Human Immune Associated Nucleotide 1: a member of a new guanosine triphosphatase family expressed in resting T and B cells

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TAL-1 is a basic helix-loop-helix oncoprotein that is expressed in up to 30% of T-cell acute lymphoblastic leukemias but not in the T lineage. We have cloned a complementary DNA, called Human Immune Associated Nucleotide 1 (hiIAN1), whose messenger RNA (mRNA) level expression is inversely correlated to the TAL-1 mRNA level in human leukemic T-cell lines. The hiIAN1 encodes a 38-kd protein that belongs to a novel family of proteins conserved from plants to humans and characterized by motifs related to, but highly divergent from, the consensus motifs found in guanosine triphosphate (GTP)-binding proteins. Despite these divergent amino acids at positions involved in GTP/guanosine diphosphate (GDP) binding and guanosine triphosphatase (GTPase) activities, we found that hiIAN1 specifically binds GDP (Kd = 0.47 μM) and GTP (Kd = 6 μM) and exhibits intrinsic GTPase activity. Among mature hematopoietic cells, hiIAN1 is specifically expressed in resting T and B lymphocytes, and its expression level tremendously decreased at the protein but not the mRNA level during B- or T-lymphocyte activation, suggesting a specific role for this new type of GTPase during the immune response. (Blood. 2002;99:3293-3301)

Introduction

The most common genetic alteration found in T-cell acute lymphoblastic leukemia (T-ALL) involves the tal-1/SCL/tcl-5 (hereafter referred as tal-1) gene, which codes for a basic helix-loop-helix transcription factor, TAL-1 (see Begley and Green1 for a review). In adults, TAL-1 expression is restricted to hematopoietic precursor erythrocytic, megakaryocytic, and mastocytic cells; as well as some endothelial cells2; yet TAL-1 is never expressed in the T lineage. Gene-targeting experiments have established that TAL-1 is necessary for primitive and definitive hematopoiesis in mice and have also demonstrated a TAL-1 function in blood vessel formation.3-5

The role of TAL-1 in T-cell leukemogenesis is still poorly understood. In humans, activation of the tal-1 gene occurs by chromosomal translocations (5% of T-ALLs) or by an interstitial 90-kilobase (kb) deletion (25% of T-ALLs) (see Begley and Green1 for a review). In both cases, the TAL-1 coding sequence is not affected by these genetic rearrangements, which result in forced TAL-1 expression in the T-cell lineage. Indeed, unscheduled TAL-1 expression in the T-cell lineage of transgenic mice results in aggressive T-cell malignancies that appear relatively late in life and exhibit incomplete penetrance.5 However, concomitant expression of TAL-1 and LMO1 or LMO2, 2 proteins that interact with TAL-1 and are encoded by genes translocated in some T-ALLs, leads to leukemia early in life with a high degree of penetrance, suggesting a collaboration between these oncogenes in the establishment of T-cell malignancies.7,8

The Jurkat T-cell line is derived from a human T-ALL and expresses high levels of the TAL-1 oncoprotein. We have previously derived a clonal subline of the Jurkat T-cell line, Jurkat-ΔCOOH, which produces only a mutant TAL-1 protein that exhibits a dramatic decrease of protein-binding activity to the TAL-1 DNA consensus sequence (E box).9 Growth curves indicate that the mutant subline exhibits a premature apoptosis upon medium depletion, and this phenomenon can be partially reverted by the expression of the wild-type TAL-1 protein in Jurkat-ΔCOOH.9 In this study, we used the Jurkat/Jurkat-ΔCOOH cell lines to isolate genes that might be regulated by TAL-1 during T-cell leukemogenesis. Using complementary DNA (cDNA) subtraction by representational difference analysis (RDA), we characterized a messenger RNA (mRNA) that is expressed only in Jurkat-ΔCOOH. This mRNA encodes a protein, Human Immune Associated Nucleotide 1 (hiIAN1), which belongs to a novel family of proteins characterized by an unconventional guanosine diphosphate/guanosine triphosphate (GDP/GTP)-binding domain. Here we describe the biochemical properties and the expression pattern of hiIAN1 in hematopoietic cells.

