A Specific Isoform of Nonmuscle Myosin II-C Is Required for Cytokinesis in a Tumor Cell Line*

Siddhartha S. Jana, Sachio Kawamoto, and Robert S. Adelstein

From the Laboratory of Molecular Cardiology, NHLBI, National Institutes of Health, Bethesda, Maryland 20892-1762

Received for publication, May 12, 2006
Published, JBC Papers in Press, June 21, 2006, DOI 10.1074/jbc.M604606200

Nonmuscle myosin IIs play an essential role during cytokinesis. Here, we explore the function of an alternatively spliced isoform of nonmuscle myosin heavy chain (NMHC) II-C, called NMHC II-C1, in the A549 human lung tumor cell line during cytokinesis. NMHC II-C1 contains an insert of 8 amino acids in the head region of NMHC II-C. First, we show that there is a marked increase in both the mRNA encoding NMHC II-C1 and protein in tumor cell lines compared with nontumor cell lines derived from the same tissue. Quantification of the amount of myosin II isoforms in the A549 cells shows that the amounts of NMHC II-A and II-C1 protein are about equal and substantially greater than NMHC II-B. Using specific siRNAs to decrease NMHC II-C1 in cultured A549 cells resulted in a 5.5-fold decrease in the number of cells at 120 h, whereas decreasing NMHC II-A with siRNA does not affect cell proliferation. This decreased proliferation can be rescued by reintroducing NMHC II-C1 but not NMHC II-A or II-B into A549 cells, although noninserted NMHC II-C does rescue to a limited extent. Time lapse video microscopy revealed that loss of NMHC II-C1 leads to a delay in cytokinesis and prolongs it from 2 to 8–10 h. These findings are consistent with the localization of NMHC II-C1 to the intercellular bridge that attaches the two dividing cells during the late phases of cytokinesis. The results suggest a specific function for NMHC II-C1 in cytokinesis in the A549 tumor cell line.

Nonmuscle myosin IIs belong to the conventional class II myosins, which form bipolar filaments at relatively low ionic strength and share a number of biological properties with skeletal, cardiac, and smooth muscle myosins (1). Nonmuscle myosin IIs are expressed in both muscle and nonmuscle cells and are hexamers, consisting of a pair of heavy chains (200 kDa) and two pairs of light chains (20 and 17 kDa). They are one of the major motor proteins interacting with cytoskeletal actin and are involved in regulating cytokinesis, cell motility, and cell polarity in many eukaryotic cells (1, 2).

To date, three isoforms of nonmuscle myosin heavy chain (NMHC) II, termed NMHC II-A, NMHC II-B, and NMHC II-C, have been identified in vertebrates (3–6). In humans, the genes (MYH9, MYH10, and MYH14) encoding these myosin heavy chains are located on different chromosomes: 22, 17, and 19, respectively (7, 8). All NMHC IIs are conserved with a 64–80% identity in amino acids among the various isoforms (6), suggesting that they might share some cellular functions, such as a role in cytokinesis and cell adhesion. For example, decreasing NMHC II-B in COS-7 cells results in multinucleation, a defect that can be rescued most efficiently by NMHC II-B but also to a significant extent by NMHC II-A and NMHC II-C (9). On the other hand, studies from a number of laboratories have also revealed that the different isoforms of nonmuscle myosin II have distinct tissue and subcellular distributions and apparently play different roles, particularly during development (10–16). Ablation of NMHC II-A in mice leads to a defect in cell adhesion during early development (15), whereas ablation of NMHC II-B results in defects in the heart and brain, two organs known to be enriched for this isoform (16).

Both NMHC II-B and II-C, but not NMHC II-A, undergo alternative splicing at homologous amino acids located in loop 1 and in loop 2 of their heavy chains. In the case of NMHC II-B, an exon encoding 10 amino acids is incorporated into loop 1 near the ATP binding region at amino acid 212 (NMHC II-B1), and an exon encoding 21 amino acids is inserted into loop 2 near the actin binding region at amino acid 622 (NMHC II-B2) (17). These inserted isoforms are only expressed in neuronal tissues, and recently, the results of ablating each of them in mice have been reported (18). For NMHC II-C, an alternative exon encoding 8 amino acids is incorporated into loop 1 (NMHC II-C1), and another alternative exon encoding 41 amino acids is introduced into loop 2 (NMHC II-C2) at locations homologous to the NMHC II-B inserts. Whereas the tissue distribution of NMHC II-C2 is similar to that of II-B1 and II-B2 in being confined to neuronal tissues,3 NMHC II-C1 is expressed in a variety of tissues, such as liver, kidney, testes, brain, and lung (6). In a recent report, it was demonstrated that the presence of the C1 insert in NMHC II-C increases both the actin-activated MgATPase activity and the in vitro motility of heavy meromyosin derived from this isoform (19). However, at present, little is known about the function of NMHC II-C1 at the cellular level.

