 expression is enhanced by pro-inflammatory cytokines and bacterial components via NFκB, a mechanism that may contribute to the amplification of the innate immune response [5]. Furthermore, much remains to be discovered to better elucidate the expression and function of these genes within the hematopoietic compartment. Ipaf is highly expressed in the bone marrow and among circulating blood leukocytes the highest mRNA levels have been found in monocytes [6]. It has been suggested that this CARD-containing protein may contribute to a number of intracellular processes, including apoptosis, cytokine processing, and NFκB activation [1,6,7].

* Corresponding author. Fax: +34-942-200952.
E-mail address: fluna@humv.es (J.L. Fernandez-Luna).

Abbreviations: TNFα, tumor necrosis factor-α; JNK, c-Jun N-terminal kinase; NFκB, nuclear factor κB; EMSA, Electrophoretic mobility shift assay; RACE, rapid amplification of cDNA ends

In an attempt to improve our understanding of the regulation of Ipaf in hematopoietic cells, we investigated its expression in response to different stimuli in primary cells and cell lines. We show that Ipaf expression is increased during differentiation of CD34$^+$ progenitors to granulocytes and monocytes, and that DNA damage and treatment with tumor necrosis factor-α (TNFα) induce transactivation of Ipaf in human leukemia HL-60 cells.

2. Materials and methods

2.1. Cells

Mobilized peripheral blood progenitors and peripheral blood subpopulations were isolated as previously described [5]. Purified CD34$^+$ cells (more than 95%) were seeded into 24-well culture plates at 5×10^4 cells/mL in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA), containing 20% fetal calf serum (FCS) and recombinant human stem cell factor (SCF), interleukin-3 (IL-3) and interleukin-6 (IL-6) (Immunex, Seattle, WA) at a final concentration of 100 ng/ml. Granulocyte (Gr) and monocyte/macrophage (Mo) cell populations were generated as described [5].

Human leukemia cell lines Jurkat, HL-60, K562, U937, THP-1 and TF1 and the breast cancer cell lines MCF7 and SUM159 were maintained as described elsewhere [5,8]. HEK293T cells were cultured in RPMI with 10% FCS. When indicated, HL-60 cells were treated with lipopolysaccharide (LPS), TNFα (both from Sigma, St Louis, MO) or irradiated with UV rays.

2.2. RT-PCR and rapid amplification of cDNA ends (RACE) analysis

Total RNA was prepared using TRIZOL reagent (Invitrogen). To quantitate mRNA expression, a quantitative real-time PCR method was used as previously described [5]. The generated cDNA was amplified by using primers for human Ipaf (^{5}CCAGTCCCCTCACCATAGAAG$^{3'}$, and $^{5'}$ACCCAAGCTGTCAGTCAGACC$^{3'}$) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [9]. The ratio of the abundance of Ipaf transcripts to that of gapdh transcripts was calculated as 2^n, where n is the C_T (threshold cycle) value of gapdh minus the C_T value of Ipaf, and normalized by the value of the sample with the lowest expression level of Ipaf. Specificity of the desired PCR products was determined with melting curve analysis.

Semiquantitative RT-PCR was used as previously described [5]. After 20 (GAPDH) or 32 (Ipaf) amplification cycles, the expected PCR products were size fractionated onto an agarose gel and stained with ethidium bromide.

For analysis of sequences at the 5′ end of Ipaf mRNA, a 5′-RACE kit was used according to the manufacturer's instructions (Roche, Mannheim, Germany), with the following Ipaf primers: ^{5}TGTTCTGGATGAAAGCTTCCCACC^3 and ^{5}CTCTTCATTCTGGCTGAGCAATCC^3.

2.3. c-Jun N-terminal kinase (JNK) immunoassay

JNK activity was detected by an immunocomplex assay as described elsewhere [10] with slight modifications. In brief, cell lysates were

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cleared by centrifugation at 13 000 rpm for 10 min. Soluble proteins were incubated with anti-JNK1 (sc-474) (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4 °C and then incubated with Gammabind Sepharose beads (Amersham Biosciences, Piscataway, NJ). The immune complexes were washed and resuspended in kinase buffer containing [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) and 1 μg of GST-ATF2 (SIGMA). After incubation, samples were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography. To ensure an even immunoprecipitation, part of the immune complexes were run on a 10% SDS–PAGE, transferred to nitrocellulose filters and developed with anti-JNK antibody.

