Mutant Rab24 GTPase is targeted to nuclear inclusions
William A Maltese*1, Gwendolyn Soule1, William Gunning2, Edward Calomeni2 and Brandy Alexander

Address: 1Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, OH 43614, USA and 2Department of Pathology, Medical College of Ohio, Toledo, OH 43614, USA
E-mail: William A Maltese* - wmaltese@mco.edu; Gwendolyn Soule - gsoule@mco.edu; William Gunning - wgunning@mco.edu; Edward Calomeni - ecalomeni@mco.edu; Brandy Alexander - balexander@mco.edu
*Corresponding author

Abstract

Background: Members of the Rab GTPase family regulate intracellular protein trafficking, but the specific function of Rab24 remains unknown. Several attributes distinguish this protein from other members of the Rab family, including a low intrinsic GTPase activity.

Results: The functions of other Rab proteins have been defined through the use of dominant-negative mutants with amino acid substitutions in the conserved N(T)KxD nucleotide binding motif. Surprisingly, when such Rab24 constructs were expressed in cultured cells, they accumulated in nuclear inclusions which disrupted the integrity of the nuclear envelope. The inclusions reacted positively with antibodies against ubiquitin and Hsp70, similar to protein aggregates observed in polyglutamine disorders. They also appeared to sequester importin-β and GFP-coupled glucocorticoid receptor. Other Rab GTPases with similar mutations in the N(T)KxD motif were never found in inclusions, suggesting that the unusual localization of Rab24 is not related solely to misfolding of its nucleotide-free form. Studies with Rab24/Rab1B chimeras indicated that targeting of the mutant protein to inclusions requires the unique C-terminal domain of Rab24.

Conclusion: These studies demonstrate that mutations in Rab24 can trigger a cytopathic cellular response involving accumulation of nuclear inclusions. If the N(T)KxD mutants of Rab24 function as dominant suppressors, these studies may point to a unique role for Rab24 in degradation of misfolded cellular proteins or trafficking of proteins to the nuclear envelope. However, we cannot yet eliminate the possibility that these phenomena are related to unusual non-physiological protein interactions with the mutant form of Rab24.

Background
Rab proteins comprise a large family of Ras-related GTPases that function in the anterograde and retrograde trafficking of proteins in mammalian cells [1–3]. Different Rab proteins reside in specific subcellular membranes or organelles, where they mediate vesicular transport between discrete donor and acceptor compartments in the endocytic or exocytic pathways [4,5]. Detailed studies of a few Rab proteins (e.g., Rab1, Rab5 and Rab9) have led to a general model wherein Rab proteins are presumed to cycle on and off donor and acceptor membranes in connection with changes in their guanine nucleotide state [6–8].
In the active GTP-bound state, Rab proteins associate with budding transport vesicles [9–11] and appear to participate in the assembly of membrane docking complexes [12–14]. Following vesicle fusion with the acceptor compartment and GTP hydrolysis, the inactive GDP-bound Rab is extracted from the membrane by a carrier protein termed guanine nucleotide dissociation inhibitor (GDI) [15–17]. The GTPase can then re-enter the transport cycle through nucleotide exchange promoted by a specific exchange factor at the donor vesicle membrane [18–20]. In addition to changes in guanine nucleotide state, the post-translational prenylation of Rab proteins plays an important role in the cycling mechanism. Non-modified Rabs have a low affinity for GDI [21,17] and do not associate effectively with membranes [22,23].

Rab24, originally identified by Olkkonen et al. [24], remains one of several members of the Rab family without a confirmed biological function. We have described several unusual features of Rab24 that distinguish it from all other members of the Rab family studied to date [25]. Rab24 has a markedly reduced GTPase activity, due primarily to the presence of a serine residue instead of the usual glutamine at the position equivalent to Q61 in H-Ras. Furthermore, Rab24 is not efficiently geranylgeranylated and does not form a detectable complex with GDI. These unique properties of Rab24 suggest that it may not function according to the accepted cyclical model.

In the present study we set out to learn about the biological function of Rab24 by using a well-established paradigm in which a dominant-negative Rab construct is expressed in cultured cells, anticipating that it will suppress the trafficking step(s) mediated by its endogenous counterpart. Amino acid substitutions at the position cognate to N116 in H-Ras typically yield soluble Rab proteins that have a reduced affinity for guanine nucleotides. Such constructs have been used successfully to define the functions of Rab1A and Rab1B [26–28], Rab4 [29], Rab5 [30,31] and Rab6 [32]. Unexpectedly, we found that expression of the mutant forms of Rab24 in cultured cells resulted in their accumulation in a striking array of insoluble intranuclear inclusions which disrupt the integrity of the nuclear envelope.

**Results**

**Mutant forms of Rab24 accumulate in nuclear inclusions**

In all of the known Ras-related GTPases the guanine nucleotide binding site is surrounded by a set of highly conserved sequence elements [33,34]. One of these elements, termed the G2 motif, consists of NxxD (with the asparagine located at position 116 in H-Ras). In Rab24, and in some members of the Rho family, threonine occurs instead of the asparagine. Amino acid substitutions in the N(T)KxD motif cause a drastic reduction in the affinity of Ras-related GTPases for guanine nucleotides [35,36]. When overexpressed in cultured cells, these mutant GTPases function as dominant-suppressors of their endogenous counterparts [26,27,31], presumably because they can compete for interaction with effectors, exchange factors or docking proteins, but cannot cycle on and off membranes in a nucleotide-dependent manner. We set out to assess the function of Rab24 by making substitutions in its TKxD motif, starting with D123-->I. To facilitate localization of the proteins, the wild-type and mutant constructs were made with a myc-epitope tag at the amino terminus. The addition of such tag sequences to Ras-related proteins, including several members of the Rab family, does not appear to alter their function or subcellular localization [36–38].