In this study, we report that NMHC II-C1 expression is markedly increased in two different tumor cell lines and is the only NMHC II-C isoform present in a number of tumor cell lines. Our studies show that NMHC II-A and II-C1 are the only NMHC II-C isoform present in a number of tumor cell lines. Our studies show that NMHC II-A and II-C1 are the

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: NMHC, nonmuscle myosin heavy chain; NMHC II-C0, noninserted isoform of NMHC II-C; NMHC II-C1, inserted isoform of NMHC II-C; siRNA, small interfering RNA; GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; RT, reverse transcription.
2 The abbreviations used are: NMHC, nonmuscle myosin heavy chain; NMHC II-C0, noninserted isoform of NMHC II-C; NMHC II-C1, inserted isoform of NMHC II-C; siRNA, small interfering RNA; GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; RT, reverse transcription.
3 S. S. Jana, unpublished observation.
major isoforms in the A549 lung tumor cell line, each contributing ~45% of the total nonmuscle myosin II at the protein level. Decreasing nonmuscle myosin II isoforms using siRNA had a different effect on cytokinesis. Whereas decreasing nonmuscle myosin II-A had a significant effect on cell proliferation, lowering NMHC II-C1 decreased the number of cells 5.5-fold at 120 h by prolonging cytokinesis from 2 to 8–10 h. Decreasing NMHC II-C1 slowed the rate of cell proliferation by delaying the formation and retraction of the intercellular bridge that connects the two nascent daughter cells. Expression of exogenous NMHC II-C1-GFP, but not GFP-NMHC II-A or GFP-NMHC II-B, in the NMHC II-C1-depleted cells rescued the decrease in cell proliferation. These findings suggest a specific function for NMHC II-C1 in cytokinesis in the A549 tumor cell line.

**EXPERIMENTAL PROCEDURES**

Plasmid Constructs and siRNAs—The cytomegalovirus promoter from pEGFP-C3 (Clontech, Palo Alto, CA) was inserted into the AseI/AgeI sites, replacing the pTRE promoter in the expression plasmids containing the GFP-tagged, full-length cDNAs of human NMHC II-A and NMHC II-B (9, 20). Mouse noninserted NMHC II-C0 and inserted NMHC II-C1 (6) were cloned into the SalI/BamHI sites of the pEGFP-N3 expression vector (Clontech). Expression of all four full-length cDNAs encoding NMHC IIs in cell lines was confirmed by immunoblot analysis and fluorescence microscopy. An siRNA (II-C1 siRNA) specific for the human C1 inserted sequence (6) and siRNA (II-C siRNA) specific to the 3′-untranslated region of NMHC II-C were chemically synthesized by Qiagen (Valencia, CA). A pool (SMARTpool) of siRNAs specific for human NMHC II-A (Ref Seq accession number NM_002473) or NMHC II-B (Ref Seq accession number NM_005964) was chemically synthesized by Dharmacon Research, Inc. (Lafayette, CO). Fluorescein-labeled nonspecific siRNA from Qiagen were used to determine the efficiency of transfection and as a nonspecific control for siRNA experiments. Duplex siRNAs obtained from Qiagen were as follows: II-C1 siRNA duplex, sense strand (5′-r(CGUCAGCACCUGUCUUUA)d(TT)-3′) and antisense strand (5′-r(UAAAGACACGGUGUCAGC)d(GA)-3′); II-C siRNA duplex, sense strand (5′-r(GGACUGGAGCUACCUGCU)d(TT)-3′) and antisense strand (5′-r(AGCAAGGUACCUGCCAGC)d(TT)-3′); nonspecific siRNA duplex, sense strand (5′-r(UUCUCGAAGUCUGACG)d(TT)-3′) and antisense strand (5′-r(ACGGACUGUCCGGA)d(TT)-3′).

Cell Lines—Human A549 (lung carcinoma), MCF-7 and HCC1569 (breast carcinoma), HepG2 (liver carcinoma), PC-3 (prostate carcinoma), Panc-1 (pancreas carcinoma), NIH: OVCAR-3 (ovarian carcinoma), Beas-2B (nontumor lung), and MCF-10A (nontumor breast) were purchased from ATCC (Manassas, VA). All cell lines were maintained following standard ATCC protocols. For growth curves, cells were trypsinized and harvested at the indicated times, and the number of cells was counted using a hemocytometer.

The (−)-enantiomer of blebbistatin was purchased from Sigma and dissolved at 10 mM in Me2SO, 50 μM blebbistatin was added after 72 h of NMHC II-C1 siRNA treatment. Within 8 min after the addition of blebbistatin, time lapse imaging was started.

Transfection—1 μg of plasmid DNA/ml of culture medium and 200 nM siRNA were transfected using Effectene® and RNAiFect™ transfection reagents (Qiagen), respectively. Efficiency of siRNA transfection (as detected by the fluorescein signal) was >90%, and that for plasmid DNA (detected by the GFP signal) was 70–80%.

Reverse Transcription (RT)-PCR and Real Time RT-PCR—Total RNA from cell lines was isolated using the RNAasy minikit (Qiagen). 1 μg of total RNA was reverse-transcribed using random hexamers and the GeneAmp RNA PCR core kit (Applied Biosystems, Branchburg, NJ), and the resulting cDNA was amplified by PCR for individual gene products using specific primers. The primer sets were as follows: 5′-AACAGCCATCTCTGGT-GGA-3′ and 5′-ACCITGGCGCATAGTGGATA-3′ for human NMHC II-A; 5′-GCTGTTCACCAACCACATG-3′ and 5′-ACAGTTCCGCTGAACAGCTT-3′ for human NMHC II-B; 5′-ATGCTGAGGATCGTGGAG-3′ and 5′-ATGATTGCGGATCGTGGAG-3′ for human NMHC II-C; 5′-ATCTGGGACCACCACTTCTACATGAGTCGG-3′ and 5′-CGTCTAATCTTGCTGTATCCACATCTG-3′ for human β-actin. β-Actin was used to normalize the samples. PCR products were separated by 1.8% agarose gel electrophoresis and stained with ethidium bromide.

