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Myosin II is associated with Golgi membranes: identification of p200 as nonmuscle myosin II on Golgi-derived vesicles

Elina Ikonen[1], J. Bruno de Almeid[2], Karl R. Fath[3], David R. Burgess[3], Keith Ashman[1], Kai Simons[1] and Jennifer L. Stow[2,4,*]

[1]European Molecular Biology Laboratory, D-69012, Heidelberg, Germany
[2]Renal Unit, Massachusetts General Hospital, Charlestown, MA 02129, USA
[3]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, USA
[4]Centre for Molecular and Cellular Biology, University of Queensland, Brisbane, QLD 4072, Australia

*Author for correspondence at address 4 (e-mail: j.stow@mailbox.uq.oz.au)

SUMMARY

A variety of peripheral membrane proteins associate dynamically with Golgi membranes during the budding and trafficking of transport vesicles in eukaryotic cells. A monoclonal antibody (AD7) raised against Golgi membranes recognizes a peripheral membrane protein, p200, which associates with vesicles budding off the *trans*-Golgi network (TGN). Based on preliminary findings, a potential association between p200 and myosin on Golgi membranes was investigated. Immunofluorescence staining of cultured cells under a variety of fixation conditions was carried out using an antibody raised against chick brush border nonmuscle myosin II. We show that, in addition to being found in the cytoplasm or associated with stress fibres, nonmuscle myosin II is also specifically localized on Golgi membranes. Myosin II was also detected on Golgi membranes by immunoblotting and by immunogold labeling at the electron microscopy level where it was found to be concentrated on Golgi-derived vesicles. The association of myosin II with Golgi membranes is dynamic and was found to be enhanced following activation of G proteins. Myosin II staining of Golgi membranes was also disrupted by brefeldin A (BFA). Colocalization of the AD7 and myosin II antibodies at the light and electron microscopy levels led us to investigate the nature of the 200 kDa protein recognized by both antibodies. The 200 kDa protein immunoprecipiated by the AD7 antibody was isolated from MDCK cells and used for microsequencing. Amino acid sequence data enabled us to identify p200 as the heavy chain of nonmuscle myosin IIA. In addition, an extra protein (240 kDa) recognized by the AD7 antibody specifically in extracts of HeLa cells, was sequenced and identified as another actin-binding protein, filamin. These results show that nonmuscle myosin II is associated with Golgi membranes and that the vesicle-associated protein p200, is itself a heavy chain of myosin II.

Key words: Myosin, Golgi vesicle

INTRODUCTION

Populations of intracellular transport vesicles are adorned by a variety of proteins bound to their cytoplasmic surfaces. Complexes of coat proteins and different monomeric G proteins, ARFs and Rabs, bind dynamically to budding membranes for specific roles in vesicle budding, targeting and fusion (Rothman, 1994; Schekman and Orci, 1996). Molecular motors are also now known to be associated with specific membrane domains on intracellular organelles and on transport vesicles themselves. The microtubule-based motors, dynein and kinesin are well-documented as mediators of organelle and vesicle movement along microtubules (Hirokawa, 1996; Hollenbeck and Swanson, 1990; Lippincott-Schwartz et al., 1995; Schroer et al., 1989). Actin-based motors are also involved in organelle movement and several classes of unconventional myosins, particularly myosins-I (110 kDa) and myosins-V (~190 kDa) have been implicated in vesicular trafficking. MYO2p in yeast and other members of the myosin-V class in higher organisms participate in vectorial transport of vesicles on actin filaments (Cheney and Mooseker, 1992; Govindan et al., 1995; Hasson and Mooseker, 1995; Johnston et al., 1991). Myosin-I motors are able to translocate isolated vesicular membranes on actin (Adams and Pollard, 1986). Brush border myosin-I has been localized on Golgi membranes and vesicles (Fath and Burgess, 1993; Fath et al., 1994) and the expression of truncated forms of myosin-I has a dominant negative effect on the distribution of endocytic compartments in hepatoma cells (Durbach et al., 1996). The presence of a variety of motors provides transport vesicles with the potential to interact with different elements of the cytoskeleton, although the exact nature of such interactions in most trafficking pathways is not yet understood.

The myosins now constitute a broad superfamily of molecular motors (Cheney et al., 1993). The subfamily of class II myosins contains both muscle and nonmuscle forms of myosin II whose overall structure is similar, consisting of a pair of 200 kDa heavy chains which dimerize and associate non-

covalently with pairs of myosin light chains (17 and 20 kDa). The head domains of the myosin II heavy chains contain binding sites for actin and ATP, providing them with an actin-activated ATPase activity. The alpha-helical, coiled-coil tails of the myosin II heavy chains interact to form characteristic bipolar filaments. The role of myosin II as a force-generating, actin-binding motor protein in muscle contraction is well established. In other cells, nonmuscle myosin II has been widely implicated in functions ranging from cell motility and adhesion to cytokinesis (Ruppel and Spudich, 1995; Warrick and Spudich, 1987). The essential role of myosin II in cytokinesis is demonstrated by the lack of contractile ring formation and cell division in *Dictyostelium* null mutants (Manstein et al., 1989; Ruppel and Spudich, 1995). In epithelial cells, myosin II is required for contraction of the zonula adherens circumferential actin ring of epithelial cells (Broschat et al., 1983). Two vertebrate heavy chain genes give rise to different isoforms of nonmuscle myosin II (A and B) which are differentially expressed in cells and tissues (Katsuragawa et al., 1989; Kawamoto and Adelstein, 1991; Murakami and Elzinga, 1992; Simons et al., 1991; Takahashi et al., 1992). Further isoforms of myosin IIB, generated by alternately splicing, have also been demonstrated in neurons (Takahashi et al., 1992). Recently, it has been shown that the intracellular distributions, enzymatic activities and proposed functions of the myosin II isoforms vary and are often distinct (Kelley et al., 1996; Maupin et al., 1994; Rochlin et al., 1995). It appears that the full array of cellular functions involving nonmuscle myosin II is not yet fully known.

In order to identify new proteins involved in vesicle trafficking on the Golgi complex, a panel of monoclonal antibodies was raised against canine liver *trans*-Golgi network (TGN) membranes (Narula et al., 1992). One of these antibodies, AD7, showed strong immunofluorescence staining of the Golgi region and was found to recognize a 200 kDa soluble phosphoprotein (p200) in a wide variety of cells (Narula et al., 1992). Electron microscopy studies revealed that p200 associates preferentially with vesicular profiles on the *trans*-Golgi network (TGN). In NRK cells, these TGN-derived p200 vesicles are distinct from clathrin-coated vesicles and do not contain β-COP (Narula et al., 1992). In MDCK cells, p200 vesicles do not contain apically-or basally-directed viral proteins (Ikonen et al., 1996) and they are distinct from vesicles bearing another cytoplasmic protein, p230 (Gleeson et al., 1996). Brefeldin A (BFA) induces a rapid and reversible redistribution of p200 from Golgi membranes to the cytosol, and conversely, the binding of p200 to Golgi membranes is initiated by G proteins, including the pertussis-toxin sensitive heterotrimeric G protein, $G\alpha_{i\text{-}3}$ (de Almeida et al., 1993). These findings led to the proposal that p200 may be part of a novel 'coat' of peripheral membrane proteins on Golgi-dervied vesicles, although previously there was no sequence data available for the specific identification of p200, and to date, its specific role in vesicle trafficking is not known.

On the basis of preliminary sequence findings (B. Burke, K. Matlin and J. Stow, unpublished data) and the fact that p200 is a major cytosolic protein in most cells, we decided to investigate its potential relationship to myosins. In the experiments reported here we examined the intracellular distribution of nonmuscle myosin II, its association with Golgi membranes and its relationship to p200. A 200 kDa band and an additional protein of ~240 kDa in HeLa cells (Gleeson et al., 1996; Ikonen et al., 1996), both recognized by the AD7 monoclonal, were immunopurified and identified as different actin-binding proteins. Our data now provide evidence for the association of another class of molecular motors with Golgi membranes and further implicate actin-based motor proteins in vesicular transport.

MATERIALS AND METHODS

Antibodies

An antiserum to myosin II was raised against chick intestinal brush border nonmuscle myosin II which was isolated as described previously (Broschat et al., 1983); the antibody used for these studies was affinity-purified against the 200 kDa heavy chain of this myosin II. Samples of chick brush border myosin II antibody were also obtained from A. Bretcher (Cornell University, Ithaca, NY). The AD7 monoclonal antibody raised against Golgi-associated proteins recognizes a protein referred to as p200, which has been previously characterised as a peripheral membrane phosphoprotein associated with Golgi vesicle membranes (Narula et al., 1992).