When mycRab24(wt) was transiently expressed in 293 cells, immunofluorescence microscopy revealed a diffuse reticular distribution throughout the cytoplasm, with a concentration in the perinuclear region (Fig. 1A). In contrast, when the same studies were performed with cells expressing mycRab24(D123I), we observed a striking accumulation of the mutant protein in punctate structures localized in both cytoplasmic and nuclear compartments. In most cells the inclusions appeared to be concentrated predominantly within the nucleus (Fig. 1A). Identical structures were seen when immunofluorescence microscopy was performed with antibodies against the N-terminal myc epitope or the C-terminal hypervariable domain of Rab24, indicating that the immunofluorescence pattern reflected the localization of full-length mycRab24(D123I). Finally, the localization pattern of Rab24(D123I) remained the same when the protein was expressed without the myc tag (Fig. 1B).

Western blot analyses of cytosol and particulate fractions obtained from the transfected 293 cells indicated that mycRab24(wt) was present in both fractions, consistent with previous results with Rab24 and other wild-type Rab GTPases [25,39]. In contrast, mycRab24(D123I) was found exclusively in the particulate fraction (Fig. 1C). When transfected cells were disrupted in a buffer containing 0.5% NP-40, 0.5% deoxycholate and 0.1% SDS, most of the Rab24(D123I) was recovered in the detergent-insoluble fraction (Fig. 1D). This suggested that the mutant protein was concentrated in inclusion bodies rather than membrane components.

The nuclear inclusions containing Rab24(D123I) were not cell-type specific. Fig. 2A shows that similar punctate structures were formed when the D123I mutant was expressed in NIH 3T3 fibroblasts. We also verified that the unique behavior of the Rab24 mutant was not related specifically to the substitution at the aspartate position in the N(T)KxD motif. Thus, similar inclusions were observed
when Rab24 was expressed with substitutions at the N/T position; i.e., T120A (Fig. 2A) or T120I (not shown). To test the possibility that protein aggregates might be formed when any Rab GTPase is overexpressed with a mutation that impairs nucleotide binding, we examined the localization of four additional members of the Rab family containing amino acid substitutions in the N(T)KxD motif. In all cases, these proteins exhibited a general cytoplasmic localization with no evidence of nuclear inclusions (Fig. 2A).

### Figure 1
Subcellular distribution of wild-type and mutant Rab24 expressed in 293 cells. (A) Cells were transfected with vectors encoding mycRab24wt or mycRab24(D123I) and 24 h later the expressed proteins were localized by immunofluorescence microscopy, using primary antibodies against the myc epitope or the Rab24 C-terminal hypervariable domain, as indicated above each panel. At the exposure setting used for the photograph, the Rab24 antibody did not give a detectable signal in the non-transfected cells (indicated by asterisk). The bar equals 10 microns. (B) Cells were transfected with a vector encoding Rab24(D123I) without the myc epitope, and the expressed protein was localized with the Rab24 antibody. (C) Transfected cells expressing mycRab24wt or mycRab24(D123I) were lysed in buffer without detergent and fractionated by centrifugation as described in the Methods. The cytosol and particulate fractions were subjected to SDS-PAGE and immunoblot analysis, using the anti-myc monoclonal antibody to detect the expressed proteins. (D) Transfected cells expressing mycRab24wt or mycRab24(D123I) were lysed in high-detergent buffer (see Methods) and the proteins recovered in the detergent-soluble and insoluble fractions were assayed by SDS-PAGE and immunoblot analysis, using the anti-myc monoclonal antibody.

### Figure 2
Localization of different Rab GTPases with amino acid substitutions in the N(T)KxD nucleotide binding motif. (A & B) NIH 3T3 cells were transfected with pCMV5 vectors encoding different myc-tagged Rab proteins with the indicated amino acid substitutions. Twenty-four hours after transfection, cells were processed for immunofluorescence microscopy, using the anti-myc monoclonal antibody, followed by FITC-conjugated GAM IgG. Non-transfected cells cannot be seen at the exposure setting used for the photographs. The bar in the upper left panel is 10 microns. (C) Autoradiogram of a thin-layer plate showing 32P-labeled GDP and GTP eluted from the indicated immunoprecipitated proteins. The immunoblot in the lower panel compares the relative amount of mycRab24 collected in 1/10 of each immunoprecipitate.

Rab24 is known to be expressed endogenously in many cell types, including the cell lines used in these studies [24,25], and we have estimated that both mycRab24wt and D123I are transiently expressed 5–10 fold over the level of endogenous Rab24 (data not shown). Therefore, a question raised by these studies is whether the genesis of nuclear inclusions occurs indirectly, through dominant suppression of endogenous Rab24 function by the D123I and T120A mutants, or directly, through association of cellular proteins with the accumulating mutant protein. We attempted to distinguish between these possibilities by using an alternative strategy to make a dominant-negative form of Rab24. Several laboratories have shown that in addition to mutations in the N(T)KxD motif, which reduce overall nucleotide binding, dominant-negative Rab constructs can be generated by introducing amino acid substitutions (e.g., asparagine) at the position cognate to Ras S17 (i.e., S22 in Rab24) [31,40–42]. Such proteins are thought to be locked in an inactive GDP-bound state because the mutation markedly reduces their affinity for GTP, compared to GDP [42]. As shown in Fig. 2B, when mycRab24(S22N) was expressed in 3T3 cells, there was no evidence of nuclear inclusions. However, when the bound...
with Q (which occupies this position all other Rab proteins) increases the GTPase activity of Rab24 [25], resulting in a modest increase in the ratio of protein in the GDP versus GTP state (see Fig. 2C). When this mutant was expressed in 3T3 cells we again failed to observe inclusion bodies (Fig. 2B). Since neither Rab24(S22N) nor Rab24(S67Q) showed evidence of being locked in a GDP-bound state, it is unlikely that either would function as a dominant-negative. Thus, the question of whether the nuclear inclusions containing the D123I and T120A mutants are caused by dominant-negative suppression of endogenous Rab24 function remains to be resolved.

