



A Giant GTPase, Very Large Inducible GTPase-1, Is Inducible by IFNs

Thorsten Klamp, Ulrich Boehm, Daniela Schenk, Klaus Pfeffer and Jonathan C. Howard

This information is current as of August 4, 2022.

J Immunol 2003; 171:1255-1265; ;
doi: 10.4049/jimmunol.171.3.1255
<http://www.jimmunol.org/content/171/3/1255>

Supplementary Material <http://www.jimmunol.org/content/suppl/2003/07/22/171.3.1255.DC1>

References This article cites 45 articles, 21 of which you can access for free at:
<http://www.jimmunol.org/content/171/3/1255.full#ref-list-1>

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>



A Giant GTPase, Very Large Inducible GTPase-1, Is Inducible by IFNs¹

Thorsten Klamp,* Ulrich Boehm,^{2*} Daniela Schenk,* Klaus Pfeffer,[†] and Jonathan C. Howard^{3*}

The complex, partially overlapping, cellular responses to IFN type I (IFN- α and - β) and IFN type II (IFN- γ) involve several hundred genes that can be largely classified in terms of specific cellular programs functional in innate and adaptive immunity. Among these programs are previously unconsidered mechanisms of cell-autonomous resistance against various pathogens mediated by dedicated, largely novel families of GTPases. We report here the identification and characterization of a new GTPase family that contributes to the cellular response to both type I and type II IFNs. We name this family the very large inducible GTPases (VLIGs). The prototype VLIG, VLIG-1, is a strongly IFN-inducible, soluble, cytosolic and nuclear protein of 280 kDa. The open reading frame of VLIG-1 is encoded on a single very large exon, and outside the canonical GTP-binding motifs, sequence and structural prediction suggest a unique family without significant relationship to other known protein families. Within the GTPase superfamily the VLIG family is more closely related to IFN-inducible GTPases mediating cell-autonomous resistance than to other GTPase families. In addition, we provide evidence that VLIG-1 is polymorphic in mice of different genetic backgrounds and is a member of a small gene family on mouse chromosome 7 with a conserved homologue located on human chromosome 11. *The Journal of Immunology*, 2003, 171: 1255–1265.

The type I (α and β) and type II (γ) IFNs regulate partially overlapping sets of several hundred genes at the transcriptional level (1–4). Binding of IFNs to their specific cell surface receptors leads to the activation of distinct, but related, signaling pathways (5, 6). Some components of the signal transduction and transcriptional activation machinery are shared by type I and type II IFN, providing an explanation for the observed overlapping biological activities of the two IFN types (1, 6, 7).

Despite their complexity, the cellular responses to both types of IFN can be described largely in terms of specific cellular programs functional in innate and adaptive immunity, as extensively reviewed over the last few years (1, 2, 6, 8). Although IFNs regulate some proteins with cell type specificity, especially in professional cells of the lymphoid system, much of the response appears to be cell type nonspecific (1, 2, 6). The IFN response thus represents a generalized property of all or most differentiated cell types, and cells exposed to IFNs are converted to a new, complex, multidimensional state of resistance to infectious organisms.

There is now much interest in fully cell-autonomous resistance mechanisms stimulated by IFNs, which interact destructively with the intimate cell biology of intracellular pathogens. The paradigm for such a mechanism is the small family of dynamin-like large GTPases, the Mx proteins, which are induced strongly in all cell

types by type I IFNs (9). In a transfection setting, the expression of these proteins alone is sufficient to confer resistance to several RNA viruses on otherwise susceptible cells (9, 10). Two additional families of IFN-inducible GTPases have been implicated in cell-autonomous resistance phenomena (11–15). This may suggest that some common principle of action involving GTP-GDP exchange is involved in the resistance function, although there is essentially no sequence homology between the protein families. Human guanylate-binding protein (hGBP-1),⁴ a member of the p65 guanylate binding proteins, has been shown to confer cell-autonomous resistance against encephalomyocarditis virus and vesicular stomatitis virus (11), while several members of the p47 GTPase family have been shown to be required for resistance to vesicular stomatitis virus (12) and to *Listeria monocytogenes* and *Toxoplasma gondii* (13–15).

In this paper we report the discovery and characterization of a fourth family of IFN-inducible GTPases identified in cDNA libraries of mouse primary embryonic fibroblasts (MEFs) and ANA-1 macrophages stimulated with type II IFN (IFN- γ) after subtractive hybridization with cDNA from unstimulated cells. The subject of this study, very large inducible GTPase-1 (VLIG-1), is the prototype of the new GTPase family. Encoded on a strongly IFN-inducible, 9-kb transcript, the VLIG-1 protein has a molecular mass of ~280 kDa and is the largest known GTPase in any species. The entire protein coding sequence (CDS) is encoded on a single very large exon. In the mouse, VLIG-1 is one member of a cluster of at least six similar genes on chromosome 7, and the family is represented in humans by a single close homologue encoded on chromosome 11. The greatest part of the long protein sequence does not present structural features of any other protein family. However, VLIG-1 has a canonical GTP-binding motif embedded in a local sequence environment with affinities closest to the nucleotide binding domains of the other IFN-inducible GTPase families listed

*Institute for Genetics, University of Cologne, Cologne, Germany; and [†]Institute for Medical Microbiology, University of Düsseldorf, Düsseldorf, Germany

Received for publication December 9, 2002. Accepted for publication May 28, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by grants from the Deutsche Forschungsgemeinschaft (to J.C.H. (Sonderforschungsbereich 243) and to K.P. (Pf259/2-4, Sonderforschungsbereich 455 and 576)).

² Current address: Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue, Seattle, WA 98109.

³ Address correspondence and reprint requests to Dr. Jonathan C. Howard, Institute for Genetics, University of Cologne, Zùlpicher Strasse 47, D-50674 Cologne, Germany. E-mail address: jonathan.howard@uni-koeln.de

⁴ Abbreviations used in this paper: hGBP, human guanylate-binding protein; ActD, actinomycin D; BAC, bacterial artificial chromosome; CDS, coding sequence; CHX, cycloheximide; EST, expressed sequence tag; IRF-1, IFN-regulatory factor 1; MEF, mouse embryonic fibroblast; mGBP, mouse GBP; ORF, open reading frame; UTR, untranslated region; VLIG, very large inducible GTPase.

above. It therefore seems likely that VLIG-1 will prove to represent a new class of resistance GTPases that share a common mode of action with these proteins.

Materials and Methods

Mice

C57BL/6J, 129/Sv, and BALB/c mice were obtained from the animal house at Institute for Genetics, University of Cologne (Cologne, Germany). Mice deficient for IRF-1 (IRF-1^{-/-}) or the IFN- γ R (IFN- γ R^{0/0}), both with the 129/Sv genetic background, have been described previously (16, 17) and were provided by Drs. C. Weissmann and M. Aguet, respectively. All mice were maintained in a conventional animal facility.

Cells and tissue culture

MEFs from C57BL/6J, 129/Sv, BALB/c, IRF-1^{-/-}, and IFN- γ R^{0/0} mice were isolated on day 14 post coitum (18) and grown as described previously (19).

The fibroblast-like L929 cell line derived from C3H/An mice (20) was maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (ICN, Costa Mesa, CA), 100 μ g/ml streptomycin (Invitrogen), and 100 μ g/ml penicillin (Invitrogen).

The macrophage-like cell line ANA-1 (21), derived from C57BL/6J mice, was cultured in low endotoxin RPMI (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated, low endotoxin FCS (HyClone, Logan, UT) and 50 μ M 2-ME (Invitrogen).

For Northern and Western blot analyses (see below), cells were stimulated for different lengths of time with different concentrations of recombinant mouse IFN- γ (Genzyme Diagnostics, Cambridge, MA), recombinant mouse IFN- β (Calbiochem, San Diego, CA), or recombinant TNF- α (Genzyme Diagnostics) as indicated in the text.

For treatment of cells with the protein translation inhibitor, cycloheximide (CHX), (Sigma-Aldrich), CHX was solubilized in PBS and added directly to the culture medium, either alone or 30 min before the addition of 100 U/ml IFN- γ . The final concentration of CHX was 50 μ g/ml.

The transcription inhibitor, actinomycin D (ActD; Sigma-Aldrich), was solubilized in PBS and added directly to cells that had been exposed to 100 U/ml IFN- γ for 24 h. The cells were then incubated with ActD and IFN- γ for various times, as indicated in the text. The final concentration of ActD was 50 μ g/ml.

L. monocytogenes infection

Mice were infected by i.p. injection with 1/10th of the LD₅₀ of *L. monocytogenes*, strain EGD, as previously described (19).

Northern blot analysis

Total RNA from tissues or cells was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany), the DNA/RNA isolation kit (Amersham Pharmacia Biotech, Little Chalfont, U.K.), or the acid guanidinium thiocyanate-phenol-chloroform extraction method (22). The amount of RNA was quantified spectrophotometrically. The Northern blots were prepared as described previously (19) and quantified with methylene blue.

The analysis of basal VLIG-1 expression in different mouse tissues was performed using the Ambion FirstChoice Mouse Blot I (Ambion, Austin, TX), the Strip-EZ DNA kit (Ambion) for generating [α -³²P]dATP (Amersham Pharmacia Biotech)-labeled probes and the ULTRAhyb buffer (Ambion) for overnight hybridization. The blot was washed under stringent conditions following the manufacturer's instructions.

