

# Nuclear efflux of heterogeneous nuclear ribonucleoprotein C1/C2 in apoptotic cells: a novel nuclear export dependent on Rho-associated kinase activation

Hsiao-Hui Lee<sup>1</sup>, Chung-Liang Chien<sup>2</sup>, Hsin-Kai Liao<sup>3</sup>, Yu-Ju Chen<sup>3</sup> and Zee-Fen Chang<sup>1,\*</sup>

<sup>1</sup>Institute of Biochemistry and Molecular Biology and <sup>2</sup>Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, No.1 Jen Ai Road Sec.1, Taipei, 100, Taiwan, Republic of China

<sup>3</sup>Institute of Chemistry, Academia Sinica, 128, Academia Sinica Road, Nankang, Taipei, 115, Taiwan, Republic of China

\*Author for correspondence (e-mail: zfchang@ha.mc.ntu.edu.tw)

Accepted 3 August 2004

Journal of Cell Science 117, 5579-5589 Published by The Company of Biologists 2004

doi:10.1242/jcs.01482

## Summary

Using a proteomic approach, we searched for protein changes dependent on Rho-associated kinase (ROCK) during phorbol-12-myristate-13-acetate (PMA)-induced apoptosis. We found that heterogeneous nuclear ribonucleoprotein C1 and C2 (hnRNP C1/C2), two nuclear restricted pre-mRNA binding proteins, are translocated to the cytosolic compartment in a ROCK-dependent manner in PMA-induced pro-apoptotic cells, where nuclear envelopes remain intact. The subcellular localization change of hnRNP C1/C2 appears to be dependent on ROCK-mediated cytoskeletal change and independent of caspase execution and new protein synthesis. Such a ROCK-dependent translocation is also seen in TNF $\alpha$ -induced apoptotic NIH3T3 cells. By overexpressing the dominant active form of ROCK, we showed that a ROCK-

mediated signal is sufficient to induce translocation of hnRNP C1/C2. Deletion experiments indicated that the C-terminal 40-amino-acid region of hnRNP C1/C2 is required for ROCK-responsive translocation. By using nuclear yellow fluorescent protein (YFP) fusion, we determined that the C-terminal 40-amino-acid region of hnRNP C1/C2 is a novel nuclear export signal responsive to ROCK-activation. We conclude that a novel nuclear export is activated by the ROCK signaling pathway to exclude hnRNP C1/C2 from nucleus, by which the compartmentalization of specific hnRNP components is disturbed in apoptotic cells.

Key words: Rho-associated kinase, hnRNP C1/C2, Nuclear export, Apoptosis, Phorbol ester, Proteomics

## Introduction

The Rho GTPases are a family of proteins involved in multiple cellular processes, including cytoskeletal organization, gene expression and transformation (Etienne-Manneville and Hall, 2002; Hall, 1998). Among them RhoA is known to coordinate with other signal pathways to control dynamic rearrangements of the actin cytoskeleton during proliferation, differentiation and apoptosis (Aznar and Lacal, 2001; Coleman and Olson, 2002; Kaibuchi et al., 1999). Activation of RhoA is responsible for the contraction observed in the apoptotic cells induced by serum starvation, and ectopic expression of the dominant active form of RhoA is sufficient to induce apoptosis (Dubreuil et al., 2003; Esteve et al., 1998; Jimenez et al., 1995). Among the diverse downstream effectors of RhoA, Rho-associated kinase (ROCK) has been shown to mediate RhoA signaling in membrane contraction force during apoptosis. Upon binding to the GTP-bound form of RhoA, the kinase activity of ROCK is enhanced, which leads to myosin light chain (MLC) phosphorylation and increase of interaction between stabilized actin filaments and myosin, thus generating contractile force (Amano et al., 1996; Kimura et al., 1996; Kureishi et al., 1997). Additional to its activation by RhoA signaling, ROCK I can be

activated by caspase-3-mediated cleavage at its C-terminal inhibitory domain in apoptotic cells. The cleaved form of ROCK I activates MLC phosphorylation, resulting in membrane blebbing in apoptotic cells (Coleman et al., 2001; Sebbagh et al., 2001). Collectively, these findings suggest that RhoA- or caspase-dependent activation of ROCK confers a mechanism contributing to cell contraction and membrane blebbing during the apoptotic process.

Work in our laboratory has previously shown that following treatment with the phorbol ester, phorbol-12-myristate-13-acetate (PMA), myeloid leukemia cell lines TF-1 and D2, become apoptotic, where activated extracellular signal-regulated kinase (ERK) is unable to be translocated into nuclei. We have further shown that inhibition of ROCK by its specific inhibitor Y27632 (Narumiya et al., 2000) could rescue these cells from PMA-induced apoptosis and restore nuclear translocation of activated ERK, indicating that ROCK-mediated signal affects protein trafficking during apoptosis (Lai et al., 2002). In addition, we have determined that PMA treatment can induce a pro-apoptotic status, of which the RhoA/ROCK/myosin contraction pathway acts as an upstream event required for caspase activation (Lai et al., 2003). The

purpose of this study was to search, using a general proteomic screen, for an unknown molecular event that is regulated by ROCK during initiation of pro-apoptosis. We took advantage of the fact that PMA-induced pro-apoptotic cells remain in suspension with ROCK-dependent myosin-mediated contraction and that inhibition of ROCK by its specific inhibitor Y27632 protects these suspension cells from PMA-induced apoptosis. Using differential two-dimensional gel electrophoresis (2DE) analysis, we compared the cytosolic protein patterns from PMA-treated D2 cells that were pre-incubated with or without Y27632. Through this approach, we identified that hnRNP C1/C2, two nuclear restricted pre-mRNA binding proteins, are distributed in the cytoplasm of PMA-induced pro-apoptotic cells in a ROCK-dependent manner.

hnRNP C1/C2 are derived from alternative splicing with a difference in a 13-amino-acid insertion for C2 protein (Burd et al., 1989). They form stable heterotetramers that bind cooperatively to RNA (McAfee et al., 1996; Rech et al., 1995). Purified 40S hnRNP monomers contain heterotetrameric complexes of hnRNP C1 and C2, hnRNP A1 and B2, and hnRNP A2 and B1 (Barnett et al., 1989). These six proteins are the major constituents of the core hnRNP complex, and hnRNP C is the only member of the core complex that does not shuttle between the nucleus and cytoplasm (Pinol-Roma and Dreyfuss, 1992). In addition to the hnRNP particle, hnRNP C1/C2 are also the major constituents of the nuclear matrix (van Eekelen and van Venrooij, 1981; Verheijen et al., 1986). In this study, we found that translocation of hnRNP C1/C2 from nucleus to cytoplasm occurs in PMA-induced pro-apoptotic cells dependent on ROCK activation, but independent of caspase activation. We provide evidence that the cytosolic distribution of hnRNP C1/C2 is not a result of nuclear leakage, and this ROCK-regulated event is also found in TNF $\alpha$ -induced apoptotic NIH3T3 cells, where ROCK I is activated by caspase-3-mediated cleavage. Thus, hnRNP C1/C2 are no longer the nuclear restricted proteins in either PMA-induced pro-apoptotic or TNF $\alpha$ -induced apoptotic cells. We show that activation of ROCK is sufficient to induce translocation of hnRNP C1/C2 with an intact C-terminal region. Our experimental results further indicated that 40 amino acids of the C-terminal region of hnRNP C1/C2 can act as a novel transferable nuclear export signal (NES), which is activated by ROCK signaling and is distinct from other known mammalian NESs. These data suggest that activation of ROCK in the cytoplasm is linked to a novel, so far uncharacterized, nuclear export pathway, which is responsible for nuclear efflux of hnRNP C1/C2 proteins in the apoptotic cells.

## Materials and Methods

### Reagents

PMA was purchased from Sigma Chemical Co. (St. Louis, Mo., USA) and dissolved in dimethyl sulfoxide; Y-27632 [(+)-(R)-*trans*-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexane-carboxamide dihydrochloride, monohydrate] and blebbistatin were from Calbiochem-Novabiochem Corp. (San Diego, California, USA); Cy<sup>TM</sup>3 and Cy<sup>TM</sup>5 mono NHS ester were from Amersham Biosciences; goat polyclonal anti-hnRNP C1/C2, anti-hnRNP A2/B1; rabbit polyclonal anti-Sp1, and mouse monoclonal anti-c-myc, anti-lamin A/C antibodies were purchased from Santa Cruz Biotechnology (Santa

Cruz, CA). Human thymidine kinase-1 (TK-1) antibody was generated in our laboratory as described previously (Chang et al., 1998).