**Morphological characteristics of the nuclear inclusions**

Several follow-up studies supported the notion that the punctate structures containing the Rab24 mutants were inclusion bodies rather than membrane-bound organelles. For example, immunofluorescence microscopy revealed little or no overlap between these structures and markers for Golgi (Rab6, *Lens culinaris* lectin), endosome (EEA1) or lysosome (LAMP-1) membranes (data not shown). Although Rab24 has been proposed as a possible regulator of autophagic processes [24,43], the accumulation of punctate bodies in cells expressing Rab24(D123I) was not prevented by 3-methyladenine, a known inhibitor of autophagosome formation [44]. Most importantly, electron microscopy with immunogold labeling of mycRab24(D123I) in ultra-thin sections confirmed the initial impression that the inclusions are in fact localized within the nucleus. The expressed protein was concentrated in clusters of dense granules of fairly uniform diameter (80–150 nm) with no evidence of a surrounding membrane (Fig. 3A,3B). The cytoplasmic inclusions containing mycRab24(D123I) were similar to those observed inside the nucleus, except that they were larger, with a diameter 2–3 times that of the nuclear particles (Fig. 3D). Such structures were never observed in cells expressing mycRab24wt or any other over-expressed Rab GTPase.

**Disruption of nuclear architecture by inclusions containing Rab24(D123I)**

The accumulation of nuclear inclusions in cells expressing Rab24(D123I) led to progressive degeneration of nuclear structure. Figure 4 shows a series of representative photographs of transfected 3T3 cells that were co-stained with antibody against the Rab24 myc epitope, combined with DAPI to visualize the chromatin (Fig. 4A), or an antibody against lamins A and B to visualize the nuclear envelope (Fig. 4B). At 24 h after transfection, we observed many cells with mycRab24-positive inclusions that showed fairly uniform staining of the nuclear envelope with no loss of chromatin or deformation of nuclear shape. However, in some cells with larger and more numerous inclusions we observed discontinuities in the anti-lamin staining of the nuclear envelope and some co-localization of lamin

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**Figure 3**

Electron microscopy of inclusion bodies in 293 cells expressing mycRab24(D123I). Cells were harvested 24 h after transfection. Expressed protein was detected with the mouse monoclonal antibody against the myc epitope, followed by secondary antibody conjugated to 10 nm gold particles. Cells were processed for electron microscopy as described in the Methods. Panel A shows a cell where the majority of immunoreactive inclusions accumulated within nucleus (N). Panel B shows the intranuclear inclusions at higher magnification, revealing the individual gold particles concentrated in dense spherical particles of nearly uniform diameter. Panel C shows an example of a cell where disruption of the nuclear envelope was observed (between arrows) in the region near the accumulated intranuclear inclusions. Panel D shows a cell where inclusions were observed in the perinuclear cytoplasm. The dimension bars in each panel are as follows: A, 0.5 micron; B, 0.1 micron; C, 0.5 micron; D, 0.5 micron. The solid arrowheads in panels A, C and D indicate the nuclear membrane.

32P-labeled nucleotides associated with the expressed protein were analyzed by thin-layer chromatography (Fig. 2C), we were surprised to find that the S22N mutant was predominantly in the GTP-bound state. The inability of the S22N substitution to substantially reduce the affinity of Rab24 for GTP adds to the growing list of fundamental differences between Rab24 and other members of the Rab family. In a previous study we observed that replacing S67
Figure 4
Disruption of nuclear architecture by Rab24(D123I) inclusions. NIH 3T3 cells expressing mycRab24(D123I) were processed for immunofluorescence microscopy 24 h after transfection. (A) The inclusions detected by staining with the anti-myc monoclonal antibody appeared to occupy areas of the nucleus devoid of DAPI staining (arrows). In some cells (upper panels) the nucleus was well defined, and it was possible to distinguish between inclusions localized inside and outside the nucleus. In other cells (lower panels), the inclusions were more diffuse, the nucleus was distorted, and it was difficult to discern the boundaries between the nuclear and cytoplasmic compartments. (B) mycRab24(D123I) inclusions were localized with rabbit anti-myc polyclonal antibody followed by FITC-conjugated GAR IgG. The nuclear envelope was highlighted by co-staining with a monoclonal antibody against lamins A and B, followed by rhodamine-conjugated GAM IgG. In some cells with immunoreactive inclusions (upper panels) a well defined nuclear envelope was visible, whereas in others (bottom panels) the nuclear envelope appeared to be completely disrupted. Where both cytoplasmic and nuclear inclusions were present (top and middle panels), it appeared that the myc-reactive aggregates inside the nucleus contained lamins (arrows), while those outside the nucleus did not (arrowheads). For comparison, panel C shows the nuclear lamin staining typically observed in non-transfected cells. The scale bar represents 10 microns.
with mycRab24(D123I) in the intranuclear aggregates. The gaps in the nuclear membrane were clearly visible in electron micrographs of cells with large numbers of intranuclear aggregates (Fig. 3C). In the most extreme cases, the nuclear envelope was significantly distorted and fragmented and there was leakage of chromatin into the cytoplasm (Fig. 4A & 4B, lower panels). The number of cells with obvious nuclear disruption increased with time, so that by 48-h this was seen in the majority of the transfected cells (not shown).

The morphological changes occurring in cells with accumulated Rab24(D123I) inclusions did not match the hallmark features of apoptosis (e.g., membrane blebbing, condensation of chromatin at the nuclear periphery). This was confirmed by TUNEL assays where cells at various stages of nuclear inclusion body accumulation showed no evidence of DNA fragmentation, either before or after disruption of the nuclear envelope (Fig. 5A). In contrast, TUNEL-positive cells were readily detected in parallel cultures where apoptosis was induced by treatment with staurosporine (Fig. 5B).

**Rab24(D123I) disrupts nuclear import of glucocorticoid receptor**

Rab24 contains a unique arginine-rich insert, 126EEDRRRRR133 [25] in the domain cognate to loop-8 in H-Ras [34]. The latter sequence is reminiscent of unusual arginine-rich nuclear localization signals that allow proteins like HIV tet and rev to undergo nuclear translocation by association with importin-β, in the absence of importin-α [45,46]. Since the loop-8 insert in Rab24 lies adjacent to the conserved N(T)KxD nucleotide binding cassette, we hypothesized that conformational changes caused by the D123I substitution might render the Rab24 mutant capable of disrupting the nuclear import machinery [47]. To test this possibility, we co-expressed mycRab24wt or mycRab24(D123I) with GR-GFP, a fusion between the glucocorticoid receptor and green fluorescent protein. This protein contains a prototypical nuclear localization signal but remains in the cytoplasm when expressed in cultured cells in the absence of steroids. Upon addition of 1 μM dexamethasone (Dex) the receptor is translocated into the nucleus within 10 min [48]. When GR-GFP was co-expressed with the wild-type Rab24 in NIH3T3 cells, we observed normal ligand-dependent translocation of GR-GFP into the nucleus when Dex was applied (Fig. 6A). However, when GR-GFP was co-expressed with mycRab24(D123I) there was a striking perturbation of receptor localization. Instead of remaining in the cytoplasm in the absence of steroid, nearly all of the GR-GFP was found in the cytoplasmic and nuclear inclusion bodies containing the Rab24 mutant (Fig. 6A). The latter point is clearly illustrated by the overlap of GR-GFP (green) fluorescence with rhodamine-IgG staining for