Materials and methods

Cell lines and primary human hematopoietic cells

Jurkat, Jurkat-ΔCOOH, Molt-4, RPMI 8402, CEM, and DU528 T-cell lines were grown in RPMI 1640 medium supplemented with 10% (20% for DU528) heat-inactivated fetal calf serum (FCS), l-glutamine, penicillin, and streptomycin at 37°C under 5% CO2. BaF3 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin at 37°C under 5% CO2. BaF3 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin at 37°C under 5% CO2.

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1640 medium supplemented with 10% FCS, t-glutamine, penicillin, and streptomycin and 5% (vol/vol) WEHI-3B as a source of interleukin (IL–3).

T-cell purification from human peripheral blood and activation were performed according to Costello et al. Primary T cells were maintained in RPMI 10% FCS. Stimulations were performed with anti-CD28 248 (mouse immunoglobulin IgG–M), and anti-CD3 289 (mouse IgG2a) obtained from Dr Axel pinkett (Cancer Institute, Genoa, Italy) and used as ascites fluid (1/400 dilution) or as purified monoclonal antibody (mAb) (10 μg/mL), respectively. CD3 mAb was coated onto Petri tissue-culture dishes (CD3c). T-cell activation was controlled by proliferation assays and CD25/L-2Rα expression.

Highly purified B cells were obtained from human peripheral blood monocyte cells (PBMCs) by positive selection by means of anti-CD19 magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The B cells were activated for 4 days in flasks with irradiated (75 Gy) murine L cells transfected with human CD40 ligand and 10 mg/mL IL-4 (R&D Systems, Minneapolis, MN). Activated B cells were always more than 98% CD19+CD50+CD86+.

Jurkat–ΔCOOH was transfected by electroporation by a TAL–1 expressing vector previously described and stable clones were established by limiting dilution. BaF3 cells were transfected by electroporation by an hIAN1-expressing vector allowing G418 resistance and stable clones were also established by limiting dilution.

Cell cycle analysis

Cell cycle analysis was determined by fluorescence-activated cell sorting (FACS) following staining with propidium iodide. Cells were collected by centrifugation and washed with phosphate-buffered saline. The cells were permeabilized and incubated in a buffer containing 100 μg/mL propidium iodide, 50 μg/mL RNase, 0.1% Nonidet P-40, 5 mM NaCl, and 2 mM sodium citrate at 4°C for 2 hours prior to analysis with a Becton Dickinson FACSort analyzer (San Jose, CA). The cell cycle profile was analyzed by means of CellQuest software (Cale...
Nucleotide-binding assays

Binding of GDP was assessed by incubating, at 25°C, 120 nM GST-hIAN1 fusion protein with 5 μM [3H]GDP (Amersham) (11.3 Ci/mmol [41.8 × 10^4 MBq/mmol]) in 50 mM Tris [pH 7.5], 1 mM DTT, 100 μg/mL bovine serum albumin (BSA), and various concentrations of MgCl₂ (10 mM, 1 mM, or 1 μM) or 10 mM EDTA. At the indicated times, 50 μL was removed and immediately diluted in 2 mL washing buffer at 4°C (50 mM Tris [pH 7.5], 1 mM DTT, and 10 mM MgCl₂), filtered through nitrocellulose filters (NC45; pore size, 0.45 mm) (Schleicher and Schuell, Dassel, Germany), and washed 3 times with 2 mL washing buffer. Radioactivity remaining on the filter corresponded to [3H]GDP bound to protein and was measured by liquid scintillation counting.

Specificity of nucleotide binding to hIAN1

The specificity of nucleotide binding to hIAN1 was assessed by a competition assay. First, 120 nM GST-hIAN1 was incubated at 25°C for 120 minutes with 5 μM [3H]GDP in binding buffer (50 mM Tris [pH 7.5], 1 mM DTT, 100 μg/mL BSA, and 10 mM MgCl₂) and with various concentrations (0, 0.3, 3, 30, or 300 μM) of the following nucleotides: adenosine triphosphate (ATP), ribothymidine triphosphate (TTP), cytidine triphosphate (CTP), guanosine monophosphate (GMP), β,γ-imido guanosine triphosphate (GppNHp), a nonhydrolyzable analogue of GTP, and GDP. The residual amount of [3H]GDP bound was determined as above.