RNase protection assay

RNase protection assays were performed as previously described (23). The VLIG-1 probe covers a 294-bp fragment of the long protein-coding exon (nt 5310–5603). A probe protecting 160 bp of the murine TATA box binding protein served as an internal loading control and was provided by Dr. V. Steimle (Max Planck Institute of Immunology, Freiburg, Germany). [α -³²P]UTP (Amersham Pharmacia Biotech)-labeled antisense riboprobes were generated by in vitro transcription with SP6 or T3 RNA polymerase (Roche, Mannheim, Germany). The probes were gel-purified and hybridized overnight to 10 μ g of total RNA in the presence of 40 μ g of yeast tRNA in hybridization buffer (40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA, and 80% formamide) at 50°C. After digestion with RNases A and T1, the protected fragments were analyzed by electrophoresis on 5% denaturing polyacrylamide gels. Bands were visualized by autoradiography and quantified by phosphorimager analysis using Fuji BAS1000 (Fuji

Photo Film, Düsseldorf, Germany) and the evaluation software TINA 2.08a (Raytest Isotopenmessgeraete, Straubenhardt, Germany).

5'RACE

Poly(A)⁺ RNA was isolated from total RNA of C57BL/6J spleen with the Oligotex mRNA kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. 5'RACE was performed using Clontech's SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) following the manufacturer's protocol. One microgram of poly(A)⁺ RNA and the internal VLIG-1 primer 242L (5'-CTCAAAAGGAAGATGGCGGGGGTGG-3') were used for first-strand cDNA synthesis. Universal Primer A Mix and primer 242L were used for the first round of 5'RACE PCR, whereas Nested Universal Primer A and the internal VLIG-1 primer 289L (5'-CTTTCACCTCAACGAGTCCTG-3') were used for the second round. The resulting PCR product was gel-purified, cloned into the pGEM-T Easy vector (Promega, Madison, WI), and sequenced with T7 or SP6 vector primer.

Antisera and Western blot analysis

Two rabbit antisera against VLIG-1 were prepared. Antiserum VLIG-1/B was prepared by immunization with an in-frame GST fusion protein representing aa 270–541 of the VLIG-1 sequence. The VLIG-1 sequence segment was cloned into pGEX-4T-2 (Amersham Pharmacia Biotech), expressed in *Escherichia coli* BL21, recovered from inclusion bodies (24), and resolubilized using the Roti-Fold reagent (Carl Roth, Karlsruhe, Germany). Antiserum VLIG-1/218 was generated against two synthetic peptides (9–24, DEPQLQSRKHNQEM(C) (peptide 919) and 1036–1050, KSPQDKSYSHRNQQM(C) (peptide 920)) from the predicted VLIG-1 protein sequence, using Eurogentech's Double X Program (Eurogentech, Seraing, Belgium), both including an additional C-terminal cysteine for keyhole limpet hemocyanin coupling. Affinity purification of serum VLIG-1/218 showed that all the immunological activity for VLIG-1 was directed against the N-terminal peptide (peptide 919). Other primary Abs used were rabbit anti-calnexin antiserum (StressGen, Victoria, Canada) at a dilution of 1/2000 and goat anti-mouse GBP2 (anti-mGBP2) antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1/1500. As detection reagent, HRP-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany) or HRP-conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology) was used at a dilution of 1/2000. Unless otherwise noted, cell lysates were prepared according to the method described by Knittler et al. (25).

Nucleotide binding assay

MEFs derived from C57BL/6J mice were induced for 24 h with 100 U/ml IFN- γ , washed twice with ice-cold TBS containing 3 mM MgCl₂, and disrupted by freezing and thawing. The resultant suspension was centrifuged at 3,000 \times g for 15 min at 4°C. The supernatant was recentrifuged at 100,000 \times g for 1 h at 4°C. Aliquots of the high speed supernatant corresponding to 1 \times 10⁶ cells were incubated for 1 h at 4°C with 50 μ l of guanine nucleotide-agarose resins (Sigma-Aldrich). For competition experiments, 10 mM free GTP, GDP, or GMP (Sigma-Aldrich) was added to the nucleotide-agarose resins. The nucleotide-agaroses with the bound material were pelleted by centrifugation at 17,000 \times g for 1 min at 4°C and the supernatant removed. After washing, the beads were shaken for 15 min at room temperature with SDS sample buffer, followed by heating for 5 min at 65°C. Eluted proteins were separated on a 10% SDS-polyacrylamide gel and transferred to membranes for Western blots. Before blocking, the membrane was cut into two parts that were probed separately for VLIG-1 and mGBP-2.

Immunofluorescence analysis

MEFs derived from C57BL/6J mice growing on glass coverslips were stimulated for 24 h with 100 U/ml IFN- γ or were left unstimulated. The cells were then washed in PBS containing 0.9 mM CaCl₂ and 0.5 mM MgCl₂, for 5 min and fixed in 3% paraformaldehyde for 20 min at room temperature. Fixed cells were incubated for 10 min in blocking buffer (PBS, 3% BSA, 0.2% gelatin, 0.02% NaN₃, and 0.1% saponin) and incubated with anti-VLIG-1 antiserum VLIG-1/218 (diluted 1/500 in blocking buffer) for 1 h in a humid chamber. After washing with PBS containing 0.1% saponin, the cells were incubated for 30 min with the second-stage Ab, Cy3-conjugated goat anti-rabbit (Molecular Probes, Leiden, The Netherlands), diluted 1/1000 in blocking buffer. Stained cells were analyzed using an Axioplan II fluorescence microscope (Carl Zeiss, Oberkochen, Germany), a cooled Photometrics Quantix CCD camera (Roper Scientific, Trenton, NJ), and Metamorph software (version 4.5r3; Universal Imaging Corp., West Chester, PA).

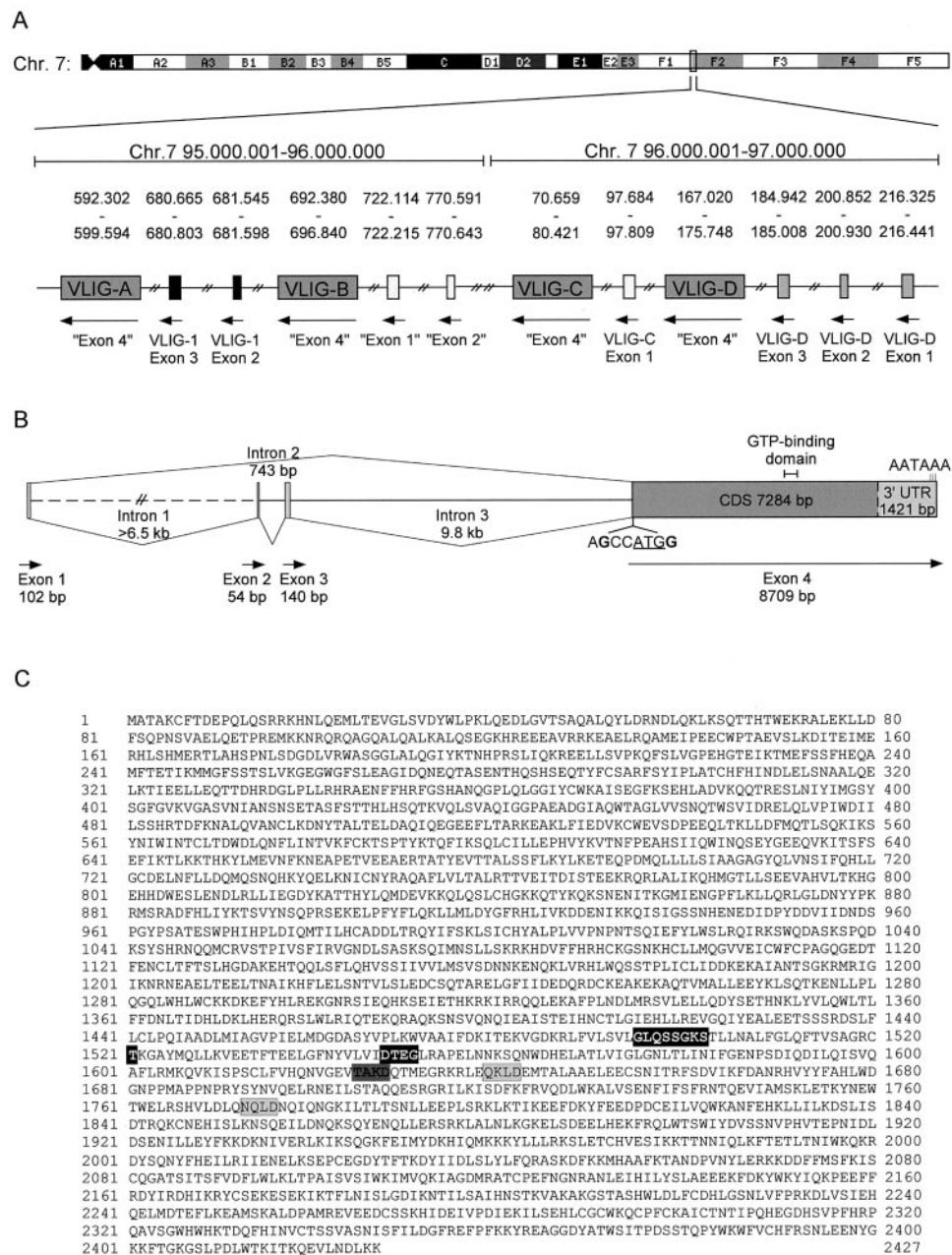


FIGURE 1. Chromosomal localization of VLIG family members, VLIG family structure, gene structure of VLIG-1, and predicted protein sequence of VLIG-1. **A**, Localization of mapped VLIG family members on mouse chromosome 7 based on the ENSEMBL database, version MGSC v12.3.1. The identified genomic region on mouse chromosome 7 between bp 95,560,000 and 96,220,000 is not yet fully sequenced, but includes several contigs. The relative positions of mapped VLIG family members within these contigs, their orientation and structure with regard to VLIG-1 full-length cDNA, as well as the position of VLIG-1, based on the positions of exons 2 and 3 (black boxes) and of mapped BACs carrying VLIG-1, are indicated. Exons 1–3 of VLIG-D and exon 1 of VLIG-C were confirmed by ESTs containing these sequences. **B**, Schematic drawing of the preliminary gene structure of VLIG-1. The genomic structure was determined by sequencing of C57BL/6J-derived BACs and P1 artificial chromosomes and by database analysis. Intron 1 is not yet fully sequenced. The alternative splice sites, the exon/intron sizes, the proposed start codon with its surrounding Kozak sequence, the putative polyadenylation signal sequences (AATAAA), and the position of the GTP-binding domain are indicated. **C**, Predicted protein sequence of VLIG-1. The identified G1 and G3 motifs and the predicted G2 threonine are printed in white in black boxes, the preferred candidate G4 motif is black in a dark gray box, and the other G4 candidates are in light gray boxes.