### Constructs

Plasmid containing cDNA encoding full-length human hnRNP C1 was constructed by PCR amplification of cDNA from HeLa cells using primers 5'-CGGGAATTCCATGGCCAGCAA CGTT-3' and 5'-CGGGAATTCTCATCTCCATTGGC-3', and cloned into the *Eco*RI site of expression vector pDsRed2-C1 (Clontech). PCR amplification reactions using pDsRed-hnRNP C1 as the template were carried out to generate DNA fragments of hnRNP C1 (88-165), hnRNP C1 (1-165) hnRNP C1 (88-290) and hnRNP C1 (1-250). Each of these various DNA fragments was individually subcloned into pDsRed2-C1. The DNA fragment of hnRNP C1 (251-290) (C-terminal 40 amino acids) was also generated by PCR and subcloned into the nuclear reporter vector pEYFP-Nuc (Clontech). The construct of myc-tagged ROCK(CAT) in the pcDNA3 was described previously (Lai et al., 2003).

### Cell culture and transient transfection

D2 cells were maintained in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) and supplemented with 10% heat-inactivated fetal bovine serum (Hy-Clone, Logan, UT, USA), 2 mM L-glutamine, 100 U/ml penicillin G and 100 U/ml streptomycin. HEK293T cells were maintained in DMEM medium containing 10% fetal bovine serum. NIH3T3 mouse fibroblasts were grown in DMEM medium containing 10% donor calf serum and were placed in serum-free medium overnight before treatment with 25 ng/ml recombinant mouse TNF $\alpha$  (Peprotech) plus 20  $\mu$ g/ml cycloheximide. For transient transfection experiments, NIH3T3 and HEK293T cells were grown on glass coverslips in 35 mm dishes in complete medium overnight before transfection. Cells were transfected with 1  $\mu$ g of each DNA construct and 3  $\mu$ l of Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

### Cell fractionation and western blotting

Cells with different treatments were harvested and washed with phosphate-buffered saline (PBS). The cytoplasmic and nuclear fractions were prepared using NE-PER<sup>TM</sup> Nuclear and Cytoplasmic Extraction Reagents (Pierce) according to the manufacturer's instructions. The protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride membrane (Millipore). The antibodies used and their dilutions were as follows: anti-hnRNP C1/C2, 1:2,000; anti-hnRNP A2/B1, 1:2000; anti-lamin A/C, 1:3000; anti-Sp1, 1:2000; anti-human TK1 (TK-1), 1:2000. Horseradish peroxidase-conjugated anti-goat immunoglobulin G (IgG), anti-rabbit IgG and anti-mouse IgG antibodies (Santa Cruz, CA, USA) were used for detection of the primary antibodies. Enhanced chemiluminescence detection of the horseradish peroxidase reaction was performed according to the vendor's instructions.

### Protein Cy3/Cy5 labeling and two-dimensional electrophoresis

The cytosolic proteins were acetone precipitated and dissolved in IEF buffer (7 M urea, 2 M thiourea, 4% Chaps and 40 mM Hepes, pH 8.5) in a final concentration of 5  $\mu$ g/ $\mu$ l. The CyDye fluors (mono NHS ester) were prepared to a final concentration of 400 pmole/ $\mu$ l in dimethylformamide. 100  $\mu$ g of proteins were labeled with 800 pmol of fluors for 30 minutes on ice in the dark. The reaction was stopped by adding 20 nmol of lysine. Equal amounts of Cy3- and

Cy5-labeled proteins were mixed and then separated in the same 2DE gel.

The Immobiline DryStrips (pH 4-7, 18-cm; Amersham Bioscience) were rehydrated with rehydration buffer (8 M urea, 2% Chaps, 2 mM tributylphosphine and 0.5% ampholyte, pH 4-7) for at least 12 hours. The protein sample mixtures (200 µg) were applied into the strip using cup loading. Isoelectric focusing was performed at 20°C using an IPGphor electrophoresis unit (Amersham Bioscience). A gradient of 300-8000 V was applied to the strips followed by constant 8000 V, with focusing complete after 50 KWhr. Prior to the second dimension electrophoresis, strips were incubated in equilibration buffer (6 M urea, 2% SDS, 50 mM Tris, pH 8.8, and 30% glycerol containing 2% dithiothreitol) for 15 minutes, then followed by another 15-minute incubation with the same buffer containing 2.5% iodoacetamide. Equilibrated strips were inserted onto the top of SDS-PAGE gels (9%-16% gradient), and sealed with 0.5% agarose. The second dimension electrophoresis was performed at 12°C (BioRad Protean IIXi) with 40 mA/gel constant current for 5 hours. The images were acquired using ProXPRESS™ image system (PerkinElmer).

#### Protein identification

Preparative 2DE gels were silver stained using the low fixation protocol, and the protein spots were excised directly from gels. The in-gel digestions were performed according to the method of Shevchenko et al. (Shevchenko et al., 1996), except that reduction and alkylation steps were omitted because cysteines were carboxylamidomethylated during gel preparation. The proteins were digested with modified porcine trypsin (Promega), and the peptides were eluted with 5% TFA/50% acetonitrile. The in-gel digests were then separated and analyzed by the Nanoflow capillary liquid chromatography-tandem mass spectrometric (LC/MS/MS) system using a capillary LC system (Ultimate, LC Packings, Amsterdam, Netherlands) coupled to a Q-TOF (quadrupole time-of-flight) mass spectrometer (QSTAR Pulsar i; Applied Biosystem/MDS Sciex, Foster city, CA, USA). Peptide product ion spectra generated by LC/MS/MS were searched against the SwissProt protein database using the Mascot search program sequence database search engine (Perkins et al., 1999).

#### Immunofluorescence staining

Cells on coverslip were fixed with 3% paraformaldehyde (Merck) in PBS at room temperature for 30 minutes, and then permeabilized with cold 100% methanol (-20°C) for 5 minutes. Cells were washed with TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) and blocked by incubation with 5% normal rabbit serum in TBST. Cells were then incubated with anti-hnRNP C1/C2 (1:100 dilution) in TBST containing 3% bovine serum albumin (BSA) in a humidified atmosphere at 4°C overnight. After washing in TBST, cells were incubated with TRITC-conjugated rabbit anti-goat IgG antibody (Sigma) at a 1:200 dilution in TBST-3% BSA containing 4',6'-diamidino-2-phenylindole (DAPI) for 1 hour at room temperature. Cells were then washed with TBST four times and mounted for analysis with a Leica TCS SP2 confocal spectral microscope.

#### Electron microscopy

Cells were fixed with 1% glutaraldehyde and 4% paraformaldehyde in PBS at 4°C for overnight, and post-fixed with 1% osmium tetroxide in PBS at room temperature. After dehydration in a graded series of ethanol and embedding in Epoxy resin, ultra-thin sections of cells were cut and stained with uranyl acetate/lead citrate for examination with an electron microscope (Hitachi 7100).

