Journal of Cellular Biochemistry 98:920–930 (2006)

Identification of Nuclear Localization Signal That Governs Nuclear Import of BRD7 and its Essential Roles in Inhibiting Cell Cycle Progression

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Abstract BRD7, a novel bromodomain gene, is identified to be associated with nasopharyngeal carcinoma (NPC). Decreased or loss of expression of BRD7 was detected in NPC biopsies and cell lines. Overexpression of BRD7 could inhibit NPC cell growth and arrest cells in cell cycle by transcriptionally regulating some important molecules involved in ras/MEK/ERK and Rb/E2F pathway, and downregulate the promoter activity of E2F3. In the present study, the subcellular localization of BRD7 was investigated. It was found that BRD7 was mainly localized in nucleus without distinct cell-specific difference between COS7 and HNE1. Furthermore, a functional nuclear localization signal (NLS) sequence ranging from amino acid 65 to 96 was identified and characterized. The NLS is composed of a cluster of four bipartite nuclear targeting sequences, which are tightly linked and extremely overlapped. We found that whether the entire NLS or the four bipartite nuclear targeting sequences could respectively determine the nuclear import of green fluorescent protein (GFP). The most important is that NLS-deleted BRD7 shifted the nuclear localization to be mostly in cytoplasm, and failed or reduced to negatively regulate the expression of cell cycle related molecules, cyclin D1 and E2F3, and cell cycle progression from G1 to S phase. In conclusion, NLS is an essential motif affecting BRD7 nuclear distribution, and the nuclear localization of BRD7 is critical for the expression of cell cycle related molecules and cell biological function. J. Cell. Biochem. 98: 920–930, 2006.

Key words: nasopharyngeal carcinoma (NPC); BRD7; subcellular localization; nuclear localization signal (NLS); cell cycle

BRD7 is a newly identified bromodomain gene which is also associated with nasopharyngeal carcinoma (NPC). Decreased or loss of expression of BRD7 was detected in NPC biopsies and cell lines, whereas high expression was found in normal nasopharyngeal epithelium [Yu et al., 2000, 2001]. Moreover, overexpression of BRD7 could inhibit NPC cell growth and arrest cells in G0/G1 phase by transcriptionally regulating some important molecules involved in ras/MEK/ERK and Rb/E2F pathway, and downregulate the promoter activitiy and mRNA expression of E2F3 [Zhou et al., 2004].

It was found that BRD7 protein could interact with some nuclear transcription factors such as BRD2 and IRF2 [Ada et al., 2000; Yu et al., 2002]. Another research group observed that BRD7 could form a triple complex with E1B-AP5 and histones, and affect the transcription

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 30300175, 30330560, 30400238, 30470367.

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Received 22 October 2005; Accepted 29 November 2005
DOI 10.1002/jcb.20788

activity of E1B-AP5 [Kzhyshkowska et al., 2003]. Co-localization was found between BRD7 and polymerase II, hyperacetylated H3 and hyperacetylated H4 by immunofluorescence assay [Ada et al., 2000]. These results suggested that BRD7 may be an important transcription regulation factor.