Determination of affinities for GDP and GppNHp

The purified GST-hIAN1 protein (120 nM) was incubated with increasing concentrations of [3H]GDP or [3H]GppNHp (25 Ci/mmol [92.5 × 10⁶ MBq/mmol]) (Amersham) in 50 μL binding buffer, at 25°C for 120 minutes. Bound [3H]GDP and [3H]GppNHp were measured as described above. The dissociation constants were calculated after performing a Scatchard analysis of the data.

Guanosine triphosphatase activity

Guanosine triphosphatase (GTPase) activity was assessed by thin-layer chromatography (TLC). First, 0, 5, or 10 μM GST-hIAN1 was incubated at 30°C with 50 μM [α-32P]GTP (4000 cpm/pmol) in 50 mM Tris (pH 7.5), 1 mM DTT, 100 μg/mL BSA, and 10 mM MgCl₂ in a total volume of 10 μL. At 0, 5, and 20 minutes, 2 μL reaction was removed and mixed with 2 μL solution containing 0.2% SDS, 5 mM EDTA, 50 mM GDP, and 50 mM GTP at 4°C. Samples were incubated at 70°C for 2 minutes to dissociate protein-bound nucleotides, and 1-μL aliquots were spotted onto polyethyleneimine-cellulose-covered TLC plates. They were developed in 0.6 M NaH₂PO₄ [pH 3.4], for 30 minutes, dried, and autoradiographed.

Results

Structural features of hIAN1, a protein translated from an mRNA selectively expressed in Jurkat-ΔCOOH

To identify genes potentially regulated by TAL-1 in leukemic cells, we performed a cDNA RDA between Jurkat and Jurkat-ΔCOOH at day 1 of culture, when the growth curves of the 2 cell lines start to differ (Leroy-Viard et al9 and Figure 1A). RDA was performed with the Jurkat-ΔCOOH representations as “testers” and an excess amount of Jurkat representations as “drivers.” After 2 rounds of subtraction, the DP2 product showed discrete bands (data not shown). Random cloning of these differential products followed by screening with a labeled DP2 probe identified a 441-bp DpnII-DpnII DNA fragment that was used to hybridize a Northern blot containing total RNA isolated from Jurkat and Jurkat-ΔCOOH cells at different days of the growth curve. As shown in Figure 1B, the DpnII-DpnII fragment hybridized to a 2-kb RNA that was present only in Jurkat-ΔCOOH throughout the culture. This result clearly identified a Jurkat-ΔCOOH–specific mRNA and prompted us to clone the full-length cDNA.

Using this DpnII-DpnII DNA fragment as a probe, we screened a Jurkat-ΔCOOH cDNA library and isolated 3 positive clones that exhibited a similar restriction-enzyme pattern. Cloning and sequencing of the longest cDNA isolated revealed an ORF of 329 amino acids with the first ATG (nucleotide [nt] 77) lying in a favorable context for translation initiation (aaATGg)12 and preceded by an in-frame stop codon (nt 51) (Figure 2).

The protein encoded by the ORF exhibited 3 motifs related to motifs found in GTP-binding proteins and a potential coiled-coil domain between amino acids 249 and 288 (Figure 2). Because the encoded protein shared significant homologies with murine Immune Associated Nucleotide 1 (IAN-1),13 it was called hIAN1.

