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Mechanisms Regulating the Positioning of Mouse p47 Resistance GTPases LRG-47 and IIGP1 on Cellular Membranes: Retargeting to Plasma Membrane Induced by Phagocytosis

Sascha Martens,† Katja Sabel,* Rita Lange,* Revathy Uthaiah,† Eva Wolf, † and Jonathan C. Howard‡∗

The recently identified p47 GTPases are one of the most effective cell-autonomous resistance systems known against intracellular pathogens in the mouse. One member of the family, LRG-47, has been shown to be essential for immune control in vivo of Listeria monocytogenes, Toxoplasma gondii, Mycobacterium tuberculosis, and Mycobacterium avium, possibly by promoting acidification of the phagosome. However, the intracellular localization of LRG-47, and the nature of its association with the phagosomal or any other membrane system is unknown. In this study, we show that LRG-47 is a Golgi-associated protein in the IFN-stimulated cell, which is rapidly recruited to active plasma membrane upon phagocytosis and remains associated with phagosomes as they mature. We show that the Golgi localization of LRG-47 is dependent on the integrity of an amphipathic helix near the C terminus, whereas the plasma membrane localization depends on an unidentified signal associated with the G domain. Unlike LRG-47, but like the published p47 resistance GTPase, IGTP, a further p47 GTPase, IIGP1, is associated with the endoplasmic reticulum. However, unlike IGTP, IIGP1 is associated with the endoplasmic reticulum by an N-terminal myristoylation modification. Thus, the p47 GTPases are a diverse battery of intracellular defense factors dynamically associated with different membrane systems.


The p47 GTPases are some of the most potent, known resistance factors against intracellular pathogens in the mouse (1–3). Mice with targeted deletions of single members of the p47 GTPases showed complete loss of immune control of certain bacterial and protozoan infections. In particular, LRG-47 has been shown to be essential for resistance against Toxoplasma gondii and Listeria monocytogenes (4), and most recently against Mycobacterium tuberculosis (4) and Mycobacterium avium (5). LRG-47 has been copurified with phagosomes isolated from Mycobacterium-infected macrophages and was shown to promote acidification and thus maturation of the phagosome (4). However, the cellular localization and mode of recruitment of LRG-47 to the phagosome and the nature of its association with cellular membranes are unknown.

All p47 GTPases tested so far, including LRG-47, are abundantly induced by IFN-γ from low resting levels in vivo and in vitro (6) and have been shown to confer cell-autonomous immunity against intracellular pathogens (1, 2, 4, 7, 8). So far 6 family members, namely TGTP (9), IRG-47 (10), IIGP1 (6), GTPI (6), IGTP (11), and LRG-47 (12), have been described in the mouse, although the gene family in the C57BL/6 mouse has 23 members (C. Beckpen, J. Hunn, L. Guethlein, D. M. Dunn, E. Glowalla, I. Parvanova, and J. C. Howard, manuscript in preparation). The 6 published p47 GTPases fall into two sequence subgroups (6), GKS and GMS, defined by variation in the otherwise universally conserved G1 motif. This is the familiar GXGXGKS in IIGP1, TGTP, and IRG-47, but GXGXGMS in LRG-47, IGTP, and GTPI. Members of both subgroups are involved in cell-autonomous resistance and the function of at least one member, IGTP, is required in cells of both hemopoietic and nonhemopoietic origin (13). This is consistent with the finding that the p47 GTPases are induced by IFN-γ in all cell types tested so far (13).

Recently, the crystal structure of IIGP1 has been determined (33). The Ras-like G domain is preceded by a three-helix bundle carrying an N-terminal myristoylation motif, and is followed by a larger C-terminal domain that is also predominantly helical. Both N- and C-terminal regions of the different p47 GTPases are highly variable in sequence (6). However, colinear stretches of high homology occur throughout the molecule, and largely shared secondary structure predictions strongly suggest that all the p47 GTPases share a fundamentally similar structure (33).

Three family members, namely IGTP, IIGP1, and LRG-47, have been shown to be associated with internal membrane compartments. IGTP was found to be endoplasmic reticulum (ER) associated in mammary gland-derived C127 cells in a GTP-independent manner (14); IIGP1 has been reported to localize predominantly to...
Golgi membranes (15); and LRG-47 has been copurified with phagosomes containing mycobacteria from infected macrophages (4). However, the intracellular localization of LRG-47 and the mechanism and dynamics of membrane association of the p47 GTPases are unknown.

We show in this study that LRG-47 is normally localized to the cis-Golgi by a C-terminal amphipathic helix. Upon phagocytosis, LRG-47 is recruited locally to the plasma membrane, at the forming phagocytic cups, and (in fibroblasts) also at phagocytosis-induced membrane ruffles. This recruitment is likely to be mediated by sequences in the G domain and is probably nucleotide dependent. LRG-47 then remains associated with the maturing phagosome, reaching a late endosomal or lysosomal compartment colocalizing with lysosome-associated membrane protein 1 (LAMP-1). A second IFN-inducible member of the p47 GTPases, IIGP1, is localized to the ER membrane, and this localization is mediated by mechanisms very different from those guiding LRG-47 to the Golgi. However, like LRG-47, IIGP1 also carries a cryptic targeting signal in its G domain that can direct the free G domain to the plasma membrane. The related but distinct cell biological behaviors of these two members of the p47 GTPase family are consistent with the idea that the family represents a battery of cell-autonomous resistance factors diversified to engage effectively with the variety of intracellular pathogens.

