IIGP, a member of the IFN inducible and microbial defense mediating 47 kDa GTPase family, interacts with the microtubule binding protein hook3

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Summary

Innate immunity against intracellular pathogens is critically determined by an as yet unknown interferon (IFN)-inducible mechanism exerted by members of the 47 kDa GTPase family. The association of IGTP and IIGP with membranous compartments, the endoplasmic reticulum and, in addition in case of IIGP, the Golgi, implicate these GTPases in intracellular membrane trafficking or processing. We identified the cytoplasmic linker molecule hook3 as an interactor for IIGP by yeast two-hybrid screening. The physical complex between these molecules was present in lysates of IFNγ-stimulated macrophages as demonstrated by co-immunoprecipitation. Only a minor subfraction of total cellular IIGP or hook3 was co-purified, indicating that this interaction is either transient and/or involves distinct subpopulations of the total cellular pools of these molecules. Binding of IIGP to hook3 depends on a GTP-bound conformation. Hook3 is a microtubule-binding protein which participates in the organization of the cis-Golgi compartment. Both proteins were detected in the Golgi-membrane-enriched fraction upon subcellular fractionation. Apart from the Golgi localization of both proteins, hook3 was detected in perinuclear regions in close spatial proximity to IIGP, associated with the endoplasmic reticulum. Our experiments identify hook3 as the first cooperation partner of a member of the 47 kDa GTPase protein family and indicate that hook3 links in an IFNγ-inducible fashion to cytoskeleton-based membrane trafficking.

Key words: GTPase, Innate immunity, Golgi, Microtubule

Introduction

Multiple effector mechanisms of the innate immune system are directed against pathogens present in the extracellular space. Neutralization by antibodies, complement mediated lysis, or opsonization, restrict pathogen number and spread (Janeway et al., 2001). However, successful cellular invasion and subsequent productive replication are inherent to intracellular pathogens as are multiple intracellular defense mechanisms on the side of the host cells. In this regard, the profound role of nitric oxide (Bogdan, 1997; Nathan and Shiloh, 2000), and reactive oxygen intermediates (Kaufmann, 1999; Nathan and Shiloh, 2000), iron availability (Lieu et al., 2001), indoleamine 2,3-dioxygenase (IDO) mediated tryptophan starvation (Taylor and Feng, 1991), activation of the double-stranded RNA-dependent protein kinase (PKR) (Stark et al., 1998; Samuel, 2001) or of the 2,5-oligoadenylate synthetase/RNaseL-system (Stark et al., 1998; Samuel, 2001) in antimicrobial or antiviral defense at the cellular level are well acknowledged. These intracellular effector mechanisms are not constitutively active but rather are induced upon recognition of pathogen-specific structures or stimulation by interferons (IFN) (Boehm et al., 1997).

Among the numerous genes that are induced by IFNs are members of three protein families with GTPase activity, which participate in distinct intracellular defense mechanisms: the Mx proteins, the 65kDa guanylate-binding proteins (GBP), and the 47kDa GTPase protein family. The Mx proteins confer resistance to a variety of RNA viruses, influenza being one of the most prominent examples (Samuel, 2001; Haller and Kochs, 2002). Antiviral activity has recently been attributed to human GBP1, a member of the 65 kDa GBP family (Anderson et al., 1999). The 47 kDa GTPase family comprises, to date, six genes, namely IGTP, TGTP, LRG-47, IRG-47, GTPI and IIGP, which are strongly induced by IFNs in hematopoietic as well as non-hematopoietic cells (Gilly and Wall, 1992; Carlow et al., 1995; Lafuse et al., 1995; Sorace et al., 1995; Taylor et al., 1996; Boehm et al., 1998; Zerrahn et al., 2002). The significance of some members of this GTPase protein family in immunity against intracellular pathogens has been revealed by analysis of knockout (KO) mouse strains. Deficiency of IGTP or LRG-47 renders mice highly susceptible to infection with *Toxoplasma gondii* during the acute phase, while IRG-47 contributes to defense during the chronic phase (Taylor et al., 2000; Collazo et al., 2001). Interestingly, LRG-47-deficient KO mice succumb to sublethal infection with *Listeria monocytogenes*, while IGTP or IRG-47 are apparently not essential to resolve this infection. In contrast, all 47 kDa GTPase-deficient KO mice display normal resistance to murine CMV (Taylor et al., 2000; Collazo et al., 2001). This differential contribution to defense against different intracellular pathogens suggests that the 47 kDa GTPases (i) perform distinct functions, (ii) exert them in a cell type-specific fashion or (iii) underlie differential regulation. So far, type I and type II IFNs seem to be the prime inducers of expression

(Gilly and Wall, 1992; Carlow et al., 1995; Lafuse et al., 1995; Sorace et al., 1995; Taylor et al., 1996; Boehm et al., 1998; Zerrahn et al., 2002). Consistent with this IIGP is only transiently expressed in the spleen during the early phase of infection with *L. monocytogenes* (Zerrahn et al., 2002). Marginal metallophilic macrophages and endothelial cells throughout the spleen strongly express IIGP. A cell type-specific aspect of the effector mechanism mediated by IGTP was recently revealed by analysis of IGTP-deficient astrocytes infected with *T. gondii* (Halonen et al., 2001). While growth of *T. gondii* is significantly inhibited in wild-type cells upon IFNγ stimulation, the pathogen multiplies almost unrestrictedly in IGTP-deficient astrocytes (Halonen et al., 2001). This is consistent with previous results, that cellular resistance of murine astrocytes is independent from nitric oxide, reactive oxygen and tryptophan metabolism, which contribute efficiently to inhibition of *T. gondii* in other cell types (Halonen and Weiss, 2000). Furthermore, IGTP function in non-hematopoietic cells is essential for immunity against *T. gondii* (Collazo et al., 2002). Conceptually, these findings suggest a cell type-specific role of IGTP or its cognates in defense against intracellular pathogens. The effector mechanism(s) mediated by these 47 kDa GTPases are apparently operative at the intracellular level. This is in line with uncompromised overt immune parameters (IL-12, NO, TNF-α, T and NK cell function) in 47 kDa GTPase-deficient KO mice upon infection (Taylor et al., 2000; Collazo et al., 2001).