Tal-1 and hIAN1 mRNAs are reciprocally expressed in human T-leukemic cells

To assess any relation between the expression of tal-1 and hIAN1 genes, we first analyzed the levels of hIAN1 mRNA in T-cell lines and in T leukemic blasts expressing different levels of TAL-1. The
tal-1 gene expression in T-ALL results from 3 mechanisms: chromosomal translocations, interstitial deletion, or monoallelic activation without apparent gross abnormality of the tal-1 locus. Each mechanism leads to a different TAL-1 protein level. TAL-1 protein expression in T-ALLs harboring sil-tal interstitial deletions is often weaker than in T-ALLs harboring chromosomal translocations or monoallelic activation (our unpublished data, February, 2001). We measured the levels of hIAN1 mRNA in the following cell lines: 2 T-cell lines derived from T-ALL carrying a sil-tal interstitial deletion (CEM and RPMI 8402); 2 cell lines derived from monoallelic activation of the tal-1 locus without obvious abnormalities of the tal-1 gene (Jurkat and Molt-4); and 1 cell line (DU528) in which the tal-1 locus is activated by a chromosomal translocation.\(^1\) Northern blot analysis showed that the tal-1 mRNA level was higher in Jurkat, Molt-4, and DU528 than in CEM or RPMI 8402 (Figure 3A). Conversely, hIAN1 mRNA was present in CEM and RPMI 8402 and absent in Jurkat, Molt-4, and DU528 (Figure 3A).

Similar results were obtained with other T-cell lines and suggested a reciprocal expression of tal-1 and hIAN1 mRNAs in human T-ALLs harboring sil-tal interstitial deletions is often weaker than in T-ALLs harboring chromosomal translocations or monoallelic activation (our unpublished data, February, 2001). We measured the levels of hIAN1 mRNA in the following cell lines: 2 T-cell lines derived from T-ALL carrying a sil-tal interstitial deletion (CEM and RPMI 8402); 2 cell lines derived from monoallelic activation of the tal-1 locus without obvious abnormalities of the tal-1 gene (Jurkat and Molt-4); and 1 cell line (DU528) in which the tal-1 locus is activated by a chromosomal translocation.\(^1\) Northern blot analysis showed that the tal-1 mRNA level was higher in Jurkat, Molt-4, and DU528 than in CEM or RPMI 8402 (Figure 3A). Conversely, hIAN1 mRNA was present in Jurkat, Molt-4, and DU528 (Figure 3A).
various amounts of the wild-type TAL-1 protein (data not shown). Semiquantitative RT-PCR analysis on total RNA isolated from the 5 independent TAL-1–expressing clones was performed. Only one DNA fragment was obtained; its sequence showed that it corresponded to hIAN1 mRNA (data not shown). As shown in Figure 3B, the hIAN1 mRNA level was not affected by the expression of TAL-1 in Jurkat–ΔCOOH transfectants, suggesting that the hIAN1 gene is not a direct target of TAL-1.

hIAN1 belongs to a family of proteins encoded by genes clustered on human chromosome 7 band q36

When we screened the Genbank database with the hIAN1 GTP/GDP–binding domain, we identified partial sequences of 5 new human coding sequences and 1 previously identified coding sequence, hIAN5.18 All the encoded proteins (named hIAN1 to hIAN7) showed a high degree of homology in their putative GTP/GDP–binding domain (Figure 4), indicating that hIAN1 defines a novel protein family.

No significant homology was found elsewhere in these proteins; only 2 members (hIAN1 and hIAN7) exhibited a coiled-coil motif, while hIAN5 has a hydrophobic domain at its COOH terminus.18

We then searched for the chromosomal localization for the hIAN genes and found, by means of the NCBI Map Viewer (National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD) that all of these genes were clustered on human chromosome 7 band q36. Careful analysis of the available sequences of this region revealed that the hIAN1, hIAN2, hIAN5, and hIAN7 genes were linked within 250 kb of genomic DNA and the hIAN3, hIAN4, hIAN6 genes were linked within 60 kb of genomic DNA (data not shown).