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**Figure 5**

Accumulation of Rab24(D123I)-positive intranuclear inclusions is not accompanied by chromosomal DNA fragmentation. 293 cells expressing mycRab24(D123I) were fixed 24 h after transfection and subjected to the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling reaction (TUNEL). The transfected cells were identified by co-staining with anti-myc monoclonal antibody followed by rhodamine-conjugated GAM IgG. (A) Representative cells with mycRab24(D123I)-positive nuclear inclusions exhibit varying degrees of nuclear disruption but are uniformly negative when subjected to the TUNEL reaction. (B) Parallel cultures of non-transfected 293 cells were treated with 1 μM staurosporine for 24 h to induce apoptosis. Examples of TUNEL-positive cells are shown at the same exposure setting used to photograph the TUNEL-negative cells in panel A. Nuclei were visualized by staining with propidium iodide (PI).
mycRab24(D123I) (red) in Fig. 6B. The possibility that the extensions induced by mycRab24(D123I) contain aggregated nuclear import complexes was further supported by immuno-staining with an antibody against importin-β (karyopherin-β), which revealed focal concentrations of importin-β in these structures (Fig. 7A). However, other proteins such as Ran binding protein (Fig. 7B) and Ran GAP (not shown), which are recruited to the nuclear import complex through interactions with the Ran GTPase, did not appear to be present in the inclusion bodies.

The C-terminal hypervariable domain of Rab24 is required for the formation of inclusions

To learn more about the structural features of Rab24(D123I) that are responsible for triggering the formation of nuclear inclusions, we focused on regions of the protein that distinguish Rab24 from other members of the Rab family. We began by deleting a portion of the loop-8 insert, described in the preceding section. Four amino acids (ÆEDRR) were removed from both Rab24(wt) and Rab24(D123I). Immunofluorescent localization studies showed that this modification had no effect on distribution of the wild-type protein in the cytoplasm (Fig. 8A) and, contrary to expectations, did not interfere with the accumulation of the D123I mutant in nuclear inclusions (Fig. 8B).

The second region where the sequence of Rab24 differs substantially from other Rab GTPases is the last 38 amino acids of the C-terminal tail. This region, commonly referred to as the hypervariable domain, plays a major role in the subcellular targeting and functional specificity of each Rab protein [49]. In accord with this view, replacement of the hypervariable domain of Rab24(wt) with the corresponding domain from Rab1B, caused the resulting chimera to exhibit a discrete juxtanuclear Golgi-like fluorescence pattern typical of Rab1B in 293 cells [50,51]. (Fig. 8C). When the D123I substitution was introduced into the Rab24 portion of the Rab24/Rab1B chimera, the protein was no longer targeted to inclusion bodies (Fig. 8D), but instead exhibited a diffuse distribution similar to Rab1B(N121I) and other Rab proteins with mutations in the N(T)KxD nucleotide binding motif. To confirm the importance of the Rab24 hypervariable domain for targeting to inclusion bodies, we performed the reciprocal experiment, replacing the C-terminal domain of Rab1B(wt) or Rab1B(N121I) with the hypervariable domain from Rab24. Instead of the usual Rab1B juxtanuclear Golgi localization, the Rab1B(wt)/Rab24 chimera showed a broader cytoplasmic distribution similar to that of Rab24(wt) (Fig. 8E). Most notably, replacement of hypervariable domain of Rab1B(N121I) with the corresponding C-terminal region from Rab24 caused a major change in the localization of the chimera from a predominant cytoplasmic distribution typical of Rab1B(N121I) (Fig. 2) to a pattern of large intranuclear and cytoplasmic inclusion bodies (Fig. 8F).

To determine whether the hypervariable domain of Rab24 was sufficient to promote the formation of inclusion bodies when fused to a heterologous protein, we prepared fusion constructs with enhanced green fluorescent protein (EGFP) containing either full-length Rab24(D123I) or just the Rab24 C-terminal domain. As shown in Fig. 9A, EGFP-Rab24(D123I) accumulated in inclusion bodies similar to those described earlier for mycRab24(D123I). In contrast, the EGFP fusion protein containing only the hypervariable domain of Rab24 exhibited a diffuse cytoplasmic and nuclear localization that was essentially identical to the pattern observed with EGFP alone (Fig. 9B &9C). Therefore, it appears that although the C-terminal domain of Rab24 is required to promote its incorporation into inclusion bodies, it does so only when expressed in the context of a Rab GTPase with a mutation in the N(T)KxD nucleotide binding motif.

Nuclear inclusions generated by Rab24(D123I) contain ubiquitin and Hsp70

Although the formation of intranuclear inclusions has not been observed in previous studies of Ras-related GTPases, it is a common feature of several neurodegenerative disorders where abnormal proteins containing poly-Gln tracts are synthesized as a result of CAG codon expansions [52]. Examples include Huntington’s disease (huntingtin) [53,54], spinocerebellar ataxia (SCA) types 1 and 3 (ataxin-1 and ataxin-3) [55,56] and dentatorubral-pallidolysian atrophy (atarophin-1) [57]. These poly-Gln protein aggregates typically contain ubiquitin [58] and members of the Hsp70 chaperone family [59], consistent with a proposed pathway for cellular clearance of toxic misfolded proteins. We noted that the inclusions caused by expression of Rab24(D123I) were similar morphologically to those described in some of the poly-Gln disorders and hypothesized that they might arise via a similar pathway. An immunofluorescence study was performed to determine whether or not the intranuclear Rab24(D123I) aggregates might sequester ubiquitin and Hsp70. Both ubiquitin (Fig. 10A) and Hsp70 (Fig. 10B) showed substantial overlap with nuclear and cytoplasmic aggregates containing mycRab24(D123I). In contrast, the aggregates did not contain Hsp90 (Fig. 10C), suggesting that the sequestration of Hsp70 was specific.