However, the actual nature of the effector mechanism(s) and the molecular function(s) of these GTPases remain unknown. *T. gondii* resides permanently in and *L. monocytogenes* very rapidly transits, membranous compartments within the host cell. The 47 kDa GTPases seem to localize to intracellular membranes: IGTP is associated with the endoplasmic reticulum (ER) (Taylor et al., 1997) and for IIGP, a significant subcellular fraction localizes to the Golgi apparatus in addition (Zerrahn et al., 2002). Hence it is tempting to speculate that these GTPases participate in the modulation of intracellular membrane-dependent processes, like vesicular trafficking, vesicular cargo specification, or interactions with components of the pathogen-containing vacuolar membrane. In general, GTPases act as molecular switches that adopt either a GTP- or GDP-bound conformational state, thereby modulating downstream events by differential interactions with other effector proteins (Vetter and Wittinghofer, 2001). Elucidation of the actual molecular processes will provide deeper insights into the role of the 47 kDa GTPases in the innate immune defense against intracellular pathogens on the subcellular level.

We report here, that IIGP physically interacts with the partially Golgi-associated murine hook3 protein, a member of the recently identified family of microtubule-binding proteins (Walenta et al., 2001). The interaction of IIGP with a potential cytoplasmic linker protein, which dynamically connects defined membrane compartments to microtubules, is not only consistent with previous notions about a role of the 47 kDa GTPases in membrane-associated processes (Taylor et al., 1997; Zerrahn et al., 2002) but it also represents the first evidence that GTPase family members participate in processes underlying differential membrane trafficking. This mechanism could form the basis for the functions exerted by IIGP, which lead to defense against intracellular pathogens.

Materials and Methods

Cells, reagents and antibodies

Bone marrow-derived macrophages (BMM) from C57BL/6 mice were prepared as described elsewhere (Falk and Vogel, 1990) and used for all experiments at days 6-9. The BMM were cultured in Dulbecco's modified Eagle's medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS, 20% L cell-conditioned medium, 5×10^{-5} M 2-ME, 20 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate and antibiotics. BMM were stimulated overnight with 1000 U /ml recombinant murine IFNγ (Strathmann Biotech, Hannover, Germany). For microtubule depolymerization, cells were treated with 5 μg/ml nocodazole (NZ) for 1 hour. Antibodies used were: anti-IIGP mAb 5D9 (Zerrahn et al., 2002), ER-specific anti-calnexin rabbit serum (Stressgene, Victoria, Canada), anti-Golgi 58K mAb 58K-9 (Sigma, Missouri, USA), anti-α-tubulin mAb DM1A (Sigma, Missouri, USA), anti-IGTP mAb clone 7 (Becton Dickinson, #610880), cis-Golgi-specific anti-GM130 (BD Transduction Lab., Heidelberg, Germany), rabbit anti-α-mannosidase (kindly provided by Dr A. Haas, University of Bonn, Germany) and affinity-purified rabbit anti-hook3 serum (kindly provided by Dr H. Krähmer, University of Texas, USA).

Constructs

The full-length, and the deletion variants, encoding cDNAs of the 47 kDa GTPases IIGP, IGTP, GTPI, LRG-47, IRG-47 or of murine hook3 were generated by PCR using cDNA derived from IFNγ-stimulated C57BL/6 BMM and gene-specific primers including restriction sites suited for subcloning into the yeast binding domain vector pGILDA (Clontech) or activation domain vector pB42AD (Clontech), respectively. A GTPase-negative IIGP mutant was generated that was similar to a variety of described and well-characterized GTPase mutants. A serine/threonine to asparagine exchange in the last position of the P-loop sequence leads to the generation of variants that are assumed to be locked in a GDP-bound conformation (Dugan et al., 1995; Taylor et al., 1997; Feig, 1999). Accordingly, the mutant IIGP_S83N, was generated by site-specific mutation introduced by overlap extension PCR using wild-type IIGP cDNA as substrate. The overlap extension primers were 5′-TCAGGGAAGAACAGCTT-CATCAATACCCTGAG and 5′-ATGAAGCTGTTCTTCCCTGAT-CCCGTCTCC with the altered sequences underlined. In *E. coli* expressed recombinant IIGP_S83N protein proved to be GTPase negative in contrast to wild-type IIGP protein (data not shown). The integrity of all constructs was verified by sequencing. The following commercial control plasmids were used: pRHFM1, encoding a LexA-homeodomain of bicoid fusion (OriGene, Rockville, USA), pEG202-Max, encoding a LexA-Max fusion (Origene, Rockville, USA), pLexA-Lam, encoding a lexA-lamin fusion (Clontech), pLexA-53, encoding a lexA-p53 fusion (Clontech), p42AD-T, encoding a acidic activator B42-SV40 large T antigen fusion (Clontech).

Yeast-two hybrid screen

For the yeast-two hybrid screening the Matchmaker LexA-system (Clontech, Palo Alto, USA) was used. In principle, technical procedures were performed as recommended by the manufacturer. The yeast reporter strain EGY48(p8op-lacZ), which contains the independent reporter genes *lacZ* and *LEU2* downstream of a binding sequence for LexA, was transformed with the pGilda-IIGP construct. The level of fusion protein expression, autonomous reporter gene activation, and *lexA* operator binding indicative for nuclear transport of the fusion protein were validated prior library screening as described by the manufacturer. A murine $CD4^+$ T cell cDNA library constructed in the vector pJG4-5 (kindly provided by Dr V. R. Prasad, Albert Einstein College of Medicine, New York) was transformed by standard lithium acetate technique into pGILDA_IIGP harboring EGY48(p8op-lacZ) yeast. Plasmid amplification was achieved by incubation of the

cotransformants in glucose containing synthetic medium lacking histidine, uracil and tryptophan for 2 hours at 30°C. To get rid of trace amounts of glucose the double transformants were extensively washed and afterwards plated on galactose/raffinose-containing synthetic induction medium lacking histidine, leucine, tryptophan and uracil. Leu^+ colonies were propagated on glucose-containing selective medium and tested for Leu prototrophy and *lacZ* activity by replica-plating on galactose/raffinose-containing selective medium with X-gal and BU salts (25 mM Na_2HPO_4, 25 mM NaH_2PO_4, pH 7.0) for β-galactosidase activity detection. Plasmids from Leu^+β-gal^+ colonies were isolated and transformed into *E. coli* strain KC8 to rescue the pJG4-5-derived library plasmids. The recovered plasmids were used to retransform EGY48(p8op-lacZ)-containing pGILDA_IIGP and the transformants were validated for interaction by plating on selective medium, monitoring growth on leucine-deficient medium and β-galactosidase activity. The cDNA inserts from specific clones confirming the Leu^+β-gal^+ phenotype upon re-analysis, were sequenced. Subsequent control experiments, for analysis of the specificity of interaction in yeast, as well as further analytical experiments were performed accordingly. All pGILDA-, pJG4-5- or pB42AD-based constructs were controlled to drive expression of the respective fusion proteins in yeast EGY48 by western blot analysis using either an anti-LexA or an anti-HA mAb (Clontech, Palo Alto, USA). β-Galactosidase activities, reflecting strength of the interactions, were determined by a liquid culture assays using ONPG (ortho-nitrophenyl-β-D-galactopyranoside) as substrate as recommended by the manufacturer (Clontech yeast protocol handbook). Briefly, 5 ml galactose/raffinose-containing induction medium lacking histidine, tryptophan and uracil were inoculated to an OD_{600}=0.3-0.5 with individual cotransformants which were grown on plates with glucose-containing selective medium lacking histidine, tryptophan and uracil. After incubation for 5 hours at 30°C with shaking, suspensions in mid-log phase were normalized to OD_{600}=0.5 and 1 ml aliquots were assayed at 30°C in triplicate. β-galactosidase activity was expressed in Miller Units (Miller, 1972) and assays were repeated twice.