## Results

### A proteomic screen for ROCK-regulated changes in PMA-induced apoptosis

Our previous study has shown that inhibition of ROCK by Y27632 significantly interferes with PMA-induced apoptosis and induces cell adhesion (Lai et al., 2003). To search for molecular events that are dependent on ROCK activity, we compared protein expression patterns of PMA-treated cells that were pre-incubated with or without Y27632. To exclude the molecular changes that might arise as a result of adhesion, we plated cells onto hydrogel-coated dishes to prevent adhesion. Cells were found to induce contraction and apoptosis in serum-free medium following PMA treatment, and pre-incubation of cells with Y27632 could interfere with this PMA-induced apoptosis (Fig. 1A,B). Accordingly, cells were plated under this experimental condition and monitored for protein alterations in response to ROCK inhibition during PMA induction. Under the circumstances, we believed that the molecular changes derived from serum- and adhesion-mediated signals were significantly minimized. As ROCK is present in the cytoplasm, we specifically isolated the cytosolic fractions of cells for 2DE analysis. Cytosolic fractions from PMA- and PMA+Y27632-treated cells were prepared and acetone precipitated, followed by solubilization with IEF buffer for the subsequent labeling with Cy5- and Cy3-mono NHS ester dye, respectively. The labeled proteins from these two preparations were subjected to a single isoelectric focusing (IEF), followed by second dimension gel separation. The gel was then analyzed by an imaging system to show differential fluorescence labeling patterns. Cy3- and cy5-labeled proteins were indicated by green and red color, respectively. Two images were overlapped to show the differentially expressed spots that are Y27632 responsive (Fig. 1C). A series of spots at pI 4.8 to 5.2 with molecular masses of around 34-42 kDa were clearly seen in PMA-treated cells, but not in PMA+Y27632-treated cells. Closer examination of the 2DE profile revealed at least six spots in this region that were responsive to ROCK inhibition (Fig. 1D). Each of the spots was excised and subjected to in-gel digestion with trypsin. By mass spectrometric analysis, they all appeared to be hnRNP C1 or hnRNP C2. We speculate that these multiple spots correspond to phosphorylated isoforms of hnRNP C1/C2, since it has been shown that casein kinase II-like enzyme in nuclei can phosphorylate hnRNP C1/C2 (Mayrand et al., 1993). Because the 2DE patterns of hnRNP C1/C2 from whole cell extract of PMA- and PMA+Y27632-treated cells were essentially the same (Fig. 1E), it is unlikely that the covalent modification of hnRNP C1/C2 is affected by the ROCK-mediated pathway. Rather, a compartmentalization change of hnRNP C1/C2 is closely related to the status of ROCK activation.

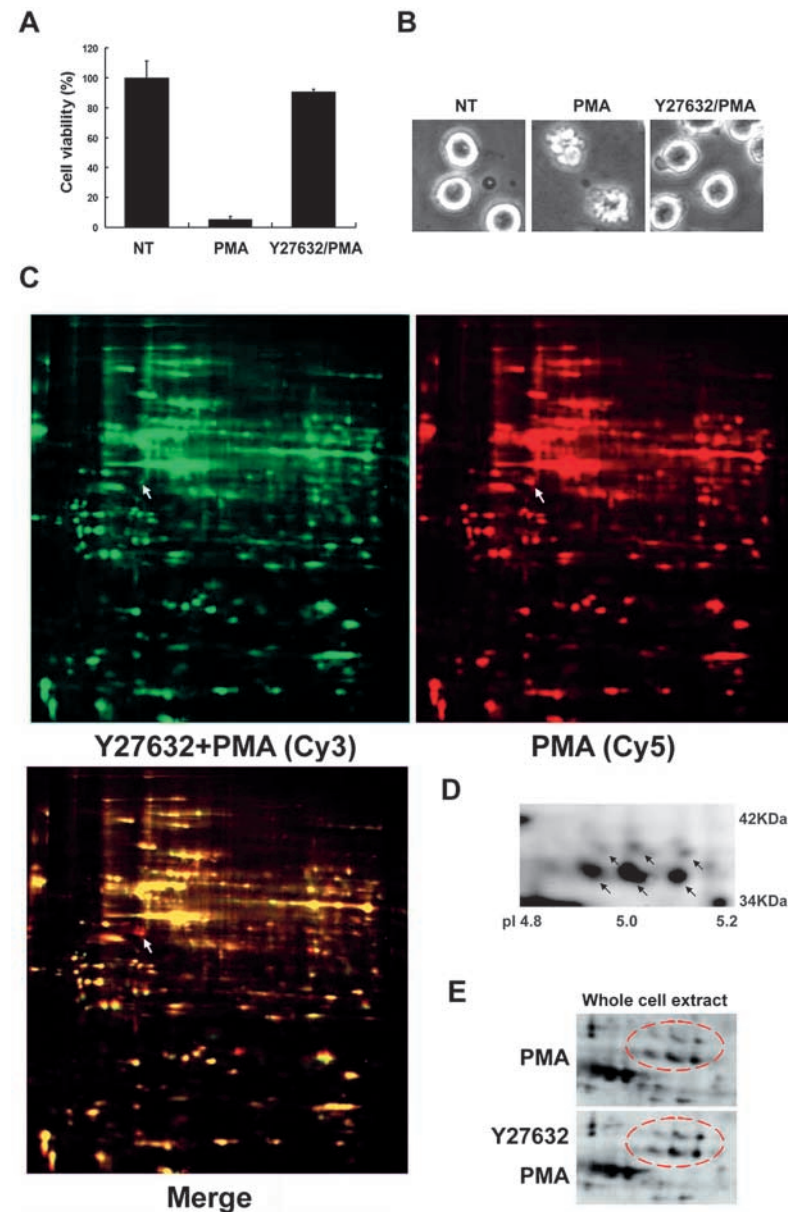
### Evidence for ROCK-dependent cytosolic translocation of hnRNP C1/C2

Our previous study had already found that following PMA treatment in the serum-containing medium, a proportion of cells attached to the dish and survived, while others remain in suspension and become apoptotic (Lai et al., 2001). Western blot analysis showed that the levels of cytosolic hnRNP C1/C2 were significantly increased in the PMA-induced pro-apoptotic

suspension cells in a time-dependent manner, whereas in the attached cells hnRNP C1/C2 were clearly restricted to the nuclear fraction (Fig. 2A). By probing the same blots with

antibodies against TK1, a cytosolic protein, and Sp1, a nuclear transcription factor, we assured that cytosolic and nuclear fractions were appropriately prepared. Of note, the protein levels of hnRNP C1/C2 in total cellular lysates remained essentially unaltered in attached and suspended PMA-treated cells. Confocal microscopic observation of cells with immunofluorescence staining with hnRNP C1/C2 antibody further confirmed that the subcellular localization of hnRNP C1/C2 was no longer restricted to the nuclei, but distributed in cytoplasm of the PMA-treated suspension cells (Fig. 2B). We have previously shown that PMA-induced apoptosis can be inhibited by treatment of cells with a general caspase inhibitor zVAD-fmk without affecting cell contraction (Lai et al., 2003). Here we found that inhibition of caspases did not affect translocation of nuclear hnRNP C1/C2 to the cytoplasm in the PMA-treated suspension cells, indicating that the translocation event is independent of apoptotic execution.

To determine whether the cytosolic distribution of hnRNP C1/C2 is a result of a defect in nuclear translocation of newly synthesized protein in the pro-apoptotic cells, we then tested the effect of pretreatment with cycloheximide, to inhibit protein synthesis, or with actinomycin D, to inhibit RNA synthesis, on their subcellular distribution during PMA induction. As shown in Fig. 2C, these two pre-treatments did not affect the cytosolic distribution of hnRNP C1/C2 in PMA-treated cells. In contrast, pre-treatment of cells with latrunculin B, an actin polymerization inhibitor, or Y27632 not only abrogated PMA-induced membrane contraction, but also prevented hnRNP C1/C2 translocation. Similar result could also be observed when cells were treated with the myosin ATPase inhibitor blebbistatin, further suggesting actomyosin-mediated contraction is required for this ROCK-dependent change (Fig. 2D). Taken together, we conclude that translocation of hnRNP C1/C2 from nuclei to cytoplasm in PMA-induced pro-apoptotic cells is dependent on ROCK-mediated cytoskeleton rearrangement. Consistent with result of confocal microscopic experiment, treatment of cells with the caspase 3 inhibitor, zDEVD-fmk, did not affect the cytosolic distribution of hnRNP C1/C2.

