The death domain protein p84N5, but not the short isoform p84N5s, is cell cycle-regulated and shuttles between the nucleus and the cytoplasm

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Abstract P84N5 is a death domain containing protein that interacts with the tumor suppressor retinoblastoma protein and induces apoptosis. We cloned and characterized two novel alternatively spliced versions of p84N5. The p84N5 short isoform (p84N5s) lacks the death domain and does not induce apoptosis. We showed that p84N5, but not p84N5s, is cell cycle regulated. We found that p84N5-GFP chimera can rapidly shuttle between the nucleus and the cytoplasm. Taken together, these observations suggest that p84N5 may transmit signals from the nucleus to cytoplasmic effectors.

Keywords: p84N5; Death domain; Apoptosis; Nucleus; Alternative splicing

1. Introduction

Numerous evidences indicate that apoptosis is tightly connected to cell cycle checkpoints; for example, DNA damage in dividing cells results in cell cycle block and often induction of apoptosis [1,2]. Although the main effectors of either pathways are well characterized, in particular the p53-dependent pathways [3,4], the details of a direct interaction between the cell cycle machinery and apoptosis deserve further investigation.

The tumor suppressor retinoblastoma protein (Rb) plays a major role in cell cycle and apoptosis [5]; in particular, hypophosphorylated Rb inhibits cell proliferation primarily through its ability to bind and modulate distinct transcription factors including E2F-1 [6]. A role for Rb in protection from apoptosis has also been demonstrated; studies on Rb deficient mice suggest that in addition to E2F, other molecules may mediate Rb-regulated apoptosis [7].

The protein p84N5 was originally identified because it interacts with Rb [8]. More recently, it was also shown to be part of the TREX complex [9]. Further studies demonstrated that p84N5 possesses a nuclear localization signal (NLS) and a death domain (DD) in its C-terminal region [10,11]. The DD is a protein binding domain found in molecules involved in inflammation and apoptosis [12,13]. A pro-apoptotic function of p84N5 was also observed and the association with Rb inhibits p84N5-induced apoptosis [11]. These data suggest that p84N5 may play a role in connecting cell cycle regulation to the apoptotic machinery. However, the regulation of the expression of p84N5 and the mechanistic details of its action are ill defined. The aim of this study was to characterize at a molecular level the protein p84N5.

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Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; RT-PCR, real time polymerase chain reaction

2. Materials and methods

2.1. Antibodies

Murine anti-p84N5 antibody (clone 5E10) was from GeneTex (San Antonio, TX); murine anti-Myc monoclonal antibody (clone 9B11) was from Cell Signaling Technology (Beverly, MA); murine anti-tubulin-α antibody was from Sigma–Aldrich (St. Louis, MO); murine anti-cyclin D1 polyclonal antibody and murine anti-cyclin B1 monoclonal antibody (clone GNS-11) were from BD Pharmingen (San Diego, CA); anti active-caspase3 antibody was from Promega (Madison, WI); Fluorolink™ Cy3™ labeled anti-mouse and anti-rabbit antibodies were from Amersham Pharmacia Biotech (Little Chalfont, UK).

2.2. Plasmids and constructs

Full-length and alternatively spliced coding sequences of p84N5 were amplified by polymerase chain reaction (PCR) from human lung, kidney and fetal brain cDNA libraries purchased from Clontech (Palo Alto, CA) and cloned into pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA) or pEGFP-C1 and pEGFP-N1 vectors (BD Biosciences Clontech, Palo Alto, CA). The V5-tagged XIAP expression plasmid was obtained from ResGen (Invitrogen Corporation, Carlsbad, CA); the AU1-tagged FADD construct has been described previously [14].

2.3. Cell lines and transfection

Normal human dermal fibroblast (NHDF) cells were obtained from PromoCell (Heidelberg, Germany), HeLa 293, U2OS and SAOS2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA).

HeLa and U2OS cells were transfected with the Lipofectamine™ 2000 kit (Invitrogen, Carlsbad, CA). 293 cells were transfected with the CalPhos Mammalian Transfection Kit (BD Biosciences Clontech, Palo Alto, CA, USA).