To determine whether ubiquitin was conjugated directly to Rab24(D123I), it was necessary to solubilize the protein aggregates in a high-detergent buffer that was incompatible with standard immunoprecipitation methods. Therefore, we generated a mammalian expression vector encoding a His$_6$-tagged version of Rab24(D123I) and used Ni$^{2+}$ affinity resin to isolate the expressed protein
from detergent extracts. When expressed in 293 cells the His<sub>6</sub>Rab24(D123I) accumulated in inclusion bodies that were morphologically identical to those formed by the myc-tagged and untagged proteins (not shown). When the His<sub>6</sub>-tagged protein was extracted from the inclusions and subjected to SDS-PAGE, we saw no evidence of higher molecular mass forms that would correspond to poly-ubiquitinated species, using either anti-His<sub>6</sub> (Fig. 11A) or anti-Rab24 (not shown) antibodies. Moreover, immunoblot analysis of the His<sub>6</sub>Rab24(D123I) isolated from cells treated with lactacystin (to block degradation of poly-ubiquitinated proteins by the 26S proteasome) showed no detectable ubiquitin associated with the protein, although the same antibody detected multiple ubiquitinat-
ed polypeptides in the whole-cell lysate (Fig. 11B). Again, these findings agree with recent studies of poly-Gln protein aggregates, which indicate that although ubiquitinated proteins are present in the inclusions, they are not conjugated directly to the initiating poly-Gln polypeptide [60].

Discussion
Here we have shown that single amino acid substitutions (e.g., D123I and T120A) in one of the conserved guanine nucleotide binding domains of Rab24 can cause the protein to accumulate in a massive array of nuclear inclusions in mammalian cells. Although members of the Ras GT-Pase superfamily have been studied for many years using

Figure 6
Effects of Rab24 wt and Rab24(D123I) on nuclear translocation of glucocorticoid receptor. (A) 3T3 cells expressing GR-GFP with mycRab24wt show predominant cytoplasmic localization of both proteins in the absence of Dex (left panels). After 10 min incubation with 1 μM Dex, most of the GC-GFP is within the nucleus in cells expressing Rab24wt (center panels). When GR-GFP was co-expressed with mycRab24(D123I) in the absence of steroid, much of the GR-GFP was localized to inclusion bodies containing the Rab24 mutant (right panel). Identical results were observed when 1 μM Dex was added (not shown). (B). Higher magnification shows extensive co-localization of myc-Rab24(D123I) (red) with GR-GFP (green) in nuclear inclusion bodies. The bar represents 10 microns.
site-directed mutagenesis approaches, there have been no previous reports demonstrating cytopathic effects of this kind. In particular, as we have shown in Fig. 2, a variety of Rab proteins bearing similar mutations are localized in the cytoplasmic compartment.

It remains unclear whether the nuclear inclusions induced by Rab24(D123I) are generated de novo inside the nucleus or instead penetrate the nuclear membrane after being assembled in the cytoplasmic compartment. Rab24 does not have a classic nuclear localization signal sequence, but monomeric mycRab24(D123I) is theoretically small enough (24 kDa) to diffuse across the nuclear pores [61]. However, it is less likely that this could occur in the case of the larger EGFP-Rab24(D123I) fusion construct (50 kDa), which also formed abundant intranuclear inclusions (Fig. 9). Our preliminary observations indicate that some of the intranuclear inclusions containing Rab24(D123I) are immunoreactive for lamins, while those outside the nucleus are not (Fig. 4B). Moreover, the electron micrographs show that nuclei containing clusters of inclusions often have regions where the nuclear membrane appears to be discontinuous (Fig. 3C). Based on these findings, we believe that aggregates formed in the cytoplasm may transiently associate with and disrupt the nuclear envelope, then enter the nucleus through the resulting gaps. Ultimately, the accumulation of nuclear inclusions in cells expressing Rab24(D123I) causes a catastrophic disruption of the nuclear architecture, although this does not appear to be accompanied by classic signs of apoptotic cell death such as DNA fragmentation or caspase activation.

The molecular mechanism underlying the formation of nuclear inclusions in cells expressing the mutant forms of Rab24 remains to be defined. An extensive body of published work supports the notion that Rab proteins bearing mutations in the N(T)KxD nucleotide binding motif function as dominant-suppressors of their endogenous cellular counterparts. Our domain-exchange experiments with Rab24 and Rab1B (Fig. 8) indicate that incorporation of the mutant proteins into nuclear inclusions requires the specific C-terminal hypervariable targeting domain of Rab24. Therefore, one possibility is that the inclusions arise as a consequence of disruption of the normal physiological function of Rab24. To explore this issue further, we attempted to generate another potential dominant-negative form of Rab24, predicted to be locked in the GDP state. However, when we made the amino acid substitution customarily used to generate such Rab mutants (i.e., S22N), Rab24 unexpectedly remained in the GTP state. Therefore, pending further investigation, we cannot rule out the alternative hypothesis that mutations in the N(T)KxD motif of Rab24, combined with features in the C-terminal domain, cause Rab24 to misfold in a unique manner, attracting non-physiological protein partners that can promote aggregation and nuclear localization.