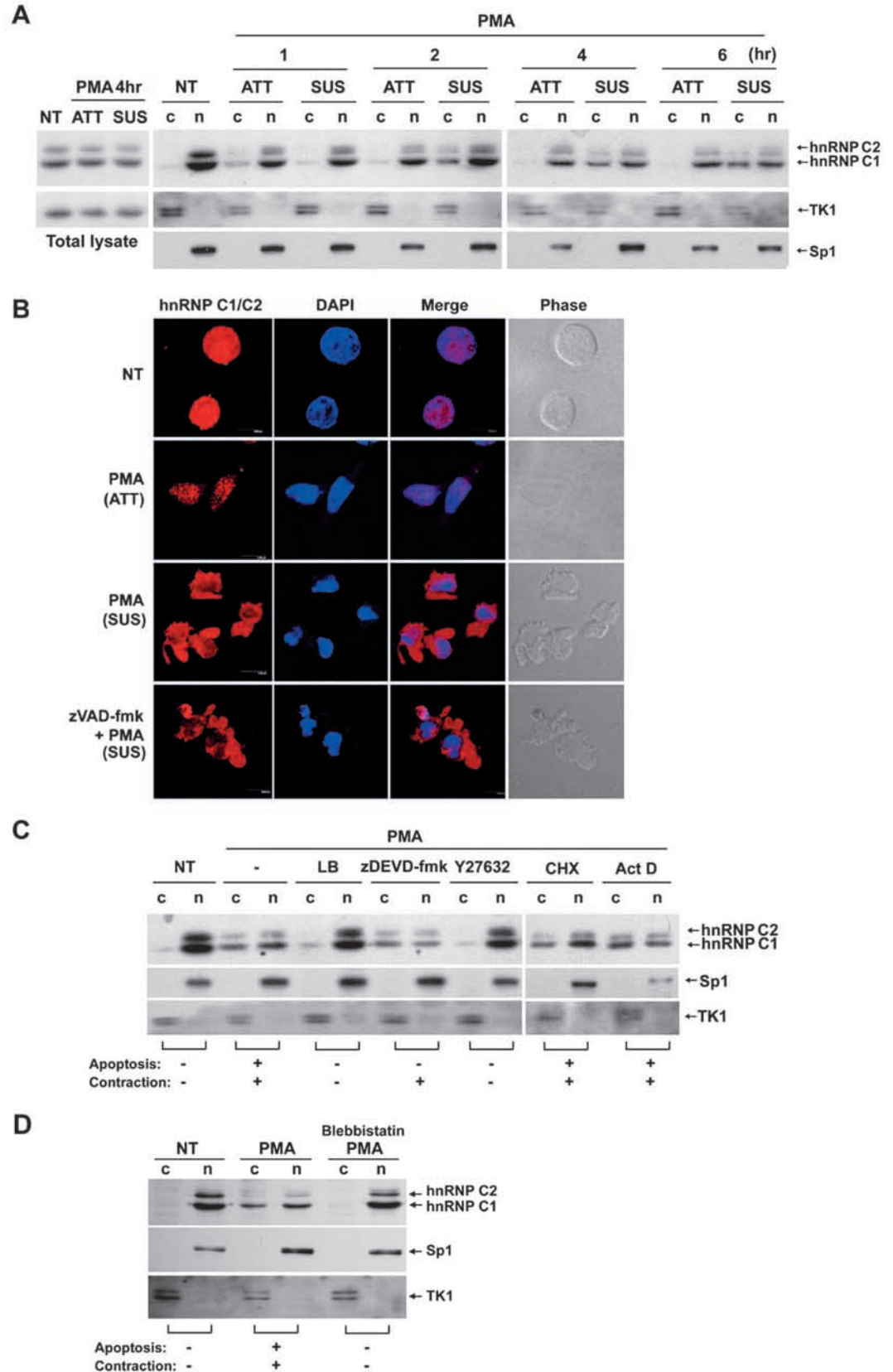


**Fig. 1.** A proteomic screen for ROCK-regulated changes during PMA-induced apoptosis. D2 cells were pre-treated with or without 20  $\mu$ M Y27632 for 1 hour in serum-free medium on hydrogel-coated dishes prior to treatment with 32.4 nM PMA. (A) After PMA treatment for 8 hours, cell viability was determined by the Trypan Blue exclusion method. NT, cells without either PMA or Y27632 treatment. (B) Phase-contrast micrographs of D2 cells after 4 hours of PMA treatment as indicated. (C) Following treatment with PMA for 4 hours, the cytosolic proteins of cells treated with and without Y27632 were prepared and labeled with Cy3 and Cy5, respectively. Two sets of these labeled lysates each containing 100  $\mu$ g of proteins were pooled together and separated on a single 2DE gel. Green indicates Cy3-tagged proteins, red Cy5-tagged proteins and yellow colocalization of the spots. (D) A higher power image of spots corresponding to hnRNP C1/C2. (E) Whole cell extracts of D2 cells treated as in C were separated by 2DE and the gels were silver stained. Red dashed line outlines the spots corresponding to hnRNP C1/C2.

#### Cytosolic distribution of hnRNP C1/C2 is not a result of nuclear leakage in PMA-induced pro-apoptotic cells.

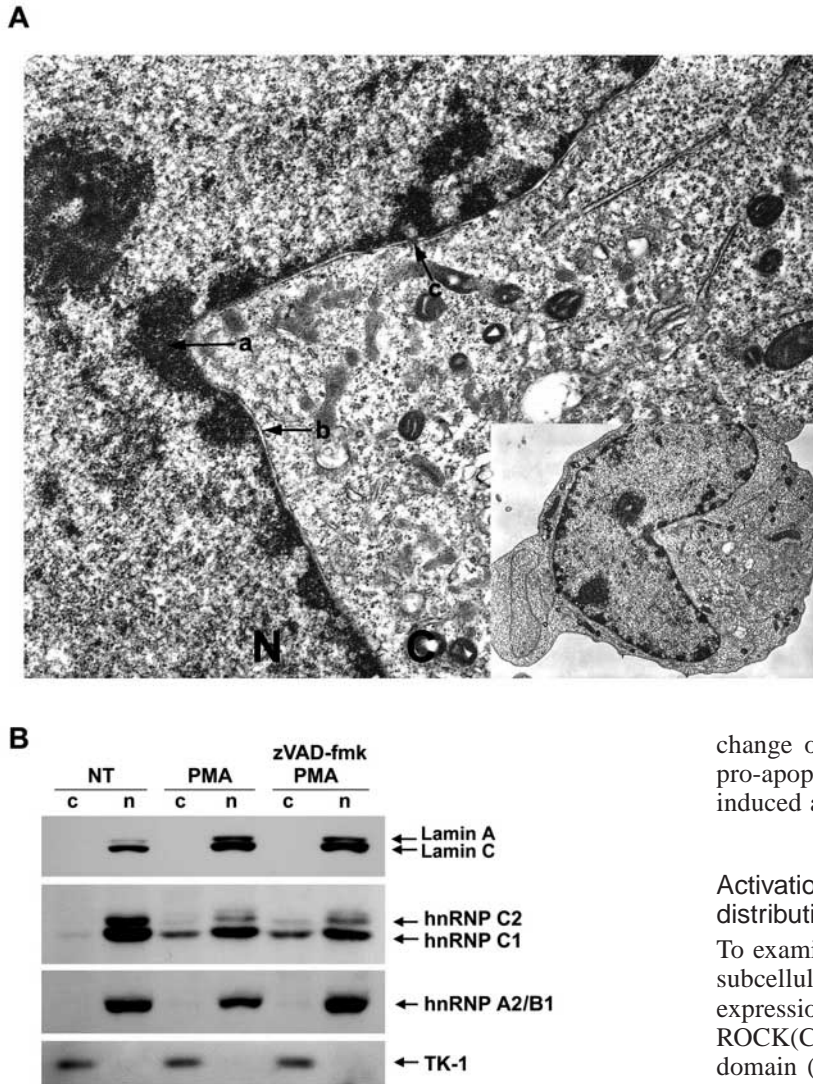
We next used electron microscopy to directly assess whether there is nuclear envelope breakdown in PMA-induced pro-apoptotic cells. The images showed that the nuclear membrane remained intact (Fig. 3A). To determine whether this efflux phenomenon also happens for other hnRNP and nuclear matrix proteins, we examined the subcellular distribution of hnRNP A2/B1, other

**Fig. 2.** HnRNP C1/C2 are translocated to cytoplasm in PMA-induced pro-apoptotic cells in a manner dependent on ROCK-mediated cytoskeleton rearrangement. (A) D2 cells were treated without (NT) or with 32.4 nM PMA in serum-containing medium on regular dishes. Cells remained in suspension (SUS) and attached to the culture dish (ATT) were harvested at the time indicated. Whole cell lysates, cytosolic and nuclear fractions were prepared and separated by SDS-PAGE, followed by western blot analysis with antibodies against hnRNP C1/C2, Sp1 and TK1. (B) D2 cells treated with 32.4 nM PMA as described above for 4 hour were fixed and incubated with antibody against hnRNP C1/C2, followed by visualization with TRITC-conjugated anti-goat antibody. A parallel PMA-induced suspension culture with 50  $\mu$ M zVAD-fmk incubation was subjected to the same analysis. Nuclei were stained with DAPI. Scale bar: 8  $\mu$ m. (C) D2 cells were incubated in serum-free medium on hydrogel-coated dishes and treated with 32.4 nM PMA for 4 hours in the presence of reagents as indicated (0.5  $\mu$ M latrunculin B, LB; 20  $\mu$ M zDEVD-fmk; 20  $\mu$ M Y27632; 50  $\mu$ g/ml cycloheximide, CHX; 5  $\mu$ g/ml actinomycin D, Act D). (D) D2 cells were treated as (C) in the presence of 50  $\mu$ M blabbistatin for 4 hours. The cytosolic and nuclear fractions were prepared for Western blot analysis as described above.