Materials and Methods

Cells and tissue culture

RAW 264.7, L929 mouse fibroblasts, and TIB-75 hepatocytes (16) were cultured in IMDM supplemented with 10% FCS (Sigma-Aldrich, Deisenhofen, Germany), 2 mM L-glutamine (Invitrogen Life Technologies, Eggenstein, Germany), 1 mM sodium pyruvate (ICN, Eschwege, Germany), 100 U/ml penicillin (Invitrogen Life Technologies), and 100 μg/ml streptomycin (Invitrogen Life Technologies). Cells were induced with mouse IFN-γ (Cell Concepts, Umkirch, Germany) at a concentration of 200 U/ml for the time spans indicated in the figures. Peritoneal macrophages were isolated from the peritoneal cavity of CB20 mice and cultured in six-well plates with coverslips. Cells were grown in IMDM and washed several times to remove nonadherent cells over a period of 36–48 h before experiments.

Antisera and Abs

Polyclonal anti-IIGP1 antiserum (165) was raised against the full-length protein. The purification of IIGP1 as a cleaved GST-fusion protein is described elsewhere (17). Rabbits were immunized against the full-length IIGP1 protein.

Serological agents used were mouse monoclonal anti-IGTP A68120 (BD Transduction Laboratories, Lexington, KY), mouse monoclonal anti-IIGP1 10D7 and 10E7 Abs (gift from J. Zerrahn, Max-Planck Institute, Berlin, Germany), anti-TGF-β A2O sc-11079 goat anti-peptide antiserum (Santa Cruz Biotechnology, Santa Cruz, CA), and LRG-47 A19 sc-11075 goat anti-peptide antiserum (Santa Cruz Biotechnology). Anti-IRG-47 2078 rabbit anti-peptide antiserum was raised against internal peptides, CKTPYHKPKFYK and CDAKLLRKTVENVA, using the double X program (Eurogentec, Seraing, Belgium), affinity-purified anti-rabbit antibody antiserum (gift from B. Goud, Centre National de la Recherche Scientifique, Paris, France), monoclonal anti-Giantin Ab (gift from H.-P. Hauf, Biocenter, Basel, Switzerland (18)), anti-ERP60 rabbit antiserum (gift from T. Wileman, BBSRC, Pirbright, U.K.), monoclonal rat anti-LAMP-1 1D4B (University of Iowa, Iowa City, IA), monoclonal anti-TAP 294 (19), monoclonal anti-FLAG M2 (Sigma-Aldrich), anti-calnexin SPA-685 rabbit antiserum (StressGen Biotechnologies, Victoria, BC, Canada), anti-caveolin-1 N20 sc-894 rabbit antibody (Santa Cruz Biotechnology), goat anti-mouse Alexa 546/488, goat anti-rabbit Alexa 546/488, donkey anti-goat Alexa 546/488, donkey anti-mouse Alexa 488, donkey anti-rabbit Alexa 488 (Molecular Probes, Eugene, OR), goat anti-mouse HRP (Amersham Biosciences, Piscataway, NJ), donkey anti-rabbit HRP (Amersham Biosciences), and donkey anti-goat HRP (Santa Cruz Biotechnology).

Cosedimentation assay of rIIGP1

rIIGP1 was produced as previously described in Ref. 17. Liposomes were prepared from synthetic phosphatidylycerine (1,2-dioleoyl-sn-glycero-3-phospho-L-serine); Avanti, Alabaster, AL). Lipids dissolved in chloroform were dried under nitrogen and additionally in a rotary evaporator to remove residual chloroform. The resulting lipid cake, hydrated by addition of HEPES-buffered saline, was left undisturbed for 1 h at room temperature and finally vortexed to resuspend the lipids. The lipid suspension was extended 21 times through polycarbonate membranes with a pore size of 100 nm (Avestin, Ottawa, ON, Canada). For cosedimentation assays, 0.25 mM phosphatidylserine lipids and 0.25 μM IIGP1 in HEPES-buffered saline were incubated for 30 min at 37°C followed by centrifugation for 30 min with 125,000 × g at 4°C. The samples were further processed as described for the cellular membranes.

Bead uptake experiments

A volume of 500 μl of 2-μm carboxylated latex beads (Polysciences, Warrington, PA) were coated with collagen as follows. Beads were washed two times with 0.1 M NaHCO3 (pH 9.6), followed by three washes with 0.02 M phosphate buffer (pH 4.5). The beads were resuspended in 675 μl of phosphate buffer (pH 4.5), and 675 μl of 2% carbodiimide (Sigma-Aldrich) in phosphate buffer (pH 4.5) was added. After an incubation of 4 h on a rotator at room temperature, the beads were washed three times with phosphate buffer (pH 4.5), resuspended in 1.2 ml of borate buffer (pH 8.5) containing 500 μg of collagen (type I from rat tail; Sigma-Aldrich), and incubated overnight at room temperature on a rotator. A volume of 50 μl of 0.25 M ethanolamine was added, and the beads were incubated for another 30 min. The beads were pelleted and resuspended in borate buffer (pH 8.5) and 10 mg/ml BSA, and stored in 500 μl of 0.02 M phosphate buffer (pH 7.4), 0.15 M NaCl, 1% BSA, 5% glycerol, and 0.1% NaN3.

L929 cells were grown on coverslips and induced with IFN-γ. Twenty hours after induction, the collagen-coated beads were added, and the cells were incubated for an additional 4 h. Finally, the cells were fixed with 3% paraformaldehyde for 20 min at room temperature. A total of 3 × 106 RAW 264.7 cells was induced with 200 U/ml IFN-γ for 48 h and incubated with 2-μm latex beads not coated with collagen diluted 1/1000 in IMDM for 30 min to 4 h.