Immunoprecipitation

IFNγ-stimulated and non-stimulated BMM were dislodged from Petri dishes by incubation in cold PBS with 0.5 mM EDTA. The cells were washed twice with ice-cold PBS and after centrifugation the cell pellets were resuspended in lysis buffer (120 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 10 mM CHAPS and protease inhibitors). After incubation on ice for 30 minutes the insoluble material was pelleted by centrifugation at 16,000 ***g*** for 30 minutes at 4°C. The purified lysates were divided into the number of aliquots (0.8 ml/aliquot) needed. The lysates were pre-cleared by incubation with either 10 μg irrelevant murine $IgG_1κ$ antibody or rabbit pre-immune serum and 100 μl protein-G Sepharose (Pharmacia, Uppsalla, Sweden) for 30 minutes at 4°C. After centrifugation, individual lysates were left on ice or incubated for 30 minutes at 32°C with 1 mM (f.c.) GTP-γ-S or GDP-β-S (Sigma, Missouri, USA) prior to specific immunoprecipitation. Subsequently, murine hook3 or IIGP proteins were immunoprecipitated from the pre-cleared lysates by addition of 10 μg anti-IIGP mAb 5D9 or affinity purified rabbit anti-hook3 serum, respectively, and 100 μl protein-G Sepharose slurry. After incubation for 1 hour at 4°C the beads were pelleted and washed five times with lysis buffer and once with PBS. Immune complexes were eluted with 0.5 ml 6.25 mM Tris-HCl, pH 6.8, SDS, 5% 2-ME at 37°C for 45 minutes. Subsequently, the eluates were lyophilized, resuspended in H_2O containing 10% glycerol, 5% 2-ME, and boiled. SDS-PAGE and western blot analysis were performed according to standard protocols (Harlow and Lane, 1988) and the blots were developed with ECL-western blot detection reagents (Amersham, Buckinghamshire, UK).

Microscopy

IFNγ-activated BMM were fixed with 4% (w/v) paraformaldehyde in PBS for 15 minutes, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and blocked with 10% goat serum, 20 μg/ml anti-Fc receptor mAb 2.4G2, 1% BSA, 0.05% Tween 20 in PBS for 30 minutes. The cells were then incubated for 30 minutes with anti-IIGP mAb 5D9 and rabbit anti-hook3 serum in blocking solution. Subsequently, the cells were incubated with Cy2-conjugated goat anti-rabbit IgG/IgM or Cy3-conjugated donkey anti-mouse IgG/IgM (Dianova, Hamburg, Germany). Between these individual steps the cell were extensively washed with 0.05% Tween 20 in PBS. After mounting in mowiol, the cells were analyzed with a Leica TCS-SP confocal laser scanner (Leica, Deerfield, USA) equipped with a DMIRB microscope (Leica). Individual scans were analyzed using the TCS-NT software and Adobe Photoshop (Adobe Systems, Mountain View, USA).

Subcellular fractionation

A post-nuclear supernatant was prepared from BMM stimulated overnight with 1000 U IFNγ/ml. The harvested cells were washed twice with ice-cold PBS. Upon centrifugation the cell pellet was gently resuspended in 30 ml hypotonic buffer (10 mM Hepes, pH 7.5, 1 mM $MgCl_2$), centrifuged (450 ***g***, 4°C, 5 minutes), and the cells were gently resuspended in 2 ml ice-cold hypotonic buffer with 1:100 aprotonin (Sigma, #A-6279, Missouri, USA), 50 μg/ml leupeptin, and 1 mM PMSF and incubated for 5 minutes on ice. Cells were lysed by 15-20 strokes with a tight-fitting Dounce homogenizer until approximately 90% of the cells were broken. A previously described modified fractionation protocol was applied, suited for enrichment of soluble cytosolic, plasma, Golgi and ER membrane protein fractions (Vidugiriene and Menon, 1993). The lysate was cleared by centrifugation at 1000 ***g*** for 5 minutes at 4°C and the post-nuclear supernatant was adjusted to 8.5% sucrose followed by centrifugation at 10,000 ***g*** for 15 minutes at 4°C. The resulting supernatant was layered on a sucrose step gradient of 20%, 30% and 38% sucrose solution layers. The sucrose solutions contained 10 mM Hepes, pH 7.5, and 1 mM $MgCl_2$. Subcellular fractionation was achieved by ultracentrifugation at 100,000 ***g*** for 2 hours at 4°C. Five fractions were taken: fraction I, the 8.5% sucrose layer, fraction II, the 20% sucrose layer, fraction III, the interphase between the 20% and 30% sucrose layers, fraction IV, the interphase between the 30% and 38% sucrose layers and the pellet was designated fraction V. The individual fractions were subsequently diluted with 8.5% sucrose solution and the cellular material was recovered by ultracentrifugation at 100,000 ***g*** for 2 hours at 4°C. The pellets were resuspended in 100 μl hypotonic buffer and the protein content was determined with the BCA Protein Assay Reagent (Pierce, Bonn; Germany). For subsequent SDS-PAGE and western blot analysis, 10 μg total protein of each fraction were loaded per lane. The blots were probed with primary antibodies specific against IIGP, IGTP, α-tubulin, mHk3, Golgi-58K or calnexin. Secondary reagents used were either peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Dianova, Hamburg, Germany).