Many transcription factors were identified to contain one or more nuclear localization signal (NLS) sequences, which have been proved to determine nuclear localization of themselves or other heterologous proteins [Goldfarb et al., 1986; Moreland et al., 1987; Plafker and Gibson, 1998]. Typical NLS is a short region or a bipartite basic sequence [Moreland et al., 1987; Robbins et al., 1991; Cecilia et al., 2000]. Proteins entering the nucleus require importin molecules to recognize NLS sequences, allowing nuclear pore docking. Then cargo protein and import molecular complex are permitted to transport through the nuclear pore complex, followed by release inside the nucleus [Gorlich et al., 1994; Pemberton et al., 1998; Gorlich and Kutay, 1999]. Some transcription factors may not be constitutively localized in the nucleus and exist in two forms, a nuclear form and a latent cytoplasmic form. Cytoplasmic retention and nuclear translocation of the transcriptional factors are mechanisms regulating their activity [Baeuerle and Baltimore, 1988; Munoz et al., 1994]. BRD7 was found predominantly in nucleus, therefore it is deduced that there may be a mechanism of nuclear importin regulated by the NLS of BRD7. To explore the molecular mechanisms of cell-specific localization of BRD7, we studied the subcellular localization of BRD7 in different cell lines. We also identified a functional nuclear localization sequence (65aa–96aa) in BRD7, which is composed of a cluster of extremely overlapped bipartite nuclear target sequences (65aa–81aa; 66aa–82aa; 67aa–83aa; 80aa–96aa). We described the roles of the NLS in the subcellular localization and functions of BRD7, and provided evidences that NLS is essential for BRD7 to inhibit cell cycle progression from G1 to S phase by negatively regulating the expression of cell cycle related molecules, cyclin D1 and E2F3.

MATERIALS AND METHODS

Materials

pEGFP-C2 and pCMV-Myc were purchased from Clontech, pGL3 enhancer vector was from Promega. pEGFP-C2/BRD7 and pCMV-Myc/BRD7 inserted full-length cDNA of BRD7 were obtained from the plasmid bank in our laboratory. pGL3-E2F3 promoter has been described previously [Zhou et al., 2004]. The antibody against c-Myc was purchased from Clontech, and the other antibodies against GFP, cyclin D1, E2F3, and α-Tubulin were from Santa Cruz. Cy3-conjugated secondary antibody and DAPI were from Sigma. Cell culture media was from Invitrogen, and other molecular biological reagents were from SABC. Oligonucleotide primers synthesis and DNA sequencing were performed in Shanghai Bioasia company. HNE1 cell line was derived from NPC and established by our laboratory. COS7 and Hela cell lines were purchased from China Center for Type Culture Collection.

Construction of Recombinant Plasmids

We predicted the NLS sequence (amino acids 65–96) of BRD7, which contains four tightly linked and overlapped bipartite nuclear targeting sequences. In order to identify whether the nuclear localization sequence could mediate the nuclear localization of the heterologous protein, pEGFP-C2/NLS recombinant was constructed. NLS of BRD7 produced by polymerase chain reaction (PCR) was linked to the 3′ end of the sequence encoding green fluorescent protein (GFP) in frame, which encoded a fusion protein containing GFP and BRD7 NLS (65aa–96aa). pCMV-Myc/BRD7 inserted full-length cDNA of BRD7 was kept in our laboratory, which was used as templates to generate DNA fragment of pCMV-Myc/BRD7ΔNLS by direct PCR. After phosphorated with T4 polynucleotide kinase, the PCR product was self-ligated with T4 DNA ligase. For the construction of pEGFP-C2/BRD7ΔNLS recombinant, the above construct, pCMV-Myc/BRD7ΔNLS, was used as templates to produce NLS-deleted BRD7 fragments by PCR. Moreover, we constructed recombinants of pEGFP-C2/NLS-1 (65aa–81aa), pEGFP-C2/NLS-2 (66aa–82aa), pEGFP-C2/NLS-3 (67aa–83aa), and pEGFP-C2/ NLS-4 (80aa–96aa) by fusing the four probably bipartite targeting sequences to pEGFP-C2 to investigate whether they could mediate the nuclear localization of GFP. We obtained the products including pEGFP-C2 and BRD7 NLS-1, pEGFP-C2 and BRD7 NLS-2, pEGFP-C2 and BRD7 NLS-3, pEGFP-C2 and BRD7 NLS-4 by using direct PCR and pEGFP-C2 vector was used as

