Interferon-γ Selectively Induces Rab5a Synthesis and Processing in Mononuclear Cells*

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Carmen Alvarez-Dominguez‡ and Philip D. Stahl§
From the Department of Cell Biology and Physiology,
Washington University School of Medicine,
St. Louis, Missouri 63110

Macrophage activation by interferon (IFN)-γ is characterized by enhanced phagocytosis and killing of internalized pathogens. We studied the effects of IFN-γ on Rab5a, a GTPase involved in both endocytosis and phagocytosis. IFN-γ induced the synthesis of Rab5a in mononuclear cells as detected by immunoprecipitation and by Western blotting. Rab5a messenger RNA levels were also increased. Elevated protein expression was detected as early as 6 h following IFN-γ and was maximal at 24 h. Following IFN-γ, membrane association of Rab5a:GTP was substantially increased. Rab5b and Rab5c, as well as Rab7 and Rab11, Rab GTPases localized in the endosomal-lysosomal pathway, were unaffected by IFN-γ. Moreover, Rab5a expression in non-macrophages was unaltered by IFN-γ. Rab5a is a prenylated protein, and newly synthesized Rab5a was rapidly processed following IFN-γ. However, elevated geranylgeranylation was not Rab5a-specific since all the Rab5 isoforms were more rapidly prenylated in vitro using cytosol from IFN-γ-treated cells. Last, guanine nucleotide exchange on Rab5a was elevated about 3-fold in the presence of cytosol from IFN-γ-treated cells. The selective effect of IFN-γ on Rab5a, synthesis, processing, and guanine nucleotide exchange suggests that Rab isoforms have closely associated but not identical functions and that selective enhancement of membrane trafficking may play a key role in intracellular killing.

Macrophages (MØs)§ occupy a central position in the immune system because of their phagocytic and antimicrobial capabilities. As a component of both acquired and innate pathways, MØs respond to lymphokine treatment by acquiring new functions. The activating effects of IFN-γ on macrophage function are well known (1) and include enhanced intracellular killing of pathogens via phagocytosis (2, 3). Similar to endocytosis, phagocytosis is regulated by the GTPase Rab5 (4–6). The Rab5 cycle is well described and involves Rab5 recruitment to membrane sites from a cytoplasmic pool where Rab5 is presumably bound to GDI. Prenylation via mono- and di-geranylgeranylation is essential for binding of Rab5 to intracellular membranes (7, 8). Rab5 is active in the GTP form, and membrane association is associated with GDP/GTP exchange via the Rab5 exchange factor (GEF) (4, 6, 9, 10). GTPase hydrolysis terminates its activity and is thought to permit release of Rab5 from the membrane. At least one Rab5 GTPase activating protein (GAP) has been described that potently acts on membrane-bound Rab5 to enhance GTP-hydrolysis (11). Rab5 is expressed as three isoforms, Rab5a, Rab5b, and Rab5c (12). Rab5 isoform messengers are ubiquitously expressed, and the three proteins have overlapping intracellular distributions. When overexpressed in living cells, all Rab5 isoforms activate endocytosis (12).

Here we report on a highly specific effect of IFN-γ on Rab5a synthesis, processing, and guanine nucleotide exchange. These findings have important implications in defining the boundaries between the endocytic and phagocytic pathways and on the mechanism of enhanced phagosome maturation following IFN-γ treatment.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Human monocyte-derived macrophages (HMDs) were prepared as described previously (13). J774E-clone, A431, L929, and A20 cells were cultured in RPMI, 5% fetal calf serum, 2 mM glutamine, 50 μg/ml gentamicin. The following antibodies were used: mouse monoclonal anti-Rab5a (4F11) (6, 12, 14); polyclonal rabbit anti-Rab5b and polyclonal rabbit anti-Rab5c, kindly provided by M. Zerial (EMBL, Heidelberg, Germany) (12); polyclonal rabbit anti-Rab7 antibody (purified IgG), a gift from A. Wandinger-Ness (Northwestern University, Evanston, IL); polyclonal rabbit anti-Rab11 antibody (purified IgG) was a gift from D. Sabatini (NYU, New York). Human and mouse recombinant IFN-γ (Genzyme) were used at 100–200 units/ml. Immunoprecipitation and Western Blots—Cells were lysed with PBS containing 1% Triton X-100, 2 mM EDTA, 10 mM α-mercaptoethanol, 10 mM sodium pyrophosphate, 1 mM Na3VO4, and 1 mM NaF followed by ultracentrifugation at 100,000 × g, for 45 min at 4 °C. For immunoprecipitation, lysates were precleared overnight at 4 °C by incubation with 20 μl of a 50% slurry of protein-A-Sepharose beads. The supernatants were then incubated with antibodies for 4–5 h at 4 °C, and at 1 h prior to washing, 20 μl of a 50% slurry of protein A-Sepharose was added. Beads were washed with lysis buffer, 0.5% sodium deoxycholate; lysis buffer, 0.5% sodium deoxycholate; 500 mM NaCl and 1/20 lysis buffer, 0.5% sodium deoxycholate. Elution was performed in 1× Laemmli sample buffer for 1 h at room temperature. For Western blots, 30 μg of total protein (measured by the BCA method, Pierce Chemicals, IL) was loaded per lane, separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Membranes were blocked in PBS, 5% nondairy milk for at least 2 h at room temperature. Primary antibodies were incubated overnight at 4 °C and washed with PBS, 0.05% Tween 20. Secondary antibodies were incubated for 45 min at room temperature using a 1:10,000 dilution.