Membrane isolation and western blot analysis

Total microsomal membranes (100,000 ***g*** pellet) and cytosol (100,000 ***g*** supernatant) were prepared from A72, MDCK, HeLa and NRK cell lines (de Almeida et al., 1993). Golgi membranes were fractionated from homogenates of NRK cells as described by Rothman and coworkers (Rothman, 1992). Golgi membranes were fractionated from homogenates of rat liver (Ehrenreich et al., 1973) and rat liver cytosol was also recovered as the supernatant from rat liver microsomal preparations as described (de Almeida et al., 1993). Samples of rat liver Golgi membranes and cytosol were incubated together in Hepes/Mg/K/Tris-buffered (pH 7.4) saline with 100 μM GTPγS or aluminium fluoride (AlF_n: added as 50 μM $AlCl_3$ +30 mM NaF); incubations were carried out at 37°C for 30 minutes and then membranes were recovered by pelleting (de Almeida et al., 1993). Samples (50-100 μg) of membranes or cytosol were separated on 5% SDS-PAGE gels and transferred to either nitrocellulose or Immobilon (Millipore) membranes. Proteins were immunoblotted with specific antibodies using 5% nonfat dried milk with 0.1% Tween-20 in phosphate buffered saline (PBS) as a blocking/wash buffer. Bound antibodies were detected with either alkaline phophatase conjugates or with HRP-conjugated secondary antibodies detected by chemiluminescence.

Immunoprecipitation

HeLa cell cytosol was prepared according to the method of Pimplikar et al. (1994) and MDCK cell cytosol as described by Ikonen et al. (1996). AD7 IgG was purified from an ammonium sulfate cut of culture supernatant by affinity chromatography on a Protein A column. Purified AD7 IgG was then coupled to Protein A-Sepharose beads by rotating overnight at 4°C in PBS, the beads were washed in PBS with 10% BSA and then with KOAc buffer. Cytosol was added to the beads and incubated for 6 hours on ice with intermittent mixing. The beads were spun down and bound proteins were analyzed by SDS-PAGE and Coomassie staining. AD7 IgG (15 μg) was used to deplete 100 μg of cytosol and, as a control, IgG P5D4 monoclonal against VSV G protein was used and found not to precipitate any bands around 200 kDa.

Amino acid sequence analysis

AD7 immunoprecipitation was carried out as described above using 8 mg of MDCK or HeLa cell cytosol as starting material. Selected protein bands were excised from Coomassie Blue stained gels; washing, reduction/alkylation and peptide extraction were carried out as described previoulsy (Jeno et al., 1995; Kurzchalia et al., 1992). The resulting peptides were separated on a 1.6 mm × 250 mm Vydac

C18 218TP column (MZ) with gradient elution at a flow rate of 120 μl/minute using a 140A pump system equipped with a 1000S diode array detector (Perkin Elmer). Fractions were collected manually and stored at –20°C until characterized. Automated Edman degradation of peptides was performed using model 494 Protein Sequencer (Perkin Elmer).

Immunofluorescence staining

MDCK strain II, PtK_1, NRK and HeLa cells were grown on coverslips. Cells treated and fixed in a variety of ways were used to visualize either cytoskeleton or Golgi staining patterns as follows: (i) cells fixed in ice-cold MEOH/acetic acid (3:1) for 10 minutes, (ii) cells fixed in 4% paraformaldehyde and then permeablized with 0.1% Triton X-100, (iii) cells treated with 0.1% Triton X-100 in PBS prior to fixation in paraformaldehyde, or (iv) cells treated for 5-30 minutes in brefeldin A (5 μg/ml) prior to fixation in paraformaldehyde or MEOH/acetic acid.

Fixed cells were incubated with primary antibodies and then with FITC or TRITC-conjugated secondary antibodies, using calf serum or BSA in blocking/washing buffers. F-actin was labelled by incubating fixed, permeabilized cells in FITC or TRITC-conjugated phalloidin (Molecular Probes). Coverslips were examined in an Axiophot microscope fitted for epifluorescence (Carl Zeiss). For some figures image analysis was performed using the Adobe photoshop program.

Immunoelectron microscopy

Immunogold labeling with AD7 and myosin II antibodies was carried out on perforated MDCK cells, by methods described previously (Ikonen et al., 1996). Briefly, monolayers of MDCK cells grown on semi-permeable filters (Transwell, Costar) were incubated at 20°C and then perforated with adherent nitrocellulose filters (Wadinger-ness et al., 1990). Cells were incubated in buffered cytosol containing 100 μM GTPγS (deAlmeida et al., 1993) to induce vesicle budding, fixed in 4% paraformaldehyde with 0.1% glutaraldehyde for 30 minutes and then blocked in buffer containing gelatin. Small pieces of filter were incubated sequentially with with AD7 antibody, anti-mouse IgG-conjugated to 10 nm gold particles, myosin II antibody, and Protein A conjugated to 5 nm gold particles. Cells on filters were postfixed, processed and embedded in Epon resin for thin sectioning. Sections were cut perpendicular to the filter, stained with lead citrate and uranyl acetate and viewed on a Jeol 1010 electron microscope.

RESULTS

Myosin II staining is associated with Golgi membranes

Myosin II was localized by immunofluorescence in a variety of cultured epithelial cell lines using an antiserum that recognizes nonmuscle myosin II. This antibody produced markedly different staining patterns in cells fixed either in methanol/acetic acid (MEOH) or cells fixed in 4% paraformaldehyde. MEOH-fixed NRK cells showed myosin II staining in punctate, filamentous arrays throughout the cells (Fig. 1a). In contrast, when NRK cells were fixed with paraformaldehyde, the predominant staining pattern was seen as intense and specific staining of the perinuclear Golgi complex, with more diffuse staining throughout the rest of the cytoplasm (Fig. 1b). The perinuclear staining of myosin II in these cells also colocalized with immunostaining of a Golgi

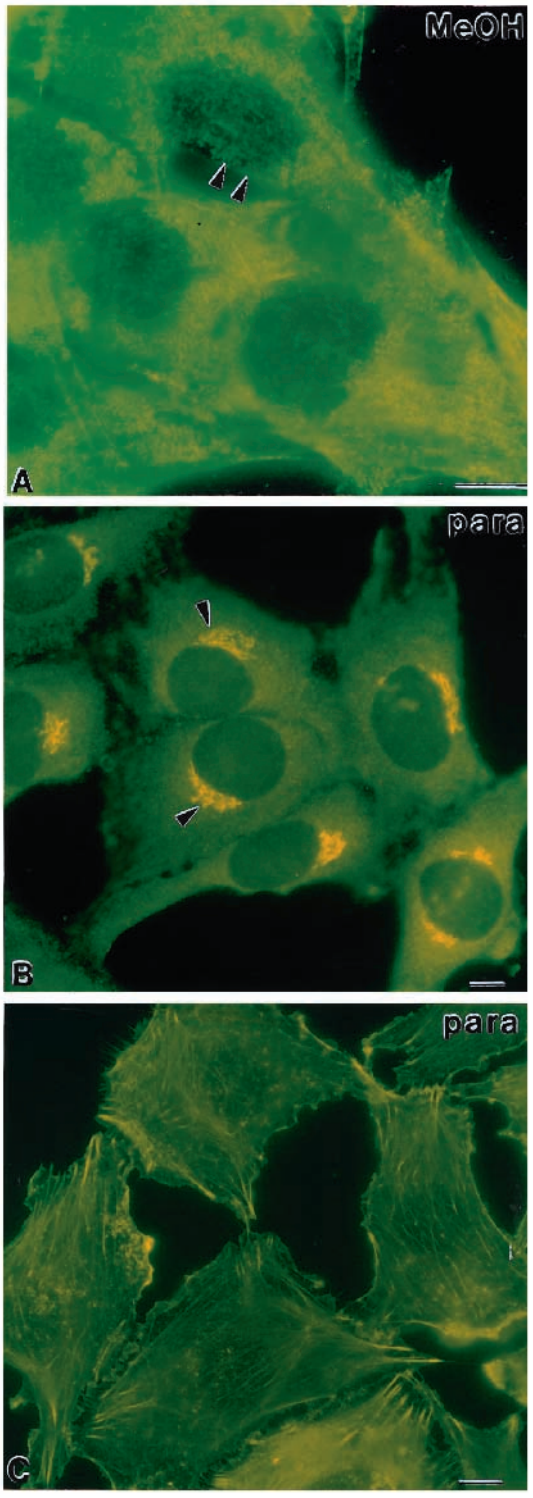

Fig. 1. Immunofluorescence staining of myosin II. NRK cells were fixed in either methanol (MEOH) or paraformaldehyde (para), as described in the text, and then stained with myosin-II antibody. (A) NRK cells fixed in MEOH show fine, punctate staining of myosin-II in stress fibre-like arrays coursing throughout the cells (arrowheads). (B) Coverslips from the same dish of NRK cells were fixed in para and stained for myosin II. Under these conditions a completely different pattern of staining is seen (arrowheads). There is intense staining of the perinuclear Golgi complex and diffuse staining throughout the cytoplasm. (C) FITC-phalloidin labeling of actin in para-fixed cells shows that, although there is no myosin II staining associated with stress fibres under these fixation conditions, there are still intact actin filaments in these cells. Bar, 20 μm.