2.4. Electrophoretic mobility shift assays (EMSAs)

HL-60 cells were lysed and nuclear fractions were resuspended in 20 mM HEPES, pH 7.9, 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 20% glycerol. Nuclear extracts (5 μg of total protein) were incubated with a ^{32}p-labeled double stranded DNA probe containing a consensus sequence for AP1 (TGAGTCA). Samples were run on a 5% non-denaturing polyacrylamide gel in 200 mM Tris-borate and 2 mM EDTA. Gels were dried and visualized by autoradiography. Supershifts were performed using antibodies specific for c-Jun, c-Fos or irrelevant GATA-1 (Santa Cruz Biotechnology, Santa Cruz, CA).

2.5. Gene reporter assays

A genomic PCR fragment of 788 bp from the promoter region of Ipaf (Ipafpt) (nucleotide positions 89533 to 90320 from GenBank Accession No. AL121653) was cloned into *Xho*I and *Hin*dIII sites of the pGL2-basic luciferase reporter vector (Promega Corp., Madison, WI). The authenticity of the construct was confirmed by sequencing. HEK293T cells were cotransfected with 1 μg of pGL2-Ipafpt and 50 ng of pRSV-β-gal in triplicate by using FuGENE 6 reagent (Roche). Twenty four hours post-transfection, cells were treated with 30 ng/mL of TNFα or briefly exposed to UV light (400 J/m^2) and then left in culture. After 12 h of incubation, cell extracts were prepared and analyzed for the relative luciferase activity by a dual-light reporter gene assay system (Applied Biosystems, Foster City, CA). Results were normalized for transfection efficiency with values obtained with pRSV-β-gal.

3. Results and discussion

We have focussed on the expression pattern of Ipaf and the identification of its inducers in a wide range of hematopoietic populations. First, we purified CD34$^+$ progenitors from peripheral blood, and the selected population was cultured with colony stimulating factors (CSF) for either granulocytes (G-CSF) or monocytes (M-CSF) for different time intervals to induce Gr or Mo maturation, as previously described [5]. CD34$^+$ progenitor cells expressed low levels of Ipaf (Fig. 1A, and data not shown), which increased about 5-fold by day 10 and 10–15-fold by day 20 of culture in both monocytic and granulocytic lineages (Fig. 1A). Thus, Ipaf is upregulated along the myelomonocytic differentiation. Then, we analyzed the expression of Ipaf in peripheral blood populations of B cells (CD19$^+$), T cells (CD3$^+$), granulocytes (CD15$^+$), monocytes (CD14$^+$), and monocyte-derived dendritic cells (CD40$^+$/CD86$^+$). Real-time PCR analysis showed that B lymphocytes expressed very low levels of Ipaf, whereas the other cell populations expressed 60–100-fold more Ipaf mRNA than B cells, with the highest levels detected in granulocytes and monocytes (Fig. 1B). A similar expression pattern has been described for Nod2, another member of the NOD family of proteins [5]. Thus, both Ipaf and Nod2 are preferentially expressed in primary myelomonocytic cells. Finally, we studied the expression of Ipaf in a number of cell lines including myelomonocytic (HL-60, TF1, U937, THP-1, K562), lymphoblastic (Jurkat), and breast cancer epithelial (MCF7, SUM159) cells. As shown in Fig. 2, the highest expression levels of Ipaf were observed in HL-60, TF1, THP-1 and U937. The expression pattern found for Ipaf partly correlates with previous data showing that among peripheral blood leukocytes, only monocytes expressed Ipaf [6]. However, we have shown that granulocytes express

