Inactive and Active States of the Interferon-inducible Resistance GTPase, Irga6, in Vivo

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Irga6, a myristoylated, interferon-inducible member of the immunity-related GTPase family, contributes to disease resistance against Toxoplasma gondii in mice. Accumulation of Irga6 on the T. gondii parasitophorous vacuole membrane is associated with vesiculation and ultimately disruption of the vacuolar membrane in a process that requires an intact GTP-binding domain. The role of the GTP-binding domain of Irga6 in pathogen resistance is, however, unclear. We provide evidence that Irga6 in interferon-induced, uninfected cells is predominantly in a GDP-bound state that is maintained by other interferon-induced proteins. However, Irga6 that accumulates on the parasitophorous vacuole membrane after Toxoplasma infection is in the GTP-bound form. We demonstrate that a monoclonal antibody, 10D7, specifically detects GTP-bound Irga6, and we show that the formation of the 10D7 epitope follows from a GTP-dependent conformational transition of the N terminus of Irga6, anticipating an important role of the myristoyl group on Irga6 function in vivo.

The biological activity of GTPases depends on the control of the GTP binding and hydrolysis cycle. For several classes of GTPase, such as the Ras family of small GTPases, the G-proteins coupled to seven-transmembrane receptors, and the translation initiation and elongation factors, the molecular mechanisms by which the GTP cycle is controlled are known in considerable detail, and the biological contexts in which the activity cycles of the proteins function are well understood. All of these proteins function in cyclical processes in cells where the binding of GTP by a GTPase initiates a conformational change that drives a forward step in the cycle, whereas the hydrolysis of GTP to GDP terminates the forward activity and reverts the GTPase to its inactive ground state. There has been considerable recent interest in the function of several anomalous large GTPase families active in cell-autonomous immune resistance. However, no systematic study of the role of the GTP binding and hydrolysis cycle has been undertaken in order to define the functions of these proteins in biological processes associated with active pathogen resistance. In this paper, we have made some progress toward defining components of the activity cycle in vivo of Irga6, an interferon-inducible 47-kDa GTPase of the IRG family in mice.

IRG³ proteins are 47-kDa, interferon-inducible GTPases intimately involved in disease resistance against intracellular parasites in mice (1–12). Some biochemical parameters and the crystal structure of one member of the family, Irga6 (formerly IIGP1), have been determined (13). Irga6 possesses a Ras-like GTP-binding domain slung between two helical modules of unknown function. The N terminus carries a myristoylation signal (14) that is active in vivo (15). The nonmyristoylated protein purified from Escherichia coli has affinities in the micromolar range for GDP (~1 μM) and for GTP (~15 μM) and a low basal turnover rate from GTP to GDP of less than 0.1/min (16). Like many anomalous large GTPases, Irga6 shows cooperativity in specific turnover rate, with a maximum of ~2.0/min. Cooperativity of Irga6 is also reflected in a tendency to form oligomers in vitro upon the addition of GTP that resolve upon hydrolysis of the substrate (16).

The mode of action of IRG proteins in vivo is not well understood. After induction by interferons, IRG proteins including Irga6 are expressed at high levels in all cell types analyzed (17). Irga6 is ~60% associated with the ER membrane and 40% freely cytosolic (15). Within a few minutes after infection by the protozoan parasite, Toxoplasma gondii, cytoplasmic Irga6 accumulates on the membrane of the parasitophorous vacuole (PVM) (8). Within 1–2 h, the PVM becomes vesiculated and ultimately disrupts, the parasite is exposed to the cytosol, and parasite replication is interrupted (6, 8). This succession of events is blocked if an interferon-stimulated infected cell is also expressing what we have previously described as a “functionally dominant negative” mutant of Irga6, Irga6-K82A, demonstrating that the cell-autonomous resistance process is dependent on the function of IRG proteins and suggesting the importance of GTP binding and hydrolysis in the biological function (8).

Next to nothing is known about the role of the GTPase function of the IRG proteins in pathogen resistance. In an early
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experiment, evidence was presented that another IRG protein, Irgm3 (formerly IGTP), isolated by immunoprecipitation from interferon-stimulated cells, co-purified with bound GTP, and the authors suggested that, unusually for a GTase, Irgm3 exists in interferon-induced cells constitutively in the GTP-bound state (18). It has so far not proved possible to effect a biochemical purification of Irgm3, so the kinetic parameters of this member of the IRG family, either as a guanylate binding protein or as a GTase, are not known.