Limiting Dilution RT-PCR—HMDs at 5 × 10^5/ml were treated with hIFN-γ (100 units/ml) for different periods of time (0, 1, 2, 8, and 18 h). Total RNA was isolated from the MØs by the phenol/guanidine thiocyanate procedure using TRI-Reagent (Molecular Research Center, OH). Complementary DNA was synthesized with a Perkin-Elmer kit using the protocol of the manufacturer. Briefly, 5 μg of total RNA in RNase-free autoclaved water was mixed with 3 μl of oligo(dT)15 primer, heated 15 min at 65 °C. After cooling on ice, the following reagents were added: 4 μl of 5× first strand buffer, 2 μl of 0.1 μM dithiothreitol (DTT), 1 μl of reverse transcriptase (20 units/ml), 1 μl of RNase inhibitor (20 units/ml), 2 μl of dNTPs (30 mM each). The mixture was incubated for 90 min at 37 °C, heat-inactivated for 5 min at 95 °C, and

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‡Recipient of a Postdoctoral Fellowship from the Formación de Personal Investigador, Ministerio de Educación y Ciencia, Madrid, Spain.
§ To whom reprint requests should be addressed. Tel.: 314-362-6950; Fax: 314-362-1490; E-mail: pstahl@cellbio.wustl.edu.

The abbreviations used are: MØ, macrophage; HMDs, human macrophages; BSA, bovine serum albumin; GGPP, geranylgeranlyphosphosphate; GST, glutathione S-transferase; IFN-γ, interferon γ; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase-polymerase chain reaction; dNTP, deoxynucleoside triphosphate; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; LM, Listeria monocytogenes; GEF, guanine exchange factor.
Fig. 1. IFN-γ selectively induces synthesis and post-translational processing of Rab5a. A, HMds were treated with hIFN-γ (150 units/ml) for 0, 2, 4, 6, 8, or 18 h. During the final 2 h, cells were metabolically labeled with 35S-translable (50 μCi/ml). Lysates were immunoprecipitated with 4F11 antibody (anti-Rab5a antibody) (1:300 dilution), and immunoprecipitates were run on SDS-PAGE. Arrows indicate the high molecular mass immature and low molecular mass mature forms, respectively. B, HMds (5 × 10⁵ cells/ml) were treated for different times with hIFN-γ (0, 1, 2, 8, and 18 h). Total RNA was extracted, and limiting dilution RT-PCR was performed as described under “Experimental Procedures.” Upper bands correspond to human Rab5a, Rab5b, or Rab5c and lower bands to β-actin. C, HMds were treated for different times with hIFN-γ (0, 2, or 4 h) in the presence (LOV) or absence (−) of lovastatin (10 μM). For the last 2 h, the cells were labeled with 35S-translable, immunoprecipitated with anti-Rab5a antibody, and analyzed by SDS-PAGE. D, GST-Rab5a, GST-Rab5b, or GST-Rab5c (10 μM) were incubated in 40 mM Hepes/KOH, pH 7, 5 mM MgCl₂, 0.5 mM Nonidet-P40, 1 mM DTT containing 12 μM [3H]GGPP and cytost (8 μM added to the reaction from a stock of 0.5 mg of protein/ml). Following incubation for 30 min (GST-Rab5a) or 60 min (GST-Rab5b and GST-Rab5c) with control cytost or with cytost obtained from HMds treated with IFN-γ (18 h, 150 units/ml), the reaction was stopped by adding a 4-fold excess of SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE, and dried gels were exposed to film for 4 days and then developed.