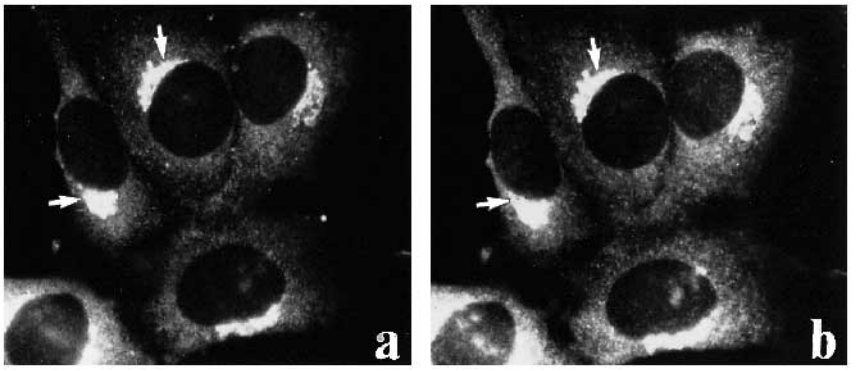

Fig. 2. Colocalization of a Golgi marker and myosin II in paraformaldehyde-fixed NRK cells. The same cells shown in Fig. 1b were double labeled with an antibody to mannosidase II (a) in addition to the myosin II antibody (b) followed by TRITC-conjugated anti-mouse and FITC-conjugated anti-rabbit IgGs. Intense staining (arrows) is obtained with both antibodies over the perinuclear Golgi complex.

marker, mannosidase II, as shown in Fig. 2. FITC-phalloidin labeling carried out on paraformaldehyde fixed NRK cells shows that, although there is no filamentous staining of myosin II visible in these cells, the cells under these conditions do still have intact actin filaments (Fig. 1c). Paraformaldehyde fixation thus preferentially highlights a membrane-associated pool of myosin II associated with the Golgi complex.

Activation of G proteins induces myosin II binding to Golgi membranes

The association of myosin II with Golgi membranes was also demonstrated by immunoblotting the myosin II protein (200 kDa) in fractions of isolated Golgi membranes. Large amounts of myosin II were detected in the cytosol and on total microsomal membranes prepared from NRK cells (Fig. 3a). Fractions enriched in Golgi membranes were isolated from homogenates of NRK cells and this fraction was also found to contain myosin II (Fig. 3a). Myosin II can also be detected by western blotting in fractions of highly purified Golgi membranes prepared from rat liver (Fig. 3b) or from intestinal epithelial cells (Fath et al., 1994). In vitro binding assays have been used to demonstrate that the binding of several other cytosolic proteins to Golgi vesicle membranes is initiated by G protein activation (de Almeida et al., 1993; Donaldson et al., 1991; Robinson and Kries, 1992). Rat liver Golgi membranes were incubated with cytosol or with cytosol plus GTPγS or aluminium fluoride. In the presence of either activator, there was significantly more of the cytosolic myosin II bound to the Golgi membranes compared to the control incubation (Fig. 3b). Thus the association of myosin II with Golgi membranes is able to be regulated and is enhanced by the action of G proteins, in manner consistent with other Golgi-associated proteins.

Myosin II association with Golgi membranes is sensitive to brefeldin A (BFA)

The drug brefeldin A (BFA) has been shown to inhibit the membrane binding of peripherally attached coat proteins to Golgi membranes (Donaldson et al., 1990; Narula et al., 1992; Robinson and Kries, 1992). The effect of BFA on the Golgi staining of myosin II was therefore tested. Treatment (>5 minutes) of NRK cells with BFA resulted in a rapid disappearance of the Golgi-associated myosin II staining, with a concomitant increase in the diffuse cytoplasmic staining (Fig. 4a,b). However, in the presence of BFA under the same conditions, the staining pattern of myosin II associated with actin filaments was unchanged (Fig. 4c,d). BFA-resistant cells, such as PtK_1 cells, show no redistribution of Golgi-associated proteins or membrane compartments in the presence of BFA (Donaldson et al., 1990). Similarly, we found that there was no alteration in the Golgi staining of myosin II in PtK_1 cells treated for up to 30 minutes with BFA (Fig. 4e,f). PtK_1 cells have particularly abundant stress fibres and in paraformaldehyde-fixed cells, myosin II staining associated with both the Golgi and with actin filaments could be seen simultaneously, and both patterns remained unchanged in untreated and BFA-treated cells (Fig. 4e,f).

Taken together, these data suggest that a pool of cytosolic myosin II can associate dynamically and peripherally with Golgi membranes. The association of myosin II with Golgi membranes is enhanced by activation of G proteins and disrupted by BFA, similar to the binding of several other peripheral Golgi proteins, but its association with actin filaments is unaffected by BFA.

The AD7 and myosin II antibodies produce the same Golgi staining patterns

Previous data has shown that, in paraformaldehyde-fixed cells, the AD7 antibody gives intense staining of p200 on the Golgi complex and more diffuse staining in the cytoplasm (Ikonen et al., 1996; Narula et al., 1992). The staining patterns obtained with the AD7 antibody and with the myosin II antibody (Fig. 1b) under this fixation protocol thus appear to be similar. To test

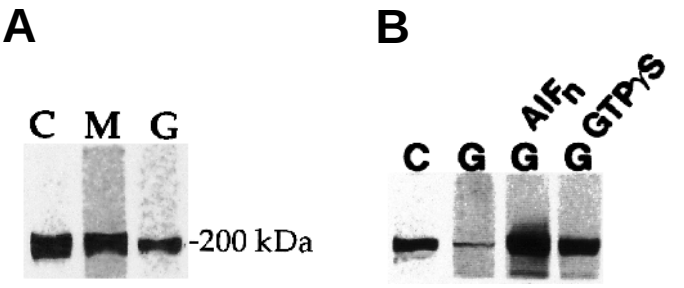

Fig. 3. G protein regulated-binding of myosin II binding to Golgi membranes. (A) Western blotting of cell fractions with the myosin II antibody. Samples of cytosol (C; 100 μg protein), total microsomal membranes (M; 100 μg protein) and Golgi membranes (G; 50 μg protein) from NRK cell homogenates all contained the 200 kDa band recognized by the myosin II antibody. (B) Rat liver Golgi membranes (G) and cytosol (C) fractions blotted for myosin II. Golgi membranes were incubated in vitro with cytosol, or with cytosol plus aluminium fluoride or GTPγS, as described in the text. Increased amounts of myosin II from the cytosol are bound to membranes in the presence of the these two G protein activators (lanes 3,4).