In this report, we have begun an analysis of the nucleotide-bound state of Irga6 in vivo in uninfected and infected cells. Our experiments strongly suggest that the resting state of Irga6 in the IFN-induced but uninfected cell is GDP-bound, whereas the Irga6 that accumulates on the PVM after T. gondii infection is in the GTP-bound state. Furthermore, our data suggest that the GDP-bound state of Irga6 in uninfected cells is actively maintained by a further interferon-inducible protein or proteins. We have shown elsewhere that this regulation is an active property of certain IRG proteins themselves (19). These results thus suggest that the resistance properties of Irga6 will prove to be properties of the GTP-bound, active state of the protein at the parasitophorous vacuole membrane. During the course of these studies, we demonstrated that a monoclonal antibody can detect the GTP-bound state of Irga6 with high specificity, and we show that the target epitope of this reagent is subject to conformational influences both from local structural elements and distantly from the nucleotide binding site.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—The pGW1H-Irga6cTag1 construct was generated by amplification of the Irga6cTag1 sequence from pGEX-4T-2-Irga6cTag1 (former pGEX-4T-2-IIGP-m) (16) by using Irga6cTag1 forward (5’-cccccccccgtcaggtgggcgtccagctttactctctctcaatgg-3’) and reverse (5’-cccccccccgtcaggtgggcgtccagctttactctctctcaatgg-3’) primers and cloned into pGW1H vector (British Biotech) by Sall digestion. Mutations were introduced into the coding region of pGW1H-Irga6wt (15), pGW1H-Irga6cTag1, and pGEX-4T2-Irga6wt (16) according to the QuikChange site-directed mutagenesis kit (Stratagene) using the following forward and corresponding reverse primers: G2A, 5’-gagtcgacccagccatcagctgctcttctctcaatgg-3’; Δ7–12, 5’-gagtcgacccagccatcagctgctcttctctcataatggcgc-3’; Δ7–25, 5’-ccacgtcgacccagccatcagctgctcttctctctcaatggg-3’; Δ20–25, 5’-gaaagatttgcctccagcagaaatattgagggagacgggatcagggaaga-3’; F20A, 5’-gaaagatttgcctccagcagaaatattgagggagacgggatcagggaaga-3’; T21A, 5’-gaaagatttgcctccagcagaaatattgagggagacgggatcagggaaga-3’; G22A, 5’-gctcctccagcagaaatattgagggagacgggatcagggaaga-3’; Y23A, 5’-gctcctccagcagaaatattgagggagacgggatcagggaaga-3’; F24A, 5’-gctcctccagcagaaatattgagggagacgggatcagggaaga-3’; K25A, 5’-gctcctccagcagaaatattgagggagacgggatcagggaaga-3’; K26A, 5’-gctcctccagcagaaatattgagggagacgggatcagggaaga-3’; S83N, 5’-gctcctccagcagaaatattgagggagacgggatcagggaaga-3’; E106A, 5’-gctcctccagcagaaatattgagggagacgggatcagggaaga-3’.

**Cell Culture and Serological Reagents**—L929 (CCL-1) and gs3T3 (Invitrogen) mouse fibroblasts were cultured in IMDM or Dulbecco’s modified Eagle’s medium (both Gibco) supplemented with 10% fetal calf serum (Biochrom). Hybridoma 10D7 and 10E7 cells were grown in IMDM, supplemented with 5% fetal calf serum. Cells were induced with 200 units/ml IFN-γ (Cell Concepts) for 24 h and transfected using FUGENE6 transfection reagent according to the manufacturer’s protocol (Roche Applied Science). Propagation of T. gondii strain ME49 was done as described previously (8). gs3T3 cells were infected for 2 h with T. gondii ME49 strain at a multiplicity of infection of 8–24 h after IFN-γ stimulation. The following serological reagents were used: anti-Irga6 mouse monoclonal antibodies 10D7 and 10E7, anti-Irga6 rabbit polyclonal serum 165, anti-cTag1 rabbit polyclonal serum 2600 (8), donkey-anti-mouse Alexa 546, donkey anti-rabbit Alexa 488 (all from Molecular Probes), goat anti-mouse κ light chain (Bethyl), goat anti-mouse κ light chain horseradish peroxidase (Bethyl), goat anti-mouse κ light chain-fluorescein isothiocyanate (Southern Bio-tech), 4’,6-diamidino-2-phenylindole (Roche Applied Science), and donkey anti-rabbit, donkey anti-goat, and goat anti-mouse horseradish peroxidase (all from Amersham Biosciences).