Nucleotide sequencing, sequence alignment, and analysis

Sequencing was performed in the sequencing facility of the Institute for Genetics. DNA and protein sequence database searches were performed using BLAST, PSI-BLAST, and TBLASTN on publicly available databases. Searches for protein motifs were performed with the ProfileScan program using PROSITE and Pfam databases. The GenBank accession numbers for VLIG-1 are AY167974 (clone A), AY167973 (clone B), and AY167972 (predicted full-length sequence). Protein sequences used for Figs. 1 and 2 are VLIG-1 (NP_083276), Mx1 (NP_034976), IIGP (CAA07798), hGBP-1 (P32455), and H-Ras (P01112).

Results

Identification and molecular cloning of VLIG-1, a novel member of the GTPase superfamily

In a previous report (19), we showed striking over-representation of cDNA fragments of members of the IFN-inducible p47 and p65 resistance GTPase families in PCR-Select libraries from IFN- γ -induced C57BL/6 MEFs and ANA-1 macrophages, subtracted

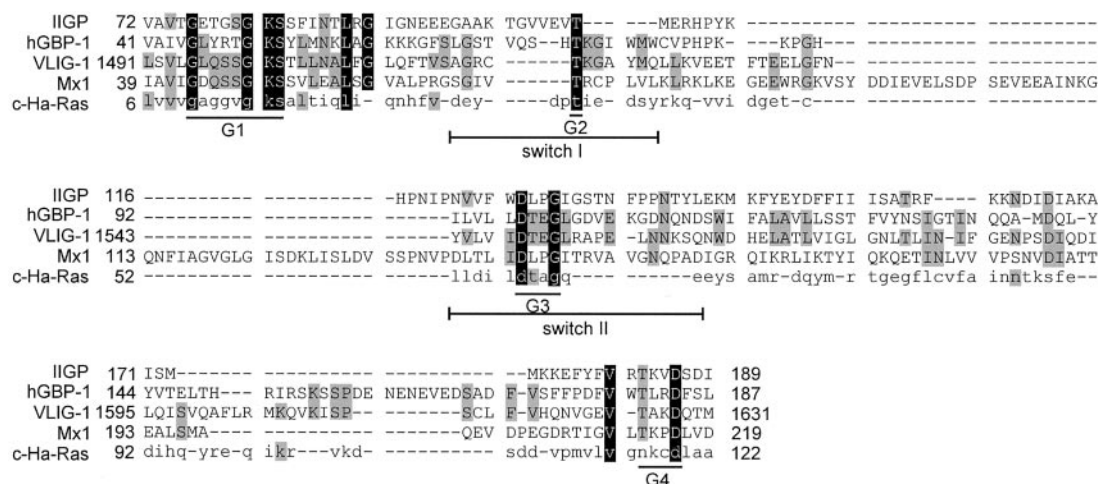


FIGURE 2. Manual alignment of the putative GTP-binding domain of VLIG-1 with members of the other three IFN-inducible families and with H-Ras. The following sequences were used for the alignments: IIGP (CAA07798), hGBP-1 (P32455), VLIG-1 (NP_083276), Mx1 (NP_034976), and H-Ras (P01112). The beginning and end of the aligned sequences are indicated in each case. The alignment of Ras with the IFN-inducible sequences follows the structure-based alignment with hGBP-1 given previously (29), including identification of the switch regions. The four recurrent motifs characterizing GTPase domains are indicated as G1–G4. The identification of the G2 threonine residue in IIGP is conjectural, while that of VLIG-1 follows from the similarity with hGBP-1 in this region. Residues printed in white in black boxes are shared by all five sequences; residues in gray boxes show further identities between VLIG-1 and the other four GTPases.

with resting cells in each case. Initial screening revealed a 743-bp cDNA fragment of unknown sequence derived from the ANA-1 library, which hybridized in Northern blots to an IFN- γ -inducible transcript of ~ 9 kb. Two additional nonoverlapping cDNA fragments of unknown sequence, 464 and 641 bp long, respectively, were isolated from the MEF differential library and hybridized to an inducible transcript of similar length. The 743-bp fragment was used to screen a size-selected cDNA library prepared from IFN- γ -stimulated C57BL/6J MEFs (19). Several hybridizing cDNAs longer than 8 kb were recovered, of which the largest was 8988 bp (clone A; GenBank accession no. AY167974). Clone A included all three PCR-Select cDNA fragments and contained a single open reading frame (ORF) of 7358 nt. 5'RACE performed on spleen RNA from C57BL/6J mice extended clone A by 16 additional 5' nucleotides. After analysis of the expressed sequence tag (EST) database, an apparent error in clone A cDNA was corrected by inserting a single base into the sequence in the 3'-untranslated region (3'UTR). The corrected full-length VLIG-1 sequence of 9005 bp has been submitted as GenBank accession number AY167972. The protein CDS of 7281 bp from the first potential translation initiation codon (Fig. 1B) corresponds to a putative protein of 2427 aa (Fig. 1C) with a calculated relative molecular mass (M_r) of 280814 and an isoelectric point of 6.12. Since this protein has not previously been identified, we name it VLIG-1. Clones of VLIG-1 with different 3' UTR lengths corresponding to three separate polyadenylation signals (Fig. 1B) were recovered from the cDNA library. An additional cDNA clone of 8794 bp corresponding to VLIG-1 (clone B; GenBank accession no. AY167973) was recovered from the cDNA library, differing from clone A only in the length of the 5'UTR (Fig. 1B).

Bacterial artificial chromosome (BAC) clones containing VLIG-1 were identified from 129/J and C57BL/6 genomic libraries (see supplemental Table I for clone identifiers).⁵ Our partial genomic sequence from a C57BL/6 BAC clone RP23-454A11 was confirmed by the mouse ENSEMBL database. The VLIG-1 gene is subdivided into four exons, and the entire CDS of VLIG-1 is lo-

cated on the very large fourth exon of 8709 bp (Fig. 1B). The first nucleotides of this exon form a reasonably good Kozak sequence (26). The different 5'UTR lengths of clones A and B are due to exclusion of the second and third exons by alternative splicing in clone B (Fig. 1B). The predicted AUG start codon was the same for both clones, preceded in both cases by a stop codon in the respective 5'UTRs. Thus, both isoforms encode the same protein product.

The GTP-binding domain of VLIG-1 is related to that of other IFN-inducible resistance GTPases

The predicted amino acid sequence of VLIG-1 showed the canonical G1 ($G(X_4)GKS$) and G3 ($D(X_2)G$) motifs conserved in most GTPases (27, 28). No canonical G4 motif ($(N/T)KXD$) was present, but three related sequences (TAKD, 1625–1628; QKLD, 1639–1642; and NQLD, 1773–1776) were noted (Fig. 1C). No other similarity to known proteins or protein domains was detected, and no recurrent sequence elements indicating internal duplication were seen. VLIG-1 thus represents a novel class of IFN-inducible GTPases. The only homology to other proteins could be found at the canonical GTP-binding motifs and in their immediate sequence environment. Comparison of VLIG-1 with the extensive classification of GTPases recently published by Leipe et al. (28) suggested that the G domain of VLIG-1 has the closest similarity to two other families containing IFN-inducible GTPases, namely the dynamin-related Mx proteins and the p65 guanylate-binding proteins. Fig. 2 shows a manual alignment of the GTP-binding domain of VLIG-1 compared with that of mouse Mx1, human GBP-1 for which a high resolution crystal structure is available (29) as well as a member of the mouse p47 IFN-inducible GTPases, IIGP (19). VLIG-1 shares with IIGP and hGBP-1 as well as p21-Ras a relatively short region between G1 and G3 motifs, compared with the long insertion in the Mx proteins. In particular, VLIG-1 shows a number of points of resemblance to hGBP-1. These include the sequence environment of the putative G2 threonine (T1512) and the somewhat extended sequence before the G4 motif, which neither Mx1 nor IIGP possesses. This latter region contributes an additional short helix and an unstructured loop to

⁵ The on-line version of this article contains supplemental material.

the hGBP-1 structure relative to the canonical p21-Ras structure. Interestingly, the DENENEVED sequence, which further extends the loop in hGBP-1 at residues 159–167, but not in VLIG-1, in this region, is also not present in some other p65 GBPs. However, perhaps the strongest point of resemblance between VLIG-1 and hGBP-1 is the absence of a canonical G4 (N/T)KXD motif. If the manual alignment of Fig. 2 is to be trusted in this region, then the unusual hydrogen-bonding network to the guanine base of the R183 of hGBP-1 would be implemented by K1627 of VLIG-1, while the aspartate D1628 would correspond to aspartate D184 of hGBP-1, which also hydrogen-bonds to the base.