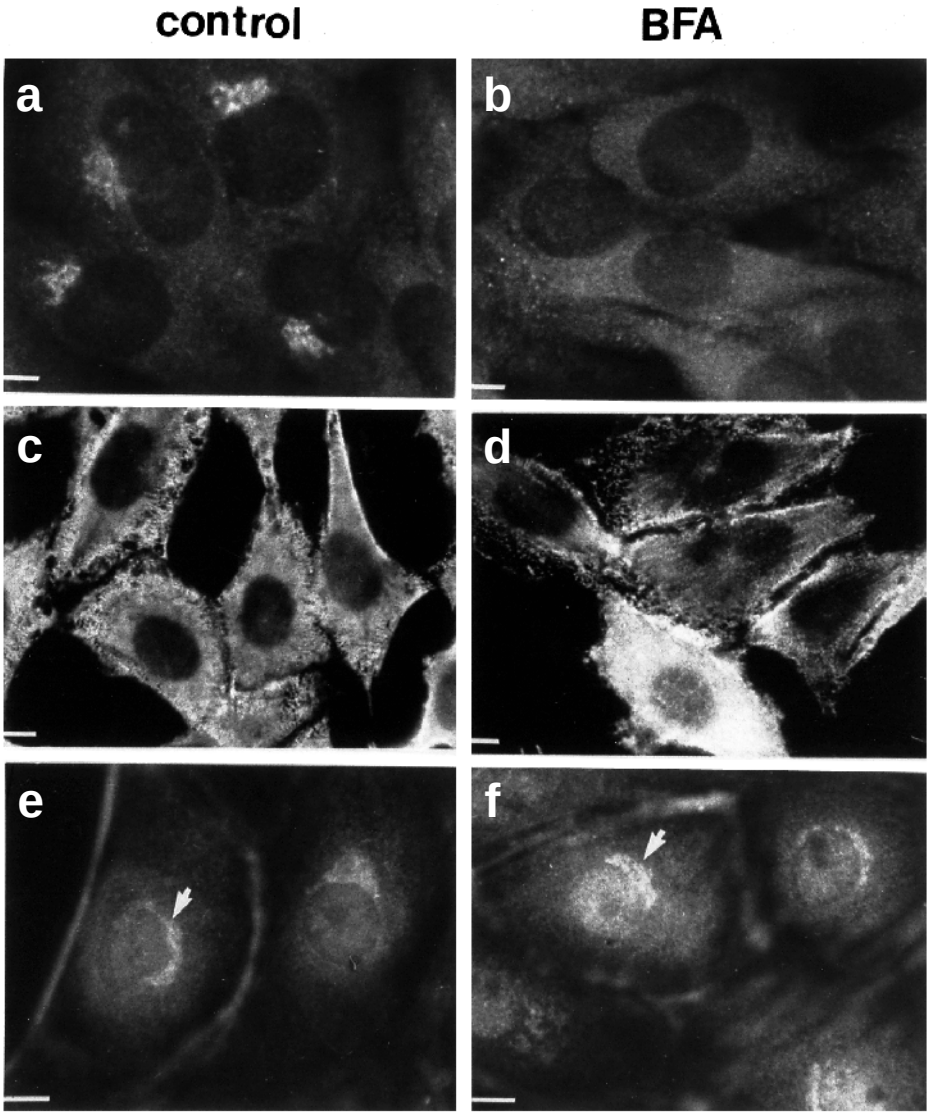

Fig. 4. Myosin II staining on Golgi membranes is sensitive to BFA. NRK cells were fixed in paraformaldehyde (a,b) or in MEOH (c,d) and stained for myosin-II. The Golgi-associated staining in control cells (a) is dispersed into the cytoplasm by pretreatment of cells with BFA (b). Myosin II staining associated with actin in stress fibers is not disrupted by BFA treatment (c,d). BFA-resistant, PtK1 cells were fixed in paraformaldehyde and stained for myosin II. Pretreatment of the cells with BFA (f) did not alter the staining pattern seen in control cells (e). PtK1 cells have large stress fibers and myosin-II staining on Golgi membranes and on stress fibers at the cell edges can be seen simultaneously (arrows). Bars, 20 μm.

whether the AD7 antibody also stains the cytoskeleton, MDCK cells were treated briefly with detergent prior to paraformaldehyde fixation, leaving the cytoskeleton intact. Cells stained under these conditions (Fig. 5a), or after MEOH fixation (not shown) had AD7 staining on stress fibres and actin filaments, in addition to the perinuclear Golgi staining. Thus both the AD7 and the myosin II antibodies label epitopes in the perinuclear Golgi complex and associated with the actin cytoskeleton.

A series of double-labeling experiments was performed to directly compare the staining patterns obtained with both antibodies. Double-labeling of untreated, paraformaldehyde fixed MDCK cells with the AD7 and myosin II antibodies showed immunofluorescence staining on the perinuclear Golgi complex and more diffuse staining in the cytoplasm (Fig. 5b,c). Within the bounds of resolution offered by immunofluorscence at the light microscopy level, the Golgi staining produced by both antibodies appeared near identical over perinuclear Golgi regions (Fig. 5b,c). Images of cells stained with the AD7 antibody show staining of distinct patches within the Golgi complex and the same patches are stained more intensely with the myosin II antibody. Double-labeling by immunofluorescence was also performed on NRK cells. The separate patterns obtained with AD7 and myosin II antibodies show similar staining over the Golgi complex (Fig. 6). To judge the level of level overlap in staining, the color images obtained with FITC-labeled AD7 staining and TRITC-labeled myosin II staining were superimposed. The resulting image (Fig. 6) shows coincident (yellow) labeling of the perinuclear Golgi complex, with somewhat more disparate labeling in the cytoplasm. Together, these results show that, at the light microscopy level, the AD7 and myosin II antibodies appear to colocalize over the Golgi complex in different cell types.

Double-labeling with the AD7 and myosin II antibodies was then performed at the electron microscopy level. Immunogold labeling was carried out on perforated MDCK cells, incubated with cytosol and GTPγS to maximize vesicle budding on the Golgi complex (Fig. 7). Examination of the sections showed gold labeling around the remnant Golgi complexes in the perinuclear area (Fig. 7), in wider views of the cell there was no labeling of any other membranes or organelles. There was heavy gold labeling of vesicles budding from the Golgi stacks and most vesicles were labeled with both sized gold particles

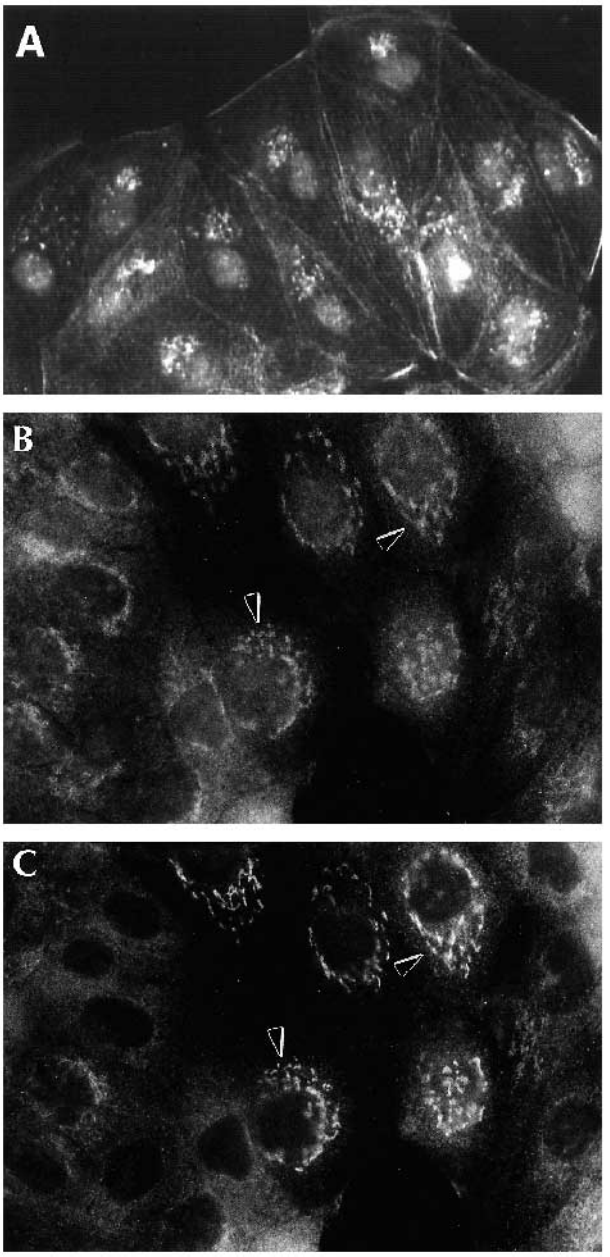

Fig. 5. Immunofluorescence staining with the AD7 and myosin II antibodies. MDCK cells were treated with 0.1% Triton-X 100 prior to fixation in paraformaldehyde (A), or were untreated before paraformaldehyde fixation (B,C). Staining with the AD7 antibody on extracted cells (A) shows filamentous staining of stress fibers in addition to staining of the Golgi complex. In B and C, untreated fixed cells were double labeled with AD7 (B) and myosin II (C) antibodies. Both images show staining of the scattered patches of the perinuclear Golgi complex and faint diffuse staining of the cytoplasm. Within the Golgi complex, the antibodies both stain the same structures (arrowheads) and overall have very similar staining patterns.