For quantitation, real time RT-PCR was performed. The cDNA was amplified by PCR using a Quantitect SYBR green PCR kit (Qiagen) using the primers described above. The amount of cDNA from tumor and nontumor cell lines was normalized using β-actin. For each experiment, a standard curve was generated using 4-fold serial dilutions of plasmid containing each of the cDNAs. When PCR products fell within the range of the standard curve, the amount of cDNA of each gene was calculated relative to the standard curve using the Opticon Monitor program. Samples were run in triplicate using a PCR program with an initial cycle of 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 54 °C (or 60 °C for II-C), and 1 min (or 30 s for II-C) at 72 °C. After each run, a melting curve was examined to ensure that no primer dimers or secondary products were formed. S.E. was calculated from three independent experiments.

**Immunoblot Analysis**—Extracts of various cell lines were prepared as described previously (6). Briefly, cells on tissue culture plates were washed twice with cold phosphate-buffered saline and directly lysed with Laemmli sample buffer. Proteins were separated by SDS-PAGE on 4–12% polyacrylamide gradient Tris–glycine gels or 6% polyacrylamide Tris–glycine gels (Cambrex, Rockland, ME or Invitrogen), transferred to a polyvinylidene difluoride membrane (Invitrogen), and blocked in 5% nonfat milk in phosphate-buffered saline. The upper part of the blot was incubated with antibodies to the carboxyl terminus of NMHC II-C (1:10,000), the amino terminus of NMHC II-B (1:5,000 or 1:2,000), NMHC II-A (anti-platelet II-A, 1:100,000 or 1:50,000), or panmyosin (1:5,000; Covance Research Products, Inc., Berkeley, CA) at 4 °C overnight (6, 21). The lower part of the blot was incubated with either anti-β-actin (1:10,000) (Sigma) or anti-α-tubulin (1:10,000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody. The blot was washed and...
then incubated with horseradish peroxidase-conjugated secondary antibodies (Pierce) at room temperature for 1 h. The blot was treated with SuperSignal West Pico or Femto luminal enhancer solution (Pierce). Luminescence signal was captured on Biomax MR film (Eastman Kodak Co.). Films were scanned using a densitometer (Molecular Devices, Sunnyvale, CA). Band intensity was calculated using ImageQuant software after normalizing with actin band intensity.

**Immunofluorescence Microscopy**—A549 cells grown on chamber slides were rinsed with phosphate-buffered saline and fixed with 4% paraformaldehyde at room temperature for 30 min and permeabilized with 0.5% Triton X-100 for 10 min. For antibody staining, the samples were blocked with 0.1% bovine serum albumin and 10% normal goat serum in phosphate-buffered saline for 1 h at room temperature, incubated with affinity-purified polyclonal rabbit antibodies against NMHC II-C or NMHC II-A at 4 °C overnight. The secondary antibody, Alexa 594 goat anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) was incubated with cells at room temperature for 1 h. DAPI was used for nuclear staining. After washing, chamber slides were mounted using a Prolong anti-fade kit (Molecular Probes). The images were collected using a Leica SP1 confocal microscope (Deerfield, IL).

**Time Lapse Imaging**—Time lapse imaging of cytokinesis was performed using an Olympus IX-70 microscope supported by the Metamorph program (Molecular Devices). Images were recorded every 5 min for 2 h for nonspecific siRNA or every 10 min for 10 h for NMHC II-C1 siRNA-treated A549 cells using a Photometric Cool Snap Camera. Images of blebbistatin-treated cells were recorded every 2 min using an Olympus IX-70 microscope supported by the Andor iQ program and Andor iXon DV887 camera (Andor Technology, Belfast, Northern Ireland). All time lapse imaging was performed under 5% CO₂ and at 37 °C in a stage incubator.

**Statistical Analysis**—Data were expressed as the means ± S.E. Statistical significance was tested with a two-way analysis of variance followed by the Bonferroni test. The differences were considered to be significant if p was <0.05.

## RESULTS

**Expression of C1 Inserted mRNA in Human Epithelial Tumor Cell Lines**—Golomb et al. (6) reported that the gene encoding NMHC II-C contains an alternatively spliced exon that inserts 24 nucleotides encoding 8 amino acids into loop 1 of NMHC II-C near the ATP binding region of the NMHC. Here, we investigated the expression of this alternatively spliced isoform of NMHC II-C, NMHC II-C1, in human tumor and related nontumor cell lines by RT-PCR, using primers flanking the inserted C1 exon. Fig. 1A shows that NMHC II-C1 is expressed in a number of different tumor cell lines, as shown by the generation of a nucleotide fragment of 269 bp instead of 245 bp (for the noninserted isoform). Note also that the noninserted isoform of NMHC II-C (Fig. 1A, II-C0) is not expressed in any of these cell lines at the mRNA level.

In contrast to the lung and breast tumor cell lines (denoted as T in Fig. 1B), which express significant amounts of NMHC II-C1, Fig. 1B shows that a nontumor cell line derived from the same tissue, Beas-2B (lung) and MCF-10-2A (breast, denoted as N in Fig. 1B), express very little mRNA encoding either the NMHC II-C1 or noninserted NMHC II-C (Fig. 1B, top). We also determined the relative amounts of mRNA encoding NMHC II-A and II-B using isoform-specific primers. Fig. 1B shows that mRNAs encoding both isoforms are expressed in all four cell lines with no major difference in expression between the nontumor or tumor cell lines. These data were confirmed using real time PCR (see Table 1).