With respect to the putative catalytic mechanism, again VLIG-1 clearly assorts with the other IFN-induced GTPase families and away from the Ras paradigm, because VLIG-1 lacks the critical catalytic Q61 of Ras that participates directly in the formation of the catalytic intermediate after stabilization by the arginine finger of GTPase activating protein (30). VLIG-1 shares with the other IFN-inducible GTPases a large hydrophobic residue (L1552) in this key position.

Taken together, the features of the putative nucleotide binding domain of VLIG-1 strongly suggest that this GTPase will have catalytic properties similar to those of the other IFN-inducible GTPase families in general and to the p65 GBPs in particular. It is therefore likely that VLIG-1 will prove to be a self-activating GTPase whose intrinsic catalytic activity is enhanced by oligomerization, as has now been shown to be the case for all the IFN-inducible GTPase families (31–33).

VLIG-1 is a member of a small gene family on mouse chromosome 7, and a homologue is located on human chromosome 11

A total of 222 VLIG-1-related EST clones of C57BL/6 origin have been recovered from public databases. The EST sequences could be assembled into several contigs (see supplemental Table II), indicating the presence of five VLIG related genes in addition to VLIG-1. We provisionally name these VLIG-A to VLIG-E. In the ENSEMBL mouse genome database (ENSEMBL MGSC v12.3.1), sequences corresponding to VLIG genes were mapped to chromosome 7 regions F1 and F2. Closer analysis showed four fully mapped VLIG sequences in this region, namely, VLIG-A, VLIG-B, VLIG-C, and VLIG-D (Fig. 1A). VLIG-1 itself maps into the same region of chromosome 7. Exon 2, intron 2, and exon 3 are located 81 kb telomeric to VLIG-A. Exon 4 is not connected to this region in ENSEMBL, but a genomic position adjacent to the two mapped upstream exons is confirmed from the map positions of BACs identified as carrying the gene (see above and supplemental Table I). The genomic contig carrying VLIG-E is not yet mapped.

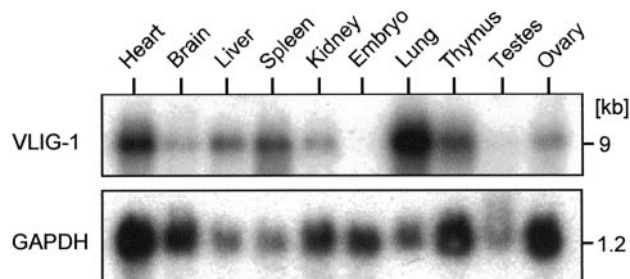


FIGURE 3. Expression of VLIG-1 mRNA in various mouse tissues as analyzed by Northern blot using a full-length VLIG-1 cDNA probe. Each lane was loaded with 2 μ g of poly(A) RNA, isolated from different tissues of mixed sex Swiss-Webster mice. Autoradiographic exposure time of the VLIG-1 image was >400 times longer than that of the GAPDH image.

As noted in supplemental Table II, there is evidence for the existence of at least one additional VLIG gene. Thus, like Mx, p65, and p47 GTPases, VLIG-1 is a member of a small gene family in mice. Many of the mouse ESTs were derived from immune cells or tissues rich in hemopoietic cells, i.e., thymus, spleen, or lymph node (see supplemental Tables II and III and Fig. 1). Only transcripts corresponding to VLIG-1 were recovered from our IFN- γ -stimulated PCR-Select or cDNA libraries of macrophage and fibroblast origins, suggesting that of the known family members, VLIG-1 plays a dominant role in the IFN response. The IFN inducibility of the other VLIG homologues is unknown. Most of the contigs defining VLIG homologues show multiple frameshifts, and definitive ORFs have not yet been determined. It is therefore unclear whether all the VLIG genes are able to encode functional proteins. The fourth exon sequences of the six available full-length or nearly complete VLIG family member of the mouse show 80–92% identity at the nucleotide level in all two-way comparisons, suggesting recent diversification (see supplemental Table IV).

ESTs homologous to VLIG-1 were identified from human, rat, pig, bovine, *Xenopus*, salmon, and zebrafish (see supplemental Table V). In humans the VLIG-1-related ESTs were derived from germinal center B cells, Jurkat T cells, spleen, lymph node, placenta, and lung tumor (see supplemental Table VI). We were able to identify the human homologue of VLIG-1 (hVLIG-1) in the public databases on the short arm of chromosome 11, band p15 + 4. The human protein must have, at least in part, a different structure from that of the mVLIG-1 gene, since there are frameshifts in the 5' end of the long fourth exon, leading to early termination. Whether these frameshifts are avoided by excision of further introns from the long fourth exon in humans cannot yet be established from the available human EST collection. 3' of the putative frame shifts the human and mouse VLIG proteins were found to be 69% identical and 74% similar over 2123 aa; the highest degree of identity was found in the conserved GTP-binding region.

Tissue distribution of constitutively expressed VLIG-1 mRNA

By Northern blot analysis, VLIG-1 was expressed at low basal levels in lung, heart, thymus, and spleen; at still lower levels in liver, ovary, kidney, and brain; and only marginally in testis (Fig. 3). VLIG-1 was undetectable in embryonic tissue. This tissue distribution was consistent with the tissue origins of the VLIG-1 ESTs recovered from the databases (see supplemental Table II) and suggested that constitutive expression of VLIG-1 is predominantly, although not exclusively, in organs containing abundant cells of hemopoietic origin. Weak, but unambiguous, signals corresponding to VLIG-1 were always apparent in Northern blots of RNA from uninduced fibroblasts.

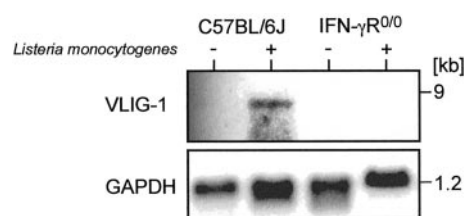


FIGURE 4. Inducibility of VLIG-1 in different mouse strains upon infection with *L. monocytogenes*, as shown by Northern blot analysis of liver RNA hybridized with a full-length VLIG-1 cDNA probe. Mice were infected with 1/10th of the LD₅₀. Livers were isolated 24 h postinfection. Ten micrograms of total RNA was loaded per lane. GAPDH hybridization is shown as the loading control (shorter exposure).