2.4. Cell cycle and apoptosis analysis

Analysis of cell cycle distribution was conducted by propidium iodide staining and flow cytometry by using FACScan (Beckton Dickinson, Franklin Lakes, NJ). Cell cycle subpopulations (G1, S and G2/M) were quantified with the software ModFit LT v3.0, purchased from Verity Software House (Topsham, ME, USA).

Apoptosis induction was confirmed and quantified by scoring of apoptotic nuclear morphology after staining with 4,6-diamidino-2-phenylindole (DAPI). Alternately, cells were stained with anti active-caspase3 antibody and quantified by scoring of active-caspase3 positive cells after staining with DAPI.

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2.5. Real-time PCR

Real time PCR (RT-PCR) was amplified using the Applied Biosystems "real time" version of the assay on the ABI Prism 7900 thermalcycler and Sybr green MasterMix (Applied Biosystems, Foster City, CA). Full-length p84N5 amplicon was amplified using the following primers: 5′-AGC AATCACTTTGGATTGAAGATA-3′ (forward) and 5′-GGGGGTTTTCAGATA GTAGTTGAT-3′ (reverse); the short isoform p84N5s amplicon was amplified with the following primers: 5′-AGCAATCACTTTGGATTGAAGA-3′ (forward) and 5′-CATGGGTTTGTTGCGTGT-3′ (reverse). The analysis of RT-PCR output data followed the manufacturer-suggested ΔΔCt method, that provides the target gene expression value as unitless fold changes in the unknown sample compared to a calibrator sample, represented by a cDNA mix of 12 human tissues, retro-transcribed from RNA (Clontech, Palo Alto, CA). mRNA levels were normalized on the global expression of four housekeeping genes (18S, GUS, CPH and actin).

2.6. Immunofluorescence and time lapse microscopy

Fluorescence photomicrographs were obtained by means of an Axiovert S100 inverted microscope (Zeiss, Göttingen, Germany), equipped with a charge-coupled device (CCD) camera VarioCam (PCO Computer Optics, Germany).

Time lapse microscopy was performed with the Cell Observer system (Zeiss, Göttingen, Germany) based on a inverted fluorescence microscope Axiovert 200M (Zeiss, Göttingen, Germany), equipped with an AxioCam CCD camera (Zeiss, Göttingen, Germany). The software AxioVision (Zeiss, Göttingen, Germany) was used to acquire the images and to process the movies.

3. Results

3.1. Identification of alternatively spliced versions of p84N5

Screening of public cDNA databases revealed the existence of distinct transcripts encoding for different isoforms of p84N5. Two novel p84N5 alternative splicing isoforms were cloned by PCR from three human cDNA libraries (GenBank Accession Nos. AY573302 and AF573303, respectively). We named these two novel short isoforms as p84N5s and p84N5s2 (Fig. 1).

Sequence analysis of p84N5s revealed the presence of an additional 100 bp sequence within the exon 14 containing an in-frame stop codon. Evidences support the fact that the sequence is introduced by alternative splicing: first, sequence ends follow the canonical "GT-AG rule" usually valid for splicing consensus motifs [15]. Second, this extra sequence was previously annotated in the genomic locus of p84N5 (Locus ID: 9984).

Intriguingly, the coding sequence of p84N5s (1185 nucleotides) lacks the Rb-binding domain, as well as the NLS and the DD. Therefore, p84N5s is predicted to encode for a natural p84N5 truncated form, which has a unique C-terminal sequence of 15 amino acids (VSSTRNKPMIEKMEI).

The junction sequence between the exon 14 and the extra exon unique to p84N5s (ATGAGCAATCACTTTGGATT-