indicating staining by the AD7 and myosin II antibodies. These results show that the nonmuscle myosin II antibody labels Golgi-derived vesicles in the same pattern as that previously shown for p200 (Gleeson et al., 1996; Ikonen et al., 1996;

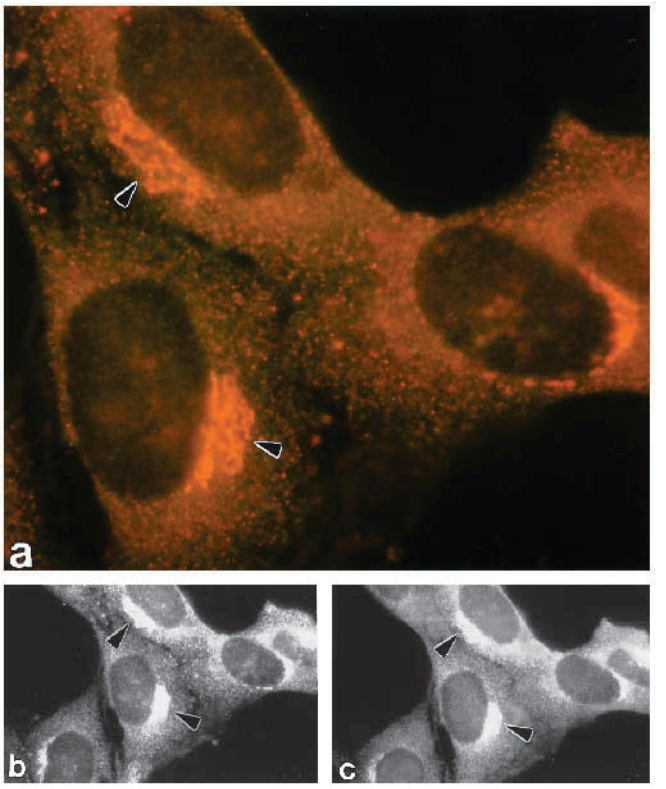

Fig. 6. Double-labeling of paraformaldehyde-fixed NRK cells with the myosin II antibody (TRITC-labeled; red) and with AD7 (FITC labeled; green). In the color image of the double labeling (a) the convergance of the two staining patterns produces the combined yellow fluorescence (arrowheads). Independent images of the staining patterns obtained with the myosin II antibody (b) and the AD7 antibody (c) are shown also. Both antibodies show prominent staining of the perinuclear Golgi complexes which have completely overlapping yellow-coloured staining, with more diffuse staining of the cytoplasm.

Narula and Stow, 1995). This direct double labeling further shows that the antibodies specifically label the same population of Golgi-derived vesicles. In the images shown in Fig. 7, the AD7 antibody was applied first giving more abundant labeling but this trend was reversed if the myosin II antibody was applied first (not shown), consistent with the two antibodies competing for closely-positioned epitopes on vesicle-associated protein(s). The likelihood of them seeing the same, or closely-related proteins, was therefore further investigated.

Amino acid sequencing identifies the protein(s) recognized by the AD7 antibody

The AD7 antibody has been used to screen a wide range of cell types by western blotting (de Almeida et al., 1993; Ikonen et al., 1996; Narula et al., 1992; Narula and Stow, 1995). The antibody primarily recognizes a soluble phosphoprotein, p200, that migrates at 200 kDa on SDS-PAGE gels and is present in many cell types (Figs 3 and 8). It has previously been noted that in the HeLa cell line the AD7 antibody uniquely and primarily recognizes a higher molecular mass protein (Gleeson et al., 1996; Ikonen et al., 1996). In extracts of HeLa cells, an additional, slower migrating band which is estimated to be ~240 kDa on the current gel system, is labeled by western

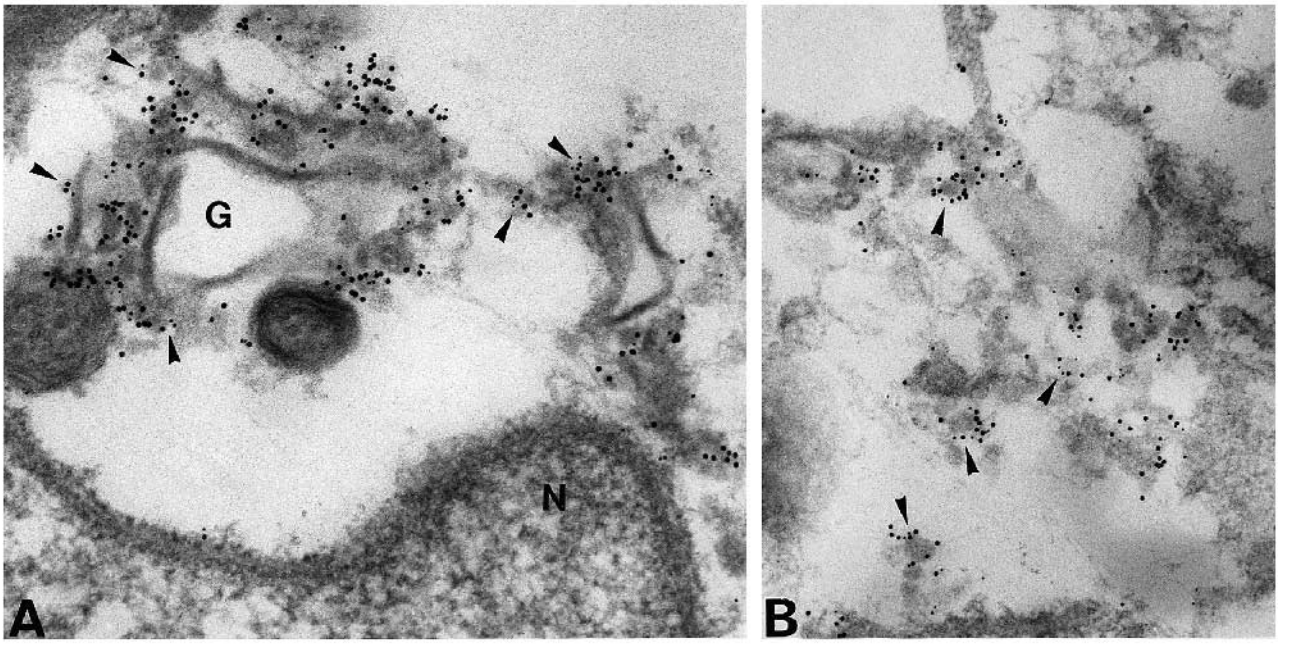

Fig. 7. Immunogold labeling on perforated MDCK cells. Perforated cells were incubated to induce vesicle budding, as described in the text, and then used for immuno-labeling with AD7 and myosin II antibodies. Membranous organelles in the perforated cells can be seen in the sections and these images show magnified areas containing the perinuclear Golgi stacks with clusters of budding vesicles. (A) Perinuclear Golgi (G) stacks show cisternae and clusters of budding vesicles. Double-labeling with AD7 antibody (10 nm gold) and myosin II antibody (5 nm gold) shows labeling around the budding vesicles. Many of the vesicles are labeled with both antibodies (arrowheads). There is no specific labeling on the nucleus (N) or mitochondria shown in this panel. (B) This view shows clusters of budding vesicle profiles which were seen adjacent to a Golgi complex. Many of the vesicles are labeled on their cytoplasmic surfaces by both antibodies (arrowheads).

blotting with the AD7 antibody (Fig. 8). Western blotting of cytosol from two different canine cell lines and HeLa cells with the myosin II antibody confirms that this antibody sees a 200 kDa band in all cells and that it does not recognize the 240 kDa band in HeLa cells (Fig. 9). Analysis of total protein patterns of MDCK and HeLa cytosol shows that the 200 kDa band is most abundant in MDCK cells, while the 240 kDa band is unusually abundant in HeLa cell cytosol (Fig. 10). The AD7 antibody immunoprecipitated only the 200 kDa band from MDCK cell cytosol, while from HeLa cells it immunoprecipitated predominantly the 240 kDa band with a smaller amount of the 200 kDa band (Fig. 10).