**Antibody Purification and Papain Digestion**—10D7 and 10E7 antibodies were purified from corresponding hybridoma supernatants over a Protein A-Sepharose column (Amersham Biosciences). Antibody was eluted with 50 mM sodium acetate, pH 3.5, 150 mM NaCl and pH-neutralized to 7.5 with 1 M Tris, pH 11. Buffer was exchanged five times subsequently by dilution of antibody-containing sample in papain buffer (75 mM phosphate buffer, pH 7.0, 75 mM NaCl, 2 mM EDTA) and concentrated in a centrifugal concentrator (Vivaspin20; Sartorius) with a 10 kDa cut-off filter at 2000 × g at 4 °C. The concentration of the antibodies was determined by using formula, concentration of antibody (mg/ml) = 0.8 × A280. Papain digestion was done according to Ref. 20. The papain-digested antibodies were further purified on a HiLoad 26/60 Superdex 75 preparation grade column (Amersham Biosciences) in papain buffer. Samples were incubated in SDS-PAGE sample buffer under nonreducing conditions and subjected to SDS-PAGE. Proteins were detected by colloidal Coomassie staining.

**Treatment with Aluminum Fluoride**—AlCl₃ (Sigma) was added to 10 ml of IMDM containing no fetal calf serum to a final concentration of 300 μM and mixed by vigorous shaking. Subsequently, NaF (Sigma) was added to a final concentration of 10 mM and mixed, and the final solution was applied to confluent L929 cells previously induced with IFN-γ or transfected for 24 h. Cells were incubated in aluminum fluoride complex (AlFx) solution for 30 min at 37 °C and then washed with cold PBS and collected by scraping. Cell pellets were lysed in 0.1% Thesit/PBS containing 300 μM AlCl₃ and 10 mM NaF in the presence or absence of 0.5 mM GTP for 1 h at 4 °C.

**Immunoprecipitation and Immunofluorescence**—Immunoprecipitation was modified from Ref. 21. 1 × 10⁶ L929 fibroblasts/sample were induced with IFN-γ and/or transfected for 24 h (or left untreated) and harvested by scraping. Cells were lysed in 0.1% Thesit, 3 mM MgCl₂, PBS, Complete Mini protease inhibitor mixture without EDTA (Roche Applied Science) for 1 h at 4 °C in the absence of nucleotide or in the presence of 0.5 mM GDP, GTP, GTPγS, or 300 μM AlCl₃ and 10 mM NaF in the presence or absence of 0.5 mM GTP (all from Sigma). Protein A-Sepharose™ CL-4B beads (Amersham Biosciences) were incubated with 10D7 monoclonal mouse anti-Irga6 antibody or 2600 (anti-cTag1) polyclonal rabbit serum for 1 h at 4 °C. Bound proteins were eluted by boiling for 10 min in elution buffer (100 mM...
Tris/HCl, pH 8.5, 0.5% SDS) with SDS-PAGE sample buffer (50 mm Tris/HCl, pH 6.1, 1% SDS, 5% glycerol, 0.0025% bromphenol blue (w/v), 0.7% β-mercaptoethanol). Immunofluorescence was performed as previously described (15).

**Colloidal Coomassie Staining**—Gels were washed 30 min with H}_2O and subsequently placed in incubation solution (17% ammonium sulfate (w/v), 20% MeOH, 2% phosphoric acid). After a 60-min incubation, solid Coomassie Brilliant Blue G-250 (Serva) was added to the solution to a concentration of 330 mg/500 ml and incubated 1–2 days. The gels were destained by incubation in 20% MeOH for 1 min and stored in 5% acetic acid. All was done at room temperature and while shaking.