Results

Identification of hook3 as IIGP interacting protein

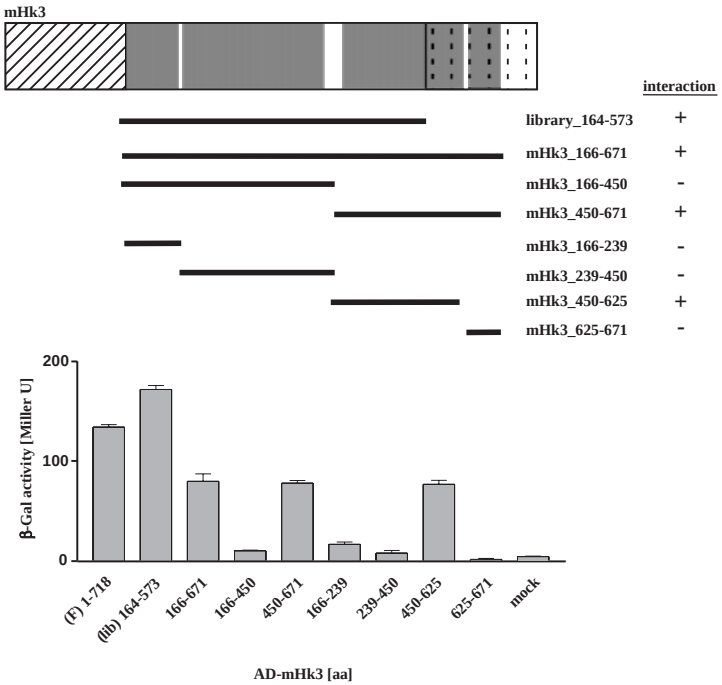
In order to identify cellular proteins that interact with the 47 kDa GTPase IIGP we employed a yeast two-hybrid screen with the complete protein sequence of IIGP fused to the lexA-binding domain as bait and a $CD4^+$ T cell-derived cDNA library as prey. After re-transformation into the yeast reporter strain and positive indication of interaction (Leu^+β-gal^+), the screening led to the identification of a cDNA encoding a portion of the murine hook3 protein. The human homologue of hook3 (hHk3) was recently described as a constitutively expressed, microtubule-binding protein (Walenta et al., 2001). The conserved N-terminal domain is involved in binding to

A.

BD	AD	-Leu/lacZ
IIGP	mHk3	+
IIGP_S83N	mHK3	-
IIGP	-	-
-	mHk3	-
lamin	mHk3	-
dros.bicoid	mHk3	-

BD	AD	-Leu/lacZ
LRG-47	mHk3	-
IRG-47	mHk3	-
TGTP	mHk3	-
IGTP	mHk3	-
GTPI	mHk3	-

B.



C.

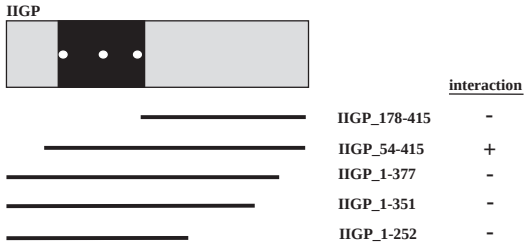


Fig. 1. Yeast two-hybrid interaction between IIGP and mHk3. A, The interaction between IIGP, the GTPase negative mutant IIGP_S83N, controls (lamin, *Drosophila* Bicoid) or the other members of the 47 kDa GTPase protein family and full-length mHk3 were analyzed by growth on selective medium lacking leucine or for *lacZ* reporter activity. A $Leu^+\beta\text{-}gal^+$ (Leu^+lacZ^+) phenotype is indicated by +, while – indicates no detectable reporter activity. (B) Schematic depiction of the domains in mHk3. The N-terminal region (hatched) harbors a microtubule binding domain. The four putative central coiled-coil regions are indicated (dark gray) and the C terminus (dotted) depicts the region involved in Golgi membrane association (Walenta et al., 2001). Truncated mutants of mHk3 were generated and analyzed for interaction with IIGP. β-Galactosidase activities, reflecting strength of the interactions, were determined by a liquid culture assay and values are given in Miller Units. (C) Schematic depiction of IIGP with the localization of the guanylate-binding domain (black) indicated. Deletion variants of IIGP were tested for interaction with full-length mHk3 by growth on selective medium lacking leucine or for *lacZ* reporter activity. All analytical experiments were repeated twice with almost identical results.

microtubules. An extended coiled-coil motif characterizes the central domain of this molecule, which mediates homodimerization, while the C-terminal part of this molecule contributes to the subcellular localization. The cDNA sequence of murine hook3 (mHk3) was inferred by analysis of available EST and genomic sequence databases (NCBI; Celera) and a full-length mHk3 cDNA was cloned based on the database sequence information for further analysis (accession number AY223806). The complete hook3 protein and IIGP strongly interacted with each other (Fig. 1A,B). In contrast, prototrophic growth on induction medium lacking leucine and β-gal activity was not observed when mHk3 or IIGP were expressed alone, or mHk3 in combination with unrelated control bait proteins, such as lamin C, *Drosophila* Bicoid, p53 or Max (Fig. 1A). In analogy to other well-characterized point mutations, locking small GTPases such as p21 ras, rab proteins or IGTP into an inactive GDP-bound conformation (Dugan et al., 1995; Taylor et al., 1997; Feig, 1999), a $serine_{83}$ to asparagine exchange was introduced into the (phosphate-binding) P-loop of IIGP. Analysis of the LexA-IIGP_S83N fusion revealed that this subtle point mutation in the first of the three characteristic elements of GTP-binding proteins abrogated detectable interactions between IIGP and mHk3 (Fig. 1A). Thus, IIGP interacted in a conformation-dependent fashion with mHk3, most likely when present in a GTP-bound conformation. Furthermore, analysis of the other 47 kDa GTPase family members for interaction with the hook3 protein revealed their inability to bind to mHk3 (Fig. 1A) demonstrating the specificity of the interaction between mHk3 and IIGP.

Next, we tried to define more precisely the regions of mHk3 and IIGP involved in binding. The library-derived cDNA of mHk3 encodes most of the central part of the molecule including the first three out of four putative coiled-coil domains. Therefore, various deletion variants of mHk3, representing the individual or consecutive putative coiled-coil domains were tested for interaction with IIGP. Fusions harboring the third putative coiled-coil domain revealed significant interactions (aa 450-625), while all other variants remained negative (Fig. 1B). This result points to the third putative coiled-coil domain of mHk3 as an integral part of the interface that mediates binding to IIGP. Conversely, we analyzed terminal deletion variants of IIGP, because the presence of functional modules distinct from the conserved

guanylate-binding region within IIGP have not been revealed so far. Deletion of the first 53 aa of IIGP had no effect. This indicates that the N-terminal portion of the protein preceding the actual GTPase domain is irrelevant for mHk3 binding (Fig. 1C). In contrast, increasing deletions of the C-terminal regions of IIGP, including one comprising as few as the last 38 aa, completely abrogated the interaction with mHk3. However, the complete region distal to the guanylate-binding domain failed to associate with mHk3, demonstrating that the last 38 aa of IIGP are not involved in mHk3 binding. Based on these results we assume that conformation-dependent binding of IIGP to mHk3 requires integrity of the complete GTPase protein, with the exception of its N-terminal 53 aa. Taken together, yeast-two hybrid analysis provides strong evidence that the 47 kDa GTPase IIGP interacts in a specific and conformation-dependent fashion with the central region of mHk3.