Immunofluorescence analysis

Cells were washed with PBS and fixed in 3% paraformaldehyde for 20 min at room temperature. Cells were permeabilized with 0.1% saponin and blocked with 3% BSA (Roht). The cells were analyzed using a Zeiss (Oberkochen, Germany) Axioplan II fluorescence microscope equipped with a cooled charge-coupled device camera (Quantix; Photometrics, Tucson, AZ). Image processing and two-dimensional deconvolution was done with the MetaMorph software (version 4.5r3; Universal Imaging, Downingtown, PA). For three-dimensional (3D) deconvolution, the Auto Deblur software (version 6.001; AutoQuant Imaging, Watervliet, NY) was used.

Generation of IIGP1 and LRG-47 expression constructs

The coding regions of IIGP1 and LRG-47 were amplified either by PCR from full-length cDNAs described in Ref. 6 from IFN-γ-stimulated mouse embryonic fibroblasts according to standard procedures using Pfu-polynu-merase (Promega, Madison, WI) and primers from Invitrogen Life Technologies. Restriction enzymes were from New England Biolabs (Beverly, MA). The PCR fragments were cloned into the Sfi I site of pEGFP-C3 (Clontech, Palo Alto, CA), pEGFP-N3 (Clontech), pGWH1 (British Biotechnology, Oxford, U.K.), or pGEX-4T-2 (Amersham Biosciences). Mutations of LRG-47 and IIGP1 were introduced into the open reading frames according to the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) protocol. All constructs were verified by sequencing.

Test of induction of p47 GTPase in L929 and TIB-75 cells

L929 or TIB-75 cells were lysed at a concentration of 106 cell/ml in PBS, 1% TX100 (Sigma-Aldrich) including complete mini protease inhibitors (Roht, Basel, Switzerland), incubated for 1 h on ice, and centrifuged for 10 min at 23,000 × g at 4°C. Equivalent volumes were loaded onto 10% polyacrylamide gels.
supernatant was stored on ice, and the pellet was washed as described above and finally resuspended in PBS and 0.5% SDS. Equal amounts were loaded on a 12% polyacrylamide gel.

**Tx114 partitioning assay**

L929 cells were lysed in PBS, 1% Tx114 (Sigma-Aldrich) including complete mini protease inhibitors (Roche) 24 h after IFN-γ induction and 48 h after transfection for 1 h on ice. Lysates were centrifuged for 15 min at 3,000 × g at 4°C to remove nuclei. Supernatants were incubated for 5 min at room temperature and subsequently centrifuged for 1 min at 23,000 × g at room temperature. The aqueous phase was transferred into a new tube and washed by an additional centrifugation step. The detergent phase was washed by addition of PBS followed by an additional centrifugation step and finally brought to the same volume as the aqueous phase by addition of PBS. Equal amounts were loaded on a 12% polyacrylamide gel.

**Transfections**

Cells growing on heat-sterilized coverslips or cell culture dishes were transiently transfected with FuGene 6 (Boehringer, Ingelheim, Germany) according to the manufacturer’s protocol.

Prediction of secondary structure was done with programs found at http://us.expasy.org/. The helical wheels were drawn with a program found at http://marqusee9.berkeley.edu/kael/helical.htm.

**Results**

The IFN-inducible p47 GTPases show different levels of membrane association

All five p47 GTPases tested were abundantly induced from very low or undetectable resting levels in the mouse fibroblast-like cell line, L929, and in TIB-75 mouse hepatocytes by IFN-γ. Expression of p47 GTPases was determined by Western blotting of Triton X-100 post nuclear supernatants (Fig. 1a). IFN-γ-induced L929 cells were disrupted in a hypotonic buffer without detergent and centrifuged at 100,000 × g. Each p47 GTPase partitioned in characteristic ratio between pellet and supernatant fractions (Fig. 1b). LRG-47 was found exclusively in the pellet fraction, whereas IRG-47 was largely in the supernatant. IIGP1 partitioned roughly equally between the two fractions.

**LRG-47 localizes to the Golgi apparatus**

L929 fibroblasts, TIB-75 hepatocytes, and RAW 264.7 macrophages were induced with IFN-γ and stained for LRG-47. All the cells showed a focused adnuclear signal accurately colocalizing with Golgi markers Rab6 (20) (Fig. 2, a–c) and giantin (18) (d–g), as well as showing a weaker diffuse signal throughout the cytoplasm (a, d, and h) partially colocalizing with the ER markers calnexin and ERP60 (data not shown). No LRG-47 was detected in cells not induced with IFN-γ (Fig. 1, c and d). Less precise colocalization was seen with α-mannosidase II in RAW 264.7 cells (Fig. 2, h–j) and no colocalization with the cation-independent mannose 6 phosphate receptor in L929 and RAW 264.7 cells (not shown) (21). Therefore, LRG-47 seems to be associated with cis- and medial-Golgi rather than with the trans-Golgi and the trans-Golgi network. We could detect no colocalization of LRG-47 with the endocytic compartments defined by rab11 and endocytosed transferrin (not shown). The localization of LRG-47 is different from that of its GMS relative IGTP, which shows an exclusive ER association (14).