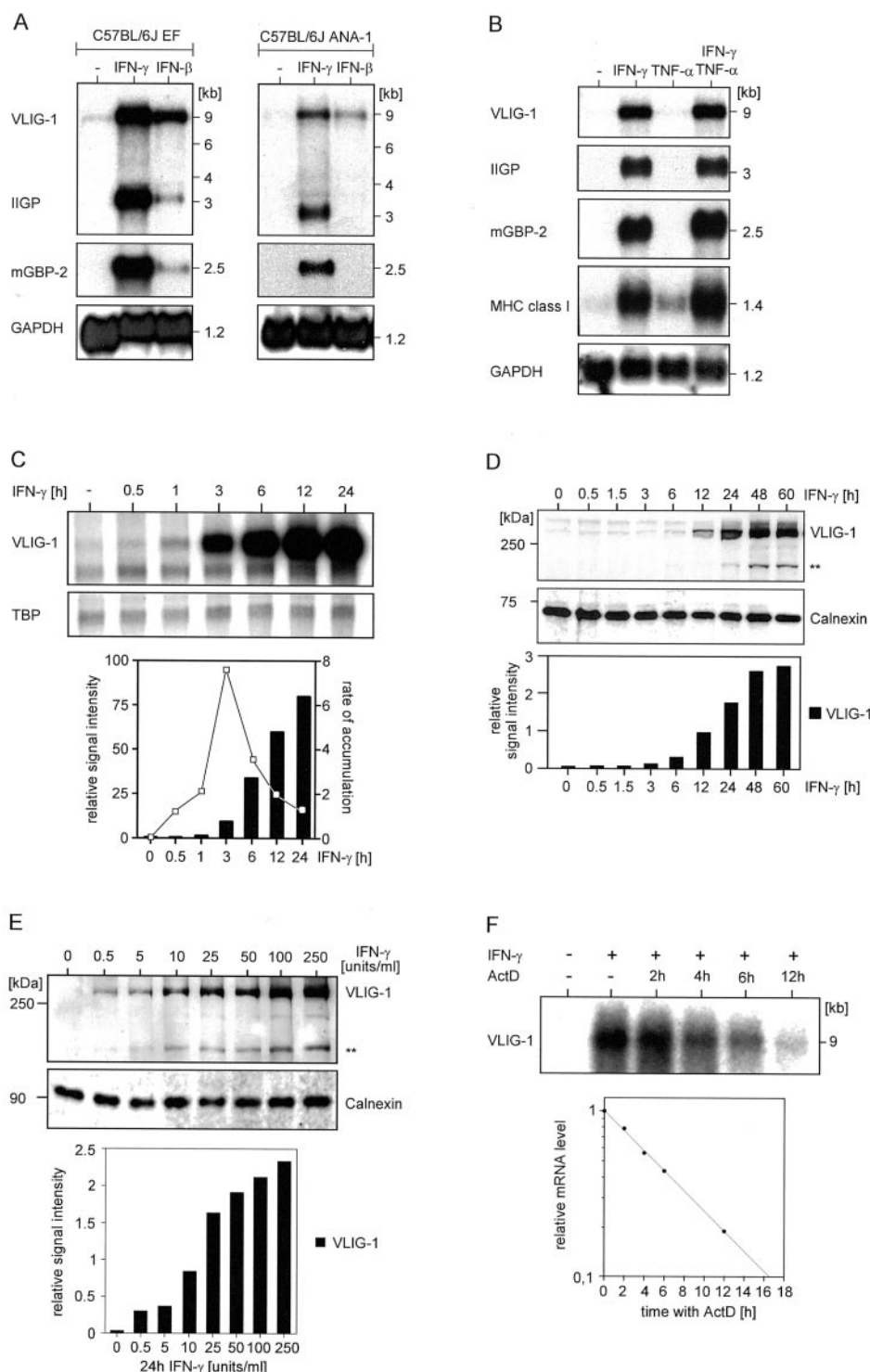


FIGURE 5. Expression studies of VLIG-1, the p65 family member mGBP-2, and the p47 family member IIGP as shown by Northern blot, RNase protection analysis, or Western blot on total RNA or cell lysates from tissue culture cells. **A**, IFN type I and type II inducibility of VLIG-1, IIGP, and mGBP-2 as shown by Northern blot using full-length VLIG-1, IIGP, or mGBP-2 cDNA probes. MEFs or the macrophage cell line ANA-1, both of C57BL/6J genetic background, were stimulated for 24 h with either 100 U/ml IFN- γ or 100 U/ml IFN- β . Ten micrograms of total RNA were loaded per lane. Hybridizations of VLIG-1 and IIGP were performed simultaneously. GAPDH hybridization (shorter exposure) is shown as the loading control. **B**, Inducibility by TNF- α and synergistic effects between IFN- γ and TNF- α on the induction of VLIG-1, IIGP, and mGBP-2 as shown by Northern blot analysis. MEFs of C57BL/6J mice were stimulated for 24 h with either 100 U/ml IFN- γ or 2000 U/ml TNF- α alone or simultaneously. Ten micrograms of total RNA was loaded per lane. Northern blots were hybridized with a full-length VLIG-1, IIGP or mGBP-2 cDNA probe. Hybridization with MHC class I H-2 K^b served as a positive control for TNF- α stimulation as well as for synergism between IFN- γ and TNF- α . GAPDH hybridization is shown as the loading control. **C**, Induction kinetics of VLIG-1 upon IFN- γ stimulation as shown by RNase protection analysis. MEFs of C57BL/6J mice were stimulated for the indicated times with 100 U/ml IFN- γ . Ten micrograms of total RNA was used for overnight hybridization with a riboprobe corresponding to $\sim 5 \times 10^5$ cpm. TBP was used as an internal total RNA loading control for quantification on a phosphorimager. The bar graph shows the calculated relative signal intensity, and the line graph indicates the rate of accumulation. **D**, Time-dependent induction of VLIG-1 and IIGP upon IFN- γ stimulation as shown by Western blot analysis. MEFs of C57BL/6J mice were stimulated for the indicated times with 100 U/ml IFN- γ . Cells were lysed (*Figure legend continues*)

Induction of VLIG-1 in the livers of *L. monocytogenes*-infected mice

The mRNA of VLIG-1 was strongly induced in the liver of wild-type C57BL/6J mice 24 h after i.p. infection with *L. monocytogenes*, (Fig. 4, tracks 1 and 2). The VLIG-1 transcript was not induced in IFN- γ ^{0/0} mice (Fig. 4, tracks 3 and 4), showing that the induction of VLIG-1 by *L. monocytogenes* infection is mediated exclusively by IFN- γ . Longer exposures of the blot showed a weak constitutive expression of VLIG-1 in the livers of all mice, including IFN- γ ^{0/0} mice, showing that basal VLIG-1 expression is independent of secreted IFN- γ .

Induction of VLIG-1 by cytokines in tissue culture

In cultured MEFs and ANA-1 macrophages from C57BL/6J mice, VLIG-1 mRNA levels were greatly increased 24 h after stimulation with 100 U/ml IFN- γ and to a somewhat lesser extent with 100 U/ml IFN- β (Fig. 5A). Under identical conditions, the strongly IFN- γ -inducible p65 GTPase, mGBP-2, or the p47 GTPase, IIGP, were much less strongly induced by IFN- β (Fig. 5A). The pleiotropic, inflammatory cytokine TNF- α , either alone or in synergy with IFN- γ , had virtually no effect on mRNA levels of VLIG-1, mGBP-2, or IIGP (Fig. 5B). This contrasts with the situation for many IFN- γ inducible genes (34, 35) including the MHC class I H-2 K^b gene which served as a positive control (Fig. 5B), where TNF- α acts synergistically with the IFN.

In RNase protection assays VLIG-1 mRNA was already above basal levels within 1 h of IFN- γ exposure (Fig. 5C). Levels of VLIG-1 mRNA peaked at 12 h and remained high at least to 24 h. The highest accumulation rate took place between 1 h and 3 h of stimulation (Fig. 5C). Comparable kinetics, but at a lower level of expression, were observed after treatment with 100 U/ml IFN- β (data not shown). VLIG-1 protein increase was not detectable in Western blots until 12 h after induction with IFN- γ (Fig. 5D), perhaps reflecting different sensitivities of the different detection methods. IFN- γ concentrations as low as 0.5 U/ml were sufficient to increase the expression level of VLIG-1 protein significantly after 24 h of stimulation (Fig. 5E). On VLIG-1 Western blots additional induced signals of lower apparent m.w. than VLIG-1 itself were also seen (Fig. 5, D and E). Whether these bands represent degradation products, post-translationally processed forms of VLIG-1, or possibly also products of different VLIG-1-like genes remains to be resolved.

The half-life of the VLIG-1 mRNA was determined by exposing C57BL/6 MEFs to 100 U/ml IFN- γ for 24 h and then to the transcriptional inhibitor ActD for various times. The decay of the accumulated mRNA was followed by Northern blotting (Fig. 5F). Under these conditions VLIG-1 mRNA decayed with a half-life of ~5 h. The mRNA half-life of VLIG-1 was similar to the mRNA half-life of 4.5 h determined for the IFN-inducible p47 GTPase family member, IGTP, in RAW264.7 macrophages (36).

Polymorphism of VLIG-1 expression in mice of different genetic backgrounds

IFN- γ -inducible mRNA hybridizing to a full-length VLIG-1 probe was detectable in Northern blots of fibroblasts from all four mouse

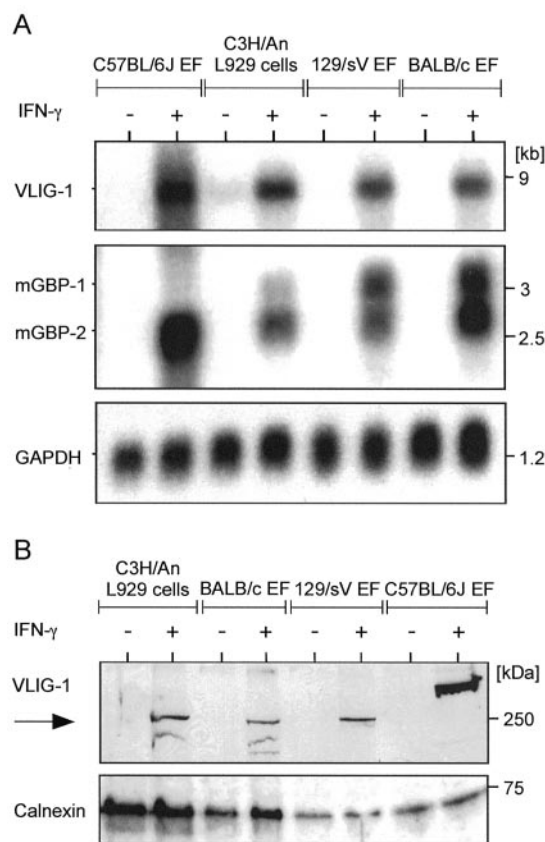


FIGURE 6. Expression analysis of VLIG-1 in different mouse strains as shown by Northern and Western blot analyses. *A*, MEFs from C57BL/6J, 129/sv, or BALB/c mice as well as the C3H/An-derived fibroblast-like cell line L929 were exposed for 24 h to 100 U/ml IFN- γ . Ten micrograms of total RNA was loaded per lane. The Northern blot was hybridized with a full-length VLIG-1 and mGBP-2 cDNA probe. GAPDH hybridization is shown as the loading control (shorter exposure). *B*, MEFs from C57BL/6J, 129/sv, or BALB/c mice and L929 cells were incubated for 24 h with 100 U/ml IFN- γ . Cells were lysed in Triton X-100-containing lysis buffer, and aliquots of the lysate corresponding to $\sim 7 \times 10^4$ cells/lane were loaded and analyzed in Western blots probed for VLIG-1 using the anti-VLIG-1 antiserum VLIG-1/B. Probing for calnexin served as the loading control. The arrow indicates the variant VLIG-1 signal.

strains tested. Apparent expression levels were noticeably higher in C57BL/6J than in the other three strains (Fig. 6A), but no difference in the apparent size of the mRNA was observed between the mouse strains. However, in Western blots the induced VLIG-1 cross-reactive signals from C3H, BALB/c and 129/Sv cells were ~40 kDa smaller than VLIG-1 from C57BL/6J mice (Fig. 6B) in addition to being markedly weaker in intensity.