Interaction between mHk3 and IIGP in vivo

To verify that the interaction identified in yeast reflects association between IIGP and mHk3 in vivo, we performed co-immunoprecipitations to determine whether a physical complex of endogenous IIGP and mHk3 is detectable in lysates of IFNγ activated BMM. Western blot analysis revealed the presence of mHk3 in immunoprecipitates from IFNγ-activated BMM using the anti-IIGP mAb 5D9 (Fig. 2). In contrast, the constitutively expressed mHk3 was not detectable in immunoprecipitates from unstimulated BMM indicating that the protein was not unspecifically pulled down in lysates from IFNγ-stimulated cells. Likewise, neither IIGP nor mHk3 were detected upon pre-clearing of the lysates with irrelevant, but isotype matched antibodies prior to specific immunoprecipitation (Fig. 2A,B). Reciprocal analysis confirmed the physical in vivo interaction between IIGP and mHk3 in IFNγ-activated BMM (Fig. 2B). The GTPase was co-purified employing a polyclonal rabbit antiserum against mHk3. In comparison to the total amount of IIGP or mHk3 present in the lysates, in both cases only a minor fraction was recovered in complexed form.

Our interaction analysis in yeast provided evidence that IIGP binds to mHk3 in a conformation-dependent fashion. Binding of the non-hydrolysable analogs GTP-γ-S or GDP-β-S locks GTPases in distinct conformational states. Frequently, adopting either a GTP-bound or GDP-bound conformation correlates with different functional properties. Therefore, we assessed the influence of either GTP-γ-S or GDP-β-S on the interaction between IIGP and mHk3. Prior to specific immunoprecipitation, an excess of either non-hydrolysable analog was added to the lysates followed by incubation at 32°C. Addition of GDP-β-S induced complete dissociation of the complex composed of mHk3 and IIGP (Fig. 2A). In contrast, a comparable amount of mHk3 complexed to IIGP, as revealed in untreated lysates, was detected upon addition of GTP-γ-S (Fig. 2A). Hence a fraction of IIGP associated with mHk3 in IFNγ-stimulated cells. The dissociation of the complex induced in the presence of GDP-β-S suggests, that the interaction of IIGP with mHk3 is conformation dependent. This finding is consistent with our initial observation that the GTPase negative IIGP_S83N mutant did not interact with mHk3 in the yeast-two hybrid analysis.

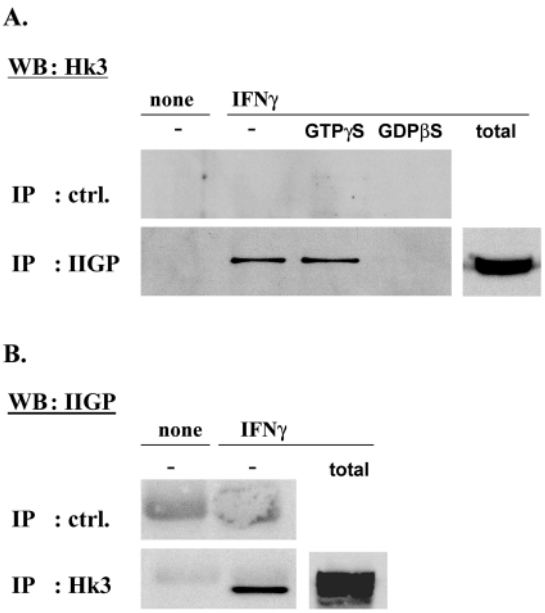


Fig. 2. Physical interaction of endogenous IIGP and mHk3. Immunoprecipitation of endogenous IIGP or mHk3 from lysates of IFNγ-stimulated or non-stimulated BMM. (A) The lysates were pre-cleared by immunoprecipitation with isotype matched control mAbs. Subsequently, IIGP was immunoprecipitated with the anti-IIGP mAb 5D9 either prior or after incubation for 30 min at 32°C with addition of GTPγS or GDPβS. Purified proteins were subjected to SDS-PAGE and western blotting. Co-immunoprecipitated mHk3 was revealed with an affinity-purified rabbit anti-hook3 serum. (B) The lysates were pre-cleared by use of a non-immune rabbit serum and mHk3 was subsequently purified from lysates of BMM stimulated with IFNγ or not. IIGP was revealed on western blots using the anti-IIGP mAb 5D9. To determine the total amount of IIGP (B) or mHk3 (A) recovered by specific immunoprecipitation, 1/5 of the samples that were probed for co-purification were analyzed in parallel by western blotting with the respective antibodies.

Subcellular localization

We have previously shown that IIGP localizes to the ER of IFNγ-stimulated cells (Zerrahn et al., 2002). In addition, a significant subcellular fraction is associated with the Golgi. The typical subcellular distribution of IIGP in IFNγ-activated BMM analyzed by two-color confocal microscopy is shown in Fig. 3A,D, revealing the reticulate distribution of IIGP throughout the cell reflecting its association with the ER. Colocalization of IIGP with the Golgi resident protein α-mannosidase is evident in the perinuclear region (Fig. 4A-C). A markedly defined accumulation of mHk3 in the same area is apparent (Fig. 3B,C,E,F), which is reminiscent of the position of the microtubule organizing center (MTOC) and the Golgi (Fig. 4D-F). Furthermore, mHk3 was detected proximal to this accumulation and in perinuclear regions in a fine reticulate to vesicular pattern. Identical distribution and staining intensity of mHk3 was observed in BMM not stimulated with IFNγ (data not shown), indicating that neither the expression level nor its subcellular distribution overtly change upon stimulation with IFNγ. A fraction of hHk3 was found to be associated with the cis-Golgi compartment, and accumulated in a microtubule-dependent way around the MTOC after disruption of the Golgi compartment by brefeldin A (Walenta et al., 2001).