**LRG-47 is recruited to the plasma membrane upon phagocytosis**

To engage with pathogens entering via the phagosomal system (2, 4), LRG-47 might translocate to plasma membrane during phagocytosis. Peritoneal macrophages and the macroage cell line RAW 264.7 were induced with IFN-γ for 24 h (Fig. 3). Phagocytosis of 2-μm diameter latex beads was then followed by fluorescence microscopy for LRG-47 and F-actin. We observed a marked recruitment of LRG-47 around forming phagocytic cups identified by F-actin accumulation in proximity to latex beads in peritoneal macrophages (Fig. 3, a–e) and in RAW264.7 macrophages (not shown). Moreover, LRG-47 apparently remained associated with the phagosomes as they matured to phagolysosomes, as demonstrated by the simultaneous labeling for LAMP-1 and LRG-47 (Fig. 3, f–j). To determine whether the phagocytosis-stimulated relocation of LRG-47 was a distinctive property of professional phagocytes, we initiated phagocytosis in IFN-induced L929 fibroblasts using latex beads precoated with collagen, exploiting the ability of fibroblasts to phagocytose collagen (22). Again, LRG-47 was found at F-actin-rich phagocytic cups and subsequently colocalized with the ingested beads in a LAMP-1-rich compartment (Fig. 4, a–f). This colocalization was restricted to phagosomes (Fig. 4, d–f); no colocalization of LRG-47 with LAMP-1 was seen in cells not engaged in phagocytosis in L929 cells (g–i). The results of these experiments were essentially identical in L929 cells and the two macrophage populations. Thus, the phagocytic process itself appears more important as a stimulus for the relocation of LRG-47 than does identity of the membrane receptor or cell type.

**FIGURE 1.** Induction of p47 GTPases in L929 and TIB-75 cells and quantification of the soluble and membrane-bound fraction. a, Cells were stimulated with 200 U/ml IFN-γ for 24 h and lysed in PBS containing 1% Triton X-100. The equivalent of 10⁵ cells was loaded in each track and subjected to SDS-PAGE. b, L929 cells stimulated with 200 U/ml IFN-γ for 24 h were lysed in a hypotonic buffer followed by a 100,000 × g centrifugation step. Equal amounts of the supernatant and pellet fractions were subjected to SDS-PAGE. Proteins were detected by Western blot analysis using specific Abs or antisera. c and d, L929 cells were induced with 200 U/ml IFN-γ or left untreated for 24 h, fixed with 3% paraformaldehyde, and stained with the anti-LRG-47 A19 antibody. Bound A19 was detected with a donkey anti-goat-Alexa 546 anti-serum. In IFN-γ-induced cells, an adnuclear signal and a diffuse granular background throughout the cell fainting at the periphery was detected. No signal was detected in noninduced cells.
involved. Apparently, LRG-47 is recruited very early during the phagocytic process, because LRG-47 could be detected at sites where beads were attached to the cell surface before formation of the phagocytic cup (Fig. 3, a–e).

IIGP1 is an ER-associated protein in fibroblasts, hepatocytes, and macrophages

IIGP1 has been reported to colocalize with Golgi and to a lesser extent with ER in mouse bone marrow-derived macrophages (15), but the mechanism and dynamics of its membrane association are unknown. In contrast to LRG-47, IIGP1 partitions between the cytosol and membrane compartments (Fig. 1b). We analyzed the subcellular localization of membrane-bound IIGP1 in IFN-γ-treated L929, TIB-75, and RAW 264.7 cells, and fresh peritoneal macrophages. IIGP1 was detected in a reticular pattern throughout IFN-γ-induced cells, growing fainter at the periphery (Fig. 5, a, d, g, and j). No signal was seen in resting cells. This pattern does not correspond with the proposed Golgi localization previously reported for IIGP1 (15), but rather overlaps with ER proteins as shown for TAP (23) and calnexin in peritoneal macrophages and ERP60 in TIB-75 cells (Fig. 5, a–i). The most accurate colocalization with IIGP1 was observed for TAP. We observed only insignificant colocalization with the Golgi protein, giantin, in L929 cells and primary cultures of peritoneal macrophages (not shown). Marginal, possibly significant colocalization with giantin was seen in RAW 264.7 macrophages (Fig. 5, j–m), although in this cell line, too, the vast majority of IIGP1 localized in an ER-like reticular pattern.
Therefore, we conclude that, at least in the cell types studied here, IIGP1 is predominantly associated with the ER.

Golgi and ER association of LRG-47 and IIGP1 are independent of GTP binding: plasma membrane binding of LRG-47 requires bound nucleotide

To examine the nucleotide dependence of membrane binding by LRG-47, a mutation analogous to that previously introduced into IGTP (14) was generated, changing the sequence in the GI motif from GX₄GMS to GX₄GMN. Both N-and C-terminal tags cause mislocalization of LRG-47 to transferrin-positive recycling endosomes and other membranes of unknown identity (S. Martens and J. C. Howard, unpublished results). Therefore, we had to express untagged LRG-47 in a noninduced background. For this purpose, wild-type and mutated forms of LRG-47 were expressed in resting L929 cells and detected with A19 by indirect immunofluorescence. Wild-type LRG-47 localized to an adnuclear region in a pattern indistinguishable from the induced, endogenously expressed LRG-47 (compare Figs. 6, a and b, and 2) colocalizing with the Golgi proteins giantin and Gm130 (24) (not shown). Apparently, positioning of LRG-47 in the cell is independent of other IFN-γ-induced factors at physiological expression levels. When overexpressed at levels higher than those induced by IFN-γ, however, LRG-47 translocated completely from endogenous membranes to the plasma membrane leaving the Golgi region dark (Fig. 6, c and d). The Golgi apparatus remains intact in these cells (Fig. 6, e and f). When LRG-47 S90N was expressed in L929 cells, a staining pattern similar to the wild-type protein expressed at normal levels was observed (compare Fig. 6, g and h, with a and b), although the reticular stain throughout the cell was noticeably increased. The adnuclear signal colocalized with Gm130 and giantin (not shown). However, unlike the wild-type case, no localization to plasma membrane was seen even at the highest expression levels of the mutant sequence. Therefore, we conclude that Golgi and ER membrane association of LRG-47 is essentially independent of GTP binding, but plasma membrane association is nucleotide dependent.