In order to obtain amino acid sequence of the 200 kDa band recognized by the AD7 antibody, this band was immunoprecipitated from large scale preparations of MDCK cell cytosol, cut out of gels and used for microsequencing. Sequence data obtained from four peptides derived from the 20 kDa band all correspond to the published sequence of the heavy chain of nonmuscle myosin IIA (Saez et al., 1990; Simons et al., 1991). The four peptides mapped to both the actin-binding head and to the coil-coil tail regions of myosin II (Fig. 11A.) The identity of p200 as myosin IIA was also confirmed by performing matrix assisted laser desorption mass spectrometry (MALDI-MS) on the peptide mixture obtained from an in-gel tryptic digest of the 200 kDa protein. Ten peptides with masses within 0.1 Da of the expected mass were found, covering in total 176 amino acids (9%) of the protein sequence (data not shown).

The 240 kDa band from AD7 immunoprecipitates of HeLa extracts was also cut out and used for sequencing. Amino acid sequence shows that the 240 kDa band is a different protein, identified as nonmuscle filamin (Fig. 11B). This identification

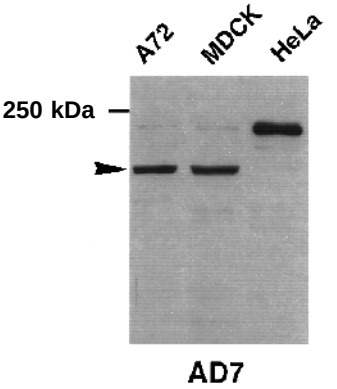

Fig. 8. Western blotting of 200 kDa and 240 kDa proteins with the AD7 antibody. Homogenates of A72, MDCK and HeLa cells run on SDS-PAGE gels were transferred and blotted with the AD7 monoclonal antibody. In A72 and MDCK cells a prominent band at 200 kDa is recognized (arrowhead), whereas in HeLa cells an alternative band at ~240 kDa is labelled by the same monoclonal antibody.

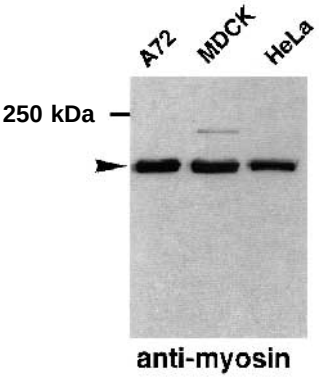

Fig. 9. Western blotting of myosin II. Cell extracts from A72, MDCK and HeLa cell extracts were blotted with the myosin II antibody showing that the 200 kDa band (arrowhead) is detected in all cell lines.

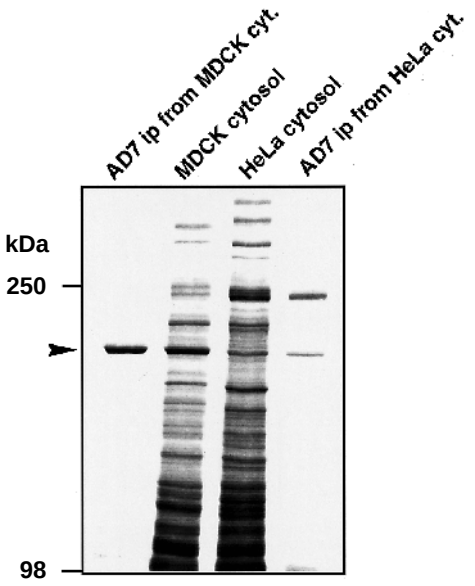

Fig. 10. AD7 recognizes distinct, abundant proteins in MDCK and HeLa cells. Coomassie stained gels of the cytosol fractions from MDCK and HeLa cells were compared with stained AD7 immunoprecipitates from the two cytosols. AD7 recognizes the 200 kDa band in MDCK cells (arrowhead), which is an abundant protein in this cytosol. In HeLa cell cytosol the 240 kDa band is especially prominent and this protein is preferentially immunoprecipitated by the AD7 antibody.

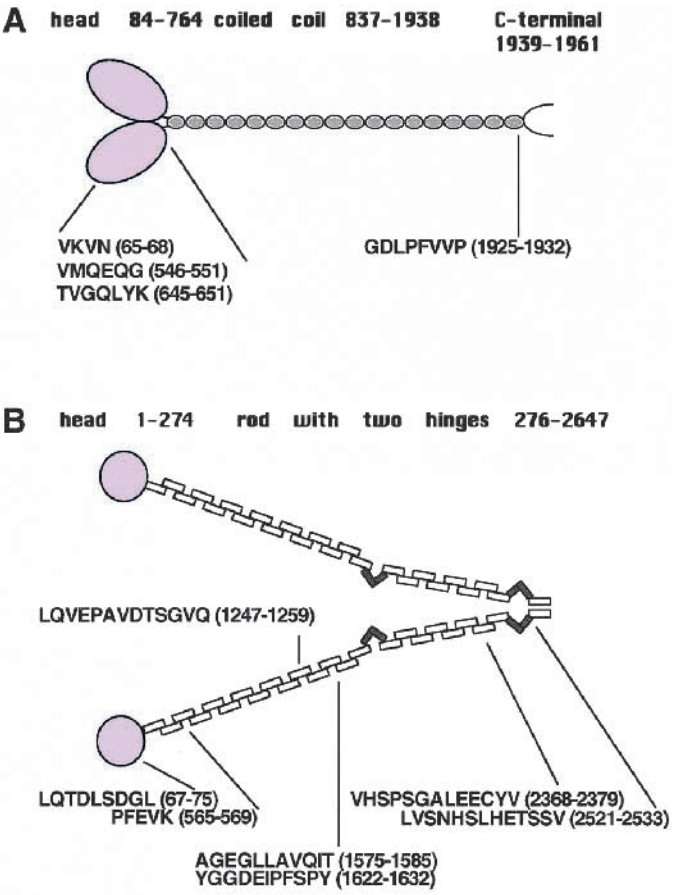

Fig. 11. Peptide sequences obtained from proteins immunoreactive with the AD7 antibody. Amino acid sequence data obtained from peptides isolated from the 200 kDa and 240 kDa proteins immunoprecipitated by the AD7 antibody. (A) Peptides from the 200 kDa protein immunoprecipitated from MDCK cells by AD7 correspond to regions in the globular head and coiled-coil tail of the heavy chain of nonmuscle myosin IIA. Sequences of 4 matching peptides are shown. (B) Peptides from the 240 kDa band immunoprecipitated by AD7 from HeLa cells correspond to sequences in the head, rod and hinge regions of the actin-binding protein, filamen. The sequences of 7 matching peptides are shown.

was based on sequence from 7 peptides ranging from 5-13 amino acids each, derived from different parts of the protein, including the head, rod and hinges. Nonmuscle filamin (ABP-280), 280 kDa, is another actin-binding protein which cross-links actin filaments into networks in the cortical web and serves as a link to membrane glycoproteins (Gorlin et al., 1990). These data indicate that, in addition to its main reactivity to p200/myosin II in all cells, the AD7 monoclonal antibody also cross-reacts with filamin in HeLa cells. Amino acid sequence from the 200 kDa band in HeLa cells shows that it is also myosin II (data not shown). However, the main reactivity of the AD7 antibody in most cells is against the 200 kDa, p200 /myosin II protein and the staining patterns obtained with the AD7 antibody in all cells is consistent with its recognition of myosin II.

DISCUSSION

The data presented here support the novel finding that nonmuscle myosin II is specifically and dynamically associated with Golgi membranes. Pools of myosin II associated with both actin filaments and with Golgi membranes were individually detected by immunofluorescence under different fixation conditions. The more traditional methods for visualizing cytoskeleton in cells, using either alcohol fixatives or treatment with detergent prior to fixation, resulted in punctate staining of myosin II associated with actin filaments. However, dramatically different staining was seen in paraformaldehyde-fixed cells where this filamentous staining was replaced by diffuse cytoplasmic staining and intense staining of the perinuclear Golgi complex. In some cells, especially PtK_1 cells which have very prominent stress fibres, myosin II staining on both actin filaments and the Golgi complex could be seen simultaneously, confirming that nonmuscle myosin II does exist simultaneously in both locations. The most cohesive interpretation of the staining results is that different fixation conditions unmask different epitopes on myosin II residing on either the Golgi or on stress fibres.