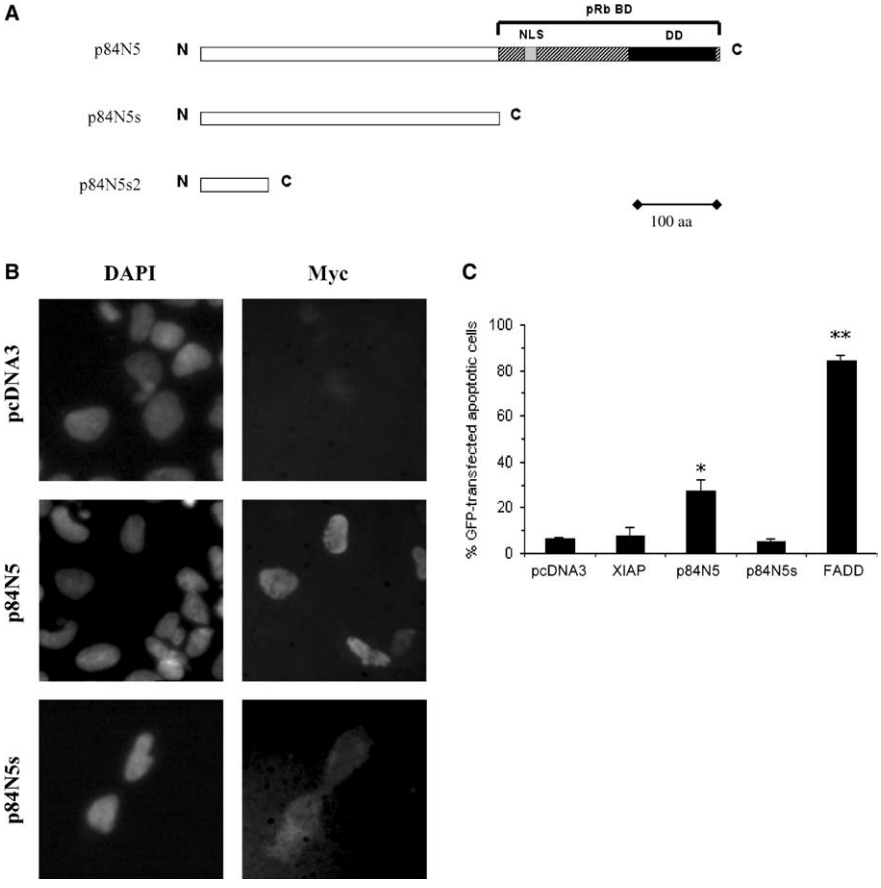

Fig. 1. (A) Schematic representation of p84N5, p84N5s and p84N5s2; the pRb binding domain (pRb BD), the NLS and the DD are indicated. (B) 293 cells were transfected with the indicated constructs, fixed and stained for indirect immunofluorescence using anti-Myc antibody and DAPI. (C) U2OS cells were transfected with the indicated constructs together with pEGFP. Apoptosis was assessed by scoring of GFP-transfected cells with apoptotic nuclear morphology (the means ± S.D. of 3 replicates are shown; *, $P < 0.05$ versus pcDNA3 group; **, $P < 0.005$ versus pcDNA3 group).

GAAGATACTACAAAATCAGTTTATCAAGTAAGTTCA-ACACGCA ACAAACCCATGATTGAAAAAATGGAAAT-TTAG) was used as a query to screen ESTs databases with BLAST algorithm: several human mouse and rat ESTs matched the query, confirming that p84N5s is conserved in mammalians (data not shown).

P84N5s2 contains an additional sequence of 43 nucleotides between the exons 4 and 5 (Locus ID 9984). An in-frame stop codon is present in this sequence, resulting in a putative coding sequence of 258 nucleotides. The predicted product is a truncated p84N5 form, containing the first 85 amino acids and a unique alanine in C-terminus. So far, only one EST has been identified in public database for this p84N5 novel short isoform (GenBank Accession No.: BG723448).

3.2. Characterization of the short isoform p84N5s

Immunostaining analysis showed that p84N5s localizes to the cytoplasm; on the contrary, p84N5 primarily localizes within the nucleus (Fig. 1B and [8]).

p84N5 has been characterized as a pro-apoptotic factor: when over-expressed, it induces apoptosis in vitro [11]. We checked if p84N5s retains any pro-apoptotic activity. The osteosarcoma cell line U2OS was transfected with expression vectors encoding for the following constructs: pcDNA3.1, the inhibitor of apoptosis protein XIAP (as a negative control), p84N5, p84N5s or FADD (as a positive control), in the presence of pEGFP. After 24 h, apoptosis was quantified by manual scoring of green fluorescent protein (GFP)-transfected cells with nuclear apoptotic morphology (Fig. 1C). Cells transfected with p84N5 showed low but significant apoptosis induction (more than 25%), whereas apoptosis levels of cells transfected with XIAP or p84N5s were comparable to negative controls (pcDNA3.1), below 8%. Interestingly, the apoptotic index of p84N5-transfected cells was considerably lower, in comparison with apoptosis induced by FADD transfection (about 80%). This evidence indicates that although both proteins contain a DD and induce apoptosis, p84N5 mechanism or kinetic may be different with respect to FADD.