**FIGURE 3.** LRG-47 is recruited to phagocytic cups and remains associated with the maturing phagosome in macrophages. CB20-derived peritoneal macrophages (a–e) or RAW 264.7 cells were induced with 200 U/ml IFN-γ for 48 h, incubated with 2-μm latex beads for 1 h, fixed, and stained. LRG-47 is recruited very early during phagocytosis. Close proximity of F-actin and LRG-47 could already be observed on not completely developed phagocytic cups (c–e, middle arrow). Colocalization of LRG-47 and LAMP-1 was observed in peritoneal macrophages (not shown) and RAW 264.7 cells (f–j). In RAW 264.7 cells, the reticular distribution throughout the cytoplasm is more pronounced than in fibroblasts, hepatocytes, and peritoneal macrophages. Nuclei were labeled with 4′,6′-diamidino-2-phenylindole.
When transfected into resting L929 cells, IIGP1 expressed at low levels was localized in a reticular, ER-like pattern resembling the endogenous protein (not shown). However, cells expressing higher levels tended to contain IIGP1 aggregates, often of filamentous appearance, preferentially accumulating in the adnuclear region (Fig. 6, i and j). Aggregates have not been observed in any IFN-γ/H9253-induced cell line tested. Because IIGP1 forms GTP-dependent multimers in vitro (17), we asked whether the formation of the observed accumulations requires GTP-binding activity. A nucleotide-binding-defective IIGP1, with the G1 motif mutated from GX4GKS to VX4VKS (17), was expressed in L929 cells. Transfected IIGP1 G76, 81V was associated with intracellular membranes but showed no aggregate formation regardless of expression level (Fig. 6, k and l). The C terminus of IIGP1 has been reported to contribute to the regulation of oligomerization of IIGP1 in vitro (17). Using the recently determined crystal structure of IIGP1 (33), a deletion mutant of IIGP1 was generated, lacking the whole C-terminal domain following the Fα helix (αF) (see Fig. 9a). In L929 cells, the truncated mutant IIGP1 aa 1–287 still bound to intracellular membranes (Fig. 6, m and n), although the pattern of localization was distinct from endogenous protein with a marked accumulation on adnuclear endomembranes. Like the G76, 81V mutant, no aggregates were observed regardless of expression level. Thus, the C-terminal domain of IIGP1 is essential for the formation of the aggregated structures seen in transfected cells and contributes in some way to the specificity of membrane association but is not required for membrane binding as such.

IIGP1 is associated with the ER via N-terminal myristoylation

It is not known how the p47 GTPases bind to membranes. Of the published p47 GTPases, only IIGP1 carries a predictable lipid modification motif, namely an N-terminal myristoylation site (within the N-terminal sequence MGQLFS, glycine 2 is predicted to be myristoylated (25)). IFN-induced L929 cells were lysed in Triton X-114, and the p47 GTPases IIGP1 and LRG-47 were assayed in the aqueous and detergent phases after temperature shift (Fig. 7a). Consistent with lipid modification, ~50% of the IIGP1
protein was found in the detergent phase despite the lack of well-marked hydrophobic regions (6). In agreement with the lack of any predicted lipid modification site, LRG-47 was found exclusively in the aqueous phase (Fig. 7a), as were IGTP, TGTP, and IRG-47 (not shown). To determine whether the predicted N-terminal myristoylation site of IIGP1 is indeed used, we mutated glycine 2 to alanine (G2A) and expressed the mutated protein in L929 cells with wild-type IIGP1 as a control. IIGP1 G2A was found exclusively in the Triton X-114 aqueous phase (Fig. 7a). Transfected wild-type IIGP1 partitioned in Triton X-114 like the endogenous, IFN-γ/h9253-induced, protein.

To determine whether the N-terminal domain of IIGP1 is sufficient to target a soluble protein to membranes, we fused the N-terminal 68 aa, representing the N-terminal helical domain of IIGP1 including αC, to the N terminus of enhanced GFP (EGFP), expressed the construct in L929 cells, and lysed the cells in a hypotonic buffer followed by ultracentrifugation. Partitioning into the pellet and supernatant was monitored by Western blotting (Fig. 7b). EGFP alone was found exclusively in the supernatant. The IIGP1 aa 1–68 EGFP construct, in contrast, partitioned between the supernatant and the pellet. The G2A mutant N terminus of IIGP1 fused to EGFP was exclusively found in the supernatant, suggesting that the N terminus of IIGP1 does not carry any myristoylation-independent membrane localization signal. These results were confirmed by immunofluorescence (not shown). The N terminus is therefore sufficient for membrane association in a myristoylation-dependent manner. However, the myristoylated N terminus is not absolutely necessary for membrane association of IIGP1 itself, in contrast to the N-terminal EGFP construct, because the transfected full-length IIGP1 G2A mutant bound to membrane

**FIGURE 5.** IIGP1 is associated with the ER in L929 cells, TIB-75 cells, and peritoneal macrophages, and shows only minor Golgi association in RAW 264.7 macrophages. L929 cells (a–c), peritoneal macrophages (d–f), and TIB-75 (g–i) or RAW 264.7 cells were stimulated with 200 U/ml IFN-γ for 24 h, fixed, and stained for the indicated proteins by indirect immunofluorescence. IIGP1 accurately colocalizes with TAP (a–c). Colocalization was also observed with Calnexin in peritoneal macrophages (d–f) and ERP60 in TIB-75 cells (g–i), although IIGP1 regularly showed a more extended expression pattern than classical ER proteins, indicating that IIGP1 might not be restricted to subcompartments of the ER. j–m, Shown is an IFN-γ-induced RAW 264.7 macrophage. The cell was stained with anti-IIGP1 165 antiserum and anti-giantin mAb. Sites of possible colocalization of IIGP1 and giantin are marked with arrows. Note that the majority of IIGP1 does not colocalize with giantin. d–f and j–l, Planes of 3D deconvoluted z-series are shown. Nuclei were labeled with 4′,6′-diamidino-2-phenylindole.
Nonmyristoylated IIGP1 is a peripheral membrane protein