As a framework for future studies we can envision at least three models that could explain the accumulation of inclusion bodies in cells expressing Rab24(D123I). Our im-

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**Figure 7**

**Inclusions generated by Rab24(D123) contain importin-β, but not Ran binding protein.** (A) mycRab24(D123I) expressed in transfected NIH 3T3 cells was detected with rabbit antibody against the myc epitope followed by FITC-conjugated GAR IgG. Importin-β was detected with mouse monoclonal antibody followed by rhodamine-conjugated GAM IgG. The lines point to aggregates staining for both myc and importin-β. (B) mycRab24(D123I) was detected as in A, and Ran BP was detected with a monoclonal antibody, followed by rhodamine-conjugated GAM IgG. The scale bars represent 10 microns.
munofluorescence studies (Fig. 10) suggest that the inclusions are complex protein assemblies containing Hsp70 and ubiquitin. Therefore, the first possibility is that the presence of Hsp70 and ubiquitin reflects a failed attempt by the cell to eliminate misfolded protein aggregates via the proteasome pathway. Indeed, a similar explanation has been invoked to explain the presence of Hsp70 and ubiquitin in nuclear aggregates observed in cells expressing abnormal gene products with poly-Gln tracts, such as huntingtin [54] and ataxin-1 [59]. Based on reports that overexpression of chaperones [59,62] or a dominant-negative form of the ubiquitin-conjugating enzyme [63] can suppress the formation of poly-Gln protein aggregates, Kaytor and Warren [58] proposed a three-stage model wherein (i) molecular chaperones initially associate with the misfolded protein, (ii) failure to diminish the pool of misfolded protein leads to aggregation and ubiquitination and (iii) inefficient degradation of the aggregate by the proteasome is manifested by the accumulation of inclusion bodies. Although this model implies that the misfolded poly-Gln protein might be the primary substrate for ubiquitination, a recent study by Suhr et al. [60] suggests that this may not always be the case. Specifically, they found that when aggregates from 293 cells expressing poly-Gln proteins were dissociated, the ubiquitin polymers were not conjugated directly to the poly-Gln protein or the sequestered Hsp70.

Figure 8
Localization of Rab24 deletion mutants and Rab24/Rab1B chimeras. 293 cells were transfected with expression vectors encoding (A,B) Rab24wt or Rab24(D123I), with deletions in the loop-8 insert region, or (C-F) a series of chimeric proteins composed of combinations of the N-terminal and C-terminal domains of Rab24 and Rab1B, with or without the D123I substitution (see Methods for detailed descriptions). After 24 h, the transfected cells were processed for immunofluorescence microscopy using the anti-myc monoclonal antibody and FITC-conjugated GAM IgG to localize the expressed proteins. Panels A-F show representative cells from cultures expressing the indicated proteins. The bar represents 10 microns.
An alternative mechanism for the formation of inclusion bodies in protein conformation disorders is suggested by a recent study showing that clearance of poly-Gln and poly-Ala inclusions is accelerated by compounds like rapamycin [64]. Along similar lines, the work of Kegel et al. [65] hints that poly-Gln huntingtin may be degraded mainly in autophagic vacuoles containing lysosomal proteases. Interestingly, Rab24 has been implicated as a possible mediator of autophagy in a recent study where wild-type GFP-Rab24 was observed to accumulate on large autophagic vacuoles in cells subjected to amino acid starvation [43]. Thus, if Rab24(D123I) functions as a true dominant suppressor of endogenous Rab24, the inclusions observed in the present study may consist of misfolded endogenous proteins that cannot be degraded because basal activity of the autophagosome-lysosome pathway is blocked.

Although the two preceding models may explain the presence of Hsp70 and ubiquitin in the Rab24(D123I)-induced aggregates, the incorporation of glucocorticoid receptor (Fig. 6) and importin-β (Fig. 7) may point to a third distinct scenario. Aside from its well-characterized
role in protein folding [66]. Hsp70 is known to associate with the GR in the cytoplasm, where it promotes formation of a heterocomplex between the inactive GR and Hsp90 [67]. In addition, Hsp70 becomes concentrated in the nucleus when cells are exposed to environmental stress [68]. Of particular note, the nuclear accumulation of the Hsp70, Ssa4p, in response to nutrient deprivation in yeast appears to occur by a novel mechanism that involves its direct interaction with Mnd5p, a member of the importin-β family [69]. A role for Rab24 in nuclear trafficking would be unanticipated, since the only GTPase known to function in nuclear import/export (Ran) is structurally distinct from the Rab subgroup of the Ras superfamily [70,71]. Nevertheless, we can speculate that if Rab24 participates in a stress-induced pathway for nuclear import of Hsp70 in mammalian cells, the constitutive overexpression of Rab24(D123I) might stimulate aberrant accumulation of Hsp70/importin-β aggregates at the nuclear pore complex. This might ultimately result in disruption of the nuclear envelope and penetration of the aggregates into the nucleus. If the Hsp70 in these aggregates retains the capacity to bind GR, this could account for the sequestration of nascent GR in these structures.

It is well established that mutations affecting the guanine nucleotide state of Ras are associated with neoplastic transformation of mammalian cells [35,72,73]. However, the potential role of Rab mutations in disease is just beginning to be explored. Menasche et al. [74] found mutations in the Rab27A gene in 16 patients with Griscelli’s syndrome, an autosomal recessive disorder characterized by defects in skin pigmentation and T-lymphocyte and macrophage activation. While several of the mutations were deletions, a few were missense mutations that changed single amino acids in conserved regions of the Rab structure. Mutations in the Rab27A gene have also been detected in mice with the coat color mutation ashen, which is regarded as a model for Griscelli’s syndrome [75,76]. Rab24 can be detected in many tissues and cell lines [24,25], but expression appears to be highest in neuronal cells [25]. At present there are no available data specifically linking Rab24 mutations to human disease. However, there are many case reports describing patients with progressive neurodegenerative disorders of unknown etiology, collectively referred to as neuronal intranuclear inclusion disease [77]. While these may consist mainly of unidentified poly-Gln or poly-Ala disorders, the present study raises the possibility that some forms of this disease may arise through unexpected mechanisms, perhaps involving mutations in Rab24 or other poorly characterized members of the Rab GTPase family that mediate trafficking of misfolded proteins within autophagosomes and lysosomes.

Conclusions

Previous studies have shown that Rab24 exhibits several unusual characteristics that distinguish it from other members of the Rab GTPase family [25]. The studies described in this report demonstrate that putative domi-
nant-negative mutant forms of Rab24, bearing single amino acid substitutions in the conserved N(T)xKxD nucleotide binding motif (e.g., D123I and T120A), are targeted to numerous inclusion bodies that disrupt the nuclear envelope and enter the nuclear compartment. The inclusions bear a superficial resemblance to those described in some of the polyglutamine disorders, and appear to sequester Hsp70, ubiquitin, importin-β, and glucocorticoid receptor. Studies of Rab24/Rab1B chimeras indicate that the unusual cytopathic effects induced by the D123I mutant require sequence elements within the Rab24 C-terminal hypervariable targeting domain. Similar mutations in the N(T)xKxD motif of other Rab proteins typically yield soluble cytoplasmic proteins. Confirmatory studies with other potential dominant-negative mutants, such as those bearing substitutions at the position cognate to Ras S17, were not possible with Rab24, because such mutants remained in the GTP state. The present results further highlight the unique nature of Rab24 and raise the possibility that it may function in autophagic degradation of misfolded proteins, or perhaps play an unexpected role in nuclear protein trafficking or maintenance/assembly of the nuclear envelope.