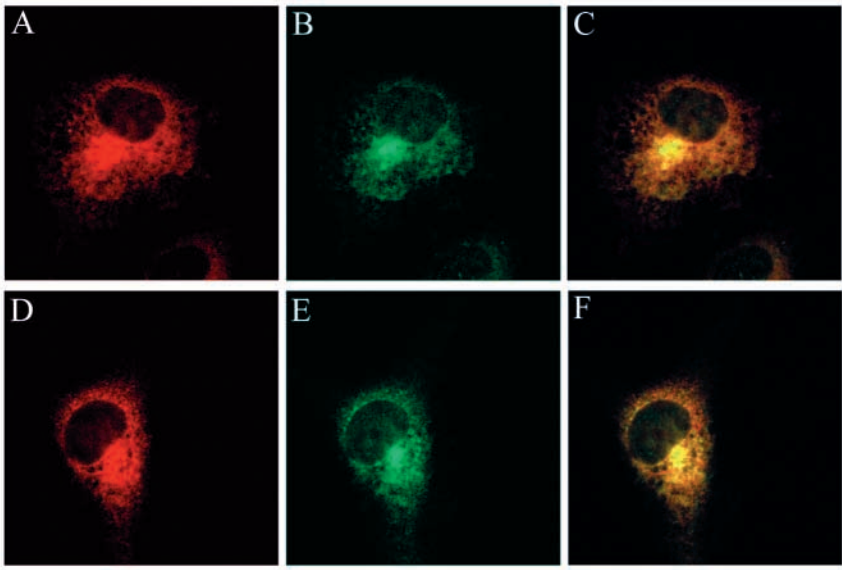


Fig. 3. Subcellular localization of IIGP and mHk3 in IFNγ-stimulated BMM. The cells were stained with the anti-IIGP mAb 5D9 revealing the typical distribution of IIGP, which associates with the ER and the Golgi (A,D). The localization of mHk3 was revealed by employing an affinity-purified rabbit anti-hook3 serum (B,E). Cy2- (mHk3) and Cy3- (IIGP) labeled secondary reagents were used. In murine BMM mHk3 strongly accumulated in a juxtanuclear region reminiscent of the position of the MTOC and Golgi. In addition, a reticulate staining for mHk3 was observed in the perinuclear region. Close spatial proximity of IIGP and mHk3, which partially colocalized, was evident in the merged images (C,F).

Furthermore, cytoplasmic vesicles of unknown identity were labeled with hHk3-specific antibodies (Walenta et al., 2001). The strong juxtanuclear accumulation of mHk3 (Fig. 3B,C,E,F) does not completely colocalize with IIGP. Rather, it seems to be distinct from the defined localization of IIGP in this confined subcellular region. However, the alignment of the reticulate and vesicular pattern of mHk3 in the perinuclear region resembles the subcellular distribution of IIGP, suggesting close spatial proximity of both proteins (Fig. 3). Upon depolymerization of microtubules by nocodazole much of the hHk3 distributes throughout the cytoplasm (Fig. 4K,L) and, as has been reported previously, is associated with the dispersed Golgi compartment (Walenta et al., 2001). In contrast, association of IIGP with the Golgi seems to depend on an intact microtubule architecture, because colocalization of IIGP with the Golgi marker α-mannosidase was abrogated by nocodazole (Fig. 4G-I). These findings suggest that the mode of Golgi association of IIGP and mHk3, is distinct and, furthermore, that IIGP recruitment to the Golgi requires integrity of the microtubule network. Alternatively, but not mutually exclusively, IIGP and mHk3 interact in the more distal perinuclear regions were the staining pattern of both proteins significantly alignment to one another. While these observations provide further evidence for an interaction between IIGP and mHk3, they are also consistent with our finding that only a minor fraction of mHk3 co-purifies in complex with IIGP.

Subcellular fractionation

IIGP, IGTP and most likely the other 47 kDa GTPase cognates associate with intracellular membranes, such as the ER and Golgi in case of IIGP (Zerrahn et al., 2002). Likewise, a fraction of hHk3 interacts with Golgi membranes and is present in association with, as yet unidentified, vesicles (Walenta et al., 2001). To gain further insights into the membrane-dependent subcellular localization of both proteins we analyzed their distribution in enriched fractions of plasma, Golgi and ER membranes prepared from IFNγ-stimulated BMM. A post-nuclear lysate was fractionated on a 8.5, 20, 30, and 38% sucrose step gradient and individual fractions were analyzed by western blot analysis (Fig. 5). IIGP was detected in fractions II-IV, being most prominent in the fractions III and IV. The Golgi enriched fractions, namely fraction II and III, were identified with the anti-Golgi 58K mAb, detecting the Golgi-associated protein formiminotransferase cyclodeaminase (FTCD) (Bashour and Bloom, 1998; Gao et al., 1998). The ER-enriched fractions IV and V were revealed by detection of the ER-resident membrane protein calnexin. Enrichment of IIGP in fraction III and IV is in line with our previous finding that IIGP not only associates with ER membranes but also with the Golgi (Zerrahn et al., 2002). The mHk3 overlapped with IIGP in the dense Golgi-enriched fraction III but was also present in significant amounts in the light Golgi-enriched fraction II where only minute amounts of IIGP were found. In contrast to IIGP, the 47 kDa GTPase cognate IGTP was identified only in the ER-enriched fractions, which is consistent with a previous report (Taylor et al., 1997). Although both, IIGP and IGTP, associated with the ER, IIGP was not detected in fraction V. The reason for this differential distribution has not been further addressed, but it could reflect differential association of the respective GTPases with ER membranes, the association with ER membranes with differing properties, or the association with other non-ER dense membranes. The α-tubulin was detected in almost all fractions, being most prominent in the cytosolic/plasma membrane fraction I. While the latter most likely includes soluble cytoplasmic and/or plasma membrane associated α-tubulin, the presence in the other fractions except fraction V, indicates association with membranous compartments enriched in these fractions. Taken together, analysis of the membrane-dependent subcellular distribution of IIGP and mHk3 reveals a subpopulation of both proteins in Golgi membrane-enriched subcellular fractions.

Discussion

Our data demonstrate that IIGP, a member of the 47 kDa GTPase family, which participates in defense against