**Expression and Purification of Irga6 Proteins from E. coli**—pGEX-4T-2-Irga6 constructs were transformed into BL-21 E. coli strain. Cells were grown at 37 °C to an A_600 of 0.8 when the expression of glutathione S-transferase-fused Irga6 proteins was induced by 0.1 mm isopropyl-β-D-thiogalactoside at 18 °C overnight. Cells were harvested (5000 × g, 15 min, 4 °C); resuspended in PBS, 2 mM DTT, Complete Mini protease inhibitor mixture without EDTA (Roche Applied Science) and lysed using a microfluidizer (Emulsiflex-C5; Avestin) at a pressure of 150 megapascals. The lysates were cleared by centrifugation at 50,000 × g for 60 min at 4 °C. The soluble fraction was purified on a glutathione-Sepharose affinity column (GSTrap) and aggregated targets. Following papain digestion, monovalent Fab fragments were unable to bind bivalently, they should bind equally, whether weakly or strongly, to distributed and aggregated targets. Following papain digestion, monovalent fragments were separated from residual bivalent material by size exclusion chromatography (Fig. 2, A and B, and supplemental Fig. 1). The binding activities of two fractions (B8 and B15, apparent molecular weights of 55,000 and 45,000, respectively) were compared by dilution in a Western blot against bivalently purified Irga6 protein (Fig. 2C). The titer of the later eluting fraction (B15) was higher than that of the earlier eluting fraction (B8), showing that the activity detected was not derived from trailing of residual bivalent material down the column. This result already indicated that monovalent 10D7 Fab fragment (fraction B15) has an affinity for Irga6 on a Western blot comparable with that of the native 10D7. It did not, however, prove that the monovalent affinity of 10D7 for Irga6 at the PVM is also high. We therefore examined the binding of Fab fragments from 10D7 to Irga6 accumulated on the T. gondii PVM in interferon-induced cells. The fragments were detected with a fluorescent conjugate of an anti-mouse κ light chain second stage reagent and the intensities of 10D7 Fab and the bivalent native 10D7 antibody signals examined at constant exposure times. Fig. 2D shows that strong signals were detected at the PVM from both 10D7 Fab and intact 10D7, and the exposure times required for equivalent signal strength were essentially identical. Thus, 10D7 binds with high affinity to Irga6 at the PVM because Irga6 in this site adopts a conformation distinct from that present in the distributed cytoplasmic pool. Subsequent experiments were dedicated to showing that the two distinct in vivo conformations represent GTP-bound and GDP-bound states, respectively.

**The Formation of Irga6 Oligomers in Vivo**—Since purified Irga6 forms enzymatically active oligomers in vitro in the presence of GTP (16), we were interested to find out whether such oligomers could be detected in the uninfected, interferon-induced cell. To this end, we transfected IFNγ-induced fibroblasts expressing wild-type Irga6 with an Irga6 construct modified at the C terminus with an 11-residue peptide tag, cTag1.
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FIGURE 2. 10D7 is a high affinity antibody. Papain-cleaved 10D7 antibody was separated on a Superdex 75 column. Fractions were subjected to SDS-PAGE on 7.5% gels under nonreducing conditions, and protein was detected by colloidal Coomassie staining (A) or Western blot (B) using goat anti-mouse k light chain and donkey anti-goat horseradish peroxidase antibodies as primary and secondary stage detection reagents, respectively. The apparent molecular weight of native 10D7 IgG on SDS-PAGE was dependent on gel conditions. In relatively short runs in 7.5% gels, 10D7 ran in a complex band pattern below 130 kDa (A and B). However, the same material run longer in a 10% gel behaved rather normally, reaching an average position at or even above 150 kDa (see supplemental Fig. 1). Papain-cleaved fragments were shown to have an apparent molecular mass of ~40 kDa in the 7.5% gel system (A and B). C, relative affinity of putative Fab fragments B8 and B15 of papain-cleaved 10D7 was estimated by binding to recombinant Irga6 fixed to the nitrocellulose membrane. 10 μg/ml antibodies was considered as 1:1 and further dilutions were made, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64. A similar dilution series of uncleaved native 10D7 was made as positive controls. Detection of 10D7 and 10D7 fragments was done as in B. Monovalent 10D7 Fab fragment (fraction B15) has an affinity for denatured Irga6 on a Western blot comparable with that of the native 10D7. D, g53T3 fibroblasts were induced with IFNγ 24 h followed by infection with T. gondii ME49 strain for 2 h. Irga6 was detected with 10 μg/ml of 10D7 antibody (10D7) or 10D7 Fab; as secondary detection reagent, goat anti-mouse k light chain-fluorescein isothiocyanate (αLkappa) was used. This secondary reagent detects papain-cleaved Fab and intact 10D7 with the same efficiency. The arrows indicate positions of T. gondii vacuoles. PC, phase-contrast images.