major hnRNP proteins and lamin A/C, which are major nuclear matrix proteins, in PMA-induced pro-apoptotic cells. It appeared that unlike hnRNP C1/C2 these nuclear proteins

stayed in nuclei without translocation to the cytoplasm (Fig. 3B), confirming that the cytosolic distribution of hnRNP C1/C2 is not the result of nuclear membrane leakage. Thus, hnRNP



**Fig. 3.** No nuclear leakage occurs in PMA-induced pro-apoptotic cells. (A) Electron micrograph of D2 cells that were treated with 32.4 nM PMA for 2 hours in the presence of 50  $\mu$ M zVAD-fmk. Cells were then washed with PBS and fixed with 4% paraformaldehyde and 1% glutaraldehyde at 4°C for overnight. N, nucleus; C, cytoplasm; a, heterochromatin; b, nuclear envelope; c, nuclear pore complexes. (B) Western blot of the cytosolic and nuclear fractions, as described in the legend to Fig. 2C, with antibodies against hnRNP C1/C2, hnRNP A2/B1, lamin A/C and TK1.

C1/C2 are no longer nuclear restricted proteins in the cells undergoing ROCK-mediated contraction.

#### Subcellular distribution of hnRNP C1/C2 in TNF $\alpha$ -induced apoptotic cells

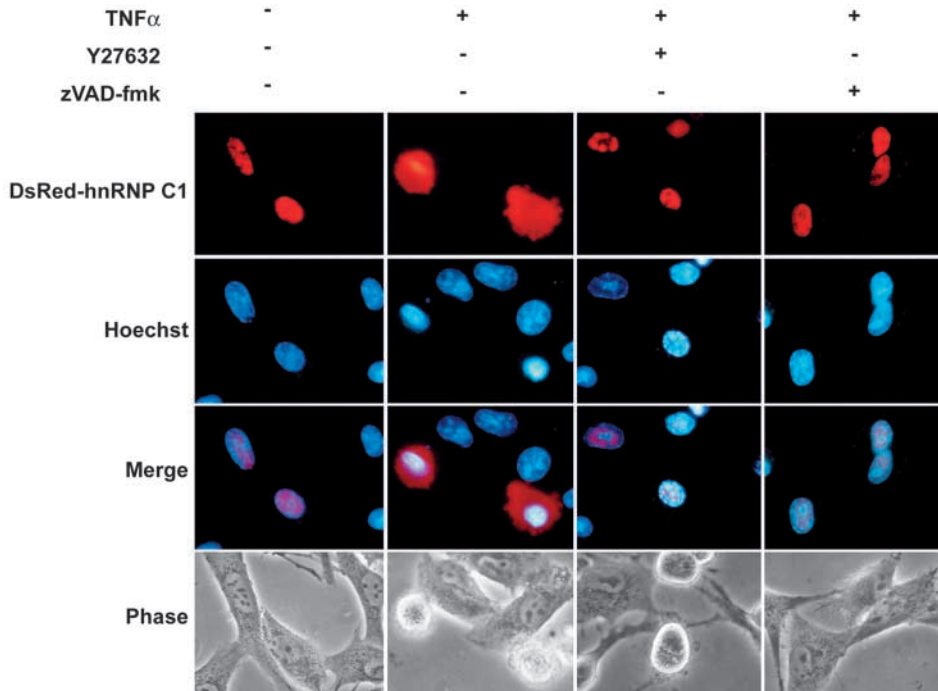
To determine whether ROCK-regulated cytosolic translocation of hnRNP C1/C2 also occurred in other apoptotic cell systems, we examined the subcellular localization of hnRNP C1 in the TNF $\alpha$ -induced apoptotic cells, where ROCK I is activated by caspase-3-mediated cleavage and is required for membrane blebbing (Coleman et

al., 2001). To this end, NIH3T3 cells were transfected with red fluorescent protein tagged-hnRNP C1 expression construct (pDsRed-hnRNP C1) for convenient observation. The transfected cells were serum-starved for 12 hours, followed by treatment with cycloheximide for 2 hours in the presence or absence of Y27632. Apoptosis was then induced by addition of TNF $\alpha$  for 3 hours. These cells were then stained with Hoechst, and examined microscopically. As expected, the DsRed-hnRNP C1 was localized in the nuclei of cells without apoptotic induction. Following TNF $\alpha$ -induced apoptosis, DsRed-hnRNP C1 was distributed in the cytoplasm in NIH3T3 cells. Inhibition of ROCK activity by Y27632 treatment markedly reduced membrane blebs and blocked cytosolic distribution of DsRed-hnRNP C1 in these cells (Fig. 4). In this apoptotic system, inhibition of caspase activity by zVAD-fmk abrogates both apoptosis and ROCK I activation; as a result, cells were rescued from apoptosis and DsRed-hnRNP C1 remained in the nuclei. Therefore, ROCK-dependent compartmentalization

change of hnRNP C1/C2 occurs not only in PMA-induced pro-apoptotic cells but also in the execution phase of TNF $\alpha$ -induced apoptotic NIH3T3 cells.

#### Activation of ROCK is sufficient to induce cytosolic distribution of hnRNP C1/C2

To examine whether activation of ROCK can directly induce subcellular distribution change of hnRNP C1/C2, the expression vector of the myc-tagged dominant active form of ROCK(CAT), which contains only an amino-terminal kinase domain (Izawa et al., 1998), was transfected into HEK293T cells. After transfection for 20 hours, cells became rounded and contracted, presumably due to actin-myosin-mediated contraction. However, these cells remained viable without the detection of caspase 3 activation (data not show). Interestingly, hnRNP C1/C2 were also detected in the cytoplasm of HEK293T cells expressing ROCK(CAT), while in the cells transfected with control vector they were confined to the nuclei (Fig. 5A). Thus, activation of ROCK is sufficient for inducing cytosolic translocation of hnRNP C1/C2. Similar to that in PMA-induced pro-apoptotic cells, fractionation experiments showed that other major hnRNP proteins, hnRNP A2/B1, still remained in the nuclear fraction regardless of ROCK(CAT) expression (Fig. 5B). Furthermore, we cotransfected the expression constructs of GFP-tagged active forms of RhoA (GFP-RhoAV14) and pDsRed-hnRNP C1 in HEK293T cells. As shown in Fig. 5C, overexpression of GFP-RhoAV14 also resulted in cell contraction and cytosolic distribution of hnRNP C1. In contrast, expression of RhoAV14 E40L, which is a dominant active form of RhoA defective in interaction with ROCK (Sahai et al., 1998), induced neither cell contraction nor nuclear export of hnRNP C1. Taken together these results suggest that activation of the RhoA/ROCK pathway is sufficient to induce efflux of hnRNP C1/C2 in HEK293T cells.



**Fig. 4.** The cytosolic distribution of hnRNP C1/C2 in TNF $\alpha$ -induced apoptotic NIH3T3 cells is also dependent on ROCK activation. NIH3T3 mouse fibroblasts on 35 mm dishes were transfected with 1  $\mu$ g of pDsRed-hnRNP C1. Cells were serum-starved for 12 hours, after which cells were pre-treated with cycloheximide (20  $\mu$ g/ml) for 1 hour, followed by incubation with or without Y27632 (20  $\mu$ M) or zVAD-fmk (50  $\mu$ M) as indicated for another 1 hour. Apoptosis was initiated by adding 25 ng/ml TNF $\alpha$  to the medium for 3 hours. The nuclei were stained with Hoechst.