Induction of VLIG-1 by IFN- γ is independent of IRF-1 and is only partly dependent on other secondary transcription factors

The induction of VLIG-1 was largely independent of the secondary transcription factor of the IFN response, IRF-1; only a slightly

in Triton X-100-containing lysis buffer, and lysate was loaded corresponding to $\sim 5 \times 10^4$ cells/lane. VLIG-1 was identified using the anti-VLIG-1 antiserum VLIG-1/B. Calnexin detection served as the loading control. **, Additional induced signals detected by the antiserum. *E*, Dose-dependent induction of VLIG-1 by IFN- γ as shown by Western blot analysis. MEFs of C57BL/6J mice were stimulated for 24 h with the indicated U/ml IFN- γ . Cells were lysed in Triton X-100-containing lysis buffer, and lysate was loaded corresponding to $\sim 5 \times 10^4$ cells/lane. VLIG-1 was detected using VLIG-1/B antiserum. Calnexin detection served as the loading control. **, Additional induced signals detected by the antiserum. *F*, mRNA stability of VLIG-1 in IFN- γ -treated cells as examined by Northern blot analysis. C57BL/6J-derived MEFs were stimulated for 24 h with 100 U/ml IFN- γ before addition of ActD at an end concentration of 50 μ g/ml. Total RNA was harvested at the indicated time points, and 10 μ g was loaded per lane. The Northern blot was hybridized with a full-length VLIG-1 cDNA probe. The half-life of VLIG-1 mRNA turnover was calculated from the above data.

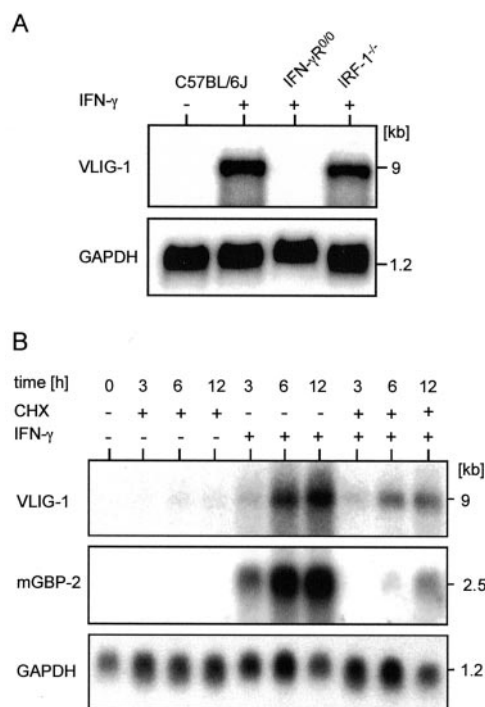


FIGURE 7. Response classification of VLIG-1 induction upon IFN- γ stimulation, as shown by Northern blot analysis. **A**, MEFs derived from C57BL/6J wild-type mice, IFN- γ R^{0/0} mice, and IRF-1^{-/-} mice, both with the 129/sV genetic background, were stimulated for 24 h with 100 U/ml IFN- γ . Ten micrograms of total RNA was loaded per lane. The membrane was hybridized with a full-length VLIG-1 cDNA probe. GAPDH hybridization is shown as a loading control. **B**, C57BL/6J-derived MEFs were incubated for the indicated times with 50 μ g/ml CHX, 100 U/ml IFN- γ , or CHX plus IFN- γ . Ten micrograms of total RNA was loaded per lane and subjected to Northern blotting with a full-length VLIG-1 and mGBP-2 cDNA probe. mGBP-2 hybridization is shown as a control for the CHX effect. GAPDH hybridization is shown as the loading control (shorter exposure).

weaker VLIG-1 signal was seen in Northern blots of IFN- γ -induced MEFs from mice lacking a functional IRF-1 gene (Fig. 7A). No induction could be detected in MEFs from IFN- γ R^{0/0} mice (Fig. 7A).

To determine whether other secondary transcription factors participated in the up-regulation of VLIG-1 mRNA level, MEFs derived from C57BL/6J mice were incubated with the translation inhibitor CHX before and during their exposure to IFN- γ . CHX did not completely inhibit the induction of VLIG-1 mRNA by IFN- γ (Fig. 7B), although the level was clearly reduced, especially at later time points.

VLIG-1 is a guanine nucleotide-binding protein

The well-structured GTPase-like sequence motifs of VLIG-1 suggested that VLIG-1 should be able to bind to guanine nucleotides. To test this we analyzed the binding of VLIG-1 to nucleotide-agaroses. IFN- γ -stimulated MEFs were lysed in TBS/MgCl₂ buffer, and the supernatants containing free VLIG-1 were adsorbed on nucleotide agaroses. After washing, eluted guanine nucleotide-binding proteins were detected in Western blots. The p65 IFN-inducible GTPase, mGBP-2, present in the same lysates served as a positive control for these experiments. The nucleotide-binding properties of mGBP-2 have been referred to previously (37) and were confirmed by our results.

VLIG-1 bound strongly to GDP-agarose and very weakly, if at all, to GTP- and GMP-agaroses (Fig. 8). The functionality of all

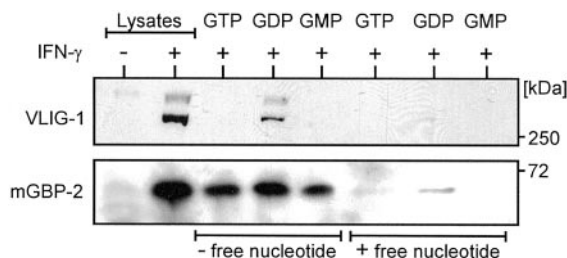


FIGURE 8. Guanine nucleotide-binding properties of VLIG-1 compared with mGBP-2. MEFs from C57BL/6J mice were stimulated for 24 h with 100 U/ml IFN- γ and harvested in TBS/MgCl₂ buffer. Aliquots of the lysates corresponding to 1×10^6 cells were incubated with different nucleotides bound to agarose. The binding specificity was confirmed by competition experiments with free guanine nucleotides. After repeated washing with lysis buffer, bound proteins were eluted with SDS-containing sample buffer and analyzed in Western blots probed with VLIG-1 antiserum VLIG-1/218 or mGBP-2 antiserum. Lysate not incubated with agarose-immobilized nucleotides is shown as an induction control.

nucleotide-agaroses was verified by strong binding of mGBP-2. The specificity of VLIG-1 as well as that of mGBP-2 binding were confirmed by competition experiments with free guanine nucleotides (Fig. 8).

Subcellular localization of VLIG-1

Immunofluorescence studies on the subcellular localization of endogenous VLIG-1 using the anti-VLIG-1 antiserum VLIG-1/218 suggested that basal expressed VLIG-1 protein is located predominantly in the nucleus (Fig. 9). After stimulation with IFN- γ , VLIG-1 protein could be detected either predominantly in the nucleus or in the cytoplasm in different cells (Fig. 9). An N-terminal enhanced green fluorescent protein-tagged construct also showed cytoplasmic and nuclear localization in different cells after transient transfection (data not shown). A largely free cytosolic localization was also supported by the good recovery of IFN- γ induced VLIG-1 in the supernatant of an aqueous cell lysate (see also Fig. 8). Approximately 50% of the cells showed nuclear localization of the signal. The explanation for this behavior is under investigation.

Discussion

In this paper we describe VLIG-1, the prototype of a new IFN-inducible GTP-binding protein family in the mouse. VLIG-1 is greatly up-regulated transcriptionally and at the protein level by IFN treatment of macrophages and primary embryonic fibroblasts

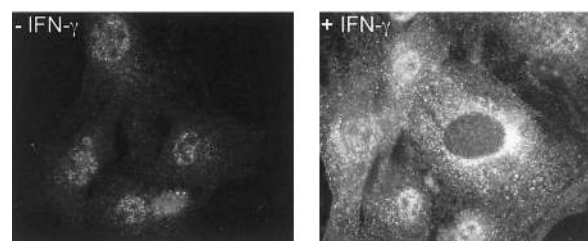


FIGURE 9. Subcellular localization of VLIG-1 in unstimulated and IFN- γ -stimulated cells as shown by immunofluorescence analysis. C57BL/6J-derived MEFs were induced for 24 h with 100 U/ml IFN- γ or were left unstimulated. Cells were fixed with 3% para-formaldehyde, permeabilized with saponin, and stained with anti-VLIG-1 antiserum VLIG-1/218, followed by incubation with the secondary stage Ab.

in vitro (Fig. 5) as well as in the livers of mice infected with *L. monocytogenes* (Fig. 4). This latter induction appears to be mediated entirely by in vivo production of IFN- γ , since it is not seen in *Listeria*-infected mice carrying a disabled IFN- γ R gene. The VLIG-1 gene thus seems to belong to one of the non-cell type-specific programs induced by IFNs (1, 2, 6).

The transcript of ~9000 bp in the C57BL/6J mouse encodes a protein with a predicted M_r of >280 kDa. The VLIG-1 protein, induced in IFN-stimulated cells and detected in the Western blot, corresponded in apparent size with this expectation (Fig. 5, *D* and *E*). Thus, at least in this mouse strain, the VLIG-1 protein is expressed at full length. We observed, however, that despite an induced transcript of similar size, the serologically detectable protein product was ~40 kDa smaller in three other inbred mouse strains (Fig. 6*B*). The basis for this apparent size difference remains to be clarified.