TABLE I. The Primer Couples Used to Construct Various Recombinant Vectors

Name of constructs	Primer couples
pEGFP-C2/NLS	5′-cgactcgagcaagagaaagaaaggagagaag-3′ (sense); 5′-cctgaattcccggtctcgatctcgcttctt-3′ (anti-sense)
pCMV-Myc/BRD7ΔNLS	5′-gtggagaatgaggcagaaaaagactccag-3′ (sense); 5′-ccccttttcttccc ctggaatctgcttctc-3′ (anti-sense)
pEGFP-C2/BRD7ΔNLS	5′-aatctcgagtatgggcaagaagcacaagaagcacaag-3′(sense); 5′-atactgcagtcaactttcaccaggtccacactc-3′ (anti-sense)
pEGFP-C2/NLS-1	5′-ccaggggaagaaaaggggagaaaattcgaattctgcagtcgacgg-3′ (sense); 5′-aatctgcttctctcctttctttctcttgcttgagctcgagatctgagt-3′ (anti-sense)
pEGFP-C2/NLS-2	5′-ccaggggaagaaaaggggagaaaacggttcgaattctgcagtcgacgg-3′ (sence); 5′-aatctgcttctctcctttctttctgcttgagctcgagatctgagt-3′(anti-sense)
pEGFP-C2/NLS-3	5′-ccaggggaagaaaaggggagaaaacggagattcgaattctgcagtcgacgg-3′(sence); 5′-aatctgcttctctcctttcttgcttgagctcgagatctgagt-3′(anti-sense)
pEGFP-C2/NLS-4	5′-gataaaaagaagcgagatcgagaccggttcgaattctgcagtcgacgg-3′ (sence); 5′-ctccttaactcttctccgttttctgcttgagctcgagatctgagt-3′ (anti-sense)

templates. All these PCR products were self-ligated as described above.

All primer couples were listed in the Table I. DNA sequencing and expression assay were employed to detect correct insertion of all above fragments.

Cell Culture and Transfection

HNE1 cells and Hela cells were grown in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS). COS 7 cells were grown in Dulbecco' modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS. Cells seeded in 6-well plate or 35-mm dish were transfected with a total of 2 μg per well or 3 μg per dish by using the LIPOFECTAMINE™ Plus Reagent according to the manufacturer's instructions.

Detection of GFP Fluorescence and Immunofluorescence

Cells were grown on the coverslips in the wells of 6-well plates 12 h prior to tansfection, and cells were washed twice with phosphate buffered saline (PBS) 36 h after transfection, fixed with acetone/methanol (1/1) for 30 min at room temperature, permeabilized with 0.05% Triton X-100 in PBS for 20 min, and incubated for another 20 min with normal serum. After incubating overnight at 4°C with the monoclonal anti-Myc (1:1,000, Clontech), cells were washed thrice with PBS for 40 min, and incubated for another 60 min with Cy3-conjugated anti-mouse second antibody, which was diluted 1:100 in blocking buffer. After extensive washing with PBS, cells were further stained with 4,6-diamidino-2-phenylindole (DAPI) for 10 min, then washed twice with PBS and mounted on a glass slides with Prolong anti-fade medium. The fluorescence signal was observed with an AX-80 analytical microscope system (Olympus, Japan) and a Leica confocal microscope (Leica TCS-NT, Germany). GFP fluorescence was directly observed after fixed and stained with DAPI.