In contrast, p84N5s does not induce apoptosis at any concentration tested; this may be due to either mislocalization to the cytoplasm or lack of a functional DD.

3.3. P84N5 and p84N5s expression in normal and tumoral tissues

It has been previously shown that p84N5 is ubiquitously expressed in normal tissues [8].

The expression levels of p84N5 and p84N5s were quantified in a panel of 28 cell lines. Fibroblasts (NHDF, BJ, BJ/5TA), an embryo intestine cell line (I407) and an embryo kidney cell line (HEK293), and 23 tumoral cell lines from 13 different tissues were analyzed: prostate carcinoma (LNCAP, DU145), osteosarcoma (U2OS, SAOS2), lung carcinoma (A549), colon carcinoma (HCT116, HCT116/E6N7), colon adenocarcinoma (COLO 205, HT29, LOVO), cervix carcinoma (HeLa), cervix adenocarcinoma (C33A), ovary carcinoma (A2780), breast adenocarcinoma (MCF7), gastric carcinoma (GTL16), pancreas adenocarcinoma (BX-PC3), pancreas carcinoma (PANC-1), bladder carcinoma (HT1197), kidney carcinoma (CAKI 2), skin carcinoma (A431, A431/ST2) and T cell lymphoma (U937).

All cell lines analyzed expressed significant levels of either p84N5 or p84N5s (Fig. 2). It is noteworthy that p84N5 and p84N5s are differentially expressed in distinct cell lines, suggesting a specific regulation of each isoform within a cellular context.

3.4. P84N5, but not the short isoform p84N5s, is cell cycle regulated

P84N5 has been reported to bind Rb protein that plays a crucial role in cell cycle entry and progression [5]. We therefore investigated expression levels of p84N5 and p84N5s during cell cycle in NHDF. NHDF cells were starved by serum deprivation and released in serum-containing medium in order to obtain synchronous populations of different cell cycle phases. The cells were collected before starvation (As), during quiescence (Qui) and 24, 32 and 56 h after release. Samples were processed for flow cytometry analysis of DNA content to confirm synchronization and further analyzed by Western blot and RT-PCR.

More than 90% of serum starved cells were in G1 phase. 24 h after release, the cell population was enriched in S-phase cells. 32 h after release, the population was enriched in G2/M-phase cells and 56 h after release the cell population was undistinguishable from an asynchronous cell population (Fig. 3). The expression of p84N5, cyclin D1 and cyclin B1 in the same samples was quantified by Western blot (Fig. 3). As expected, cyclin D1 is specifically expressed during G1–S transition, whereas cyclin B1 is highly expressed in G2–M transition [16]. P84N5 protein significantly decreased during starvation, but it was restored to physiological levels 24 h after release, without any further modulation at the following time points; its expression followed a kinetic similar to cyclin D1.

The same samples were analyzed by RT-PCR to detect mRNA expression levels of either p84N5 or the short isoform p84N5s. As shown in Fig. 3, the analysis confirmed that cyclin D1 and cyclin B1 mRNAs are cell cycle regulated. Similarly, p84N5 mRNA levels significantly decreased during starvation, but they were restored to physiological levels 24 h after release, corresponding to the re-entry into S-phase. On the contrary, p84N5s mRNA levels did not significantly change at any time points. These observations suggest that full-length p84N5 mRNA, but not p84N5s mRNA, is regulated during cell cycle progression.

3.5. P84N5-GFP and GFP-p84N5 chimeras shuttle between the nucleus and the cytoplasm

Since its identification, p84N5 has been characterized as a nuclear protein [8]. By immunofluorescence analysis of p84N5-Myc transfected cells, we detected nuclear localization of p85N5 in the majority of cells. However, in a small but reliable fraction of transfected cells, both overexpressed and endogenous p84N5 localized to the cytoplasm (data not shown).