**Immunoblot Analysis of NMHC II-C in Tumor and Nontumor Cell Lines**—We then quantified the relative difference in protein expression among the NMHC II isoforms in the lung and breast tumor and nontumor cell lines using antibodies specific for each of the isoforms. Fig. 2 shows immunoblots probed with antibodies to NMHC II-A, II-B, and II-C, and Table 1 quantifies the relative expression of each isoform, setting the quantity of NMHC II in the nontumor cell line as 1 for each isoform. We used a series of three different immunoblots and dilutions, one example of which is shown in Fig. 2. For NMHC II-C1 (third blot from the top), the protein expression analysis correlates with the increase in mRNA expression (Fig. 1B) and shows a
4-fold increase in the breast tumor cell line and a 13.5-fold increase in the lung tumor cell line over the respective nontumor cell lines. In contrast, the immunoblots detecting NMHC II-A and II-B show a marked reduction in each of the isoforms at the protein level in the tumor epithelial cell lines, differing from the mRNA results, which showed no significant change between the tumor and nontumor cell lines for both lung and breast (Fig. 1B and Table 1). These results suggest that expression of NMHC II-A and II-B protein was decreased at the translational level or due to protein instability, whereas NMHC II-C1 expression was increased transcriptionally or due to increased mRNA stability or both in the tumor epithelial cell lines.

NMHC II-A and NMHC II-C1 Are the Major Isoforms in Lung Tumor A549 Cells—To compare the percentage of each NMHC II isoform at the protein level in the A549 lung tumor cell line, we made use of specific siRNAs and a panmyosin II antibody. As noted under “Experimental Procedures,” we lowered the mRNA encoding each NMHC II isoform using the appropriate siRNA and then analyzed the NMHC II protein levels using antibodies specific to each isoform as well as a panmyosin antibody. We correlated the change in the immunoblot seen following siRNA treatment with that seen following detection with the panmyosin antibody. Fig. 3 shows that siRNA targeting NMHC II-A specifically lowered the II-A protein (II-A, compare control lanes 1 and 2 with lanes 3 and 4) and also significantly reduced the amount of total NMHC as detected in the panmyosin immunoblot (Pan-Myosin, lanes 3 and 4). On the other hand, lowering II-B protein with siRNA (II-B, lanes 5 and 6) had little effect on the panmyosin blot (Pan-Myosin, compare control lanes 1 and 2 with lanes 5 and 6). Lowering NMHC II-C1 (see Fig. 4A, II-C) also significantly lowered the total NMHC signal on the panmyosin blot (Fig. 3, lanes 9 and 10). Quantification of the panmyosin blot for three different immunoblots revealed that NMHC II-B is less than 8% of the total NMHC II, and NMHC II-A and II-C comprise about 45% each of the total NMHC II.

The Effect of Lowering NMHC II Isoforms on Cell Proliferation—Since myosin II-A and II-C1 are the major nonmuscle myosin II isoforms in the A549 lung tumor cell line, we were interested in exploring the effect of lowering each of these two isoforms on cell proliferation. We found that lowering NMHC II-C1 had only a small effect (although statistically significant) on cell proliferation compared with the effect of transfecting nonspecific siRNA (quantified in Fig. 4B, hatched bars compared with the black bars for nonspecific siRNA). In contrast, using siRNA directed specifically against the NMHC II-C1 mRNA lowered NMHC II-C1 protein (Fig. 4A) and markedly decreased cell proliferation (open bars) compared with the effect of nonspecific siRNA (Fig. 4B). At 120 h, there was almost a 5.5-fold difference in the number of NMHC II-C1 siRNA-treated cells compared with nonspecific siRNA-treated cells (Fig. 4B).

When NMHC II-C1 siRNA-treated cells and nonspecific siRNA-treated cells were replated at 156 h after siRNA transfection and the cells were counted at 24, 48, and 72 h after replating, there was no longer a difference in the rate of cell proliferation between them (see Fig. 4C, Passage 2). This correlated with increased expression of NMHC II-C1 protein following replating, which was most likely due to the loss of siRNA. Fig. 4D quantifies the expression of NMHC II-C1 protein in the siRNA-treated cells compared with nonspecific siRNA-treated cells at the times indicated as determined by scanning immunoblots. It demonstrates that by 180 h after siRNA transfection, the effects of siRNA have decreased, resulting in increased expression of NMHC II-C1 to about 40% of control cells and restoring proliferation of NMHC II-C1 siRNA-treated cells to that found for nonspecific siRNA-treated cells. This shows that the effects of siRNA treatment are reversible and account for the decrease in cell proliferation. Of note is that there was no evidence for binucleation in the siRNA-treated cells.

| Isoform | Lung (T:N) | Breast (T:N) |
|---------|------------|--------------|
| Real time PCR |          |              |
| II-A    | 0.69 ± 0.16:1 | 1.42 ± 0.29:1 |
| II-B    | 1.31 ± 0.01:1 | 0.66 ± 0.02:1 |
| II-C1   | 30.8 ± 5.1:1 | 7.33 ± 1.22:1 |
| Immunoblot analysis |          |              |
| II-A    | 0.13 ± 0.01:1 | 0.33 ± 0.01:1 |
| II-B    | 0.03 ± 0.02:1 | 0.30 ± 0.01:1 |
| II-C1   | 13.5 ± 0.8:1  | 4.16 ± 0.05:1 |