Biochemical characterization of hIAN1

As the hIAN members are characterized by a conserved unconventional GTP/GDP–binding motif, we studied the biochemical properties of hIAN1. GTP/GDP binding and GTPase activities usually require 5 motifs, G1, G2, G3, G4, and G5, that are highly conserved throughout the different families of GTP-binding proteins (see Valencia et al19 for a review). The G2 motif contains only a threonine residue involved in binding the Mg2+ ion complexed with GDP or GTP, whereas the other 4 domains share several amino acids. The G1, G3, and G4 motifs of hIAN1 share the consensus residues important for nucleotide binding with other known G proteins, but they contain, relative to Ras and Gα substitutions at 3 conserved positions, Gly12Ras3 Thr39hIAN1, Gln61Ras3 Ile89hIAN1, and Asn116Ras3 Arg171hIAN1 (Figure 5A). Finally, no sequence clearly corresponding to the G5 region can be found in the hIAN1; it should, however, be noted that this domain exhibits little strict conservation across G-protein families (Ras-related, Gα). Thus, hIAN1 protein, like the other members of the IAN protein family, contains notable substitutions of residues considered key in other GTPases.

Since the activity of GTP-binding proteins is regulated by their ability to bind GTP and/or GDP and to hydrolyze GTP, we first tested whether hIAN1 exhibited these properties. The hIAN1 was expressed as a GST fusion protein in Escherichia coli and purified on glutathione-sepharose beads to near homogeneity, as revealed by Coomassie blue staining after SDS-PAGE (Figure 5B). The ability of hIAN1 to bind guanine nucleotides was first studied by incubating the recombinant fusion protein with [3H]GDP and analyzing its association kinetics by means of a filter-binding assay. GST-hIAN1 was able to efficiently bind GDP (Figure 5C) with saturable kinetics; the proportion of active fusion protein calculated...
Figure 5. hIAN1 is a GDP/GTP binding protein. (A) Sequence of nucleotide-binding motifs G1, G3, and G4 from Ras, G\_hIAN1, and mIAN-1. The amino acids written in bold letters have been shown to be conserved in all GTP-binding proteins; positions 12, 59, and 61 of Ras are the amino acids involved in GDP/GTP binding and GTPase activities. (B) Affinity purification of GST-hIAN1. After purification on glutathione-Sepharose beads, material from a noninduced (lane 1) and induced culture of recombinant bacteria (lane 2) was loaded on a 10% SDS-PAGE, followed by staining with Coomassie blue. (C) Kinetics of GDP binding on hIAN1 protein in the presence of 10 mM MgCl\_2. First, 60 nM GST-hIAN1 was incubated with 5 \[^3H\]GDP in 50 mM Tris (pH 7.5), 1 mM DTT, 100 \(\mu\)g/mL BSA, and 10 mM MgCl\_2 at 25°C. The binding of \[^3H\]GDP was measured by filtration as described in “Materials and methods.” All measures were performed in triplicate. (D) Specificity of nucleotide binding to GST-hIAN1. First, 60 nM of GST-hIAN1 was incubated at 25°C for 120 minutes with 5 \(\mu\)M \[^3H\]GDP in 50 mM Tris (pH 7.5), 1 mM DTT, 100 \(\mu\)g/mL BSA, and 10 mM MgCl\_2 in the presence of the indicated concentrations of various nucleotides. The amount of \[^3H\]GDP-bound protein was measured as described in panel B. □, GppNHp; ●, GDP; △, GTP; –, ATP; ○, TTP; ▲, CTP.

Figure 6. Determination of the hIAN1 affinity for GDP and GppNHp. First, 60 nM GST-hIAN1 protein was incubated for 120 minutes at 25°C with various concentrations of GDP (0.175, 0.3, 0.7, 1.5, 3, 5, and 15 \(\mu\)M) or GppNHp (2, 4, 8, 10, 16, 30, 60, 80, 100, and 120 \(\mu\)M); then, the amounts of bound nucleotide were determined by filter-binding assays. All measures were performed in triplicate. Panels A and C show the concentration-dependent binding of GDP (panel A) and GppNHp (panel C) to GST-hIAN1. Scatchard representations are shown in panel B (GDP) and panel D (GppNHp). r indicates bound nucleotide/total protein (pmoles); F is the concentration of free nucleotide (micromolar).