Western Blot Analysis

Cell lysates were extracted according to the instruction of Molecular Cloning Laboratory Manual 36 h after transfection, then separated by SDS/polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham). After blocking with PBST (PBS containing 0.1% Tween-20) added with 5% low fat dry milk for 1 h, the membranes were incubated overnight at 4°C with the primary antibodies in PBS. Antibodies used were: anti-c-Myc monoclonal antibody (1:1,000, Clontech), anti-GFP monoclonal antibody (1:300, Santa Cruz), anti-Cyclin D1 monoclonal antibody (1:300, Santa Cruz), anti-E2F3 polyclonal antibody (1:300, Santa Cruz), or anti-α-Tubulin monoclonal antibody (1:300, Santa Cruz). Membranes were extensively washed with PBS and incubated with horseradish peroxidase (HRP) conjugated secondary anti-mouse antibody or anti-rabbit antibody (dilution 1:1,000, Santa Cruz). After further extensive washes with PBS, antigen–antibody complexes were visualized with the enhanced chemiluminescence (ECL) kit (Pierce). In some cases, cell lysates were fractionated to cytosolic and nuclear fractions as the instruction of NE-PER™ nuclear and cytoplasmic extraction reagents (Pierce). In brief, cytoplasmic protein extracts were harvested and nuclei were collected by centrifugation at 16,000*g* for 5 min after cells were treated with ice-cold CER I and CER I and II, respectively. The insoluble nuclear fraction was resuspended and homogenized in ice-cold

NER, and nuclear extracts were harvested by centrifugation at 16,000*g* for 10 min. Both cytoplamic and nuclear fractions were detected by Western blotting.

Luciferase Reporter Assay

Transfections were performed in 6-well plates with a total of 5 μg DNA containing 2 μg reporter plasmid (pGL3-E2F3 promoter or pGL3 enhancer), 3 μg expression DNA (pCMV-Myc/BRD7ΔNLS or pCMV-Myc/BRD7) as the cells were 40% confluence. Transfection efficiencies were normalized by co-transfection of β-galactosidase expression DNA. Thirty six hours after transfection, cells were harvested for luciferase assay. Cells were washed twice with PBS, then scraped into 200 μl 1× cell lysis buffer (Pharmingen) and incubated at room temperature for 10–15 min. Hundred microliters cell extract was added into an assay cuvette, followed by the manual addition of 100 μl of substrate A and B to mix in order. Emitted light was quantified for 60 s in a TD-20/20 luminometer (Turner). For β-galactosidase enzyme assay, 30 μl of cell extract was mixed with 20 μl of 1× reporter lysis buffer, then 50 μl of assay 2× buffer was added to each well. Following incubating at 37°C for 30 min, the samples were read the absorbance at 420 nm. Values represent the mean of three independent experiments plus standard deviations (SD).

Construction of Clone Pools

HNE1 cells were seeded in 6-well plates and grown in RPMI-1640 medium supplemented with 10% (v/v) FBS. At 40% confluence, cells were transfected with contructs of pEGFP-C2/BRD7, pEGFP-C2/BRD7ΔNLS or pEGFP-C2, then transferred to 50 ml flasks 48 h after transfection. At the confluence of 20–30%, cells were grown with medium containing G418 at a final concentration of 300 μg/ml for 2 weeks to screen positive clones. Western blotting was preformed to detect the expression of GFP, GFP-BRD7 or GFP-BRD7ΔNLS in three clone pools with resistance to G418, and α-Tubulin was used as an internal control.

Flow Cytometry

HNE1 cells expressing GFP-BRD7 or GFP-BRD7ΔNLS were subjected to investigate the percentage of cells in G_0-G_1, S, and G_2-M phase of cell cycle, and HNE1 cells expressing GFP tag were used as negative control. All collecting, various HNE1 cells were washed twice with PBS and fixed in 70%(v/v) ethanol overnight. Cells were centrifuged at 1,000 rpm for 10 min, resuspended in 50 μg/ml propidium iodide (Sigma) in PBS, and immediately subjected to flow cytometry using a FACStar (Becton-Dickinson, Mountain View, CA). Appropriate settings of forward and side scatter gates were used to examine 10,000 cells per experiment. Data were analyzed with Modfit software (Verity Software House, Inc., Topsham, ME). Values were expressed as mean and SD of three independent experiments.