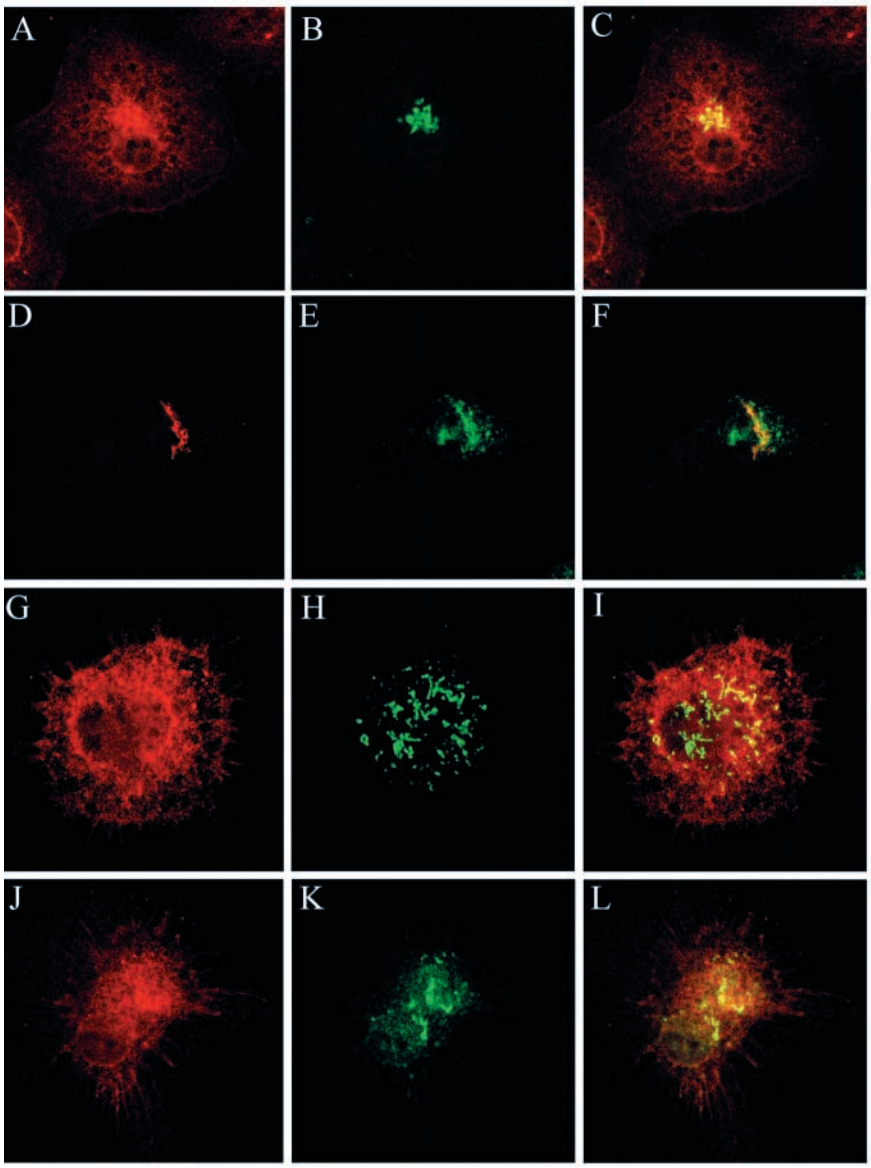


Fig. 4. Microtubule-dependent localization of IIGP to the Golgi. (A) subfraction of IIGP (A,C) localizes to the Golgi in IFNγ-stimulated murine BMM revealed by co-localization with the Golgi-resident protein α-mannosidase (B,C). Imaging specifically the juxtanuclear accumulation of mHk3 (E,F) reveals co-localization with the cis-Golgi matrix protein GM130 (D,F) as well as nearby accumulation which is reminiscent to the position of the MTOC (data not shown). The IFNγ-stimulated BMM were treated for 1 hour with nocodazole to disrupt microtubules (G-L). The fine reticulate localization of IIGP in untreated cells (A and Fig. 3), appears contracted upon nocodazole treatment, most probably reflecting the loss of the microtubule-dependent extension of the ER into the cellular periphery (G,I,J,L) (Terasaki et al., 1986). Dispersion of the α-mannosidase staining reflects the formation of Golgi-like structures at transitional ER exit sites (H,I) and colocalization with IIGP is not evident (G,I). Likewise, nocodazole treatment induces a scattered distribution of mHk3 staining (K,L).

intracellular pathogens, interacts with the microtubule-binding linker protein mHk3. The association of these GTPases with intracellular membranes suggests that these molecules modulate membrane-dependent processes (Taylor et al., 1997; Zerrahn et al., 2002). The herein identified interaction between IIGP and mHk3 provides the first evidence for a participation of IIGP in intracellular trafficking which could form the molecular basis for effector mechanisms directed against intracellular pathogens.

The recently described human hook protein family comprises three members that associate differentially with compartments of the secretory and endocytic pathways. A single hook protein (dHK) is expressed in *Drosophila*. Localized to endosomes, dHk participates in the formation or maturation of multivesicular bodies (MVB) (Kramer and Phistry, 1996), and loss of function results in a marked reduction in the number of these intermediate compartments within the endosomal pathway (Sunio et al., 1999). It has been proposed that hook proteins in general participate in proper assembly and/or positioning of membranous compartments, the cis-Golgi in the case of hHk3, and consequently contribute to the ordered dynamic formation, maturation and trafficking of these entities (Walenta et al., 2001). A common feature of all human hook proteins is the presence of an amino-terminal microtubule binding domain. The carboxy-terminal region of hHk3 confers association with Golgi membranes (Walenta et al., 2001), suggesting that hHk3 tethers endomembranes to microtubules. In accordance with these analyses, we observed a strong juxtanuclear accumulation of mHk3 and partial localization with the Golgi. In close proximity to the Golgi, the distinct accumulation of mHk3 reflects an association around the MTOC (data not shown). A subfraction of IIGP is also associated with the Golgi (Zerrahn et al., 2002). Therefore, it is conceivable that the physiologically relevant interaction between IIGP and mHk3 takes place at the Golgi. This notion is consistent with the distribution of both IIGP and mHk3 in the Golgi enriched fractions upon fractionation of subcellular membranes. In terms of the dynamics underlying the continuous formation and organization of the Golgi (Allan, 1996; Thyberg and Moskalewski, 1999), we assume that binding of IIGP to mHk3 is transient rather than permanent. An alternative, not mutually exclusive, hypothesis is based on our observation that mHk3 also localizes in the perinuclear region in a fine reticulate and/or punctuate pattern. Recent reports indicate profound interactions between the ER and endocytic or phagosomal compartments (Haj et al., 2002; Gagnon et al., 2002). Furthermore, the ER resides in close contact with the microtubule cytoskeleton and its extension into the cellular periphery is microtubule dependent (Terasaki et al., 1986; Allan, 1996). Since small vesicles of unknown identity have been stained with antibodies against hHk3 (Walenta et al.,

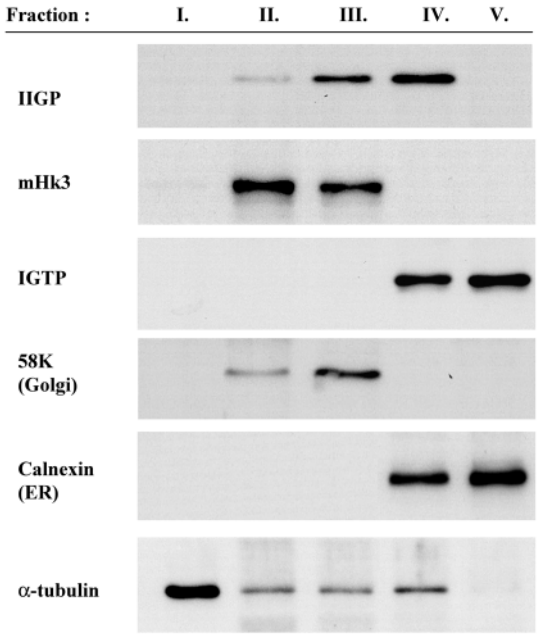


Fig. 5. Subcellular fractionation of IFNγ-stimulated BMM. Membrane enriched fractions from post-nuclear supernatants of IFNγ-stimulated BMM were prepared by separation on a sucrose-step gradient. Fraction I is the 8.5% and fraction II the 20% sucrose layer. The 20-30% and the 30-38% sucrose layer interphases constitute fraction III and IV, respectively. The pellet was designated as fraction V. Golgi and ER membrane-enriched fractions were identified upon SDS-PAGE and western blotting with mAbs against Golgi-associated FTCD and the ER resident protein calnexin. The differential distributions of IIGP, IGTP, mHk3, and α-tubulin were analyzed with specific antibodies as described in Materials and Methods.