To further investigate this phenomenon, we created p84N5-GFP and GFP-p84N5 chimera proteins (Fig. 4). As expected, both p84N5-GFP and GFP-p84N5 transfected cells showed nuclear localization (data not shown); this confirmed that the addition of GFP portion to p84N5 protein did not affect localization. Nevertheless, in a reliable fraction of transfected cells both p84N5-GFP and GFP-p84N5 presented cytoplasmatic localization (data not shown). Both p84N5-GFP and GFP-p84N5 induced significant programmed cell death in comparison to GFP-transfected cells, at levels comparable to p84N5 alone (data not shown). These results indicate that

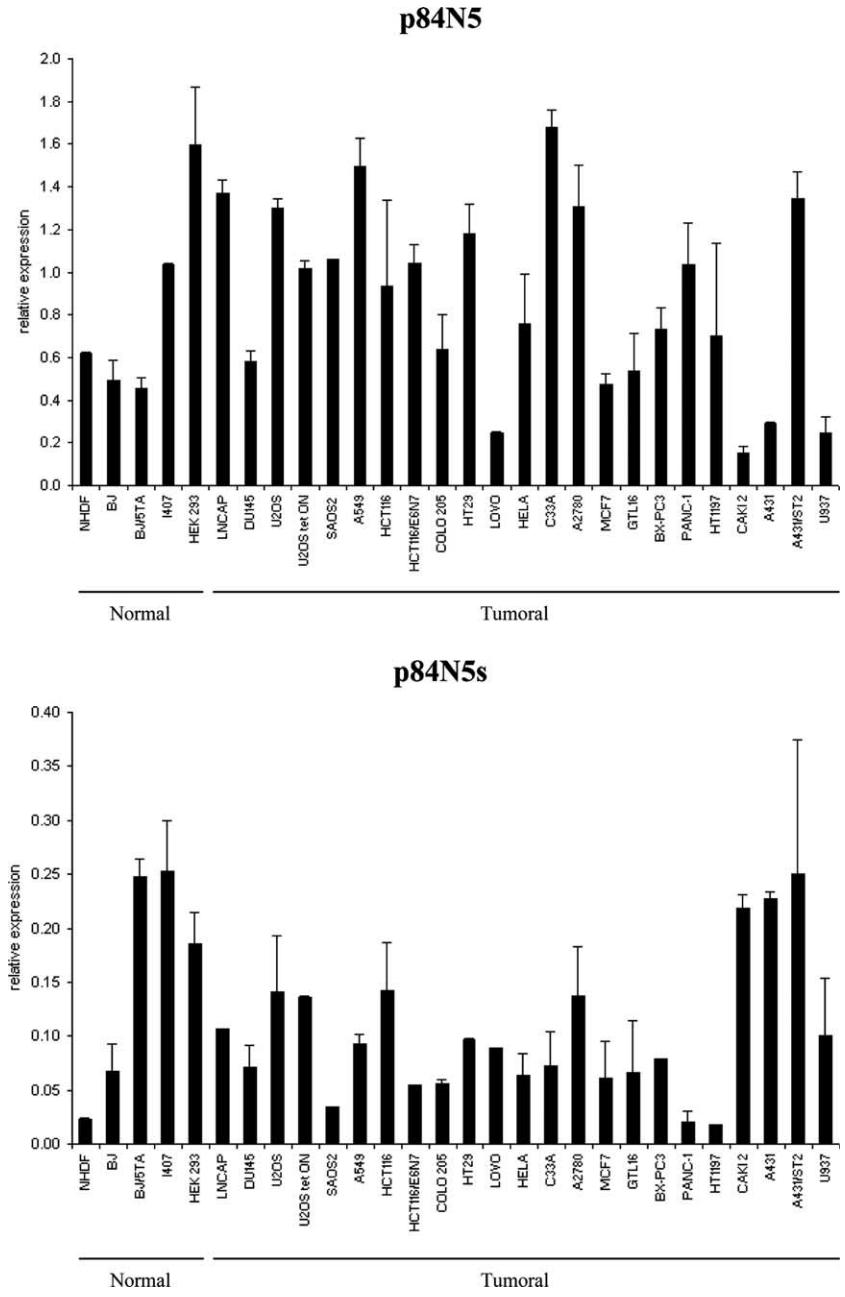

Fig. 2. p84N5 (upper panel) and p84N5s (lower panel) mRNA relative expression was quantified by RT-PCR in 28 cell lines. Data represent the target gene expression values expressed as unitless fold changes as compared to a calibrator sample, represented by a cDNA mix of 12 human tissues, both normalized by the relative housekeeping gene expressions. Data reported represent the average of two replicates with respective error bars.

p84N5 functionality, in terms of apoptosis induction, was not affected by fusion with GFP.