RESULTS

Subcellular Localization of BRD7 in Different Cells

To study the subcellular localization of BRD7 in different cells, GFP direct fluorescence assay was used to investigate the subcellular localization in COS7 and HNE1. As shown in Fig. 1A, BRD7 was mainly diffused throughout the nucleus in a thin dotted or striped pattern without distinct cell-specific difference between COS7 and HNE1 (Fig. 1A). Moreover, Myc tagged immunofluorescence assay was further performed to investigate the subcellular localization of BRD7, it was also found that BRD7 localized in the nucleus in COS7 (Fig. 1B), and the distribution pattern was consistent with that detected by GFP direct fluorescence. Together, the nucleus distribution pattern of BRD7 is some associated with mitotic chromosomes. Bromodomain-deleted mutant was also localized in nucleus but altered the localization to be more dotted or granular, with no regulation (data not shown).

Prediction and Identification of NLS of BRD7

To identify whether BRD7 contains NLS, we used the web-based program and input the complete amino acid sequences encoded by BRD7 into ScanProsite in "www. Expasy. Ch". A cluster of probable bipartite nuclear targeting sequences ranging from amino acid 65 to 96 were found, and each of them was all tightly linked and extremely overlapped in sequence (Fig. 2). So we presumed this region from amino acid 65 to 96 as a putative NLS sequence in BRD7, and also regarded the region from aa 65 to 81 as NLS-1, 66 to 82 as NLS-2, 67 to 83 as NLS-3, and 80 to 96 as NLS-4.

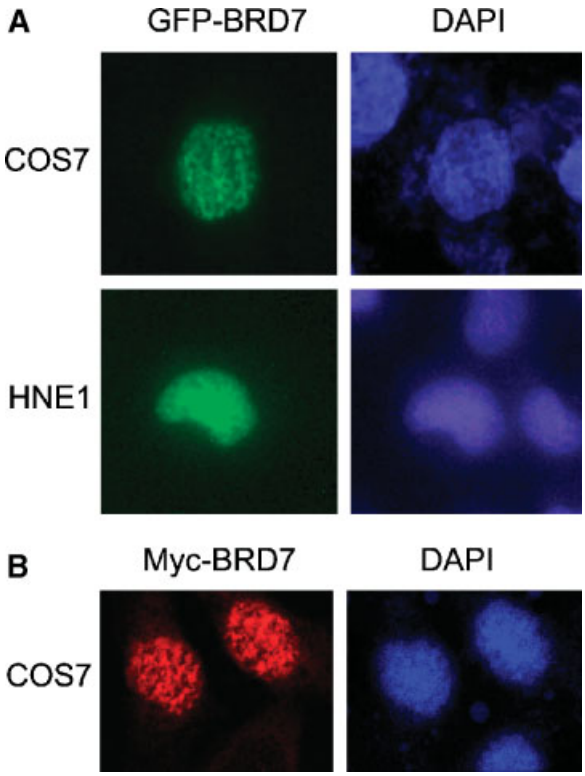


Fig. 1. Two methods, GFP direct fluorescence and indirect immunofluorescence tagged with Myc assays were used together to investigate the subcellular localization of BRD7. DAPI staining of nuclei was shown in the right column. **A**: Recombinant wild-type BRD7 tagged with GFP mainly localizes in nucleus with thin dotted or striped pattern in COS7 and HNE1 cells detected by direct GFP fluorescence. **B**: Recombinant wild-type BRD7 tagged with Myc also localizes in nucleus detected by indirect immunofluorescence in COS7, and the distribution pattern is consistent with that by direct GFP fluorescence.

To conform the putative NLS in BRD7, we firstly examined their capability to direct GFP to the nucleus. We constructed the pEGFP-C2/NLS recombinant vector and transfected it into COS7 cells. As expected, GFP with a carboxyl-terminal fusion of BRD7 NLS translocalized to nucleus, whereas GFP without any fusion peptide was visualized diffusely throughout nucleus and cytoplasm (Fig. 3A,B). The similiar results were also shown in different cells, including Hela and HNE1 (data not shown). To further confirm the finding, Western blotting analysis was performed. The results showed that GFP-NLS was detected predominantly in the nuclear fraction, whereas GFP without any fusion peptide was detected both in cytoplasm and nuclear fraction (Fig. 3C). These results provided direct evidence that the motif of residues 65–96 could direct GFP to the nucleus and function as a NLS in BRD7.