(see “Experimental Procedures”), which causes a detectable size shift in SDS-PAGE. Twenty-four hours after transfection and interferon induction, Irga6cTag1 was immunoprecipitated from detergent lysates of the cells, and the product was resolved on SDS-PAGE and analyzed by Western blot for Irga6 (Fig. 3A). In the absence of added nucleotides, no co-precipitated wild-type Irga6 could be detected (lane 1); thus, there is no stable Irga6–Irga6 association in IFNγ-induced cells. The addition of GDP or GTP to the lysate also failed to permit detectable co-precipitation of native Irga6 (lanes 2 and 3), but in the presence of the nonhydrolyzable GTP analog, GTPyS (lane 4), native Irga6 was strongly co-precipitated. These results suggested the possibility that Irga6cTag1 may indeed occur in cells in GTP-dependent oligomers with native Irga6 but that these rapidly hydrolyze GTP and dissociate again. However, the addition of nucleotides to the lysate risks the formation of artificial postlysison oligomerization, and the results may not reflect the situation in the cell before lysis. It was shown earlier that the addition of AlFx and GTP to bacterially purified Irga6 in vitro resulted in the formation of irreversibly locked oligomers (16). The affinity of Irga6 for GDP is not altered by the presence of AlFx, and the addition of GDP and AlFx to Irga6 does not cause oligomerization. Thus, the AlFx binding site is accessible only during the catalytic step immediately after cleavage of the γ-phosphate from GTP. AlFx is cell-permeant and therefore able to trap active Irga6 complexes in vivo. Although the addition of both AlFx and GTP during the immunoprecipitation procedure resulted in the co-precipitation of a significant amount of native Irga6 (lane 5), preincubation of the cells in AlFx before lysis failed to trap any mixed oligomers containing native Irga6 (lane 4). This result suggested, first, that no active, GTP-dependent oligomers are present in the uninfected, interferon-induced cells, and, second, that the complexes that were co-precipitated following the addition of nucleotides and AlFx to the lysate may have been formed postlysison. Proof that all of the mixed oligomers detected in Fig. 3A were indeed formed postlysison during the immunoprecipitation procedure itself was provided by the results of the experiment shown in Fig. 3C. Two separate cell populations were mixed at the time of lysis, one interferon-induced and the other transfected with Irga6cTag1. The mixed lysate was then immunoprecipitated for Irga6cTag1 in the presence of various nucleotides and AlFx (Fig. 3C, (i) + (t)) and compared with similar immunoprecipitates from the lysate of a single cell population that was both IFNγ-induced and transfected with Irga6cTag1 (Fig. 3C, (i) + (t)). Fig. 3C shows that the addition of GTPγS or GTP + AlFx (lanes 5 and 6) to the mixed lysates ((i) + (t)) resulted in strong co-precipitation of the native Irga6, quantitatively equivalent to the co-precipitates found in transfected and IFNγ-induced (i) + (t) cells. In summary, the results shown in Fig. 3, A and C, taken together provide strong evidence that Irga6 expressed in IFNγ-induced cells is not present in the form of GTP-dependent oligomers of any size, including dimers.

We have reported that Irga6 is substantially mislocalized when expressed in cells that have not been induced with IFNγ (15, 19). The mislocalization is seen as small cytoplasmic aggregates instead of the smooth ER-related localization seen in IFNγ-induced cells, and it can be prevented if the transfected

5 R. Uthaiah, unpublished results.
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**FIGURE 3.** In uninfected cells, Irga6 forms GTP-dependent oligomers only in the absence of IFNγ. L929 fibroblasts were simultaneously induced with IFNγ and transfected with Irga6cTag1 24 h before lysis (A), not induced with IFNγ but simultaneously transfected with both Irga6wt and Irga6cTag1 24 h before lysis (B), or either simultaneously (i + t) or separately (i) induced with IFNγ and transfected with Irga6cTag1 24 h before lysis (C). Irga6 was immunoprecipitated from lysates with rabbit anti-cTag1 serum. Cells were either preincubated with AlFx and subsequently lysed in the absence of nucleotides (lane 4) or without preincubation lysed in the presence of nucleotides with or without AlFx (lanes 2, 3, 5, and 6). Cells in lane 1 were immunoprecipitated without preincubation and without additives during the immunoprecipitation. Irga6 proteins in immunoprecipitates were detected with 10D7 antibody in a Western blot. A second band above wild-type Irga6cTag1 (marked with a single asterisk) is always found in Western blots of transfected Irga6; the nature of this presumed modification is unknown.