The whole predicted protein sequence of VLIG-1 as well as the 3'UTR is encoded on a single very large exon of 8709 bp downstream of three short 5'-untranslated exons separated by a total of at least 17 kb of intronic DNA. The first intron of >6.5 kb is not yet fully sequenced (Fig. 1*B*). The ORFs of the IFN- γ -inducible p47 GTPases are also encoded on large single exons (15) (our unpublished observations), but the ORFs of the IFN-inducible p65 and Mx GTPases are underlain by a complex intron-exon structure (38, 39). Phylogenetically, the VLIG family is relatively old, since representatives in zebrafish and salmon were recovered from the databases (supplemental Table V). In the mouse, however, the gene has probably radiated recently into a gene family of at least six closely linked members related to each other at the level of ~85% identity at the nucleotide level (supplemental Table IV). Only a single VLIG gene is apparently present in the human. This gene closely resembles a VLIG gene, but the ORF on the main exon is interrupted twice near the N terminus by frameshifts. Therefore, either the human VLIG gene is a pseudogene or the ORF is preserved by splicing across the frameshifts. The small collection of human VLIG ESTs presently available (supplemental Table VI) does not allow these alternatives to be resolved. There is at present no direct evidence that a human VLIG protein is produced or that the human VLIG gene is transcriptionally induced by IFNs.

The potential GTP-binding activity of VLIG-1 was identified from its possession of a classical GTP-binding sequence motif. The P-loop, (G(X₄)GKS) (aa 1495–1502), and G3, (D(X₂)G) (aa 1548–51), motifs are both present in canonical form. The G₄ motif (N/TKxD), associated in canonical GTPases with contact to the guanine base, is not immediately apparent (Figs. 1*C* and 2). The G₄ motif in canonical form is also absent from the IFN-inducible hGBP-1, but in this case the contact with the guanine base is provided with a different geometry by a truncated motif, -RD (40). We suggest that a related sequence in VLIG-1 (K1627/D1628) may function similarly. Several features suggest that the closest structural relationship for the G domain of VLIG-1 will prove to be with the GBP family (Fig. 2). From the analysis of binding to nucleotide agaroses, VLIG-1 appeared to show an unusual preference for GDP over GTP (Fig. 8), but this result must be confirmed in a more quantitative assay. The IFN-inducible p47 GTPase, IIGP, also shows a markedly higher affinity for GDP than for GTP (31).

The VLIG genes described here are the fourth distinctive family of GTPases associated with the IFN response. Previously described families are the dynamin-like Mx genes (9), the p65 guanylate-binding proteins, and the p47 GTPases (19), and all have been implicated in one system or another in the mediation of cell-autonomous resistance against intracellular pathogens (9, 11–13, 15). It is tempting to consider the VLIG family in the same con-

text. The general similarity of the sequence of the VLIG-1 GTP-binding domain with these other GTPase families has been noted above. A more detailed analysis of the biochemical properties of VLIG-1 and its differences or similarities to other IFN-inducible GTPases will be the subject of future studies and may help to clarify the relationship of VLIG-1 to these GTPase families.

The pattern of induction of VLIG-1 by IFNs does not correspond exactly with any of the other resistance GTPase families. VLIG-1 is induced rapidly by type I and type II IFN, and the initial induction is resistant to CHX-induced inhibition of new protein synthesis (Figs. 5 and 7*B*) and therefore is mediated by preformed transcription factors, while de novo protein synthesis of secondary transcription factors (although probably not IRF-1; Fig. 7*A*) may be required for the continued increase in VLIG-1 transcription and for sustained mRNA levels. Such a mixed response seems also to be the case for the p47 GTPase family members IGTP and IIGP (36) (our unpublished observations), while the induction of p65 family members in the mouse is completely dependent on IRF-1 (19). VLIG-1 is markedly more efficiently stimulated by type I IFN than is the p47 GTPase IIGP or the p65 GTPase mGBP-2 (Fig. 5*A*) (41, 42). Thus, VLIG-1 appears to be a major contributor to the cellular response to both types of IFN, while the p65 and p47 GTPase families are more specialized for the IFN- γ response, and Mx is more specialized for the type I response.

The highest constitutive expression level of VLIG-1 at the mRNA level appears to be in organs rich in immune cell populations (thymus and spleen) or fulfilling a barrier function of the host (lung; Fig. 3). Furthermore, ~76% of the ESTs identical or highly homologous to VLIG-1 originated from organs such as spleen and lymph node or from cell types such as T cells or macrophages (supplemental Table II). A similar basal expression pattern was observed for the p47 GTPase family members, IGTP and TGTP/Mg21 (36, 43) as well as for the p65 GTPase mGBP-3 (44). It is possible that some basal expression of VLIG and other inducible GTPases may be caused by local type I or type II IFN production in vivo. However, the basal expression of VLIG-1 seen in normal liver (not apparent in Fig. 4, but visible on longer exposures of the same blot) was similar in the liver from IFN- γ R-deficient mice, showing that IFN- γ at least is not responsible for this basal expression.

The IFN-inducible GTPases involved in cell-autonomous resistance programs tend to be grouped into small gene families. There is evidence in some cases that different family members within a species do not fulfill redundant functions (9, 13). Nevertheless, membership of the gene families is not stable over even short periods of evolutionary time. Thus, in the p47 family, where non-redundancy has been clearly shown among the six published family members of mouse (19), only a single homologous sequence is present in man (our unpublished observations). In the p65 GTPases, a polymorphism exists in mice between those strains expressing all five family members and those not expressing mGBP-1, but expressing the other four members (45) (Fig. 6*A*). In the case of Mx, mice and humans have two genes, but, unlike wild mice, most laboratory strains of mice fail to express one or both of these (46), and one of the human genes has not yet been shown to have antiviral activity (47). In the case of VLIG-1, at least six genes could be identified from the mouse databases (supplemental Tables II and IV), while only a single human homologue was found, which may, indeed, be a pseudogene. Furthermore, a polymorphism of VLIG-1 was found among four mouse strains (Fig. 6). The remarkable instability of the IFN-inducible GTPase families even within the mammals is consistent with a resistance function against rapidly

evolving pathogens. On the information available to date, the VLIG GTPases appear to belong in the same category.

The inducibility by IFNs of the mouse VLIG-1 homologous genes has not been demonstrated. To date, VLIG-1 is the only gene of this family that could be recovered from IFN- γ -stimulated primary MEF cell λ -cDNA or PCR-Select library. The failure of further attempts to document the inducibility of other VLIG-1 genes by RT-PCR (data not shown) and the lack of additional induced transcript signals within the RNase protection analysis (Fig. 5C) suggest that VLIG-1 might be the only member of this GTPase family induced by IFNs in MEFs. A recent gene chip analysis of IFN- γ -stimulated MEFs or bone marrow-derived macrophages claimed to have detected a sequence that by our analysis is VLIG-C (48). However, the mRNA signal may have been due to induced VLIG-1 cross-hybridizing to the highly homologous VLIG-C EST (AA177731, derived from C57BL/6J spleen) located on the gene chip. No other VLIG-1 sequence was reported to be present on the gene chip.

There is no direct evidence for a function of the VLIG family GTPases. However, the identification of this novel and unusual protein family associated with the IFN response and its suggestive similarities to members of the p47, p65, and Mx GTPase families justify a concentrated attempt to implicate VLIG proteins in resistance to infectious disease.

Acknowledgments

We are grateful to Agnes Fütterer (Institute of Microbiology and Immunology, Technical University of Munich, Munich, Germany) for the initial identification of the 743-bp fragment from the ANA-1 subtractive library, and to Rita Lange (Institute for Genetics, University of Cologne, Cologne, Germany) for technical assistance. We also thank Dr. Viktor Steimle (Max Planck Institute for Immunology, Freiburg, Germany) for his help in developing the RNase protection assay, Dr. Libby Guethlein (Department of Structural Biology, Stanford University, Palo Alto, CA), for her assistance with the first assembly and phylogenetic analysis of VLIG-1, and Dr. Alfred Wittinghofer (Max Planck Institute for Molecular Physiology, Dortmund, Germany) for helpful remarks on the sequence of VLIG-1.

References

- Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to interferon- γ . *Annu. Rev. Immunol.* 15:749.
- Klamp, T., U. Boehm, M. Groot, and J. C. Howard. 1997. A list of genes regulated by IFN- γ (<http://www.annurev.org/sup/material.htm>).
- Ehrt, S., D. Schnappinger, S. Bekiranov, J. Drenkow, S. Shi, T. R. Gingeras, T. Gaasterland, G. Schoolnik, and C. Nathan. 2001. Reprogramming of the macrophage transcriptome in response to interferon- γ and *Mycobacterium tuberculosis*: signaling roles of nitric oxide synthase-2 and phagocyte oxidase. *J. Exp. Med.* 194:1123.
- Der, S. D., A. Zhou, B. R. Williams, and R. H. Silverman. 1998. Identification of genes differentially regulated by interferon α , β , or γ using oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA* 95:15623.
- Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264:1415.
- Stark, G. R., I. M. Kerr, B. R. Williams, R. H. Silverman, and R. D. Schreiber. 1998. How cells respond to interferons. *Annu. Rev. Biochem.* 67:227.
- Takaoka, A., Y. Mitani, H. Suemori, M. Sato, T. Yokochi, S. Noguchi, N. Tanaka, and T. Taniguchi. 2000. Cross talk between interferon- γ and α/β signaling components in caveolar membrane domains. *Science* 288:2357.
- de Veer, M. J., M. Holko, M. Frevel, E. Walker, S. Der, J. M. Paranjape, R. H. Silverman, and B. R. Williams. 2001. Functional classification of interferon-stimulated genes identified using microarrays. *J. Leukocyte Biol.* 69:912.
- Haller, O., and G. Kochs. 2002. Interferon-induced mx proteins: dynamin-like GTPases with antiviral activity. *Traffic* 3:710.
- Pavlovic, J., T. Zurcher, O. Haller, and P. Staeheli. 1990. Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein. *J. Virol.* 64:3370.