We then analyzed both p84N5-GFP and GFP-p84N5 localization in live cells by time lapse microscopy. Three cell lines, namely HEK293, U2OS and HeLa, were transfected with p84N5-GFP or GFP-p84N5 and followed by phase contrast and fluorescence microscopy. Surprisingly, we observed that in all cell lines investigated, to different extent, the localization of both p84N5-GFP and GFP-p84N5 alternatively changed between the nucleus and the cytoplasm, indicating that p84N5 is able to cross the nuclear envelope as a shuttle (data not shown, Supplementary information). As we found the same shuttling mechanism in cells transfected with both p84N5-GFP and GFP-p84N5, we will refer only to p84N5-GFP in the next paragraphs.

Fig. 4 reports frames, exported from complete movies of 24 h showing representative live U2OS and 293 cells transfected with p84N5-GFP. P84N5-GFP chimera was exported several times from the nucleus to the cytoplasm and subsequently reimported, being nuclear localization by far the most frequent. To note, the nuclear export inhibitor leptomycin B [17] did not affect p84N5 localization (data not shown). Further experiments are needed to clarify the detailed mechanistic insights of the observed p84N5 shuttling and eventually to discover pro-

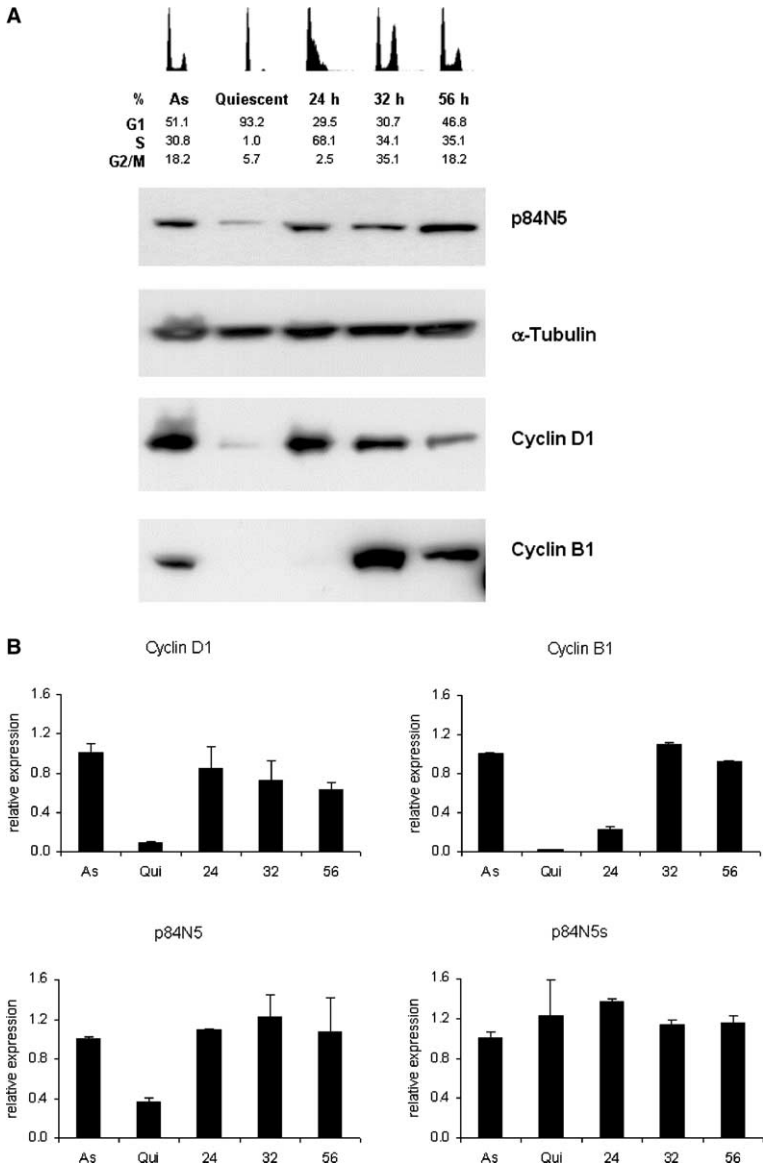

Fig. 3. (A) NHDF cells were serum starved and released; cells were collected at the indicated times after release and processed for flow cytometry analysis of DNA content. The percentages of cells in G1, S and G2/M phases are reported. p84N5, α-Tubulin, cyclin D1 and cyclin B1 were detected by western blot. As indicates asynchronous. Data represent the target gene expression values expressed as unitless fold changes as compared to a calibrator sample, normalized by the relative housekeeping gene expressions; all time points are reported as compared to asynchronous cells values set at one. Data reported represent the average of two replicates with respective error bars. (B) Relative mRNA expression levels of p84N5, p84N5s, cyclin D1 and cyclin B1, in the same samples of panel A, were quantified by RT-PCR. Qui indicates quiescent.

tein partners involved in p84N5 import/export, such as exportins.

The localization of p84N5 during apoptosis was investigated in single cells to address the question whether it undergoes cytoplasmic translocation during apoptosis. P84N5 transfected U2OS and SAOS2 cells were analyzed for two different parameters: apoptotic index and p84N5 localization. As expected, the vast majority of transfected cells showed nuclear staining of p84N5 (Fig. 4 and data not shown). However, when analysis was restricted to apoptotic cell population having caspase-3 activation (see Section 2), most of them showed cytoplasmic localization of p84N5 protein (Fig. 4). This observation indicates that p84N5 actually translocates to the cytoplasm during the early phase of apoptosis execution that is characterized by caspase-3 activation.

4. Discussion

In this letter, we report three main novel evidences on p84N5 protein that better define its mechanism of action. First, the existence of distinct alternatively spliced p84N5 isoforms. Interestingly, the p84N5 short isoform (p84N5s) encodes for a protein lacking the DD and the pRb-binding domain. This isoform localizes within the cytoplasm, was never found in the nucleus, and does not retain the ability to induce apoptosis. It is interesting to note that this same isoform was found in several