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**FIGURE 4.** In noninduced cells, 10D7 detects aggregated Irga6 as efficiently as 10E7 antibody. Untransfected gs3T3 fibroblasts were transfected with Irga6cTag1 and stained 24 h later with anti-cTag1 polyclonal serum (red) and with either 10E7 (A) or 10D7 (B) monoclonal antibodies (green). PC, phase-contrast images.

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cells are also induced with IFNγ (19). The possibility that these aggregates represented GTP-dependent oligomers was tested directly by co-immunoprecipitation from uninduced cells transfected simultaneously with both native Irga6 and Irga6cTag1 (Fig. 3B). In this case, unlike in IFNγ-induced cells (Fig. 3A), preincubation of the cells with AlFx (lane 4), before detergent lysis and in the absence of any exogenous nucleotide, allowed strong subsequent co-precipitation of wild-type Irga6 with Irga6cTag1. Since the AlFx binding site is inaccessible on GDP-bound Irga6, these results show that in the absence of other IFNγ-induced proteins intracellular Irga6 exists at least partially in hydrolytically active, GTP-containing oligomers, which can be stabilized by AlFx in the living cell. These experiments showed that Irga6 can exist in two alternative states in vivo, namely monodispersed hydrolytically inert in the IFN-

induced cell or assembled in GTP-dependent hydrolytically active oligomers in the transfected, uninduced cell. The Conformation-sensitive Monoclonal Antibody, 10D7, Binds Irga6 in the GTP-bound Form—Since 10D7 binds very inefficiently to the monodispersed, cytoplasmic form of Irga6 expressed in IFNγ-induced cells, it was of interest to find out whether the GTP-dependent Irga6 aggregates forming in transfected cells are recognized by 10D7. Figs. 4 and 5A show that 10D7 indeed binds as strongly to Irga6 aggregates in transfected cells as does the indiscriminate monoclonal antibody, 10E7 (Fig. 4), or the anti-cTag1-specific serum 2600 (Fig. 5A). Thus, the Irga6 conformation recognized by 10D7 is the GTP-dependent, hydrolytically active conformation, implying that Irga6 detected by 10D7 around the PVM in IFN-induced cells is itself in the GTP-bound state. The binding of 10D7 to transfected Irga6 was largely eliminated if transfected cells were simultaneously induced with IFNγ, especially in cells expressing relatively little of the transfected protein (Fig. 5B, arrow). This result is consistent with other evidence that the presence of other IFNγ-induced IRG proteins normally maintains Irga6 in the inactive state (19).

That 10D7 binds specifically to the GTP-bound, active state of Irga6 in vivo was further supported by the behavior of two nucleotide-binding site mutants of Irga6, the “functionally dominant negative” P-loop mutant, Irga6-K82A, and a further mutant that has lost affinity for both GDP and GTP, Irga6-S83N (19). We have shown elsewhere that transfected Irga6-K82A is functionally dominant negative in vivo, co-localizing in smaller or larger cytoplasmic aggregates with IFNγ-induced wild-type Irga6 and inhibiting the accumulation of the latter on the PVM of infecting Toxoplasma (8, 19). We have further shown that, although behaving functionally as a dominant negative, Irga6-K82A binds GTP with wild-type affinity but fails to hydrolyze it (19). Consistent with the suggestion that 10D7 binds to the GTP-bound active form of Irga6, 10D7 also bound strongly to Irga6 in cells transfected with Irga6-K82A, whether they were IFNγ-induced (Fig. 5C) or not (Fig. 5D), while binding weakly to cells transfected with Irga6-S83N, again independent of simultaneous IFNγ induction (Fig. 5, E and F).