Expression of hIAN1 mRNA in normal tissues

We studied hIAN1 mRNA expression in several normal tissues by Northern blot analysis (Figure 8A). The hIAN1 mRNA was highly expressed in spleen and peripheral blood leukocytes that contain mostly B and T lymphocytes. The hIAN1 mRNA was also detected, but at a lower level, in thymus, small intestine, colon, and ovary, but not detected in prostate or testis.

Figure 7. GTPase activity of GST-hIAN1. Intrinsic GTPase activity was assessed by TLC analysis. Different amounts of GST-hIAN1 were incubated with \[^{32}P\]GTP, and GTP hydrolysis was analyzed by TLC. As shown in Figure 7, a radioactive spot corresponding to GDP appeared; its intensity increased with time and with the amount of GST-hIAN1 protein in the reaction. No spot corresponding to GMP was detected, showing that GDP formation was not due to a contaminating phosphatase activity in the reaction.

Thus, this experiment demonstrated that hIAN1 exhibits an intrinsic GTPase activity, despite the divergent amino acids thought to be involved in the mechanisms of the GTPase activity of Go and Ras.

from such saturation curves was found to be from 30% to 38% depending on the protein preparation used in the experiments. The ability of the protein to bind GDP was highly dependent on the concentration of Mg\(^2+\) since it dramatically decreased at low (10 \(\mu\)M and 1 mM) free concentrations of MgCl\_2 or in the presence of 10 mM EDTA (data not shown).

The specificity of nucleotide binding to hIAN1 was examined in a competition–binding assay (Figure 5D). Both GDP and the nonhydrolyzable analogue of GTP, GppNHp, were efficient competitors of \[^3H\]GDP binding to hIAN1, though with different potencies: approximately 12-fold more GppNHp than GDP was required to inhibit 50% binding of \[^3H\]GDP, indicating a large difference in affinities for the diphosphate and triphosphate guanine nucleotides. In contrast, GMP was unable to compete with \[^3H\]GDP; neither were the other nucleotide triphosphates, ATP, CTP, and TTP (Figure 5D).

After establishing that the binding of GDP and GppNHp at low concentrations (0.7 and 2 \(\mu\)M, respectively) reached a plateau at 90 minutes (data not shown), we further characterized the respective affinities of hIAN1 for GDP and GppNHp. Thus, the direct binding of \[^3H\]GDP and \[^4H\]GppNHp were measured after 120 minutes at various nucleotide concentrations (Figure 6A,C).

Scatchard analysis of the data (Figure 6B,D) revealed that hIAN1 indeed bound both nucleotides to a similar extent (0.3 to 0.38 site per molecule protein, according to the preparation used) but with widely different affinities, since the dissociation constants calculated from these experiments were 0.47 \(\mu\)M for GDP and 6 \(\mu\)M for GppNHp, consistent with the competition experiment described above.

Since GTP hydrolysis plays an important role by regulating the activity of GTP-binding proteins, we investigated the intrinsic GTPase activity of hIAN1. Different amounts of GST-hIAN1 were
The use of an anti-ZAP70 antibody.25 (C) B lymphocytes were purification, and Western blot analysis was performed by means of the anti-hIAN1 antiserum. The loading control for the Western blot was obtained with

Figure 9. Modulation of hIAN1 protein level during T- and B-lymphocyte activation. (A) The hIAN1, hIAN2, and hIAN7 cDNAs were in vitro transcribed/translated as described in "Materials and methods," and the 35S-labeled protein products were run on a 10% SDS-PAGE, blotted, and revealed by the anti-hIAN1 antiserum. (B) T lymphocytes were purified from human peripheral blood and activated with anti-CD28 and anti-CD3. Before activation (lane 1), and 30 minutes (lane 2), 5 hours (lane 3), 1 day (lane 4), 4 days (lane 5), and 6 days (lane 6). Using a semiquantitative PCR assay, we showed that the hIAN1 mRNA level remained constant during this CD3/CD28 T-lymphocyte activation while the hIAN1 protein level started to decrease at day 4 and was undetectable at day 6. A similar study was performed on a CD40-ligand/IL-4 B-lymphocyte activation and showed a similar discrepancy between hIAN1 mRNA and protein levels (Figure 9C), with the decrease of hIAN1 protein level starting earlier during this B-lymphocyte activation than in the CD3/CD28 T-lymphocyte activation. These results indicate that hIAN1 gene expression is highly regulated at the posttranscriptional level during B- and T-cell activation.