Nonmuscle myosin II has been localized most commonly at the leading edges of cultured cells, in association with stress fibres and in cleavage furrows of mitotic cells (Kelley et al., 1996; Maupin et al., 1994; Rochlin et al., 1995) commensurate with its known functions in cell motility and cytokinesis. Punctate staining of myosin II in the cytoplasm of nonmuscle cells has been described in many previous studies and has been attributed to detection of clusters of myosin II filaments, while diffuse staining is thought to represent non-filamentous myosin II (Herman and Pollard, 1981; Langanger et al., 1986; Maupin et al., 1994). Nonmuscle myosin II has also been shown in association with actin as punctate staining along stress fibres in interphase cells, in addition to other specific locations (Kelley et al.,

1996; Maupin et al., 1994; Miller, 1992). All of the cell types used in the present study had these staining patterns ie, diffuse cytoplasmic staining, punctate staining and staining of stress fibres, and thus appear to have pools of myosin II in different configurations throughout the cytoplasm, in addition to Golgi-membrane associated myosin II. Isoform specific antibodies have been used in some cells to demonstrate the differential intracellular distributions of myosins IIA and B. Myosin IIB has been localized in a variety of cell types where it appears to be particularly concentrated in the leading edges of growth cones in neuronal cells (Cheng et al., 1992; Rochlin et al., 1995) and in lamellipodia in non-neuronal cells (Kelley et al., 1996; Maupin et al., 1994); whereas myosin IIA is particularly concentrated at the mitotic spindle in dividing cells (Kelley et al., 1996). In all, these studies seem to indicate diverse intracellular locations of myosin II isoforms that may differ between cell types and at different stages of the cell cycle. The association of nonmuscle myosin II with the Golgi complex represents a novel location, and potentially new functions for this motor protein.

Our results indicate that myosin II associates dynamically with Golgi membranes. We showed, for instance, that binding of myosin II to Golgi membranes can be inhibited by BFA and enhanced by G protein activators, in a similar fashion to vesicle coat proteins and other peripheral membrane proteins, including β-COP, clathrin adaptors, ARF (ADP-ribosylation factor) and spectrin (Lippincott-Schwartz et al., 1989; Robinson and Kreis, 1992; de Almeida et al., 1993; Beck et al., 1994). These binding characteristics have been attributed largely to the requirement for GTP-bound ARF, acting as an initiator of coat binding perhaps through its role in regulating phospholipase D acitivity (Boman and Kahn, 1995). At this stage, it is not known whether ARF is also directly responsible for regulating the binding of myosin II to the Golgi. Previous ultrastructural localization of p200 has established that it is associated with a particular subset of vesicles emerging from the TGN (Narula and Stow, 1995; Gleeson et al., 1996; Ikonen et al., 1996). Consistent with the other features of its Golgi association, immunoelectron microscopy confirms that the myosin II labeling is also associated with Golgi-derived vesicles; immunogold labeling was concentrated on the budding vesicles rather than on Golgi cisternae in perforated MDCK cells. The p200 and myosin II labeling were on the same population of vesicles.

It is not clear whether myosin II is bound directly to Golgi membranes or through a series of intermediary proteins such as complexes of actin-binding proteins and actin. It has been reported that the tail of myosin II can interact directly with specific phospholipids (Li et al., 1994; Murakami et al., 1994) thereby giving it the ability to bind of its own accord to membranes. Spectrin is an actin-binding protein, known for its role in attaching actin filaments to the plasma membrane via other interacting proteins and the cytoplasmic tails of membrane proteins. β-spectrin has been localized on Golgi membranes in epithelial cells (Beck et al., 1994). Since dynactin is also reported to be associated with the Golgi (Fath et al., 1994), one scenario for myosin II binding may be through the actin-related protein component of dynactin, which may also bind to the actin-binding site on spectrin.

p200 is a very abundant, ubiquitous, 200 kDa, phosphoprotein found in the cytosol of many cell types, features consistent with known attributes of nonmuscle myosin II. Our data now provide the first evidence as to the identity of the Golgi vesicle-associated protein called p200. The present study shows that the amino acid sequences obtained from p200 are identical to the heavy chain of nonmuscle myosin IIA. Importantly, these sequences span regions of both the head and tail of the myosin II molecule (as shown in Fig. 11), indicating that this homology is not based merely on a structural domain similarity. By western blotting on isolated rat liver Golgi membrane fractions we have found that the AD7 antibody and several different antibodies to nonmuscle myosin IIA stain the same protein, whereas antibodies to nonmuscle myosin IIB and other myosins do not stain the same band (K. Heimann and J. L. Stow, unpublished data). It was previously noted that p200 is heavily phosphorylated (Narula et al., 1992). This feature is also consistent with myosin II since there are multiple phosphorylation sites in both the light chains and heavy chains of myosin II (reviewed by Tan et al., 1992). The reported effects of myosin II heavy chain phosphorylation include a decrease in ATPase activity and a decrease in filament formation, although the complete range of events regulated by individual phosphorylation sites has not been fully defined. The finding that the AD7 monoclonal antibody also recognizes another actin-binding protein, filamin in HeLa cells, suggests that an antigenic epitope is shared by both proteins. As their primary structures have no homology (the longest stretch of identical amino acids is 3) the common epitope may be formed by structural motifs, such as their rod-like tails.

Detection of myosin II on the Golgi now adds another potential dimension to the range of molecular motors and cytoskeleton-associated proteins involved in vesicle trafficking. The amino acid sequence identity of p200 with the heavy chain of myosin II indicates that p200 itself can now be considered as a Golgi-associated nonmuscle myosin II. A variety of other, unconventional myosins and microtubule-based motors already been shown to associate with Golgi membranes or with specific vesicle populations. The function of this nonmuscle myosin II, which is present on a very selective population of Golgi-derived vesicles, is not yet known. Its identification now raises the question as to whether equivalent myosin motors are required on other vesicle populations for roles in budding or trafficking.

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REFERENCES

Adams, R. J. and Pollard, T. D. (1986). Propulsion of organelles isolated from Acanthamoeba along actin filaments by myosin-I. *Nature* **322**, 754-756.

Beck, K. A., Buchanan, J., Malhotra, V. and Nelson, W. J. (1994). Golgi spectrin: identification of an erythroid b-spectrin homolog associated with the Golgi complex. *J. Cell Biol.* **127**, 707-723.

Boman, A. L. and Kahn, R. A. (1995). Arf proteins: the membrane traffic police? *Trends Biochem. Sci.* **20**, 147-150.

Broschat, K. O., Stidwell, R. P. and Burgess, D. R. (1983). Phosphorylation

controls brush border motility by regulating myosin structure and association with the cytoskeleton. *Cell* **35**, 561-571.

Cheney, R. E. and Mooseker, M. S. (1992). Unconventional myosins. *Curr. Opin. Cell Biol.* **4**, 27-35.

Cheney, R. E., Riley, M. A. and Mooseker, M. S. (1993). Phylogenetic analysis of the myosin superfamily. *Cell Motil. Cytoskel.* **24**, 215-223.

Cheng, T. P. O., Murakami, N. and Elzinga, M. (1992). Localization of myosin-IIB at the leading edge of growth cones from rat dorsal-root ganglionic cells. *FEBS Lett.* **311**, 91-94.

de Almeida, J. B., Doherty, J., Ausiello, D. A. and Stow, J. L. (1993). Binding of the cytosolic p200 protein to Golgi membranes is regulated by heterotrimeric G proteins. *J. Cell Sci.* **106**, 1239-1248.

Donaldson, J. G., Schwartz, J. L., Bloom, G. S., Kreis, T. E. and Klausner, R. D. (1990). Dissociation of a 110-kD peripheral membrane protein from the Golgi apparatus is an early event in brefeldin A action. *J. Cell Biol.* **111**, 2295-2306.

Donaldson, J. G., Lippincott, S. J. and Klausner, R. D. (1991). Guanine nucleotides modulate the effects of brefeldin A in semipermeable cells: regulation of the association of a 110-kD peripheral membrane protein with the Golgi apparatus. *J. Cell Biol.* **112**, 579-588.

Durbach, A. Collins, K., Matsudaira, P., Louvard, D. and Coudrier, E. (1996). Brush border myosin-I truncated in the motor domain impairs the distribution and the function of endocytic compartments in an hepatoma cell line. *Proc. Nat. Acad. Sci. USA* **93**, 7053-7058.

Ehrenreich, J. H., Bergeron, J. J. M., Siekevitz, P. and Palade, G. E. (1973). Golgi fractions prepared from rat liver homogenates. 1. isolation procedure and morphological characterization. *J. Cell Biol.* **59**, 45-72.

Fath, K. R. and Burgess, D. R. (1993). Golgi-derived vesicles from developing epithelial cells bind actin filaments and possess myosin-I as a cytoplasmically oriented peripheral membrane protein. *J. Cell Biol.* **120**, 117-127.