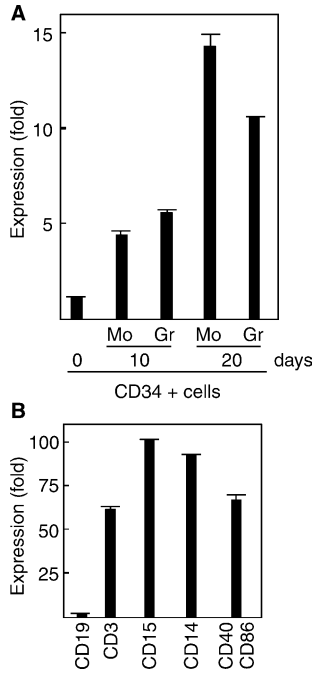

Fig. 1. Analysis of Ipaf mRNA in primary hematopoietic cells. Total RNA was purified from (A) CD34$^+$ cells cultured in the presence of M-CSF or G-CSF to induce Mo or Gr differentiation, respectively, and (B) immune-purified peripheral blood cell populations, and subjected to quantitative real-time PCR. Histograms represent the means ± S.D. of triplicate analyses.

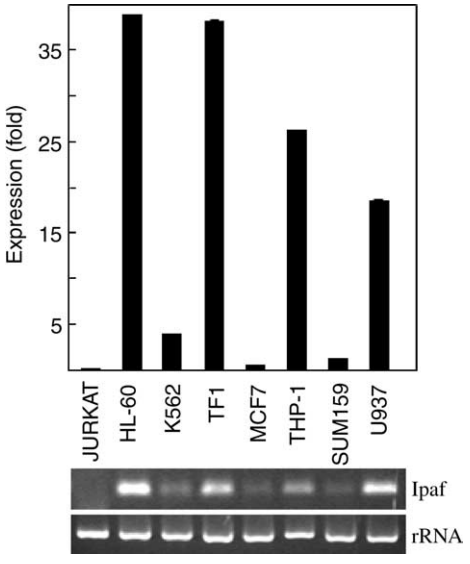

Fig. 2. Analysis of Ipaf mRNA in hematopoietic and epithelial cell lines. RNA was obtained from myelomonocytic (HL-60, TF1, U937, THP-1, K562), lymphoblastic (Jurkat) and breast cancer epithelial (MCF7, SUM159) cell lines, and subjected to quantitative real-time PCR. Histograms represent the means ± S.D. of triplicate analyses.

Ipaf at similar levels to those found in monocytes. A likely explanation for this difference is that we have used highly purified primary cell subpopulations instead of leukocytes separated by centrifugation and adherence to plastic dishes, which reduces the possibilities of cell contamination that may hinder the gene expression data when using amplification techniques such as real-time or semiquantitative PCR.

As Ipaf has been associated with activation of downstream apoptotic and inflammatory signaling pathways, we estimulated human leukemia HL-60 cells with TNFα, a major mediator of apoptosis as well as inflammation. After 24 h of treatment with TNFα, the mRNA levels of Ipaf increased about 8-fold (Fig. 3A). TNFα triggers the activation of two major transcription factors: NFκB and c-Jun [11]. However, treatment with bacterial LPS, a well known activator of the NFκB pathway, failed to modify the expression of Ipaf (Fig. 3A), which leaves c-Jun as a likely candidate for the transcriptional regulation of Ipaf. This was further confirmed by exposure of cells to UV radiation, a genotoxic stimulus that activates JNK, which in turn activates c-Jun [12]. Both TNFα and UV exposure induced the phosphorylation activity of JNK as assessed by an immune complex kinase assay (Fig. 3B), and increased the DNA binding activity of AP1 heterodimer containing c-Jun and c-Fos as determined by EMSA (Fig. 3C).