stored at −20 °C. Limiting dilution PCRs were performed as described previously (15). Briefly, cDNAs were diluted 5-fold in water before use. Primers used for PCR were prepared by the Washington University Core Facility: β-actin (β-actin-A, 5′-TGGATTGGTCTGAGGATCCGAG-3′ and β-actin B, 5′-TAAACGGCGAGTCATAAACGTCG-3′). Rab5a, 5′-ATGTTGCTGAGGATCCGAG-3′; Rab5b, 5′-TTATGTTA-CTACAAACTG-3′; Rab5c, 5′-ATGTTGCTGAGGATCCGAG-3′. Rab5a cDNA was amplified using the primers described previously (15). Briefly, cDNAs were diluted 5-fold in water before use. Primers used for PCR were prepared by the Washington University Core Facility: β-actin (β-actin-A, 5′-TGGATTGGTCTGAGGATCCGAG-3′ and β-actin B, 5′-TAAACGGCGAGTCATAAACGTCG-3′). Rab5a, 5′-ATGTTGCTGAGGATCCGAG-3′; Rab5b, 5′-TTATGTTA-CTACAAACTG-3′; Rab5c, 5′-ATGTTGCTGAGGATCCGAG-3′. Rab5a cDNA was amplified using the primers described previously (15).

Prenylation of Fusion Proteins—Prenylation was performed as described previously (4). 8 μg of GST-Rab5a, GST-Rab5b, or GST-Rab5c were incubated in 40 μl of 50 mM Hepes/KOH, pH 7, 5 mM MgCl₂, 0.5 mM Nonidet-P40, 1 mM DTT containing 12 μM [3H]GGPP for 30 min in the presence of control cytost or cytost obtained from HMds treated with IFN-γ (150 units/ml). Reaction was stopped by adding 4× SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE, and the dried gels were exposed to film for 4 days to quantitate incorporated radiolabel.

GDP/GTP Exchange—Protocol was performed as described (16). Briefly, 8 μg of GST-Rab5a was incubated with 10 μM [3H]GDP for 20 min at 37 °C in HBE-loading buffer (HBE containing 1 mM DTT, 100 mM NaCl, and 40 μg/ml BSA). A 10-fold excess of HBE-exchange buffer (HBE-loading buffer containing 1 mM GTP-15 mM MgCl₂) was added together with 5 μl of cytost (Sephadex G-25 filtered; 0.5 mg/ml) obtained from control macrophages or cytost from HMds treated with IFN-γ (18 h, 150 units/ml). GDP/GTP exchange was determined at RT by removing 5-μl aliquots at different times (0, 5, 15, and 30 min) and passing the samples through nitrocellulose filter discs coupled to a vacuum system. Filters were washed twice in ice-cold HBE-washing buffer (HBE containing 100 mM NaCl and 10 mM MgCl₂) and dried. Radioactivity was determined in a β-counter.

Fig. 2. IFN-γ selectively induces Rab5a in mononuclear cells. A, HMds were treated with hIFN-γ and metabolically labeled as in Fig. 1. Lysates were immunoprecipitated with rabbit polyclonal antibodies anti-Rab5b (lanes b, left) or anti-Rab5c (lanes c, right). B, HMds were treated for different times with hIFN-γ (150 units/ml). Cell lysates (30 μg) were separated by SDS-PAGE, transferred to NC membranes, and incubated with specific rabbit polyclonal antibodies against Rab7 or Rab11 (5) (1:300 dilution). Blots were developed by ECL. C, human epithelial cells (A431 cell line), mouse fibroblasts (L929 cell line), mouse B cells (A20 cell line), and mouse Mφ cell line (J774-E) were treated with IFN-γ for 18 h (150 units/ml) (+) or without IFN-γ (−). Cells were metabolically labeled as in Fig. 1A and immunoprecipitated with 4F11 (Rab5a, lanes a); rabbit anti-Rab5b (Rab5b, lanes b), or rabbit anti-Rab5c (Rab5c, lanes c). A representative gel is shown for the A431 cell line. The immunoprecipitated proteins were scanned, and the relative intensities of the bands were expressed as arbitrary units. Maximum levels correspond to 1.0.

Phagosome Isolation—Phagosomes were isolated from HMds with or without IFN-γ treatment (18 h, 150 units/ml), following phagocytosis of Listeria monocytogenes (LM), as described earlier with minor modifications (6, 17). After isolation, crude phagosomal fractions were loaded with IFN-γ for 18 h (150 units/ml) (+) or without IFN-γ (−). Cells were metabolically labeled as in Fig. 1A and immunoprecipitated with 4F11 (Rab5a, lanes a); rabbit anti-Rab5b (Rab5b, lanes b), or rabbit anti-Rab5c (Rab5c, lanes c). A representative gel is shown for the A431 cell line. The immunoprecipitated proteins were scanned, and the relative intensities of the bands were expressed as arbitrary units. Maximum levels correspond to 1.0.