Fath, K. R. and Burgess, D. R. (1994). Membrane motility mediated by unconventioanl myosin. *Curr. Opin. Cell. Biol.* **6**, 131-135.

Fath, K. R., Trimbur, G. M. and Burgess, D. R. (1994). Molecular motors are differentially distributed on Golgi membranes from polarized epithelial cells. *J. Cell Biol.* **126**, 661-675.

Gleeson, P. A., Anderson, T. A., Stow, J. L., Griffiths, G., Toh, B. H. and Matheson, F. (1996). P230 is associated with vesicles budding from the *trans*-Golgi network. *J. Cell Sci.* **109**, 2811-2821.,

Gorlin, J. B., Tamin, R., Egan, S., Stewart, M., Stossel, T. P., Kwiatkowski, D. J. and Hartwig, J. H. (1990). Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. *J. Cell Biol.* **111**, 1089-1105.

Govindan, B., Bowser, R. and Novick, P. (1995). The role of Myo2, a yeast class V myosin, in vesicular transport. *J. Cell Biol.* **128**, 1055-1068.

Hasson, T. and Mooseker, M. S. (1995). Molecular motors, membrane movements and physiology: emerging roles for myosins. *Curr. Opin. Cell Biol.* **7**, 587-594.

Herman, I. M. and Pollard, T. D. (1981). Electron microscopic localization of cytoplasmic myosin with ferritin-labeled antibodies. *J. Cell Biol.* **88**, 346-351.

Hirokawa, N. (1996). Organelle transport along microtubules- the role of KIFs. *Trends Cell Biol.* **6**, 135-141.

Hollenbeck, P. J. and Swanson, J. A. (1990). Radial extension of macrophage tubular lysosomes supported by kinesin. *Nature* **346**, 864-866.

Ikonen, E., Parton, R. G., Lafont, F. and Simons, K. (1996). Analysis of the role of p200 containing vesicles in post-Golgi traffic. *Mol. Biol. Cell* **7**, 961-974.

Jeno, P., Mini, T., Hintermann, E. and Horst, M. (1995). Internal sequences in proteins digested from polyacrylamide gels. *Anal. Biochem.* **224**, 451-455.

Johnston, G. C., Prendergast, J. A. and Singer, R. A. (1991). The saccharomyces cerevisiae MYO2 gene encodes an essential myosin for vectorial transport of vesicles. *J. Cell Biol.* **113**, 539-551.

Katsuragawa, Y., Yanagisawa, M., Inoue, A. and Masaki, T. (1989). Two distinct nonmuscle myosin heavy chain mRNAs are differently expressed in in various chicken tissues. *Eur. J. Biochem.* **184**, 611-616.

Kawamoto, S. and Adelstein, R. S. (1991). Chicken nonmuscle myosin heavy chains: differential expression of two mRNAs and evidence for two different polypeptides. *J. Cell Biol.* **112**, 915-924.

Kelley, C. A., Sellers, J. R., Gard, D. L., Bui., D., Adelstein, R. S. and Baines, I. C. (1996). Xenopus nonmuscle myosin heavy chain isoforms have different subcellular localizations and enzymatic activities. *J. Cell Biol.* **134**, 675-687.

Kurzchalia, T., Gorvel, J., Dupree, P., Parton, R., Kellner, R., Houthaeve, T., Gruenberg, J. and Simons, K. (1992). Interactions of rab5 with cytosolic proteins. *J. Biol. Chem.* **267**, 18419-18423.

Langanger, G., Moeremans, M., Daneels, G., Sobieszek, A., DeBrabander, M. and DeMay, J. (1986). The molecular organization of myosin in stress fibers of cultured cells. *J. Cell Biol.* **102**, 200-209.

Li, D., Miller, M. and Chantler, P. D. (1994). Association of a cellular myosin II with anionic phospholipids and the neuronal plasma membrane. *Proc. Nat. Acad. Sci. USA* **91**, 853-857.

Lippincott-Schwartz, J., Yuan, L. C., Bonifacino, J. S. and Klausner, R. D. (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell* **56**, 801-813.

Lippincott-Schwartz, J., Cole, N., Marotta, A., Conrad, P. A. and Bloom, G. S. (1995). Roles for microtubules and kinesin in membrane traffic between the ER and Golgi complex. *J. Cell Biol.* **128**, 293-306.

Manstein, D. J., Titus, M. A., De Lozanne, A. and Spudich, J. A. (1989). Gene replacement in Dictyostelium: generation of myosin null mutants. *EMBO J.* **8**, 923-932.

Maupin, P., Phillips, C. L., Adelstein, R. S. and Pollard, T. D. (1994). Differential localization of myosin-II isozymes in human cultured cells and blood cells. *J. Cell Biol.* **107**, 3077-3090.

Miller, M., Bower, Levitt, P., Li, D. and Chantler, P. D. (1992). Myosin II distribution in neurons is consistent with a role in growth cone motility but not synaptic vesicle mobilization. *Neuron.* **8**, 25-44.

Murakami, N. and Elzinga, M. (1992). Immunohistochemical studies on the distribution of cellular myosin II isoforms in brain and aorta. *Cell Motil. Cytoskel.* **22**, 281-295.

Murakami, N., Elzinga, M., Singh, S. S. and Chauhan, V. P. S. (1994). Direct binding of myosin II to phospholipid vesicles via tail regions and phosphorylation of the heavy chains by protein kinase C. *J. Biol. Chem.* **269**, 16082-16090.

Narula, N., McMorrow, I., Plopper, G., Doherty, J., Matlin, K. S., Burke, B. and Stow, J. L. (1992). Identification of a 200-kD, brefeldin-sensitive protein on Golgi membranes. *J. Cell Biol.* **117**, 27-38.

Narula, N. and Stow, J. L. (1995). Distinct coated vesicles labeled for p200 bud from trans-Golgi network membranes. *Proc. Nat. Acad. Sci. USA* **92**, 2874-2878.

Pimplikar, S. W., Ikonen, E. and Simons, K. (1994). Basolateral protein transport in streptolysin O- permeabilized MDCK cells. *J. Cell Biol.* **125**, 1025-1035.

Robinson, M. S. and Kries, T. E. (1992). Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: Effects of brefeldin A and G protein activators. *Cell* **69**, 129-138.

Rochlin, M. W., Itoh, K., Adelstein, R. S. and Bridgman, P. C. (1995). Localization of myosin II A and B isoforms in cultured neurons. *J. Cell Sci.* **108**, 3661-3670.

Rothman, J. E. (1992). *Reconstitution of Intracellular Transport*. San Diego: Academic Press.

Rothman, J. E. (1994). Mechanisms of intracellular protein transport. *Nature* **372**, 55-63.

Ruppel, K. M. and Spudich, J. A. (1995). Myosin motor function: structural and mutagenic approaches. *Curr. Opin. Cell Biol.* **7**, 89-93.

Saez, C. G., Myers, J. C., Shows, T. B. and Leinward, L. A. (1990). Human nonmuscle myosin heavy chain mRNA: generation of diversity through alternative polyadenylation. *Proc. Nat. Acad. Sci. USA* **87**, 1164-1168.

Schekman, R. and Orci, L. (1996). Coat proteins and vesicle budding. *Science* **271**, 1526-1533.

Schroer, T. A., Steuer, E. R. and Sheetz, M. P. (1989). Cyotplasmic dynein is a minus end-directed motor for membranous organelles. *Cell* **56**, 937-946.

Simons, M., Wang, M., McBride, O. W., Kawamoto, S., Yamakawa, K., Gdula, D., Adelstein, R. S. and Weir, L. (1991). Human nonmuscle myosin heavy chain are encoded by two genes located on different chromosomes. *Circ. Res.* **69**, 530-539.

Takahashi, M., Kawamoto, S. and Adelstein, R. D. (1992). Evidence for inserted sequences in the head region of nonmuscle myosin specific to the nervous system. *J. Biol. Chem.* **267**, 17864-17871.

Tan, J. L., Ravid, S. and Spudich, J. A. (1992). Control of nonmuscle myosins by phosphorylation. *Annu. Rev. Biochem.* **61**, 721-759.

Wadinger-Ness, A., Bennett, M.K., Antony, C. and Simons, K. (1990). Distinct transport vesicles mediate the delivery of plasma membrane proteins to the apical and basolateral domains of MDCK cells.J. Cell Biol. 111, 987-1000.

Warrick, H. M. and Spudich, J. A. (1987). Myosin structure and function in cell motility. *Annu. Rev. Cell Biol.* **3**, 379-421.

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