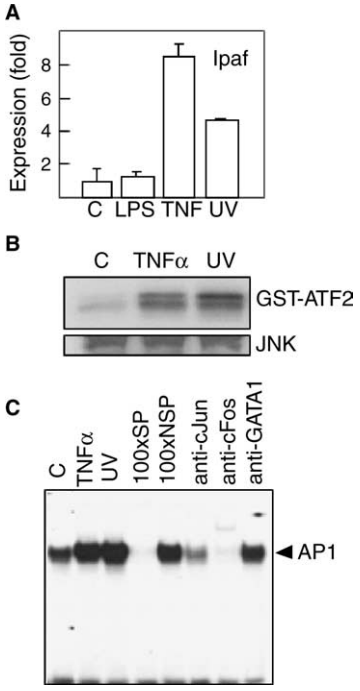

Fig. 3. Induction of Ipaf mRNA in response to UV and TNFα. (A) HL-60 cells were treated with 10 μg/mL LPS or 10 ng/mL TNFα for 24 h, or briefly exposed to UV radiation (400 J/m^2) and left in culture for 24 h. Then, total RNA was isolated and analyzed for the expression of Ipaf by real-time PCR. Histograms represent the means ± S.D. of triplicate analyses. (B) Cells were treated as in A but the incubation time was shortened to 30 min. After incubation, cells were subjected to an immune complex JNK assay with GST-ATF2 as substrate. (C) Cells were treated with 30 ng/mL TNFα for 3 h or exposed to UV (400 J/m^2) and left in culture for 1 h, and the formation of AP1–DNA complexes was determined by an EMSA using radiolabeled consensus probe. Nuclear extracts were preincubated with 100-fold molar excess of unlabeled AP1 probe (100× SP), unlabeled irrelevant probe (100× NSP), or with antibodies against c-Jun, c-Fos or GATA1.

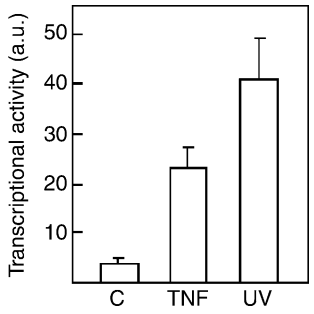

Fig. 4. Transcriptional activation of the Ipaf promoter. HEK293T cells were transfected with a luciferase reporter vector containing a 788-bp fragment from a putative promoter region of the Ipaf gene, in the presence of pRSV-β-gal. Cells were left untreated (C), or induced with TNFα or UV light for 12 h. Arbitrary units (a.u.) of luciferase activity were normalized based on values of pRSV-β-gal activity to control for transfection efficiency. Data are presented as the mean of triplicate cultures ± S.D.

Consistently, the mRNA levels of Ipaf increased more than 4-fold in reponse to UV radiation (Fig. 3A).

In order to validate these data, we attempted to study the Ipaf promoter region. However, different transcription start sites are found within a genomic sequence of about 1 kb as assessed by 5′-RACE [6] (data not shown). Furthermore, we cannot rule out the presence of other transcripts with shorter 5′ ends, as non-characterized smaller PCR products were obtained in the 5′-RACE analysis. This would be consistent with the big 5′ untranslated tale of more than 9 kb formed by three different exons as deduced from the genomic sequence of Ipaf. With the caveat that we may be missing critical regulatory sequences, a genomic fragment of 788 bp spanning a region overlapping the most 5′ transcription start site was cloned into a promoterless luciferase vector, and this construct was transiently transfected into HEK293T cells. As shown in Fig. 4, treatment with TNFα or UV exposure induced the transcriptional activity between 8- and 14-fold when compared with unstimulated cells. A search for transcriptional factor binding sites with MatInspector software (Genomatix, Munich, Germany) identified a number of putative consensus sites for transcriptional activators (CREB, Stat, E2F, forkhead-related activators) and repressors (Gfi1, E4BP4). Although in the genomic fragment analyzed there are no perfect matches to the AP1 core sequence, at least one AP1-like binding site (TGACACT) was identified. Thus, we suggest that an AP1 consensus sequence located in either this or other genomic region is a likely candidate to contribute to Ipaf expression due to the clear correlation found between AP1 activation and increase in the mRNA levels of Ipaf. Alternatively, other transcription factors may be involved. Consistent with this second hypothesis, CREB, Stat and E2F are activated by UV radiation and/or treatment with TNFα [13–16].

In conclusion, our study shows that Ipaf is upregulated during differentiation of CD34$^+$ progenitors along the granulocyte and monocyte lineages. Our data also suggest that genotoxic or pro-inflammatory stimuli induce the expression of Ipaf at the transcriptional level in leukemia cells.

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