FIGURE 3. NMHC II-A and II-C are the major isoforms in A549 cells. A549 cells were transiently transfected with NMHC II-A siRNA, NMHC II-B siRNA, NMHCII-C1 siRNA, or nonspecific (NS) siRNA. At 72 h, cells were harvested and subjected to immunoblotting using anti-II-A, anti-II-B, anti-II-C, anti-panmyosin, or anti-tubulin. Two different amounts of total cell lysate were loaded for each sample for immunoblotting. Tubulin was used for a gel loading control.
Role of Myosin II-C1 in Cytokinesis

FIGURE 4. Decreasing the NMHC II-C1 isoform reduces cell proliferation. A, immunoblot confirming that siRNA specifically inhibits only NMHC II-C1 protein expression and not II-A or II-B. Two different amounts of sample were loaded on the gel. B, an equivalent number (2.5 \times 10^4) of A549 cells were seeded and transfected with either nonspecific (NS) or NMHC II-C1 siRNA, and cell numbers were counted at 24-h time intervals until 120 h. Each assay was performed in triplicate, and the entire experiment was repeated three times. *, p < 0.01; **, p < 0.001 for siRNA II-C1 and II-A cell number versus nonspecific siRNA cell number. C, 0.75 \times 10^4 cells were transfected with either nonspecific or NMHC II-C1 siRNA (passage 1). At 156 h, cells were replated at equal numbers, and cells were counted until 228 h (passage 2). D, quantification of NMHC II-C1 protein expression using immunoblots (not shown) from passage 1 and passage 2 cells (see C). Percentage of NMHC II-C1 protein expression was calculated with respect to the protein expression of NMHC II-C1 in nonspecific siRNA-treated cells. Results are expressed as mean ± S.E. for three independent experiments.

Rescuing the siRNA-induced Decrease in Cell Proliferation Using GFP NMHC IIs—We then assessed the ability of each NMHC II isoform to rescue the decrease in cell proliferation caused by down-regulation of NMHC II-C1. Each full-length NMHC II isoform tagged with GFP was introduced into the cells that had been previously treated with NMHC II-C siRNA for 24 h. For this experiment, siRNAs to lower NMHC II-C1 were targeted to the 3′-untranslated region of NMHC II-C mRNA to prevent the siRNA from lowering mRNA derived from the NMHC II-C1-GFP construct (see “Experimental Procedures”). We previously reported (9) that, unlike NMHC II-A and II-B, it was necessary to fuse GFP to the carboxyl terminus of the NMHC II-C molecule to prevent aggregation and for it to interact properly. Following transfection of the GFP-tagged NMHC II constructs into the NMHC II-C1 siRNA-treated cells, the cells were cultured for an additional 96 h, and the cell number was counted every 24 h after cDNA transfection. These experiments were carried out under conditions that normalized the transfection efficiency of all four GFP-tagged NMHC II constructs to 70% of the cells transfected, using the GFP signal viewed by fluorescence microscopy as an indicator. Fig. 5A shows a plot of cell numbers of the NMHC II-C1-GFP following NMHC II-C siRNA treatment. These cells were able to complete cytokinesis within a time period similar to the nonspecific siRNA-treated cells. Similar to our findings for cell proliferation (Fig. 5A), noninserted NMHC II-C0-GFP was more effective than GFP-NMHC II-A and II-B. NMHC II-C0-GFP and GFP-NMHC II-C1-GFP showed the same low rate of cell proliferation as did II-C siRNA cells transfected with GFP alone (Fig. 5A). Only NMHC II-C1-GFP rescued the NMHC II-C siRNA-induced defect in cell proliferation, although the noninserted isoform, NMHC II-C0-GFP, was somewhat more effective than GFP-NMHC II-A and II-B. Fig. 5B is an immunoblot showing the decreased expression of endogenous NMHC II-C (End II-C) at 72 h in the siRNA and cDNA transfected cells. The immunoblot also detects the protein expression of exogenously introduced NMHC II-C0 and II-C1 (II-C1-GFP) using a C-terminal antibody and all four NMHC II isoforms using antibodies to GFP. Note that the exogenous NMHC II-C0-GFP and II-C1-GFP migrate more slowly than the endogenous NMHC II-C. The immunoblot shows that approximately equal amounts of each construct were expressed in the cells (GFP, top lane) and that the amount of exogenously expressed NMHC II-C0-GFP and NMHC II-C1-GFP exceeded the residual endogenous NMHC II-C and, thus, was most likely responsible for the increase of cell proliferation.

Delay in Completing Cytokinesis in NMHC II-C1 siRNA-treated A549 Cells—The marked inhibition of cell proliferation by siRNA directed against NMHC II-C1 suggested that this isoform might play a role in the cell cycle. Microscopic analysis detected that a larger percentage of the cells treated with NMHC II-C siRNA remained connected by a cytoplasmic bridge at a late stage of cytokinesis than control cells treated with nonspecific siRNA. This suggested that II-C siRNA-treated cells require a longer time to complete cytokinesis. Table 2 quantifies the percentage of cells 72 h posttransfection still connected by a cytoplasmic bridge with cells treated in a manner similar to those shown in Fig. 5A. Whereas only 2% of the cells treated with nonspecific siRNA were connected by a cytoplasmic bridge, 35% of the NMHC II-C siRNA-treated cells retained a cytoplasmic bridge. However, only 2% of the cells were connected by a cytoplasmic bridge after transfection of NMHC II-C1-GFP following NMHC II-C siRNA treatment. These cells were able to complete cytokinesis within a time period similar to the nonspecific siRNA-treated cells. Similar to our findings for cell proliferation (Fig. 5A), noninserted NMHC II-C0-GFP was more successful in rescuing this II-C siRNA-induced defect in cytokinesis than was GFP-NMHC II-A and GFP-NMHC II-B. Of note was that we observed no increase in the number of the bi- or multinucleated cells at interphase.
Role of Myosin II-C1 in Cytokinesis