The Nature of the 10D7 Epitope and Conditions for Its Formation—The 10D7 epitope of Irga6 was constitutively exposed on Western blots (Fig. 2C), suggesting that it is a linear epitope expressed by denatured protein. We further observed that the 10D7 epitope was also constitutively accessible in cells expressing the N-terminal 69 residues of Irga6 tagged at the C terminus with green fluorescent protein (data not shown). Thus, expression of the epitope is not dependent on the pres-
structure (13). This α-helix is partly exposed to solvent in the structure and partly masked by contacts to Helix B, Helix C, and Helix F. Phe<sup>20</sup>, Tyr<sup>23</sup>, and Phe<sup>24</sup> are buried in the interhelical space and not exposed on the solvent-accessible surface (Fig. 6C). In general, antibody epitopes on proteins are concentrated on loops and folds with a high degree of solvent exposure and are less common on regions of defined secondary structure (23, 24). Antibodies recognizing helical epitopes interact only with side chains on the exposed surface of the helix (25, 26). Taken together, therefore, the data suggest that the structure of the 10D7 epitope is not accurately reflected by Helix A of the crystal structure and that under conditions in which 10D7 binds to Irga6, Helix A is restructured into a solvent-exposed loop on which the linear epitope at residues 20–25 is accessible.

The expression of the 10D7 epitope in full-length Irga6 was further investigated by transfection of wild-type and certain mutant constructs into uninduced L929 fibroblasts followed by immunoprecipitation of Irga6 with 10D7 antibody in the presence and absence of GTPγS added to the detergent lysate and subsequent Western blot (Fig. 7). As predicted from the properties of 10D7 summarized above, immunoprecipitation of wild-type Irga6 from the lysate was absolutely dependent on the presence of GTPγS. The functionally dominant negative mutant Irga6-K82A, which can bind GTP with wild-type affinity but not hydrolyze it (19), and a second mutation, Irga6-
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FIGURE 7. 10D7 antibody binds to the GTP-bound form of native cellular Irga6 but not to a myristoylation-deficient mutant. L929 fibroblasts were transfected with Irga6wt, -G2A, -Δ7–12, -K82A, -S83N, or -E106A constructs. Cells were lysed 24 h later in Thesit in the absence or presence of 0.5 mM GTPγS, and Irga6 was immunoprecipitated with 10D7-Protein A-Sepharose beads. Irga6 proteins were detected in Western blot by rabbit anti-Irga6 polyclonal 165 serum. Signals were quantified using ImageQuant TLv2005, and values for immunoprecipitated proteins were normalized to the corresponding lysates. Mean values of at least three independent experiments are shown in the histogram. The 10D7 epitope is dependent on GTPγS in wild type Irga6 but constitutively expressed in functionally dominant negative mutants Irga6-K82A and -E106A. The myristoylation-deficient mutant, Irga6-G2A, cannot express the 10D7 epitope whether GTPγS is present or not. The mutant Irga6-Δ7–12 expresses 10D7 epitope independently of GTPγS.

E106A, which has similar properties, were both immunoprecipitated by 10D7 whether GTPγS was added to the lysate or not, consistent with the expectation that these mutant proteins are already at least partially reversibly GTP-bound at the time of lysis. Likewise, the nucleotide binding-deficient mutant Irga6-S83N (19) failed to bind significantly to 10D7 in absence or presence of GTPγS, consistent with the apparent GTP dependence of the epitope. Two mutants, however, failed to behave according to expectation in this assay. The mutant Irga6-Δ7–12 expressed the 10D7 epitope independently of GTPγS, whereas the mutant Irga6-G2A, which is mutated in the myristoyl attachment motif, failed to express the 10D7 epitope whether GTPγS was present or not. The behavior of these last two mutants suggested that expression of the 10D7 epitope is critically dependent on structural features at the N terminus that are not themselves part of the epitope and are also distant from the nucleotide binding site.

The apparently constitutive expression of the 10D7 epitope on the N-terminal deletion mutant, Irga6-Δ7–12, could suggest that this mutant, for unknown reasons, behaves like Irga6-K82A as a dominant negative, being constitutively GTP-bound in transfected cells. We investigated this possibility by co-immunoprecipitation from uninduced cells of transfected, untagged Irga6-Δ7–12 with transfected Irga6cTag1-Δ7–12. By this assay, Irga6cTag1-Δ7–12 (Fig. 8, lanes 3) behaved exactly like wild-type Irga6Ctag1 (lanes 1), showing complete GTPγS dependence for co-precipitation of the untagged protein and thus suggesting that the nucleotide-binding site of this mutant is not irreversibly occupied by GTP. In contrast, in the same assay, the two functionally dominant negative mutants Irga6cTag1-K82A and Irga6cTag1-E106A (lanes 4 and 6, respectively) co-precipitated their untagged equivalents weakly but constitutively, independently of GTPγS, consistent with the expectation that these proteins were already partially in irreversible GTP-dependent aggregates before cell lysis, whereas the GTP binding-deficient mutant, Irga6cTag1-S83N (lanes 5), as expected, failed to co-immunoprecipitate the untagged mutant whether GTPγS was present or not. The myristoylation-deficient mutant, Irga6cTag1-G2A (lanes 2) showed very weak GTPγS-dependent co-precipitation of the untagged mutant, confirming that absence of the myristoyl group has seriously disturbed, if not completely destroyed, the normal conformational behavior of the mutant.