2001), the question arises whether these mHk3-associated vesicles, potentially tethered to microtubules, contact the ER-associated IIGP. This would be consistent with the close spatial proximity of these proteins in perinuclear, cytoplasmic regions as revealed by microscopic analysis. Future studies are being directed to unraveling this issue in more detail.

Analysis of the primary amino acid sequence of IIGP and also of the other cognates does not provide valid information about the presence of distinct functional domains besides the guanylate binding domain. Our attempts to define more precisely the region of IIGP that is involved in binding to mHk3 revealed that the amino-terminal region preceding the guanylate binding domain is dispensable. Any other deletion abrogated interaction with mHk3 in yeast. Apparently, binding of IIGP to mHk3 depends on a specific conformation which seems to require integrity of the protein. Commonly, relaying of an effector function by GTPases goes along with conformational changes mediated by binding of GTP and subsequent hydrolysis (Vetter and Wittinghofer, 2001). The introduction of a subtle point mutation (S83N) in the guanylate binding domain of IIGP rendered the GTPase inactive and most likely induced conformational alterations. Consequently, the binding potential of the IIGP S83N mutant to mHk3 was abrogated in yeast, which is in accordance with the common scheme of GTPase functionality (Vetter and Wittinghofer, 2001). Likewise, mHk3 did not co-immunoprecipitate with IIGP if non-hydrolysable GDP-β-S was added. We assume that the interaction of IIGP with mHk3 requires a specific conformational state of the GTPase. Interestingly, the other members of the 47 kDa GTPase protein family failed to interact with mHk3. This not only emphasizes specificity of the interaction between IIGP and mHK3, but also provides further evidence that these GTPases mediate distinct effector functions.

The mHk3 protein is constitutively expressed and neither its expression level nor its subcellular location are significantly altered upon IFNγ stimulation. We conclude that mHk3 constitutes a basic element of the endomembrane trafficking pathways and/or the organization of the Golgi complex. However, a mere architectural function of mHk3 is unlikely, because the Golgi is a dynamic, steady-state system (Lippincott-Schwartz et al., 2000; Ward et al., 2001). One consequence of IFNγ encounter for the cell is to establish a common intracellular defense mode by inducing effector mechanisms that restrict intracellular pathogen replication. These IFN-induced mechanisms are in part reflected by profound changes in the dynamics and functional properties of distinct intracellular vesicles (Sidhu et al., 1999; Alvarez-Dominguez and Stahl, 1998; Tsang et al., 2000; Shurety et al., 2000). For example, the IFNγ-regulated GTPase Rab5a causes remodeling of the phagosomal environment, resulting in listeriocidal activity (Prada-Delgado et al., 2001). In analogy, IIGP could modulate processes of vesicular trafficking in which mHk3 is involved. IIGP need not regulate mHk3 function itself, but alternatively could be recruited by mHk3 to initiate antimicrobial effector mechanism at specific sites. The function of constitutively expressed small GTPases is often tightly regulated by an interplay between GTPase-activating proteins (GAPs) and GTP-exchange factors (GEFs) (Vetter and Wittinghofer, 2001). In contrast, functions of IIGP and its cognates could be primarily regulated at the transcriptional level by mere IFN stimulation. Subsequent interactions with cellular target molecules, such as mHk3, could promote expression of effector functions by IIGP. However, the exact functional significance of hHk3 for ordered vesicular trafficking or Golgi organization is not fully understood yet. Treatment of cells with brefeldin A induces disruption of the Golgi complex and redistribution of Golgi resident membrane proteins back to the ER (Dinter and Berger, 1998). Under these conditions hHk3 remains mainly localized in a juxtanuclear accumulation close to the MTOC (Walenta et al., 2001). In contrast, disruption of microtubules by nocodazole induces Golgi fragmentation and formation of scattered Golgi-like structures in close proximity to transitional ER exit sites (Dinter and Berger, 1998). In nocodazole-treated cells most of hHk3 is recruited to these Golgi fragments (Walenta et al., 2001). We observed an analogous dispersion of mHk3 staining in IFNγ-stimulated and nocodazole-treated BMM. In contrast to mHk3, we found that the association of IIGP to the Golgi depends on microtubule integrity. Apparently, IIGP association with mHk3 at the Golgi requires an intact microtubule network. The reason for this is unknown, but interactions between these proteins may depend on microtubule-dependent recruitment of IIGP to the Golgi. Our analysis provides no evidence for colocalization of IIGP with markers of the ER-to-Golgi intermediate compartment (data not shown). Hence, a conventional ER-Golgi transport of IIGP

along the secretory pathway seems unlikely. The way IIGP is being recruited to the Golgi as well as the nature of its association with the ER remains to be addressed.

Identification of mHk3 as the first interaction partner for a 47 kDa GTPase strongly emphasizes involvement in IFN-stimulated modulation of intracellular trafficking processes. Intracellular pathogens either pass through phagosomal/endosomal compartments for cytoplasmic replication or reside in specifically modified vacuoles. Whilst providing protection against lysosomal degradation, provision of nutrients for the pathogen in these vacuoles need to be ensured (Sinai and Joiner, 1997). Close association of cellular organelles with the membrane of the pathogen-containing vacuoles or intersection of cellular transport pathways are likely mechanisms employed by intracellular pathogens (Hackstadt, 2000; Dorn et al., 2002; Kagan and Roy, 2002). Interfaces between the host cell and the intracellular pathogen are well suited targets for host protection induced by IFNs. The association of IIGP with mHK3 suggests that the antimicrobial effector mechanisms are exerted in the context of modulation of intracellular trafficking by IFN. It is tempting to speculate that these alterations interfere with nutrient provision for intracellular pathogens or undermine the protective pathogen-containing niche for access of degradative pathways.

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