Distinct Localization of Myosin II-C1 and II-A in Dividing Cells—The persistence of the cytoplasmic bridge during cytokinesis prompted us to study the localization of NMHC II-A and II-C1 in the dividing cells. Fig. 6A shows that these two isoforms have a markedly different location. Whereas NMHC II-A is distributed throughout the entire cell and is more concentrated at the two opposite poles of the dividing daughter cells, NMHC II-C1 is concentrated in the cytoplasmic intercellular bridge (Fig. 6A). Interestingly, decreasing NMHC II-C1 using siRNA does not appear to alter the localization of NMHC II-A, which remains absent from the intercellular bridge (Fig. 6B). Together, these observations suggest that NMHC II-C1 may play a specific role with respect to the bridge during a late stage in cytokinesis.

Time Lapse Imaging of A549 Cells—To analyze cytokinesis in detail, time lapse imaging over a 2–10-h time period was performed. As shown in Fig. 7, we began recording cytokinesis soon after ingestion of the cleavage furrow. We found that A549 cells treated with nonspecific siRNA (n = 5) completed cytokinesis within 2 h (Fig. 7A). Careful inspection of both the images and movies (see supplemental data, Movie 1) showed that nonspecific siRNA-treated mitotic cells formed a cytokinetic ring but then flattened 30 min later, and the furrow appeared to be retracted (Fig. 7A, 0:30). The cells spread, and lamellae were formed at the two opposite poles, resulting in the two daughter cells pulling in opposite directions 180° from each other. At this time, the central portion connecting the two nascent cells formed a bridge (Fig. 7A, 1:00). The central cytoplasm then thinned out, retracted (Fig. 7A, 1:30), and severed by 2 h (Fig. 7A, 2:00). Therefore, the control cells apparently completed cytokinesis by pulling themselves apart from the opposite ends of the two daughter cells and by retracting the thinning cytoplasmic bridge. A critical step in this process appears to be the retraction and dissolution of the intercellular bridge within a 1-h period.

NMHC II-C1 siRNA-treated mitotic cells (n = 20) also first formed a cleavage furrow (Fig. 7B, 0:00; see supplemental data, Movie 2). The cells flattened and lamellae began to form, but, unlike control cells, the lamellae of the two new cells had a more random orientation (Fig. 7B, 0:30). The lamellae orientation kept changing in an uncoordinated manner, and the original cleavage furrow remained wide for a longer period (Fig. 7B, 4:00). The two nascent daughter cells did not show front-rear polarity and did not move apart efficiently in opposite directions. After an additional 2 h, the cleavage site narrowed (Fig. 7B, 6:00). The cytoplasmic bridge persisted for an additional 3 h (Fig. 7B, 9:00) and eventually severed (Fig. 7B, 10:00). Thus, the NMHC II-C1 siRNA-treated cells had defects in becoming polarized and in migrating in opposite directions and had a prolonged final abscission step. Together with the observation that myosin II-C1 is enriched in the middle portion of the dividing cells, myosin II-C1 seems to play an important role in retraction of the cytoplasmic bridge in A549 tumor cells.

Finally, to examine whether the ability of NMHC II-C1 siRNA-treated cells to carry out cytokinesis was a myosin II-dependent event, the siRNA-treated cells were exposed to the myosin II-specific inhibitor, blebbistatin (22). As shown in Fig. 7C (see also supplemental data, Movie 3), the cleavage furrow began to ingress (Fig. 7C, 0:30). However, unlike II-C1 or nonspecific siRNA-treated cells, the cells did not respread. The furrow then regressed completely (Fig. 7C, 1:30 and 1:50), and the cells rounded up and became binucleated. Thus, the spreading and crawling activities observed in the NMHC II-C1 siRNA-treated cells as well as their ability to complete cytokinesis, although belatedly, still depend on myosin II, most likely myosin II-A.

DISCUSSION

In this study, we report that decreasing NMHC II-C1 in the lung tumor cell line A549 leads to a delay in the late stages of cytokinesis, which can be rescued by introducing exogenous NMHC II-C1-GFP or partially rescued by noninserted NMHC II-C0-GFP, but not at all by GFP-NMHC II-A or GFP-NMHC II-B. We also pres-
ent evidence that NMHC II-C1 is a major myosin II isoform in a number of different epithelial tumor cell lines.

Although there is considerable information concerning the role of myosin II in cytokinesis (22–28), little is known about the individual role of each of the NMHC II isoforms in this process, since most studies have been conducted on cells with a single isoform, such as *Dictyostelium*, or have employed inhibitors, such as blebbistatin, that do not discriminate among the myosin II isoforms. For example, HeLa cells, which express NMHC II-A and II-B, show a defect in furrow ingression during cytokinesis when the motor activity of both nonmuscle myosin II isoforms is inhibited by blebbistatin (22). On the other hand, functional redundancy of the nonmuscle myosin II isoforms in cytokinesis has been reported. COS-7 cells, which express small amounts of NMHC II-C and lack NMHC II-A, become multinucleated when NMHC II-B is depleted using siRNA (9). In that study, all three NMHC II isoforms were capable of rescuing the multinucleation induced by siRNA targeted at nonmuscle myosin II-B. This is in contrast to our present findings in the A549 cell line.