In aggregate, the data strongly suggest that complex conformational changes occur in Irga6 as a consequence of GTP binding. The 10D7 epitope is exposed by GTP binding although itself distant from the nucleotide binding site. Since the epitope is partially buried in the known crystal structures of Irga6 (13) and looks inaccessible to antibody, it is likely that GTP binding results in restructuring of the Helix A, exposing the whole linear epitope probably with partial unfolding. Indeed, the 10D7 epitope is probably loosely structured, since it is well expressed on Western blots of any Irga6 protein or fragment that contains the intact 20–25 sequence of Helix A. This property is also consistent with the fact that the 10D7 monoclonal antibody was produced in mice immunized with bacterially expressed Irga6 in Freund’s complete adjuvant; the protein was probably largely denatured. In material isolated from cells, which is not intentionally denatured, the 10D7 epitope is constitutively exposed by loss of residues 7–12, which were not resolved in known crystal structures, suggesting that the presence of these residues is required to maintain Helix A in the “closed” configuration in the absence of GTP. Likewise, loss of the myristoyl moiety results in constitutive loss of the 10D7 epitope in material isolated from cells, hinting that a direct or indirect interaction between the myristoyl group and Helix A is required to enable the GTP-dependent conformational change in Helix A to occur in otherwise normally conformed molecules.
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DISCUSSION

In this study, we have provided evidence that Irga6 in interferon-induced cells must be predominantly GDP-bound (or possibly empty of nucleotide), whereas Irga6 that accumulates rapidly on the T. gondii parasitophorous vacuole in such cells is in the GTP-bound state. There are several grounds for believing that this change of state is accompanied by a significant conformational change in the Irga6 molecule. First, we present evidence that the target epitope of the anti-Irga6 monoclonal antibody, 10D7, is exposed on native wild-type Irga6 only when GTP, GTPyS, or GDP-AlFx is bound, and we show that it is strongly expressed on Irga6 associated with the T. gondii parasitophorous vacuole membrane (Fig. 1). Second, published in vitro experiments have shown that soluble monomeric Irga6 forms oligomers in the presence of GTP that can be stabilized by the presence of AlFx (16), showing that new interfaces are formed as a result of GTP binding and formation of the catalytic transition state that allow high affinity interaction between Irga6 monomers (Fig. 3). Finally, the binding of 10D7 to Irga6 molecules is influenced by at least two other elements of the protein for which unfortunately no structural information yet exists, namely amino acids 7–12 adjacent to the N terminus, which are not resolved in any crystal structure of Irga6 (13), and the myristoyl group, which was not present on the bacterially expressed protein from which the crystal structures were obtained. The individual absence of these two elements resulted in constitutive gain and constitutive loss, respectively, of the 10D7 epitope (Fig. 7).

On kinetic grounds, we should expect that cytoplasmic Irga6 will be predominantly in the GDP-bound form. The single-site equilibrium affinity constant of bacterially purified Irga6 for GDP is 15 times that for GTP (1 μM versus 15 μM) a difference accounted for entirely by the difference in off-rates (16), whereas the concentration of free cellular GTP is reported to be only 3-fold higher than that of GDP (330 μM versus 129 μM) (27). In addition, it is likely that the conformational change associated with GTP binding requires at least a dimerization of two GTP-bound Irga6 molecules (13), whereas the level of GTP-bound molecules will depend on the balance between the growth of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP. Not all of the parameters required to calculate the expected level of GTP-bound, activated oligomers and their loss through hydrolysis of the bound GTP.
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at the neck of clathrin-coated endocytic invaginations in the GTP-bound state and functions in vesicle scission by hydrolysis of GTP (32, 33), it is plausible that the effector function of Irga6 and other IRG proteins of the GKS group is fulfilled via GTP hydrolysis at the parasitophorous vacuole membrane, leading to the observed vesiculation of the membrane (6, 8) and ultimately to its rupture.

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