- Anderson, S. L., J. M. Carton, J. Lou, L. Xing, and B. Y. Rubin. 1999. Interferon-induced guanylate binding protein-1 (GBP-1) mediates an antiviral effect against vesicular stomatitis virus and encephalomyocarditis virus. *Virology* 256:8.
- Carlow, D. A., S. J. Teh, and H. S. Teh. 1998. Specific antiviral activity demonstrated by TGTP, a member of a new family of interferon-induced GTPases. *J. Immunol.* 161:2348.
- Collazo, C. M., G. S. Yap, G. D. Sempowski, K. C. Lusby, L. Tassarollo, G. F. Woude, A. Sher, and G. A. Taylor. 2001. Inactivation of LRG-47 and IRG-47 reveals a family of interferon γ -inducible genes with essential, pathogen-specific roles in resistance to infection. *J. Exp. Med.* 194:181.
- Halonon, S. K., G. A. Taylor, and L. M. Weiss. 2001. γ Interferon-induced inhibition of *Toxoplasma gondii* in astrocytes is mediated by IGTP. *Infect. Immun.* 69:5573.
- Taylor, G. A., C. M. Collazo, G. S. Yap, K. Nguyen, T. A. Gregorio, L. S. Taylor, B. Eagleson, L. Secrest, E. A. Southon, S. W. Reid, et al. 2000. Pathogen-specific loss of host resistance in mice lacking the IFN- γ -inducible gene IGTP. *Proc. Natl. Acad. Sci. USA* 97:751.
- Reis, L. F., H. Ruffner, G. Stark, M. Aguet, and C. Weissmann. 1994. Mice devoid of interferon regulatory factor 1 (IRF-1) show normal expression of type I interferon genes. *EMBO J.* 13:4798.
- Huang, S., W. Hendriks, A. Althage, S. Hemmi, H. Bluethmann, R. Kamijo, J. Vilcek, R. M. Zinkernagel, and M. Aguet. 1993. Immune response in mice that lack the interferon- γ receptor. *Science* 259:1742.
- Torres, R. M., and R. Kühn. 1997. *Laboratory Protocols for Conditional Gene Targeting*. Oxford University Press, Oxford.
- Boehm, U., L. Guethlein, T. Klamp, K. Ozbeck, A. Schaub, A. Futterer, K. Pfeffer, and J. C. Howard. 1998. Two families of GTPases dominate the complex cellular response to IFN- γ . *J. Immunol.* 161:6715.
- Sanford, K. K., W. R. Earle, and G. D. Likely. 1948. The growth in vitro of single isolated tissue cells. *J. Natl. Cancer Inst.* 9:229.
- Cox, G. W., B. J. Mathieson, L. Gandino, E. Blasi, D. Radzioch, and L. Varesio. 1989. Heterogeneity of hematopoietic cells immortalized by v-myc/v-ras recombinant retrovirus infection of bone marrow or fetal liver. *J. Natl. Cancer Inst.* 81:1492.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.
- Steimle, V., L. A. Otten, M. Zufferey, and B. Mach. 1993. Complementation cloning of an MHC class II transactivator mutated in hereditary MHC class II deficiency (or bare lymphocyte syndrome). *Cell* 75:135.
- Palmer, I., and P. Wingfield. 1995. Preparation and extraction of insoluble (inclusion-body) proteins from *Escherichia coli*. In *Current Protocols in Protein Science*, Vol. 1. J. Coligan, B. Dunn, H. Ploegh, D. Speicher, and P. Wingfield, eds. John Wiley & Sons, New York, p. 6.3.1.
- Knittler, M. R., P. Alberts, E. V. Deverson, and J. C. Howard. 1999. Nucleotide binding by TAP mediates association with peptide and release of assembled MHC class I molecules. *Curr. Biol.* 9:999.
- Kozak, M. 1987. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* 15:8125.
- Bourne, H., D. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature* 349:117.
- Leipe, D. D., Y. I. Wolf, E. V. Koonin, and L. Aravind. 2002. Classification and evolution of P-loop GTPases and related ATPases. *J. Mol. Biol.* 317:41.
- Prakash, B., G. J. Praefcke, L. Renault, A. Wittinghofer, and C. Herrmann. 2000. Structure of human guanylate-binding protein 1 representing a unique class of GTP-binding proteins. *Nature* 403:567.
- Scheffzek, K., M. R. Ahmadian, W. Kabsch, L. Wiesmuller, A. Lautwein, F. Schmitz, and A. Wittinghofer. 1997. The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science* 277:333.
- Uthaiar, R. C., G. J. Praefcke, J. C. Howard, and C. Herrmann. 2003. IIGP1, an interferon- γ inducible 47 kDa GTPase of the mouse, showing cooperative enzymatic activity and GTP-dependent multimerisation. *J. Biol. Chem.* In press.
- Praefcke, G. J., M. Geyer, M. Schwemmle, H. Robert Kalbitzer, and C. Herrmann. 1999. Nucleotide-binding characteristics of human guanylate-binding protein 1 (hGBP1) and identification of the third GTP-binding motif. *J. Mol. Biol.* 292:321.
- Flohr, F., S. Schneider-Schaulies, O. Haller, and G. Kochs. 1999. The central interactive region of human MxA GTPase is involved in GTPase activation and interaction with viral target structures. *FEBS Lett.* 463:24.
- Ohmori, Y., R. D. Schreiber, and T. A. Hamilton. 1997. Synergy between interferon- γ and tumor necrosis factor- α in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor κ B. *J. Biol. Chem.* 272:14899.
- Paludan, S. R. 2000. Synergistic action of pro-inflammatory agents: cellular and molecular aspects. *J. Leukocyte Biol.* 67:18.
- Taylor, G. A., M. Jeffers, D. A. Largaespada, N. A. Jenkins, N. G. Copeland, and G. F. Woude. 1996. Identification of a novel GTPase, the inducibly expressed GTPase, that accumulates in response to interferon γ . *J. Biol. Chem.* 271:20399.
- Vestal, D. J., J. E. Buss, S. R. McKercher, N. A. Jenkins, N. G. Copeland, G. S. Kelner, V. K. Asundi, and R. A. Maki. 1998. Murine GBP-2: a new IFN- γ -induced member of the GBP family of GTPases isolated from macrophages. *J. Interferon Cytokine Res.* 18:977.

38. Anderson, S. L., J. M. Carton, X. Zhang, and B. Y. Rubin. 1999. Genomic organization and chromosomal localization of a new member of the murine interferon-induced guanylate-binding protein family. *J. Interferon Cytokine Res.* 19:487.
39. Hug, H., M. Costas, P. Staeheli, M. Aebi, and C. Weissmann. 1988. Organization of the murine Mx gene and characterization of its interferon- and virus-inducible promoter. *Mol. Cell. Biol.* 8:3065.
40. Prakash, B., L. Renault, G. J. Praefcke, C. Herrmann, and A. Wittinghofer. 2000. Triphosphate structure of guanylate-binding protein 1 and implications for nucleotide binding and GTPase mechanism. *EMBO J.* 19:4555.
41. Vestal, D. J., V. Y. Gorbacheva, and G. C. Sen. 2000. Different subcellular localizations for the related interferon-induced GTPases, MuGBP-1 and MuGBP-2: implications for different functions? *J. Interferon Cytokine Res.* 20:991.
42. Zerrahn, J., U. E. Schaible, V. Brinkmann, U. Guhlich, and S. H. Kaufmann. 2002. The IFN-inducible Golgi- and endoplasmic reticulum-associated 47-kDa GTPase IIGP is transiently expressed during listeriosis. *J. Immunol.* 168:3428.
43. Carlow, D. A., J. Marth, I. Clark-Lewis, and H. S. Teh. 1995. Isolation of a gene encoding a developmentally regulated T cell-specific protein with a guanine nucleotide triphosphate-binding motif. *J. Immunol.* 154:1724.
44. Han, B. H., D. J. Park, R. W. Lim, J. H. Im, and H. D. Kim. 1998. Cloning, expression, and characterization of a novel guanylate-binding protein, GBP3 in murine erythroid progenitor cells. *Biochim. Biophys. Acta* 1384:373.
45. Staeheli, P., M. Prochazka, P. A. Steigmeier, and O. Haller. 1984. Genetic control of interferon action: mouse strain distribution and inheritance of an induced protein with guanylate-binding property. *Virology* 137:135.
46. Staeheli, P., R. Grob, E. Meier, J. G. Sutcliffe, and O. Haller. 1988. Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. *Mol. Cell. Biol.* 8:4518.
47. Lee, S. H., and S. M. Vidal. 2002. Functional diversity of Mx proteins: variations on a theme of host resistance to infection. *Genome Res.* 12:527.
48. Presti, R. M., D. L. Popkin, M. Connick, S. Paetzold, and H. W. t. Virgin. 2001. Novel cell type-specific antiviral mechanism of interferon γ action in macrophages. *J. Exp. Med.* 193:483.