A novel ROCK-responsive element for nuclear export resides within the carboxyl-terminus of hnRNP C1

To delineate the molecular mechanism of ROCK-induced cytosolic translocation, we next determined which region is responsible for sensing ROCK-mediated signal for translocation. The major features of the hnRNP C1 protein are, a RNP motif RNA binding domain (RBD) at the amino terminus (residues 1-104), a nuclear retention sequence (NRS) at residues 88-165, and a carboxyl-terminal auxiliary domain rich in acidic residues (residues 166-290). We generated four truncated forms of hnRNP C1 tagged with DsRed, as shown schematically in Fig. 6A. In ROCK(CAT)-expressing HEK293T cells, the distribution of wild-type hnRNP C1 was cytosolic, while the DsRed-hnRNP C1 truncated forms, 88-165 and 1-165, stayed in the nuclei. This result not only indicates that the ROCK-activated signal does not impair the nuclear retention function of NRS in hnRNP C1, but also suggests that a sequence signal present in the wild-type hnRNP C1/C2 can sense ROCK activation to override the nuclear retention function. Since expression of DsRed-hnRNP C1 (88-290) was also cytosolic in a ROCK(CAT)-responsive manner (Fig. 6B), we deduced that the C-terminal region of hnRNP C1, covering residues 166-290, should contain the element sensitive to ROCK-dependent translocation. We then expressed the C-terminus-deleted mutant form (1-250) in HEK293T cells, and found that co-expression of ROCK(CAT) did not significantly cause evacuation of this deleted hnRNP C1 from the nuclei. These results led to the conclusion that the C-terminal 40-amino-acid sequence of the hnRNP C1 is a necessary element for ROCK-responsive translocation.

Next, we determined whether the 40 amino acids in the C-terminal region of hnRNP C1 could act as a ROCK-responsive signal in export of nuclear protein. To this end, we constructed a reporter plasmid that contains the sequence covering this 40-amino-acid region (C40) fused to a nuclear YFP reporter gene,

YFP-Nuc, which contains the nuclear localization signal (NLS). After transfection, we found that both YFP-Nuc and YFP-Nuc-C40 proteins were confined in the nuclear compartments. Co-expression of ROCK(CAT) did not significantly change nuclear distribution of YFP-Nuc protein, indicating that overexpression of ROCK(CAT) does not cause a nuclear leakage or inhibit the nuclear translocation. In contrast, YFP-Nuc-C40 fusion protein translocated to the cytoplasm in response to ROCK(CAT) (Fig. 7A). This 40-amino-acid region is rich in acidic amino acid residues (Fig. 7B). Based on this observation, we define that the 40-amino-acid in C-terminal region of hnRNP C1/C2 is a novel ROCK-responsive nuclear export signal, which contributes to nuclear efflux of hnRNPC1/C2 in apoptotic cells.

## Discussion

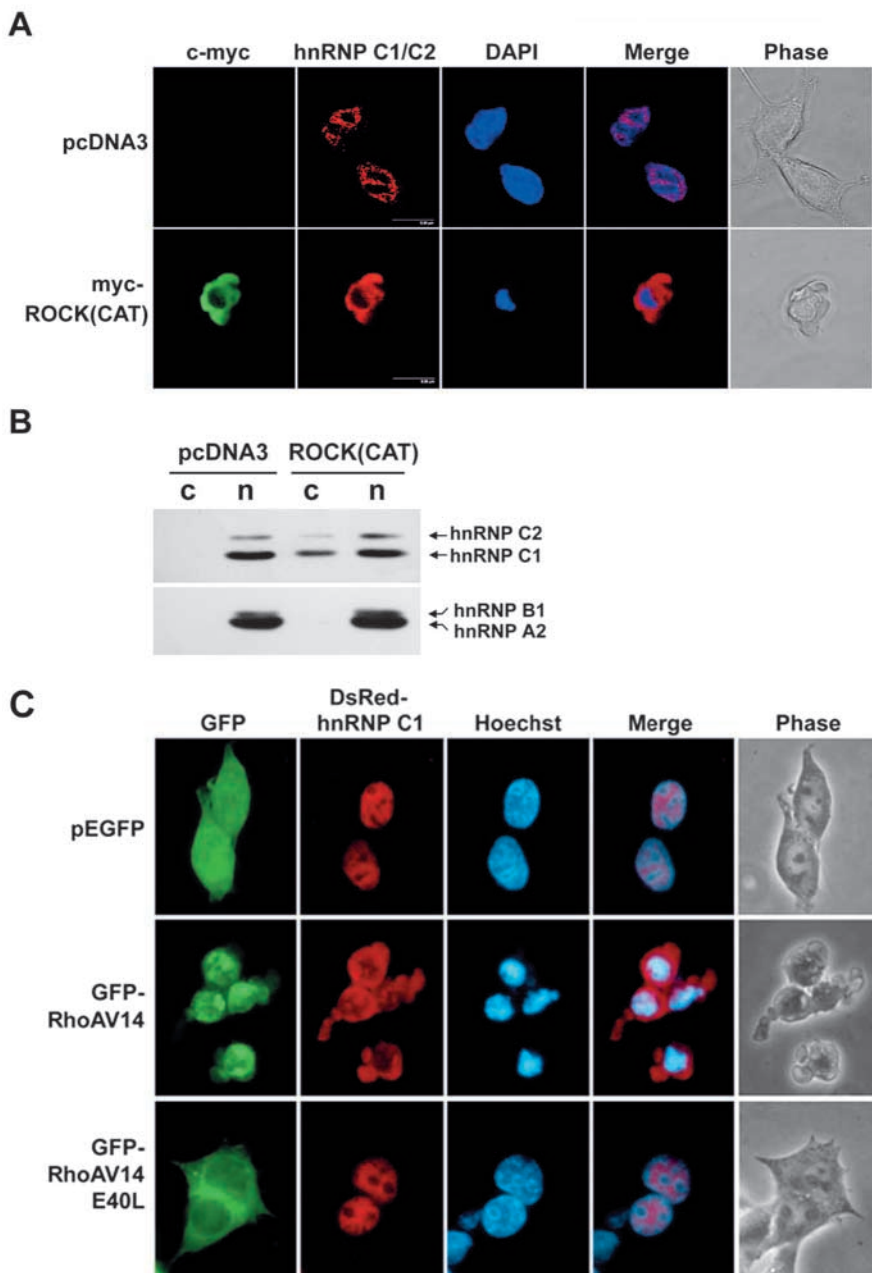
ROCK has been shown to contribute to the formation of actin cytoskeleton structures and to contractile force generation through its direct phosphorylation on MLC and inactivation of myosin phosphatase, and its activation of LIM kinase to stabilize actin filament (Amano et al., 1996; Kimura et al., 1996; Maekawa et al., 1999; Ohashi et al., 2000; Sumi et al., 2001). We have previously established that RhoA/ROCK/MLC phosphorylation is a necessary pathway in PMA-induced apoptosis in D2 and TF-1 cells (Lai et al., 2003). In this study we searched for other molecular events regulated by ROCK during PMA-induced apoptosis by a proteomic approach. We found that ROCK-dependent contraction led to an alteration of subcellular distribution of hnRNP C1/C2 in PMA-induced proapoptotic cells independent of caspase activation. From the experimental results of electron microscopy and cycloheximide treatment, we knew that cytosolic distribution of hnRNP C1/C2 is not due to the nuclear membrane breakdown, or to cytosolic retention of newly synthesized hnRNP C1/C2 proteins.

Because other major hnRNP proteins, hnRNP A2/B1, nuclear matrix proteins, lamin A/C and general transcription factor Sp1 still stay in the nuclear compartment of PMA-induced proapoptotic cells, we conclude that efflux of hnRNP C1/C2 is a rather specific event controlled by ROCK activation. Cytosolic distribution of hnRNP C1/C2 can also be seen in the TNF $\alpha$ -induced apoptotic NIH3T3 cells, where ROCK I activation is dependent on caspase-3-mediated cleavage. In summary, this study discovered that ROCK activation due to either RhoA signaling or caspase-3-mediated cleavage in the cytoplasm of the apoptotic cells results in cytosolic distribution of hnRNP C1/C2.

It should be mentioned that previous experiments using domain swapping and heterokaryon assay have shown that the NRS of hnRNP C1/C2 overrides NES function, thus prohibiting protein shuttling (Nakielny and Dreyfuss, 1996).