Results and Discussion

IFN-γ induces the synthesis of a wide variety of proteins in mononuclear cells leading to activation and enhanced intracellular killing (1). Because Rab GTPases are essential for membrane trafficking events leading to phagosome lysosome fusion, we examined the effect of IFN-γ on intracellular levels of Rab5. Incubation of human macrophages (HMds) with IFN-γ (100–200 units/ml) increased both newly synthesized Rab5a protein and Rab5a mRNA levels (Fig. 1). Newly synthesized Rab5a (Fig. 1, panel A) was detected after metabolic labeling and immunoprecipitation with a Rab5a-specific monoclonal anti-
A

B

C

**IFN-γ Induces Rab5α Synthesis**

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**Fig. 3. Rab5α function is affected by IFN-γ.** Modulation of intracellular location and GDP/GTP exchange rate. A, HMds were treated for different times with hIFN-γ (150 units/ml) and metabolically labeled as in Fig. 1. Cells were homogenized in HBE buffer, centrifuged to remove nuclei and mitochondria. The post-nuclear supernatants were subjected to differential centrifugation by layering on an 8.5% sucrose cushion and spun at 100,000 × g for 60 min to obtain membranes (m) (pellets after centrifugation) and cytosolic fractions (c) (supernatants after centrifugation). Immunoprecipitation was performed with 4F11 antibody in the presence of detergent as described under “Experimental Procedures.” To quantify m/c ratios, radioactivity associated with proteins eluted from protein A-Sepharose beads was counted in a β-counter, and the m/c ratios were calculated for each condition. Actual data collected were as follows: at 0 h, (m) 1,500 ± 122 cpm and (c) 350 ± 63 cpm; 4 h, (m) 4,500 ± 128 cpm and (c) 510 ± 12 cpm; 6 h, (m) 6,700 ± 135 cpm and (c) 740 ± 31 cpm; 8 h, (m) 9,200 ± 181 cpm and (c) 1,150 ± 39 cpm. Results correspond to triplicates and the mean ± S.D. of three different experiments. B, HMds (5 × 10⁶ cells/ml) were labeled with 0.5 mCi [³²P]orthophosphate overnight in the presence or absence of IFN-γ (150 units/ml). Cells were infected with LM³⁻. Samples labeled as membranes correspond to total phagosomal membranes from isolated phagosomes (6, 17) (see “Experimental Procedures” for protocol) from cells treated with IFN-γ (+IFN) or nontreated (−IFN). Rab5α was immunoprecipitated on ice for 10 min using 4F11 antibody, and eluted nucleotides were separated by thin layer chromatography. GTP/GDP ratio was determined as described (21). A phosphomimager was used to determine GTP/GDP ratios, taking into account that the specific activity of [³²P]GDP is two-thirds that of [³²P]GTP. GTP/GDP ratios were as follows: IFN, 0.83; +IFN, 1.66 (actual GDP and GTP cpm were: −IFN, 302 ± 11 and 361 ± 24 cpm; +IFN, 1,590 ± 55 and 3,600 ± 130 cpm). Results correspond to duplicates, and statistics reflect the mean ± S.D. of three different experiments. C, GST-Rab5α (8 μg) was incubated with 10 μM [³H]GDP (11 mCi/μmol) for 20 min at 37 °C in HBE-loading buffer. GDP/GTP exchange was performed as described under “Experimental Procedures” in the presence of cytosol from control HMds or HMds treated with or without IFN-γ (18 h, 150 units/ml). Results correspond to radioactivity ([³H]GDP) bound to nitrocellulose filter disks at the times indicated following commencement of the assay. The data are expressed as the log of [³H]GDP bound to the filters at each time. Results correspond to the mean ± S.D. of three experiments performed in triplicate. Dotted lines represent the initial rates of GDP/GTP exchange for each set of data.
the Rab5α isoform. It is interesting to note that Rab5α appears to be a significantly better substrate for the induced prenyltransferase activity compared with Rab5β or Rab5c. It is possible that other Rab5α-specific factors are induced that allow Rab5α to be more rapidly prenylated. Rab7 and Rab11, GTPases acting downstream of Rab5 and localized in the endosomal-lysosomal pathway (18–20), were not induced by IFN-γ treatment as shown by Western blot assays of whole cell lysates (Fig. 2B). Interestingly, nonphagocytic cell types such as A431 human epithelial cells, fibroblasts (L929 mouse cell line), or B cells (mouse A20 cell line), which express IFN-γ receptors, were unaffected (Fig. 2C) (1). However, in macrophage-cell lines such as J774E clone, IFN-γ clearly induced the synthesis of Rab5α.