To determine the biochemical nature of the membrane association of LRG-47 and IIGP1, we conducted a series of wash-off experiments (Fig. 8a). Isolated membranes were incubated in a variety of different buffers, and partitioning of the p47 GTPases into the supernatant and pellet was determined by immunoblotting. LRG-47 and IGTP (not shown) behaved essentially like integral membrane proteins such as Calnexin even in 100 mM sodium carbonate (pH 11.75). In contrast, IIGP1 and TGTP (not shown) were weakly associated with membranes. The strength of membrane association thus correlates with the classification into the GMS and GKS subgroups. The small amount of IIGP1 and TGTP in the Triton X-100-insoluble fraction might represent aggregated protein or possibly lipid raft- or caveolae-associated protein. Consistent with the latter possibility, we observed some colocalization of IIGP1 with Caveolin-1 in TIB-75 hepatocytes (not shown). The wash-off profile of IIGP1, and in particular the pronounced shift into the supernatant for the sodium carbonate extraction, suggest that hydrostatic and ionic interactions dominate the binding of IIGP1 to membranes. Therefore, we subjected membranes of L929 cells transfected with the nonmyristoylated IIGP1 G2A mutant to the same membrane extraction assay (Fig. 8a). As control, the wash-off profile of wild-type IIGP1 expressed in resting L929 cells was determined. Although the proportion of membrane-bound IIGP1 was significantly higher than in IFN-γ-induced cells, the wash-off profile itself was not changed. Only a minor part of transfected IIGP1 was Triton X-100 insoluble, indicating that the aggregates observed in transfected L929 cells expressing high amounts of IIGP1 (Fig. 6, i and j) are membrane-associated structures. Despite the pronounced shift into the hypotonic supernatant noted above, the residual membrane-bound IIGP1 G2A showed the same wash-off profile as the transfected wild-type protein (Fig. 8a). This suggests that the myristoyl group regulates access to membrane rather than conferring strong membrane binding. Nevertheless, the N terminus of IIGP1 is able to target EGFP to membranes in a myristoyl-dependent manner (Fig. 7b).

The binding of IIGP1 to membranes independent of lipid modification might be mediated by protein-protein interactions or by a direct interaction with lipids. To distinguish between these.
alternatives, we analyzed the lipid-binding properties of purified, bacterially expressed IIGP1 (17) in vitro. The purified protein has a nonhydrophobic N-terminal extension of 10 aa derived from the GST-fusion (17) and is not myristoylated. When incubated with artificial unilamellar 100-nm phosphatidylserine vesicles, rIIGP1 efficiently cosedimented with the lipids (Fig. 8b). The wash-off profile of this protein is very similar to that of the in vivo-expressed protein (Fig. 8c). This result strongly suggests that IIGP1 has an intrinsic ability to interact with lipids, and that this interaction is independent of other proteins. A variable quantity of Triton X-100-resistant, presumably denatured, aggregated, protein was seen with rIIGP1 incubated with artificial phosphatidylserine vesicles (Fig. 8c).

Individual domains contribute independently to the targeting of the p47 GTPases to membranes

To guide a systematic analysis of the membrane-targeting signals of IIGP1 and LRG-47, we exploited the recently determined structure of IIGP1 (33). The linear organization of the p47 GTPases is shown in Fig. 9a. Helices in the N- and C-terminal parts are shown as lines. Secondary structure elements in the G domain are not
shown (33). The numbers refer to the amino acid positions in the primary structure of LRG-47 and IIGP1, respectively. As shown in Fig. 7, the N-terminal domain does not account fully for the membrane-binding properties of IIGP1 and is not responsible for membrane association of LRG-47. To localize further membrane-targeting regions of the two GTPases, the isolated G domains, the G domains with the C-terminal eL, oF helical extensions (Fig. 9a), as well as the C-terminal domains of IIGP1 and LRG-47, were expressed in L929 cells. The first two fragments were tagged with the FLAG epitope tag at the C terminus, whereas the last was fused in frame to the C terminus of EGFP. Transiently transfected L929 cells were fixed 24–40 h after transfection and stained with the indicated Abs. Strikingly, the isolated G domain of IIGP1 showed exclusive plasma membrane association (Fig. 9i). The isolated G domain of LRG-47 was not expressed in L929 cells. Therefore, we expressed a construct encoding the G domain of LRG-47 extended at the C terminus to include the aF helix. This construct also showed exclusive plasma membrane binding (Fig. 9m) indistinguishable from the isolated G domain of IIGP1 (l). No internal membranes were stained. This localization recalls the plasma membrane recruitment of LRG-47 upon phagocytosis of latex beads (Figs. 3 and 4) and possibly implicates the G domain in redirecting LRG-47 to the plasma membrane. The plasma membrane staining is particularly marked at plasma membrane ruffles for both LRG-47 and IIGP1, again similar to the observed recruitment of LRG-47 to membrane ruffles in L929 cells engaged in phagocytosis.