Therefore, unlike other hnRNP members which shuttle between nucleoplasmic and cytoplasm compartments, hnRNP C1/C2 are restricted to the nucleus because of the presence of their NRS. In this work, we showed that overexpression of dominant active form of ROCK(CAT) in HEK293T cells is sufficient to induce efflux of hnRNP C1/C2, which could be a result of impairment of NRS function or induction of a specific export. Since various C-terminus-deleted forms of hnRNP C1, which contain NRS, are retained in nuclei irrespective of ROCK(CAT) expression, it is evident that the efflux of hnRNP C1/C2 proteins is not due to the impairment of NRS-mediated nuclear retention mechanism. Therefore, it is more likely that an active export process specific to hnRNP C1/C2 is activated by the ROCK-mediated signal and overrides NRS function, so that hnRNP C1/C2 are delocalized from the nuclear compartment. This notion is supported by the experimental

data showing that expression of ROCK(CAT) also induces translocation of a reporter, YFP-Nuc-C40, which contains a fusion with the C-terminal 40 amino acids of hnRNP C1. Thus, this sequence is a novel and transferable NES, which mediates an as-yet-unknown nuclear export mechanism in response to ROCK-mediated signaling. Because the translocation of hnRNP C1/C2 to cytosol is insensitive to leptomycin B treatment, we know that this export is a CRM1-independent process (data not shown). It is worth noting that this C-terminal region is rich in acidic amino acid residues, which is very different from other well-characterized nuclear export signals. However, it has been reported that the viral protein pUL69 of the  $\beta$ -herpesvirus human cytomegalovirus contains a unique nuclear export signal located within its C-terminal 28 amino acid region, which is also rich in acidic amino acid (Lischka et al., 2001). Since there is no further



**Fig. 5.** Expression of the dominant active form of ROCK(CAT) induces cytosolic translocation of hnRNP C1/C2. (A) HEK293T cells on 35 mm culture dishes were transfected with 1  $\mu$ g of expression plasmid of ROCK(CAT) or control vector overnight. Cells were fixed and stained with antibodies against hnRNP C1/C2 and c-myc, followed by visualization with TRITC-conjugated anti-goat and FITC-conjugated anti-mouse antibodies. Nuclear staining was performed with DAPI. (B) The cytosolic and nuclear fractions from cells as described in A were prepared for western blot analysis with antibodies against hnRNP C1/C2 and hnRNP A2/B1. (C) HEK293T cells were co-transfected with 0.5  $\mu$ g of expression plasmid of GFP-RhoAV14 or GFP-RhoAV14 E40L or pEGFP and 0.5  $\mu$ g of pDsRed-hnRNP C1. Cells were stained with Hoechst and observed by fluorescence microscopy.

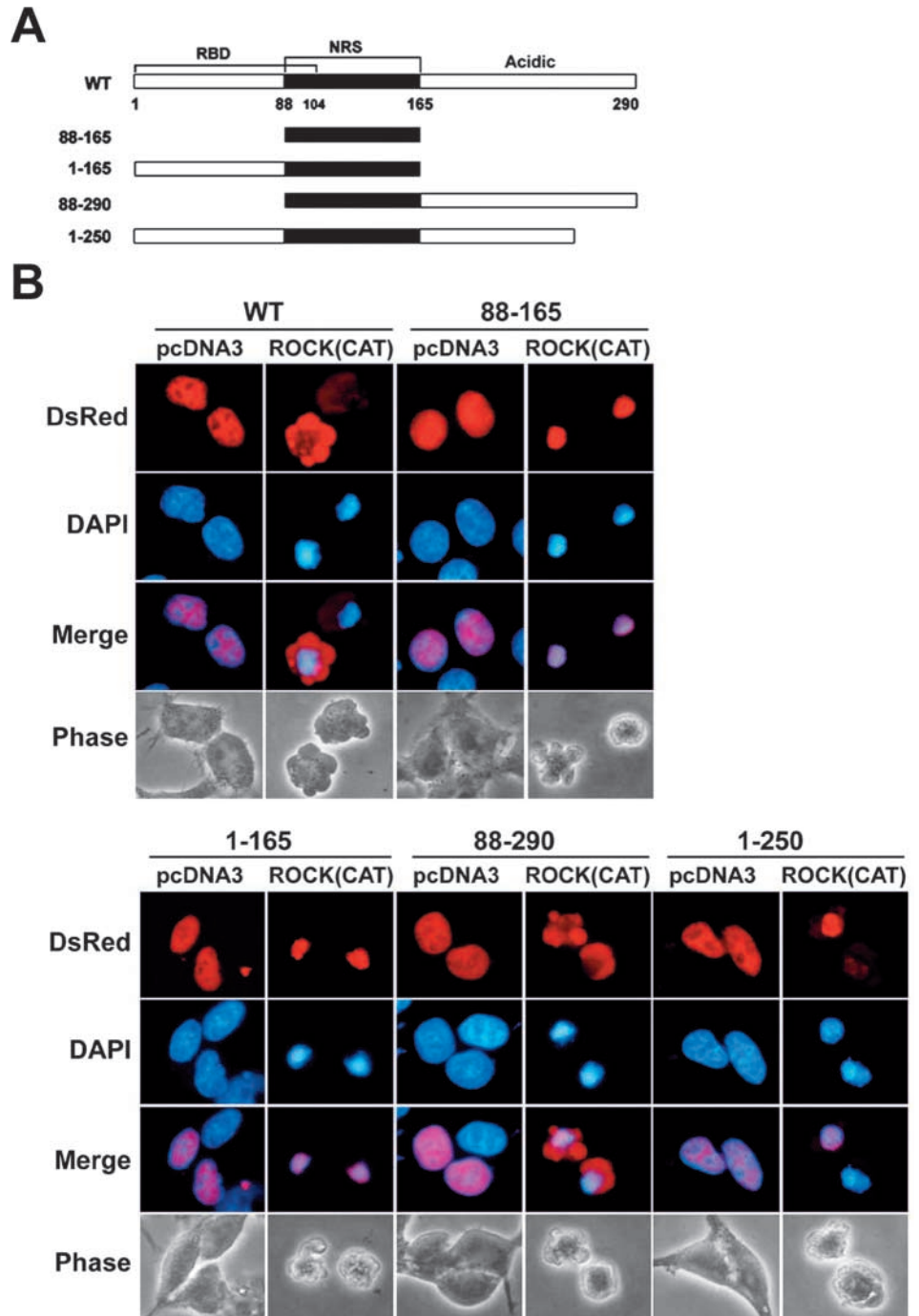


characterization of this novel viral NES, at present we are unable to define the relevance. To the best of our knowledge, this is the first report describing that hnRNP C1/C2 are specifically equipped for delocalization, which is a process not activated until overproduction of ROCK-mediated contraction force.

Since ROCK is a cytosolic kinase, the results of this study pose an interesting question: how does contraction force generated in the cytoplasm transmit the signal to the nuclear compartment? It is known that the nuclear matrix and intermediate filaments (IF) are integrated into a single cell-wide structure (Tolstonog et al., 2002). This structural integration means that nuclear shape is always coupled to cell shape (Maniotis et al., 1997; Sims et al., 1992). It has been shown that ROCK-mediated phosphorylation of vimentin causes the collapse of the vimentin IF network (Inada et al., 1999; Sin et al., 1998; Yasui et al., 2001). In this study, we found that the nuclear membrane remains intact on the deformed nuclei of PMA-induced pro-apoptotic cells. Perhaps, the contraction force works together with the altered IF network to change nuclear architecture, which in turn activates hnRNP C1/C2 export without affecting the nuclear envelope. Alternatively, it is also possible that activation of ROCK in the cytosol may induce an unknown molecule translocated to the nuclear compartment, which in turn triggers the export.

Given the fact that hnRNP C1/C2 are implicated in a variety of processes including RNA transcript package, splicing, polyadenylation, turnover, telomere regulation, nuclear retention of hnRNAs and nuclear matrix (Choi et al., 1986; Ford et al., 2002; Huang et al., 1994; Krecic and Swanson, 1999; Nakielny and Dreyfuss, 1997; Nakielny and Dreyfuss, 1999; van Eekelen and van Venrooij, 1981; Wilusz and Shenk, 1990), we have speculated that the efflux process of hnRNP C1/C2 could potentially affect RNA metabolism and nuclear function. By RNA interference experiments, however, we found that knockdown of hnRNP C1/C2 in HEK293T cells did not significantly affect

cell growth, protein synthesis and general RNA synthesis (data not shown), indicating that loss of hnRNP C1/C2 proteins alone does not exert a significant effect on these biological



**Fig. 6.** Mapping the sequence region of hnRNP C1 required for ROCK-induced cytosolic translocation. (A) Schematic presentation of various domains of hnRNP C1 and the constructs of its deletion mutants. HnRNP C1 contains a RNA binding domain (RBD: 1-104), a nuclear retention sequence (NRS: 88-165), and a C-terminal acidic region. (B) HEK293T cells on 35 mm dishes were co-transfected with 0.5 µg of expression plasmid of ROCK(CAT) or control vector together with 1 µg of pDsRed-hnRNP C1 or its deletion mutants as indicated. After expression for 20 hours, cells were fixed, stained with DAPI and observed by fluorescence microscopy for nuclei and DsRed detection.