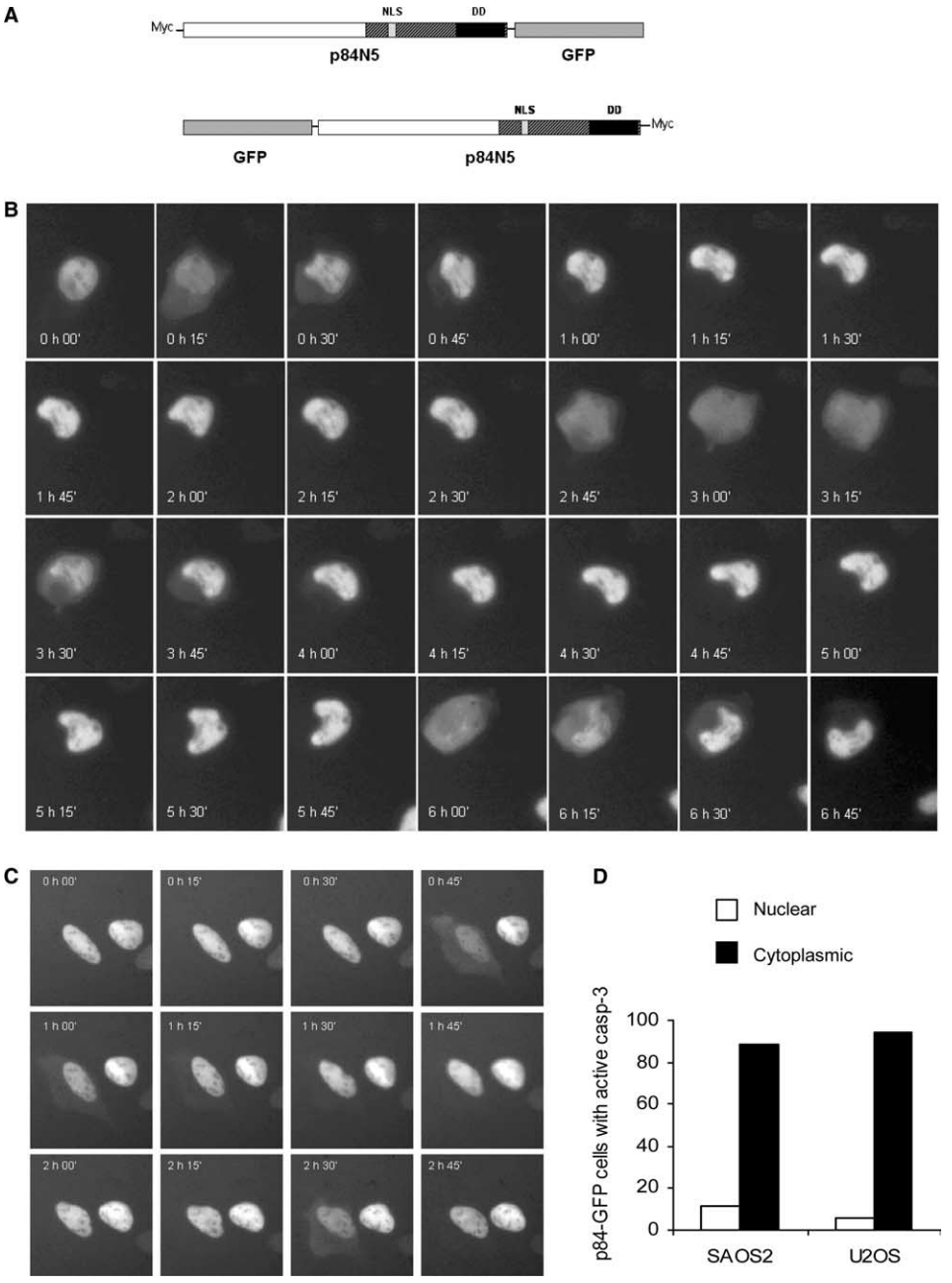

Fig. 4. (A) Schematic representation of the p84N5-GFP and GFP-p84N5 constructs. (B) CCD camera images of a representative live 293 (B) and U2OS (C) cells transfected with p84-GFP, analyzed by time lapse microscopy; frames were taken every 15 min (obj. 32×). (D) U2OS and SAOS cells were transfected with p84-GFP and analyzed for two parameters: apoptotic index and p84N5 localization.

human, mouse, and rat ESTs confirming that it is conserved in three different mammalian species. Moreover, p84N5 but not p84N5s expression is downregulated in quiescent cells. This may represent a mechanism of selective regulation of one isoform by alternative splicing that may lead to a different ratio of expression of the two isoforms during cell cycle.

Second, we show for the first time that p84N5 expression, but not p84N5s, is regulated during proliferation. To our knowledge, this is the first report of a DD containing molecule that is upregulated at the level of both RNA and protein during cell proliferation. We showed that the expression of p84N5 is markedly reduced in non-proliferating cells.

Third, in all cell lines analyzed (293, HeLa and U2OS) both p84N5-GFP and GFP-p84N5 chimeras periodically oscillate from the nucleus to the cytoplasm. The dynamic and synchronization of p84N5 shuttling was surprising: this is the first report describing the real time study of a protein shuttling from the nucleus to the cytoplasm and vice versa, repeated over time. It is important to note that under the same experimental conditions, we did not observe such a shuttling mechanism for other GFP-fused chimera proteins that can localize within the nucleus, either in stable or in transiently transfected cell lines (data not shown). Moreover, we observed a strong correlation between p84N5 cytoplasmic localization

and apoptosis execution. However, further work is needed to analyze the localization of endogenous p84N5 in live cells.

An explanation may be envisaged based on the observed shuttling mechanism; p84N5 protein may possess a nuclear export signal, or it associates with some factor that acts as nuclear-cytoplasmatic shuttle. Preliminary experiments showed that this mechanism is leptomycin B resistant (data not shown), thus indicating that the trafficking is not CRM1-dependent.

It is tempting to speculate that p84N5 may be somehow involved in an "apoptotic checkpoint". In viable proliferating cells, waves of p84N5 proteins shuttle from the nucleus to the cytoplasm. In the presence of an additional co-stimulus, p84N5 may regulate pro-apoptotic signals. This would finally result in cytoplasmatic caspases activation and apoptosis.

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