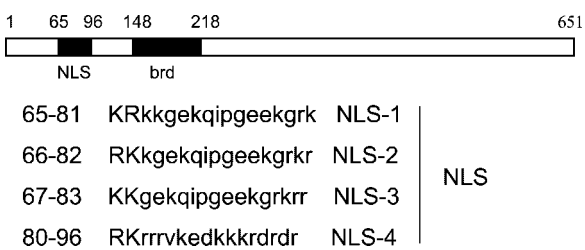
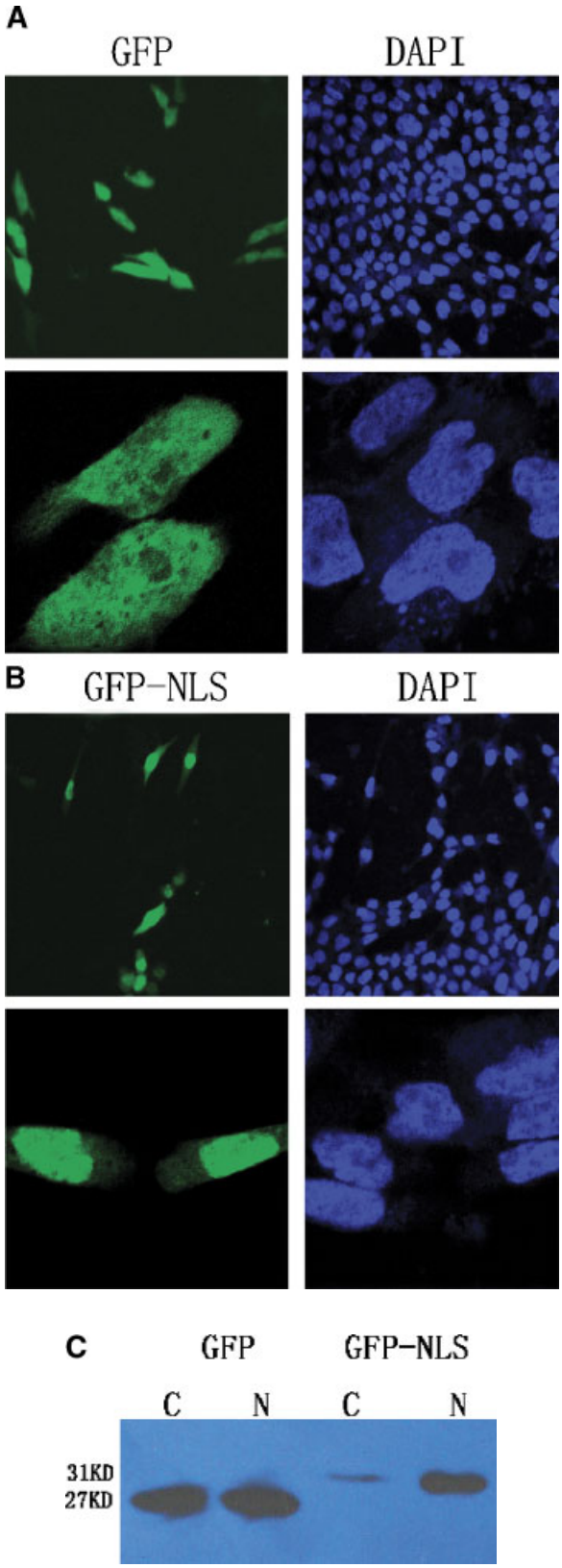


Fig. 2. The putative nuclear localization signal (NLS) sequence in BRD7 predicted by Scanprosite. **A**: Diagram of the 651-aa BRD7 protein. The position of putative NLS, aa 65–96, and bromodomain, aa 148–218, are shown. **B**: The putative NLS spans four nuclear targeting sequences, which are tightly linked and overlapped. They were named as NLS-1 (65–81), NLS-2 (66–82), NLS-3 (67–83), and NLS-4 (80–96).