- Possible involvement of the inactivation of the Rho-Rho-kinase pathway in oncogenic Ras-induced transformation. *Oncogene* **17**, 2863-2871.
- Jimenez, B., Arends, M., Esteve, P., Perona, R., Sanchez, R., Ramon y Cajal, S., Wyllie, A. and Lacal, J. C.** (1995). Induction of apoptosis in NIH3T3 cells after serum deprivation by overexpression of rho-p21, a GTPase protein of the ras superfamily. *Oncogene* **10**, 811-816.
- Kaibuchi, K., Kuroda, S. and Amano, M.** (1999). Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. *Annu. Rev. Biochem.* **68**, 459-486.
- Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K. et al.** (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273**, 245-248.
- Krecic, A. M. and Swanson, M. S.** (1999). hnRNP complexes: composition, structure, and function. *Curr. Opin. Cell Biol.* **11**, 363-371.
- Kureishi, Y., Kobayashi, S., Amano, M., Kimura, K., Kanaide, H., Nakano, T., Kaibuchi, K. and Ito, M.** (1997). Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J. Biol. Chem.* **272**, 12257-12260.
- Lai, J. M., Lu, C. Y., Yang-Yen, H. F. and Chang, Z. F.** (2001). Lysophosphatidic acid promotes phorbol-ester-induced apoptosis in TF-1 cells by interfering with adhesion. *Biochem. J.* **359**, 227-233.
- Lai, J. M., Wu, S., Huang, D. Y. and Chang, Z. F.** (2002). Cytosolic retention of phosphorylated extracellular signal-regulated kinase and a Rho-associated kinase-mediated signal impair expression of p21(Cip1/Waf1) in phorbol 12-myristate-13 acetate-induced apoptotic cells. *Mol. Cell. Biol.* **22**, 7581-7592.
- Lai, J. M., Hsieh, C. L. and Chang, Z. F.** (2003). Caspase activation during phorbol ester-induced apoptosis requires ROCK-dependent myosin-mediated contraction. *J. Cell Sci.* **116**, 3491-3501.
- Lischka, P., Rosorius, O., Trommer, E. and Stamminger, T.** (2001). A novel transferable nuclear export signal mediates CRM1-independent nucleocytoplasmic shuttling of the human cytomegalovirus transactivator protein pUL69. *EMBO J.* **20**, 7271-7283.
- Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K. and Narumiya, S.** (1999). Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* **285**, 895-898.
- Maniotis, A. J., Chen, C. S. and Ingber, D. E.** (1997). Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. *Proc. Natl. Acad. Sci. USA* **94**, 849-854.
- Mayrand, S. H., Dwen, P. and Pederson, T.** (1993). Serine/threonine phosphorylation regulates binding of C hnRNP proteins to pre-mRNA. *Proc. Natl. Acad. Sci. USA* **90**, 7764-7768.
- McAfee, J. G., Soltaninassab, S. R., Lindsay, M. E. and LeSturgeon, W. M.** (1996). Proteins C1 and C2 of heterogeneous nuclear ribonucleoprotein complexes bind RNA in a highly cooperative fashion: support for their contiguous deposition on pre-mRNA during transcription. *Biochemistry* **35**, 1212-1222.
- Nakielnny, S. and Dreyfuss, G.** (1996). The hnRNP C proteins contain a nuclear retention sequence that can override nuclear export signals. *J. Cell Biol.* **134**, 1365-1373.
- Nakielnny, S. and Dreyfuss, G.** (1997). Nuclear export of proteins and RNAs. *Curr. Opin. Cell Biol.* **9**, 420-429.
- Nakielnny, S. and Dreyfuss, G.** (1999). Transport of proteins and RNAs in and out of the nucleus. *Cell* **99**, 677-690.
- Narumiya, S., Ishizaki, T. and Uehata, M.** (2000). Use and properties of ROCK-specific inhibitor Y-27632. *Methods Enzymol.* **325**, 273-284.
- Ohashi, K., Nagata, K., Maekawa, M., Ishizaki, T., Narumiya, S. and Mizuno, K.** (2000). Rho-associated kinase ROCK activates LIM-kinase 1 by phosphorylation at threonine 508 within the activation loop. *J. Biol. Chem.* **275**, 3577-3582.
- Perkins, D. N., Pappin, D. J., Creasy, D. M. and Cottrell, J. S.** (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**, 3551-3567.
- Pinol-Roma, S. and Dreyfuss, G.** (1992). Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature* **355**, 730-732.
- Rech, J. E., LeSturgeon, W. M. and Flicker, P. F.** (1995). Ultrastructural morphology of the hnRNP C protein tetramer. *J. Struct. Biol.* **114**, 77-83.
- Sahai, E., Alberts, A. S. and Treisman, R.** (1998). RhoA effector mutants reveal distinct effector pathways for cytoskeletal reorganization, SRF activation and transformation. *EMBO J.* **17**, 1350-1361.
- Sebbagh, M., Renvoize, C., Hamelin, J., Riche, N., Bertoglio, J. and Breard, J.** (2001). Caspase-3-mediated cleavage of ROCK I induces MLC phosphorylation and apoptotic membrane blebbing. *Nat. Cell Biol.* **3**, 346-352.
- Shevchenko, A., Jensen, O. N., Podtelejnikov, A. V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Boucherie, H. and Mann, M.** (1996). Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. *Proc. Natl. Acad. Sci. USA* **93**, 14440-14445.
- Sims, J. R., Karp, S. and Ingber, D. E.** (1992). Altering the cellular mechanical force balance results in integrated changes in cell, cytoskeletal and nuclear shape. *J. Cell Sci.* **103**, 1215-1222.
- Sin, W. C., Chen, X. Q., Leung, T. and Lim, L.** (1998). RhoA-binding kinase alpha translocation is facilitated by the collapse of the vimentin intermediate filament network. *Mol. Cell. Biol.* **18**, 6325-6339.
- Sumi, T., Matsumoto, K. and Nakamura, T.** (2001). Specific activation of LIM kinase 2 via phosphorylation of threonine 505 by ROCK, a Rho-dependent protein kinase. *J. Biol. Chem.* **276**, 670-676.
- Tolstonog, G. V., Sabasch, M. and Traub, P.** (2002). Cytoplasmic intermediate filaments are stably associated with nuclear matrices and potentially modulate their DNA-binding function. *DNA Cell Biol.* **21**, 213-239.
- van Eekelen, C. A. and van Venrooij, W. J.** (1981). hnRNA and its attachment to a nuclear protein matrix. *J. Cell Biol.* **88**, 554-563.
- Verheijen, R., Kuijpers, H., Vooijs, P., van Venrooij, W. and Ramaekers, F.** (1986). Protein composition of nuclear matrix preparations from HeLa cells: an immunocytochemical approach. *J. Cell Sci.* **80**, 103-122.
- Williamson, D. J., Banik-Maiti, S., DeGregori, J. and Ruley, H. E.** (2000). hnRNP C is required for postimplantation mouse development but is dispensable for cell viability. *Mol. Cell. Biol.* **20**, 4094-4105.
- Wilusz, J. and Shenk, T.** (1990). A uridylate tract mediates efficient heterogeneous nuclear ribonucleoprotein C protein-RNA cross-linking and functionally substitutes for the downstream element of the polyadenylation signal. *Mol. Cell. Biol.* **10**, 6397-6407.
- Yasui, Y., Goto, H., Matsui, S., Manser, E., Lim, L., Nagata, K. and Inagaki, M.** (2001). Protein kinases required for segregation of vimentin filaments in mitotic process. *Oncogene* **20**, 2868-2876.