Methods

Expression constructs

The cDNA encoding Rab24 was obtained by PCR amplification from first strand cDNA template reverse-transcribed from mouse brain mRNA, using Taq DNA polymerase (Perkin Elmer). Oligonucleotide primers for the PCR reaction were based on the published sequence of murine Rab24 [24]. Kpn1 and BamH1 restriction sites were added to the 5′ and 3′ primers respectively, to facilitate cloning of the PCR product in frame with a 5′ sequence cassette encoding a myc epitope (EQKLISEEDL), previously introduced into the pCMV5 expression vector [78]. The Rab24 cDNA was altered by overlap-extension PCR [79], using appropriate mutator oligonucleotides and Pfu DNA polymerase (Stratagene Inc., La Jolla, CA), so as to introduce amino acid substitutions at D123 (D123I), T120 (T120A), S22 (S22N), S67 (S67Q), and a deletion of residues 127 through 130 (AEDDR). The cDNA encoding human Rab11 was obtained by PCR amplification from first strand cDNA template, reverse-transcribed from Hela cell mRNA. This sequence was cloned into pCMV5, in-frame with a 5′ myc epitope tag sequence, and altered to encode the N124I substitution as stated above for Rab24. Other pCMV5 expression vectors encoding Rab1B(N121I) [28], Rab6(N126I) and Rab8(N121I) [32] have been described in the indicated references. pCMV5 expression vectors containing Rab24 and Rab1B were engineered by overlap-extension PCR to encode the following chimeric proteins: Rab24/Rab1B, consisting of residues 1–165 of Rab24 with the D123I substitution, fused to residues 163–201 of Rab1B; Rab1B/Rab24, consisting of residues 1–162 of Rab1B fused to residues 166–203 of Rab24; and Rab1B(N121I)/Rab24, consisting of residues 1–162 of Rab1B with the N121I substitution, fused to residues 166–203 of Rab24. To generate Rab24 fusions with Enhanced Green Fluorescent Protein (EGFP), cDNAs encoding full-length Rab24(wt) or Rab24(D123I), or the Rab24 C-terminal hypervariable domain (residues 166–203) were generated with flanking Kpn1 and BamHI sites and subcloned into pEGFP-C1 (Clontech Inc., Palo Alto, CA) so that the Rab inserts were in-frame with a 5′ EGFP coding sequence. The sequences of all constructs were verified by automated DNA sequencing using a Beckman CEQ2000 system.

Cell culture and transfection

Human embryonal kidney (HEK) cells (line 293) and NIH 3T3 mouse fibroblasts were obtained from American Type Culture Collection (Rockville, MD) and were maintained at 37°C in a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum. On the day before transfection, cells were plated in 6 cm diameter dishes at 5 x 10^5 cells/dish or in 10 cm diameter dishes at 2 x 10^6 cells/dish. Cells were transfected with the indicated expression vectors, using Lipofectamine Plus reagent (Life Technologies, Inc., Rockville, MD) for 293 cells or Superfect reagent (Qiagen, Valencia, CA) for 3T3 cells, according to the protocols recommended by the manufacturers.

Subcellular distribution of Rab24

To determine the subcellular distribution of mycRab24(wt) and mycRab24(D123I), 293 cells were plated in 10 cm dishes and transfected with the indicated expression vectors. After 24 h, the cells were harvested in PBS, collected by centrifugation, and lysed in 5 volumes of 40 mM Tris-HCl, pH 7.4, containing complete mini-EDTA-free protease inhibitor cocktail (Sigma, St. Louis, MO). Soluble and particulate fractions were obtained by centrifuging the cell lysate at 100,000 x g for 60 min at 4°C. One quarter of each fraction was subjected to SDS-PAGE and immunoblot analysis using the 9E10 monoclonal antibody against the myc epitope (Oncogene Research Products, Cambridge, MA), followed by HRP-conjugated goat anti-mouse IgG and ECL detection reagent (Amersham Corp, Arlington Hts., IL) as described previously [50,81]. To determine the detergent solubility of the Rab24(D123I) mutant, transfected cells from a 10 cm culture dish were homogenized in 0.2 ml of 100 mM Tris-HCl, pH 7.4, containing 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA. Samples were partitioned into soluble and particulate components and the latter were subjected to SDS-PAGE and immunoblot analysis.
Rab24 guanine nucleotide state
To assess the guanine nucleotide state of selected mycRab24 proteins, cells were transfected with the indicated constructs and incubated with $^{32}$P-orthophosphate for 5 h, starting 24 h after transfection. Radiolabeled mycRab24 was immunoprecipitated and the $^{32}$P-labeled guanine nucleotides eluted from the protein were subjected to thin-layer chromatography to resolve GDP and GTP. Aliquots of the immunoprecipitated protein were also subjected to immunoblot analysis with antibody against the myc epitope, so that the amount of bound nucleotide could be related to the amount of protein. A detailed description of these procedures has been published previously [25].