An amphipathic helix near the C terminus is responsible for Golgi targeting of LRG-47

Although the plasma membrane association of the G domains is intriguing, it does not reflect the steady-state localization of the p47 GTPases observed in IFN-γ-induced cells. A good candidate for the distinctive Golgi membrane targeting of LRG-47 is the C-terminal domain, which is a highly divergent region of the p47 GTPases (6). The C-terminal domains of IIGP1 and LRG-1 and LRG-47 were fused to the C terminus of EGFP and expressed in L929 cells. For LRG-47, the C terminus was fully active in targeting EGFP to the Golgi apparatus (Fig. 9, d–f). In addition, however, we observed a marked, diffuse, reticular stain throughout the cell similar to LRG-47 S90N (compare with Fig. 6g). However, the C terminus of IIGP1 does not confer membrane association by itself, because the EGFP fusion construct appeared fully soluble (Fig. 9k). Thus, LRG-47 and IIGP1 use different domains to bind to membranes. To identify the Golgi membrane-targeting motif in the C-terminal domain of LRG-47, we made a series of N-terminal truncation mutants of the C-terminal domain (Fig. 9b and not shown). These were fused to the C terminus of EGFP and expressed in L929 cells. All LRG-47 constructs containing the region corresponding to the last two helices, aK-L, of IIGP1 showed membrane association and Golgi localization (not shown). Further truncating aK and the loop preceding eL led to complete loss of membrane association (not shown). The deleted region between the last two mutations represents 25 aa and includes an α helix in IIGP1 that is also predicted in LRG-47 2 (αK (Fig. 9, a and b)) (33). These 25 aa of LRG-47 (Fig. 9b) were fused to the C terminus of GFP and expressed in L929 cells. Apparently, this small fragment was fully competent to target GFP to Golgi (Fig. 9, g–i). In LRG-47 (but not in IIGP1), this putative helix (aa 356–369) has an amphipathic character when drawn as a helical wheel (Fig. 9c). Therefore, we inserted glutamate residues at several positions that are predicted to destroy the amphipathicity of helix K (Fig. 9c). When the αK fragment with the insertions (ins 362, 367E) was expressed in L929 cells, membrane binding and Golgi association were completely abolished (Fig. 9j). The same result was obtained for the whole C-terminal domain for which all three mutations shown (Fig. 9b) completely abolished membrane association (not shown).

To examine the effect of the αK mutations on the distribution of the whole molecule, full-length LRG-47 carrying these mutations was expressed in resting L929 cells. Unlike the isolated C-terminal fragment containing these mutations, the full-length LRG-47 protein was still membrane bound. However, in contrast to wild-type LRG-47 (Fig. 9n), none of the αK mutants accumulated on Golgi membranes (o). Thus, LRG-47 is targeted to Golgi membrane by an amphipathic helix located in the C-terminal region at a position corresponding to αK in the structure of IIGP1. In the crystal structure of IIGP1, the αK helix is positioned on the same surface as the N terminus carrying the myristoylation motif. If this location is conserved in LRG-47, the orientation of the two GTPases toward the membrane would be similar despite the different membrane attachment mechanisms.

Discussion

The p47 GTPases are critically important molecules in the mouse for resistance against intracellular pathogens, but very little is known about how they work. One member of the family, LRG-47, mediates resistance against L. monocytogenes (2), T. gondii (2), M. tuberculosis (4), and M. avium (5). Phagocytosis is a common mode of entry for pathogens of radically different biological properties. However, acidification of the phagosomal vacuole and the activation of lysosomal hydrolases usually makes the compartment uninhabitable soon after particle uptake. Therefore, many intracellular pathogens that are taken up by phagocytosis try to delay or avoid acidification and the targeting of the phagosome to lysosomes (26). The finding that LRG-47-deficient macrophages showed a measurable defect in acidification of Mycobacterium-containing phagosomes (4) suggested that LRG-47 might counteract such survival strategies by marking the phagosome for rapid maturation, as proposed for the IFN-inducible Rab GTPase, Rab5a, which confers resistance to L. monocytogenes (27). However, in this study, we show that LRG-47 in the IFN-induced cell is not normally a protein of the endolysosomal compartment. Rather, it localizes mainly to the cis-Golgi, with weak expression on the ER (Fig. 2). Recruitment of LRG-47 to regions of actin polymerization at the plasma membrane, including phagocytic cups, is apparently initiated by phagocytosis, and the protein then remains associated with phagosomes as they mature to the lysosomal compartment (Figs. 3 and 4). The transfer of LRG-47 to sites of phagocytosis occurs within minutes of addition of latex beads. There is no detectable cytosolic pool of LRG-47 (Fig. 1). Therefore, the protein either must be rapidly and transiently released from Golgi (or possibly ER) membranes before reassociating with the plasma membrane, or else the recolocalization is achieved by induced fusion of Golgi (or possibly ER) membrane with active regions of plasma membrane (28).

The relocation of LRG-47 from Golgi to actin-rich plasma membrane is not restricted to professional phagocytes; LRG-47 behaved in essentially the same way in fibroblasts and macrophages (Figs. 3 and 4). Moreover pathogen-specific signals are clearly not required to induce LRG-47 recolocalization, because in our experiments phagocytosis was initiated with uncoated (macrophages and RAW264.7 cells) and collagen-coated (L929 cells) latex beads. Instead, processes associated with actin remodeling, rather than phagocytosis as such, are more plausible triggers. Consistent with this suggestion, LRG-47 was also localized at actin-rich plasma membrane ruffles adjacent to the forming phagosomes in L929 cells, as well as at phagocytic cups themselves (Figs. 3 and 4). However, it will be important to determine the recruitment of LRG-47 and the other p47 GTPases to plasma membrane and.
phagosomes containing pathogens, because this process is significantly more complex than the uptake of latex beads (29).