Following IL-3 withdrawal, hIAN1 expression in BaF3 cells led to a G2/M cell cycle arrest

The murine pre-B hematopoietic cell line BaF3 is completely dependent on IL-3 for cell proliferation and survival. These cells did not express mIAN1 mRNA (data not shown) and were used to study a possible role for hIAN1 in cell proliferation and/or survival. We established 3 stable BaF3 clones that expressed various levels of the hIAN1 protein (Figure 10A) and studied these cells in the presence or absence of IL-3. Both the parental BaF3 cells
transfected with the empty vector (BaF3-E) and the 3 clones proliferated normally with no difference in cell cycle or apoptosis in the presence of IL-3 (data not shown). After 14 hours of IL-3 deprivation, the BaF3-E and the 3 clones displayed similar signs of apoptosis (30% to 40% of apoptotic cells), and longer deprivation led to cell death in both BaF3-E and BaF3-hIAN1 cells.

However, while the BaF3-E cells were arrested at the G_{1} phase of the cell cycle, the 3 BaF3-hIAN1 clones were arrested at the G_{1} and the G_{2}/M phases of the cell cycle. Interestingly, the ratio between the G_{2}/M and the G_{1} phase arrest seemed to be correlated with the hIAN1 expression level, suggesting that hIAN1 might be involved in the G_{1}-to-S transition in BaF3 cells deprived of IL-3.

**Discussion**

Although TAL-1 is expressed in more than 30% of human T-ALLs, most of the pathways regulated by this expression are currently not defined. To characterize genes potentially regulated by TAL-1 during T-lymphocyte leukemogenesis, we compared mRNAs expressed in Jurkat, a human T-cell line that expresses TAL-1, with those expressed in Jurkat-ΔCOOH, a Jurkat subclone that expresses a mutated TAL-1 protein that cannot bind DNA. Phenotypically, Jurkat and Jurkat-ΔCOOH growth curves started to diverge at day 1 of culture; thus, this time point was chosen for RDA analysis. Although we might have missed genes turned on or off by TAL-1 later in the culture, most of the mRNAs we have characterized showed the same expression pattern throughout the 5 days of culture (unpublished data, December, 1998). Using Jurkat-ΔCOOH representations as “testers” and Jurkat representations as “drivers,” we have characterized a human gene, hIAN1, that is specifically expressed in Jurkat-ΔCOOH and thus might be negatively regulated by TAL-1. We then showed a reciprocal expression of tal-1 and hIAN1 mRNAs in T-cell lines and in leukemic blasts from T-ALL patients that expressed different levels of TAL-1 protein, and these results extended our preliminary finding. However, the expression of the wild-type TAL-1 protein in Jurkat-ΔCOOH cells had no effect on hIAN1 gene expression, suggesting that hIAN1 is not a direct TAL-1 target gene.

The reciprocal expression of tal-1 and hIAN1 mRNAs in T-cell lines and T leukemic blasts that expressed different levels of TAL-1 protein can be linked to recent studies\(^{20,21}\) showing that the presence of oncogenic transcription factors in T-ALL induces a blockade at specific stages of thymocyte differentiation and suggests that the expression of one of these factors during T-lymphocyte leukemogenesis is correlated to a specific pattern of gene expression that reflects the stage in which the disruption of T-cell development has occurred. As the presence of TAL-1 in T-ALL leads to an arrest at the double-positive stage and as mIAN-1 started to be expressed at the double-positive to single-positive stage during T-cell differentiation,\(^{13}\) our data on hIAN1 and tal-1 mRNA expression in T-ALL suggest there might be a slight delay in the blockade of T-cell differentiation, depending on whether the tal-1 gene is activated by chromosomal translocations or by sil-tal rearrangements. The hIAN1 therefore appeared as a new marker of T-cell differentiation blockade in TAL-1–dependent T-ALL.