Our studies here focus on the formation and severing of the intercellular bridge that forms late in cytokinesis. We have found that a particular isoform of NMHC II-C localizes to this intercellular bridge and that decreasing the amount of this isoform results in a number of defects in cytokinesis. The tumor cell line under study initiated cytokinesis by rounding up and forming a furrow. The two nascent cells then flattened (as shown in Fig. 7A) and formed lamellae at the bipolar ends.
of the dividing cells. Based on our results using siRNA to lower the content of NMHC II-C1, we found that this isoform appears to play a role in establishing the bipolar symmetry that is prominent in the two nascent cells at this time (see Fig. 7A, 1:00 h, and compare with Fig. 7B, 4:00 h). This is particularly evident in the II-C siRNA-treated cells between 0:00 and 4:00 h, when cells spread and initiated migration in multiple directions but failed to become polarized so as to permit efficient cell division.

About 1 h after the initiation of furrow ingression, the cytoplasmic bridge in nonspecific siRNA-treated cells begins to form and, by 2 h, it gradually thins, retracts, and severs (Fig. 7A). The process of thinning and retraction also appears to be defective in II-C siRNA-treated cells but eventually occurs between 8 and 10 h rather than at 2 h, as shown in control cells. The localization of NMHC II-C1 and not II-A to the bridge suggests that it might play a unique role in this stage of cytokinesis. This is further supported by our finding that neither NMHC II-A nor II-B could rescue the II-C siRNA-treated cells. The prolongation of cytokinesis by 8 h in NMHC II-C1 siRNA-treated cells emphasizes the importance of the presence of this myosin in the cytoplasmic bridge in dividing cells.

It is notable that decreasing NMHC II-C1 does not cause a complete failure in cytokinesis. This could be explained by the presence of myosin II-A or a small amount of II-C, since we estimate that ~10% of this protein remains after treatment with siRNA. Evidence that cytokinesis in II-C siRNA-treated cells is still a myosin II-dependent event comes from our use of the drug blebbistatin, which abrogated this process by preventing cell spreading and reversing the initial furrow ingression, resulting in binucleated cells. We suspect the nonmuscle myosin II-A is playing a role in cell spreading and attachment, since NMHC II-A siRNA-treated cells failed to spread during cytokinesis (data not shown). Moreover, the distribution of II-A as shown in Fig. 6A would be consistent with a role in cell spreading and adhesion. Interestingly, as shown in Fig. 5A, unlike treatment with II-C siRNA, there is no change in the rate of cell proliferation in II-A siRNA-treated cells, suggesting that cell spreading and attachment are not critical events in cytokinesis. However, once cell spreading and attachment occur before the completion of cytokinesis, the establishment of cell polarity and bipolar migration of the nascent daughter cells driven by nonmuscle myosin II become essential components for cytokinesis.

Recent work by Kanada et al. (29) has provided evidence for the role of an adhesion-dependent form of cytokinesis in mammalian cells. This type of cytokinesis, referred to as cytokinesis B, occurred independently of myosin and was first shown to be operative in Dictyostelium (25, 30, 31). Our findings that blebbistatin treatment causes a loss of furrow ingression leading to binucleation suggest that the process of cell adhesion and attachment of the dividing A549 cells are myosin-dependent events and that we are not observing a myosin-independent form of cytokinesis. In a number of ways, the mechanism outlined above for myosin II playing a role in formation and retraction of the cytoplasmic bridge as well as in cell adhesion and spreading agrees with the mechanism suggested by Taylor's laboratory using Swiss 3T3 cells, where they measured traction force during the late stages of cytokinesis (32, 33). Our data support the idea that this traction force is generated by nonmuscle myosin II.

In most animal cells, cleavage furrow ingression ensures an almost complete separation of two daughter cells, which, nevertheless remain interconnected for variable times by a cytoplasmic bridge until separation takes place (34). It has been proposed that completion of cytokinesis requires three steps: stabilization of the intercellular bridge, contractile ring disassembly, and membrane remodeling for abscission (35). To date, only a few studies have been reported on the resolution of the intercellular bridge, and the involvement of nonmuscle myosin II in this process is not well documented. Our findings that NMHC II-C1, and not II-A, is localized to the intercellular bridge (Fig. 6) and that decreasing NMHC II-C1 results in persistence of the bridge (Fig. 7 and Table 2) are consistent with a role for this isoform in this process.

Here, we also show that a large percentage of the nonmuscle myosin II content of a number of different tumor cell lines is myosin II-C1. However, there are some notable exceptions, such as the MDA-MB-231 breast tumor line, which lacks II-C (36). A possible relationship between the presence of the alternatively spliced isoform NMHC II-C1 and tumorigenesis has yet to be demonstrated. (However, see Ref. 37 for a review of alternative splicing in cancer-associated genes.) It is notable that our analysis of three different primary human tumors (renal cell carcinoma, pulmonary adenocarcinoma, and breast ductal carcinoma) showed expression of significant quantities of NMHC II-C1 compared with the surrounding noncancerous tissues (data not shown). Our future studies will be directed at understanding the role of NMHC II-C1 in both tumor cell lines and in primary tumors.

Acknowledgments—We acknowledge the professional skills and advice of Christian A. Combs and Daniela A. Malde (Light Microscopy Core Facility, NHLBI, National Institutes of Health). We thank the members of the Laboratory of Molecular Cardiology for reagents and helpful discussions. We thank Catherine Magruder for editorial assistance and Mary Anne Conti, Jim Sellers, and Xuefei Ma for reading the manuscript.