The mechanism of LRG-47 recruitment from Golgi or ER to plasma membrane by phagocytosis is still unknown. However, the recent report of selective activation of Ha-Ras on Golgi membranes by signals emanating from plasma membrane provides an interesting precedent (30). There is unfortunately no information about regulated nucleotide exchange in p47 GTPases and no clear definition of what activation of these GTPases really means (17). Nevertheless, our present data suggest that regulated nucleotide binding may be relevant to the positioning of LRG-47 in the cell. Overexpression unexpectedly caused transfected wild-type LRG-47 to relocate to the plasma membrane and particularly to membrane ruffles, completely excluding Golgi membranes (Fig. 6, c and d). Interestingly, however, this behavior was dependent on the integrity of the nucleotide binding site (Fig. 6, g and h). Therefore, it is not implausible that phagocytosis-stimulated repositioning of LRG-47 caused by a signal initiated at the membrane may also entail a nucleotide-dependent modification of the GTPase.

The Golgi localization of LRG-47 is mediated by a C-terminal amphipathic helix (Fig. 9). When this is disrupted, transfected LRG-47 is localized on internal membranes but not on the plasma membrane (Fig. 9, n and o). Thus, plasma membrane localization is not a simple default state. However, the isolated G domain of LRG-47 stripped of the N- and C-terminal helical domains localizes constitutively to the plasma membrane. In the IIGP1 structure, the wild-type C terminus region interacts with the G domain (33) and is required for accelerated GTP hydrolysis (17). Loss of the C-terminal domain of LRG-47 may thus delay GTP hydrolysis in vivo, causing the protein to behave like a constitutively active GTPase. If LRG-47 is released from internal membranes before transfer to actin-rich plasma membrane, then the interaction of at least one and possibly two amphipathic helices with the membrane must be disrupted. This behavior would then be reminiscent of the regulated exchange of Arf1 between cytosol and membranes, which also entails the GTP-dependent repositioning of an amphipathic helix (31, 32). The role of nucleotide in the membrane positioning of the isolated LRG-47 G domain is presently under direct investigation.

The propensity to bind to plasma membrane may be a general feature of the p47 GTPases and not just of LRG-47, because the phylogenetically rather distinct IIGP1, a member of the GKS subfamily of p47 GTPases, also has plasma membrane-targeting activity in the conserved G domain (Fig. 9l). However, in other respects, the differences between IIGP1 and LRG-47 are more conspicuous than the similarities. Thus, the data presented above establish IIGP1 to be an ER-associated GTPase in all cell types analyzed (Fig. 5), unlike LRG-47. In this respect, our results appear to differ from those of Zerrahn et al. (15), who reported predominantly Golgi localization of IIGP1 in bone marrow-derived macrophages. Native IIGP1 is targeted to membranes by N-terminal myristoylation (Fig. 7). The myristoylation signal is present only on LRG-47 among the published p47 GTPases, but of the 23 p47 GTPase genes present in the mouse genome, 9 are predicted to encode myristoylated proteins (C. Bekpen, J. Hunn, L. Guethlein, D. M. Dunn, E. Glowalla, I. Parvanova, and J. C. Howard, manuscript in preparation). Therefore, IIGP1 is probably representative of a large subfamily of p47 GTPases in the mouse, at least with respect to its membrane targeting. However, myristoylation is not the only membrane attachment mechanism in IIGP1. Full-length IIGP1 protein mutated at the myristoyl attachment glycine, G2, was still ∼40% membrane bound in transfected cells and distributed on endomembranes (Fig. 7). Because the isolated G domain targets the plasma membrane, not endomembranes, there must be a further site of interaction with endomembranes elsewhere in the molecule. Unlike LRG-47, however, the whole C-terminal region of IIGP1 lacked membrane-targeting activity when fused to EGFP. It is presently unclear where the residual membrane interaction

![FIGURE 9.](http://www.jimmunol.org/)
sites of IIGP1 are. Also, unlike LRG-47, IIGP1 has a substantial cytosolic pool in the IFN-induced cell. The results point to IIGP1 behaving as a classical peripheral membrane protein with relatively weak interactions with the membrane. This conclusion was fully confirmed by the relative ease with which IIGP1 could be released from the membrane fraction of disrupted cells. In contrast, LRG-47 behaved in this assay in all respects like a transmembrane protein, despite partitioning in the aqueous phase in Triton-X114 (Figs. 7 and 8).

The residual membrane-binding behavior of demyristoylated IIGP1 in vivo could be reproduced by purified bacterially extracted IIGP1 binding to synthetic phosphatidylserine lipid vesicles in vitro (Fig. 8, b and c). This result suggests that IIGP1 may also interact directly with membrane lipids in the cell, to some degree independently of the local protein environment. In this respect, IIGP1 is similar to LRG-47, where the strong interactions with membrane appear to be based not on protein-protein interactions, but on the C-terminal amphipathic helix and possibly a second similar interaction with lipid that we have not yet located. The ability of p47 GTPases to bind to membranes exclusively via lipids may have some adaptive importance, because intracellular pathogens such as Toxoplasma commonly modify the protein compositions of their compartments to avoid destructive host processes such as phagosome maturation. The diversity of intracellular behavior of the p47 GTPases is very striking. Among the small number studied here, the GKS group (IIGP1, TGTP, and IRG-47) have markedly lesser association with intracellular membranes than the GMS group (IGTP and LRG-47). Furthermore, they are associated with different intracellular compartments even within a group. Thus, LRG-47 is basically a protein of the cis-Golgi membrane, whereas IGTP is an ER membrane protein. Neither has a significant cytosolic pool. IRG-47 seems to be primarily a cytosolic protein, whereas TGTP and IIGP1 also have large cytosolic pools (Table I). As we have seen, LRG-47 and IIGP1 use radically different means of associating with membranes. It is already clear that different members of the family have different profiles of activity against specific pathogens (3). The biochemical differences studied in this work, which are obtained in strikingly divergent amino acid sequence, will surely prove to be critical parameters determining the nature and mechanism of the resistance functions exercised by the p47 GTPases. It is remarkable that the existence of these powerful mediators of cell-autonomous immunity was unknown for so long.

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