Immunofluorescence microscopy
HEK293 cells or NIH3T3 cells were seeded on laminin-coated coverslips in 60 mm dishes and transfected as described above. Cells were fixed for 15 min in 3% (w/v) paraformaldehyde, rinsed for 5 min with 0.05 M NH$_4$Cl, permeabilized for 2 min with 0.1% (v/v) TritonX-100, and blocked for 30 min with 0.1% (w/v) BSA. All solutions were prepared in PBS. To detect the expressed myc-tagged Rab proteins, cells were incubated for 60 min with either the 9E10 mouse monoclonal antibody against the myc epitope or a rabbit polyclonal antibody against the same epitope (Upstate Biotechnology, Lake Placid, NY). In some experiments nuclei were visualized by counterstaining with 4',6'-diamidino-2-phenylindole (DAPI) (2 μg/ml) (Molecular Probes, Eugene, OR). Other monoclonal antibodies used in combination with the rabbit anti-myc antibody were: anti-lamin A + B and anti-ubiquitin (Zymed Laboratories, South San Francisco, CA), anti-Hsp70 and anti-Hsp90 (Stressgen, Inc., Victoria, BC, Canada), anti-GFP (Clontech, Inc. Palo Alto, CA), antikaryopherin β and anti-Ran binding protein (Transduction Laboratories, Lexington, KY). Depending on the combination of primary antibodies, cells were incubated for 60 min with goat anti-mouse (GAM) or goat anti-rabbit (GAR) IgG’s conjugated with either FITC or rhodamine (Sigma Chemical Co., Louis, MO). Photomicrographs were taken with a Nikon Eclipse 800 fluorescence microscope equipped with a digital camera and ImagePro software (Media Cybernetics, Silver Spring, MD). In some cases, gray scale images were pseudo-colored to correspond to the red (Rhodamine), green (FITC) or blue (DAPI) fluorescence, and the digital images were merged.

Nuclear translocation of glucocorticoid receptor
The expression vector pK7-GR-GFP [48], which encodes a fusion of the glucocorticoid receptor (GR) with green fluorescent protein (GFP), was provided by Dr. Ian Macara, University of Virginia, Charlottesville, USA. NIH 3T3 cells were seeded on glass coverslips in DMEM containing 10% charcoal-stripped FCS, without phenol red. Twenty four hours after transfection, the cells were changed to fresh medium with or without 1 μM dexamethasone (Dex). All cells were fixed for immunofluorescence microscopy 10 min after addition of Dex. The localization of GR-GFP was determined in comparison to mycRab24, detected with anti-myc monoclonal antibody and rhodamine-conjugated GAM-IgG, as described above.

Electron microscopy
Transfected cells were washed twice with PBS and scraped from the dish using a rubber policeman. The resulting cell suspensions were centrifuged in Eppendorf® tubes and cell pellets were fixed for 5 min at room temperature using a 1% glutaraldehyde in 0.2M sodium cacodylate, pH 7.2. Fixed cell pellets were washed twice with cacodylate buffer and twice with 0.5 M ammonium chloride. Each sample was dehydrated via a graded series of chilled ethanol solutions and infiltrated and embedded in LR White® Embedding media (London Resin Co., Ltd., Berkshire, England), also at 2°C. Polymerized cell blocks were ultra-thin sectioned with a Reichert Ultracut S ultramicrotome (Leica Microsystems, Wien, Austria) and sections collected on gold 200-mesh electron microscopy support grids. Sections were incubated with PBS for 5 min, then blocked in a solution of 10% fish gelatin in PBS for 15 minutes. Reaction with the 9E10 mouse anti-myc antibody (1:100 dilution in PBS) was carried out for 2 h, followed by 6 washes with PBS. Sections were then incubated for 1 h with GAM IgG conjugated with 10 nm colloidal gold (Ted Pella, Inc., Redding, CA), followed by 3 changes of PBS. The samples were post-fixed with 1% glutaraldehyde and stained with uranyl acetate and lead citrate. They were then blot-dried and examined with a Philips CM 10 transmission electron microscope for localization of the colloidal gold probe.

Solubilization of Rab24(D123I) from inclusions and assay for ubiquitination
The cDNAs encoding Rab24(wt) and Rab24(D123I) were subcloned into a pCMV5 vector that had been modified by PCR to encode an in-frame polyhistidine tag (His$_6$) at the amino-terminus of the Rab protein. HEK 293 cells grown in 10 cm diameter dishes were transfected with either pCMV5-His$_6$Rab24 or pCMV5-His$_6$Rab24(D123I) as described earlier. 20 μM lactacystin (Sigma, St. Louis, MO) was added to the medium 18 h after transfection and cells were incubated with the proteasome inhibitor for 4 h prior to harvest. Cells were scraped from the dish, collected by centrifugation, and homogenized in 0.25 ml Buffer A: 0.15 M NaCl, 20 mM MgCl$_2$, 0.05% (w/v) SDS, 20 mM HEPES, pH 7.4, supplemented with protease inhibitor cocktail. The cell lysate was mixed with 0.25 ml of Buffer B (Buffer A containing 1% NP-40 and 1% deoxycholate) and the insoluble material was removed by centrifugation at 100,000 × g for 30 min. The supernatant
fraction was diluted 1:1 with Buffer C (Buffer A containing 0.5% NP-40 and 0.5% deoxycholate, with no SDS) and loaded on to a Ni-NTA spin column (Qiagen). The column was washed with Buffer D (0.15 M NaCl, 20 mM MgCl₂, 0.025% SDS, 0.5% deoxycholate, 0.5% NP-40, 20 mM HEPES, pH 7.4) and the His₆-tagged protein was eluted with Buffer D containing 0.25 M imidazole. Samples of the eluted protein were subjected to SDS-PAGE and immunoblot analysis as described previously, using a polyclonal antibody against Rab24 [25], or monoclonal antibodies against the His₆-tag (Sigma) or ubiquitin (Zymed Laboratories).

Cell death assay

Cells were transfected with pCMV5 expression vectors encoding myrRab24(D123I) as described earlier. DNA fragmentation was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, using fluorescein-12-dUTP, according to the instructions supplied by the manufacturer (Promega Corp., Madison, WI). Following the TUNEL reaction, the cells were incubated with monoclonal anti-myc antibody and Rhodamine-conjugated GAM IgG to detect the expressed Rab protein. Fluorescence microscopy was used to determine the number of transfected cells that were TUNEL-positive, compared to non-transfected cells in the same culture. As a positive control for apoptosis, parallel cultures were treated for 24 h with 1 μM staurosporine.

Authors’ contributions

W.A.M. drafted the manuscript and supervised the studies. G.S. generated the Rab24 constructs and carried out the immunofluorescent and western blot studies. W.G. and E.C. performed the TUNEL assays. B.A. performed the immunogold electron microscopy. B.A. performed the TUNEL assays.

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