The study of the hIAN1 primary structure showed that it contains motifs related to those found in G proteins. However, these motifs displayed amino acid substitutions at 3 highly conserved positions (corresponding to the positions 12, 61, and 116 in the Ras protein) that are important for nucleotide binding as well as intrinsic and GTPase-activating protein–stimulated GTPase activities.\(^{22-24}\) Using this putative GTP/GDP-binding domain as a probe, we searched, *in silico*, for human proteins that contain a similar domain and found 6 other proteins encoded by genes clustered on human chromosome 7 band q36. Apart from this putative GTP/GDP–binding domain, these proteins do not display any significant homology, suggesting that this family of proteins is defined only by this domain. A recent study has shown that mIAN-4, a member of the mouse IAN family, is located in the central region of mouse chromosome 6, which is proximal to a region of synteny to human chromosome 7p15-14.\(^{18}\)

However, as noticed by the authors, there is a break in synteny between human chromosome 7p15-14 and 7q34-35 in a region where the mIAN-4 gene lies. Here, we showed that the human IAN family gene is located on chromosome 7q36, and this clarifies the region of synteny between these 2 chromosomes.

Despite the amino acid substitutions present in the putative GTP/GDP domain of hIAN1, our biochemical study showed that hIAN1 is a true GTPase and thus defines a new family of G proteins. An interesting finding of the current study concerned the different binding affinities of hIAN1 for GDP and GppNHp (about 12-fold higher for GDP) and the absolute requirement of Mg^{2+} for GDP and GppNHp binding. These properties are not found for Ras-like proteins and might be related to the particular nucleotide-binding site of hIAN1. Despite these unusual characteristics, equilibrium-binding calculations show that, given the generally accepted cellular concentrations for GDP (100 μM) and GTP (1 mM), hIAN1 should be 50% bound to GTP in the absence of...
GT-Pase activity and auxiliary proteins. This prediction is supported by a recent study on mIAN-4 indicating that part of this protein is associated with GTP in vitro.\textsuperscript{18} This strongly suggested that despite its difference in affinity for GDP and GTP, hIAN1, like most other G proteins (eg, EF-Tu, Ras-related, G heterotrimeric) could act as a molecular switch cycling between biochemically distinct GDP- and GTP-bound forms. Thus, the IAN family seems to define a novel type of GT-Pase proteins, and the identification of proteins regulating their nucleotide-binding state and GT-Pase activity will shed light on the signal transduction pathways involving IAN proteins.

As the hIAN1 or mIAN-1 expression pattern suggests a function of this protein in the immune system, we first studied the regulation of hIAN1 gene expression during B- and T-lymphocyte activation. We showed that the hIAN1 protein level decreased at an undetectable level during a CD40-ligand/IL-4 B-cell activation or a CD3/CD28 T-cell activation while the steady-state hIAN1 mRNA levels are largely unaffected during these activations. These results suggested a regulation of the hIAN1 mRNA translation and/or a specific degradation of the hIAN1 protein during B- or T-lymphocyte activation, and we are currently studying the posttranscriptional regulation of hIAN1 gene expression in mature B and T lymphocytes. Finally, we showed that, following IL-3 withdrawal, the expression of hIAN1 in BaF3 cells could lead to a G2/M arrest, while parental BaF3 cells were arrested at the G1 phase. This result, together with the expression pattern of hIAN1 protein during the B- and T-cell activation, suggested that hIAN1 might be involved at some steps of the G1 checkpoint of the B- and T-lymphocyte cell cycle.

In conclusion, the highly regulated expression of the hIAN1 protein during B- and T-lymphocyte activation, together with the coordinated expression of all the genes coding for the hIAN members in resting T and B lymphocytes, suggested the presence of currently unknown coordinated functions of this protein family during the immune response. The biochemical characterization of hIAN1 shown in this study, together with previous work on the GT-Pase structure/function,\textsuperscript{22-24} will provide a molecular basis for designing gain or loss of function hIAN proteins, which represent powerful tools for studying the function of these proteins in the immune response.

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References

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