REFERENCES
1. Sellers, J. R. (1999) Myosins, Oxford University Press, Oxford
2. Krendel, M., and Mooseker, M. S. (2005) Physiology 20, 239–251
3. Katsuragawa, Y., Yanagisawa, M., Inoue, A., and Masaki, T. (1989) Eur. J. Biochem. 184, 611–616
4. Shohet, R. V., Conti, M. A., Kawamoto, S., Preston, Y. A., Brill, D. A., and Adelstein, R. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7726–7730
5. Takahashi, M., Kawamoto, S., and Adelstein, R. S. (1992) J. Biol. Chem. 267, 17864–17871
6. Golomb, E., Ma, X., Jana, S. S., Preston, Y. A., Kawamoto, S., Shoham, N. G., Goldin, E., Conti, M. A., Sellers, J. R., and Adelstein, R. S. (2004) J. Biol. Chem. 279, 2800–2808
7. Simons, M., Wang, M., McBride, O. W., Kawamoto, S., Yamakawa, K., Gudla, D., Adelstein, R. S., and Weir, L. (1991) Circ. Res. 69, 530–539
8. Leal, A., Endele, S., Stengel, C., Huehne, K., Loetterle, J., Barrantes, R., Winterpacht, A., and Rautenstrauss, B. (2003) Gene (Amst.) 312, 165–171
9. Bao, J., Jana, S. S., and Adelstein, R. S. (2005) J. Biol. Chem. 280, 19594–19599
10. Maupin, P., Phillips, C. L., Adelstein, R. S., and Pollard, T. D. (1994) J. Cell Sci. 107, 3077–3090
Role of Myosin II-C1 in Cytokinesis

11. Kelley, C. A., Sellers, J. R., Gard, D. L., Bui, D., Adelstein, R. S., and Baines, I. C. (1996) J. Cell Biol. 134, 675–687
12. Kolega, J. (2003) Mol. Biol. Cell 14, 4745–4757
13. Kolega, J. (1998) J. Cell Sci. 111, 2085–2095
14. Murakami, N., Trenkner, E., and Elzinga, M. (1993) Dev. Biol. 157, 19–27
15. Conti, M. A., Even-Ram, S., Liu, C., Yamada, K. M., and Adelstein, R. S. (2004) J. Biol. Chem. 279, 41263–41266
16. Uren, D., Hwang, H. K., Hara, Y., Takeda, K., Kawamoto, S., Tullio, A. N., Yu, Z. X., Ferrans, V. J., Tresser, N., Grinberg, A., Preston, Y. A., and Adelstein, R. S. (2000) J. Clin. Invest. 105, 663–671
17. Itoh, K., and Adelstein, R. S. (1995) J. Biol. Chem. 270, 14533–14540
18. Ma, X., Kawamoto, S., Uribe, J., and Adelstein, R. S. (2006) Mol. Biol. Cell 17, 2138–2149
19. Kim, K. Y., Kovacs, M., Kawamoto, S., Sellers, J. R., and Adelstein, R. S. (2005) J. Biol. Chem. 280, 22769–22775
20. Wei, Q., and Adelstein, R. S. (2000) Mol. Biol. Cell 11, 3617–3627
21. Phillips, C. L., Yamakawa, K., and Adelstein, R. S. (1995) J. Muscle Res. Cell Motil. 16, 379–389
22. Straight, A. F., Cheung, A., Limouze, J., Chen, I., Westwood, N. J., Sellers, J. R., and Mitchison, T. J. (2003) Science 299, 1743–1747
23. Matsumura, F. (2005) Trends Cell Biol. 15, 371–377
24. Yamashiro, S., Totsukawa, G., Yamakita, Y., Sasaki, Y., Madaule, P., Ishizaki, T., Narumiya, S., and Matsumura, F. (2003) Mol. Biol. Cell 14, 1745–1756
25. DeLozanne, A., and Spudich, J. A. (1987) Science 236, 1086–1091
26. Diehn, M., Werner, M., and Glotzer, M. (2005) Trends Cell Biol. 15, 651–658
27. Kamijo, K., Ohara, N., Abe, M., Uchimura, T., Hosoya, H., Lee, J. S., and Miki, T. (2006) Mol. Biol. Cell 17, 43–55
28. Dean, S. O., Rogers S. L., Stuurman, N., Vale, R. D., and Spudich, J. A. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 13473–13478
29. Kanada, M., Nagasaki, A., and Uyeda, T. Q. P. (2005) Mol. Biol. Cell 16, 3865–3872
30. Neujahr, R., Heizer, C., and Gerisch, G. (1997) J. Cell Sci. 110, 123–137
31. Zang, J. H., Cavet, G., Sabry, J. H., Wagner, P., Moores, S. L., and Spudich, J. A. (1997) Mol. Biol. Cell 8, 2617–2629
32. DeBiasio, R. L., LaRocca, G. M., Post, P. L., and Taylor, D. L. (1996) Mol. Biol. Cell 7, 1259–1282
33. Burton, K., and Taylor, D. L. (1997) Nature 385, 450–454
34. McCollum, D. (2005) Curr. Biol. 15, R998–R1000
35. Schweitzer J. K., and D’Souza-Schorey, D. (2004) Exp. Cell Res. 295, 1–8
36. Betapudi, V., Licate, L., and Egelhoff, T. T. (2006) Cancer Res. 66, 4725–4733
37. Brinkman, B. M. N. (2004) Clin. Biochem. 37, 584–594