In Møs, the effects of IFN-γ are principally associated with clearance of intracellular pathogens (1, 3). Membrane trafficking pathways leading to phagosome-lysosome fusion would be obvious targets of IFN-γ action (2, 3). To delineate the effects of IFN-γ on Rab5α function, we examined the intracellular localization of Rab5α following IFN-γ treatment. Metabolically labeled HMds were homogenized in HEBE (250 mM sucrose, 0.5 mM EGTA, 20 mM Heps-KOH, pH 7.2) and fractionated by sedimentation. Rab5α was then immunoprecipitated from membrane and cytosol fractions. Fig. 3A shows that most of the newly synthesized Rab5α following IFN-γ induction is localized to the membrane fraction (m). Indeed, the membrane/cytosol (m/c) ratio of immunoprecipitated Rab5α after IFN-γ increased from four in untreated macrophages to nine in IFN-γ-treated cells. Increased membrane association may be because of accelerated geranylgeranylation of newly synthesized Rab5α as shown in Fig. 1D. Taken together these data demonstrate that IFN-γ specifically enhances Rab5α synthesis and processing (i.e. isoprenylation) in mononuclear cells, including translocation of newly synthesized Rab5α to intracellular membranes.

Similar to most low molecular weight GTPases, Rab5 is active in the GTP form (4, 6, 10). As a first approximation of the effects of IFN-γ on Rab5 function, we examined the guanine nucleotide status of Rab5α on phagosomal membranes. HMds were incubated overnight with [32P]orthophosphate. The cells were then pulsed for 10 min with L. monocytogenes, an intracellular pathogen efficiently internalized by Møs. Listeria containing phagosomes were isolated by differential sedimentation (6, 17). Rab5α was rapidly immunoprecipitated following solubilization of the phagosomal membranes. [32P]guanine nucleotides were released from the immunoprecipitates and were separated by thin layer chromatography to allow estimation of the GTP:GDP ratio. As shown in Fig. 3B, the GTP:GDP ratio of membrane-bound Rab5α increased by 2-fold in response to IFN-γ (from 0.83 to 1.66). We conclude that IFN-γ not only induces Rab5α expression and processing but also guanine nucleotide exchange or hydrolysis or both.

The GEFs that mediate exchange of GTP for GDP (9) and GAP that promotes the GTP hydrolysis (11) are two key activities that regulate the Rab5 GDP/GTP cycle. Membrane ruffling caused by guanine nucleotide exchange on Rho GTPases has recently been shown as the target of a factor produced by Salmonella typhimurium (16). To examine the effects of IFN-γ on guanine nucleotide exchange on Rab5α, we used a guanine nucleotide release assay employed by Hardt et al. (16). GST-Rab5α was preloaded with [3H]GDP by incubating the protein and [3H]GDP in the presence of low levels of Mg2+. Pre-loaded Rab5α was then incubated with cytosol from control cells and from cells pretreated with IFN-γ (18 h, 150 units/ml). Increased levels of Mg2+ and GTP were added, and the release of [3H]GDP was monitored. Analysis of the GDP/GTP exchange rate showed that the cytosol obtained from HMds treated with IFN-γ specifically increased the intrinsic GDP/GTP exchange rate of GST-Rab5α (Fig. 3C) by a factor of 3 compared with the rate observed in the presence of control cytosol. It is not clear from our studies how IFN-γ enhances nucleotide exchange on Rab5. Rabex-5 has been shown to possess Rab5 guanine nucleotide exchange activity. Enhanced prenylation activity is clearly not Rab5α-selective although Rab5α is a superior substrate for prenylation following IFN-γ treatment. Prenyltransferases and molecules that interact exclusively with Rab5 (e.g. GEF or GAP) may be responsible for the selective retention of Rab5α in the membrane fraction. We speculate that enhanced Rab5α levels and function accelerates the rate of phagosome maturation and intracellular killing that accompanies IFN-γ treatment.

The selective effect of IFN-γ on Rab5α suggests that each Rab5 isoform regulates a different function along the phagocytic-endocytic pathway. Subdomains of the endocytic-phagocytic pathway were first suggested by the discovery and localization of different Rab GTPases. Our study suggests that this concept can now be extended to a newer level of refinement. Moreover, the results presented in this study constitute the first report that small GTPase action can be modulated by lymphokine signaling.

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