The motif of residues 65–96 in BRD7 is not a typically short region or bipartite basic sequence, but composed of several tightly linked and extremely overlapped bipartite nuclear targeting sequences. So the same assays were performed to study the effects of these probably bipartite nuclear targeting sequences including NLS-1 (aa 65–81), NLS-2 (aa 66–82), NLS-3 (aa 67–83), and NLS-4 (aa 80–96) on the localization of GFP in COS7 cells. The results showed that they could respectively direct the nuclear localization of GFP (Fig. 4), consistent with the integral NLS. So these tightly linked and overlapped bipartite nuclear targeting sequences (NLS-1, NLS-2, NLS-3, and NLS-4) existing in BRD7 NLS could also function as NLSs.

Translocation of BRD7 Protein to Nucleus Depends on the NLS

BRD7 is a putative transcriptional regulation protein, which is mainly localized in nucleus. To study the effect of putative NLS on the nuclear distribution of BRD7, we constructed the pEGFP-C2/BRD7ΔNLS and pCMV-Myc/BRD7ΔNLS vectors and transfected them into COS7 cells, respectively. As shown in Fig. 5, NLS-deleted BRD7 changed its nuclear localization to cytoplasmic compartments mediated whether by direct GFP fluorescence (Fig. 5A) or by Myc tagged immunofluorescence assay (Fig. 5B) in COS7 cells, which existed in two distribution forms in cytoplasm, dotted and diffused. The ratio of the two distribution forms is about 50%. Similar results were obtained in Hela and HNE1 cells (data not shown). Immunoblot analysis were also used to detect the protein expression of Myc-BRD7 and Myc-BRD7ΔNLS in cytoplasm and nucleus fractions. The results showed that Myc-BRD7 was almost



totally present in the nuclear fraction, whereas Myc-BRD7ΔNLS was predominantly present in cytoplasm fraction (Fig. 5C). These results indicate that this putative NLS, spanning from aa 65–96 in BRD7, could predominantly govern the nuclear import of BRD7 and play essential roles in nuclear localization of BRD7.

Effect of BRD7 NLS on the Cell Cycle Progression in NPC Cells

BRD7 was found to be associated with NPC, and could inhibit NPC cell growth and cell cycle progression from G1 to S phase [Zhou et al., 2004]. The motif of amino acids 65–96 was identified as a NLS motif of BRD7 in the above experiments, which could target its own nuclear localization of itself. So clone pools of HNE1 cells stably expressing GFP, GFP-BRD7, and GFP-BRD7ΔNLS were constructed, and Western blot was used to detect the expression of GFP, GFP-BRD7, and GFP-BRD7ΔNLS, respectively (Fig. 6). Flow cytometry assays were preformed to investigate the effect of BRD7 NLS on the cell cycle progression from G1 to S in HNE1 cells. Wild BRD7 could inhibit the cell cycle progression from G0/G1 to S phase, and the cell number of G0/G1 delayed more than 20%, similar to the previous results. But no obvious effect on the cell cycle progression was observed in the NPC cells transfected with NLS deleted BRD7 (Fig. 7). So BRD7 NLS is also required for its biological function in NPC.

BRD7 NLS Is Essential in Negative Regulation of the Promoter Activity of E2F3

Previous study revealed that BRD7 could downregulate the promoter activity of E2F3 one- to twofold in COS7 [Zhou et al., 2004]. It was also regarded as a putative transcriptional regulation factor. The nuclear localization sequence, spanning from aa 65–96 in BRD7

Fig. 3. The putative NLS of BRD7 (amino acids 65–96) could target GFP to nucleus in COS7 cells. **A**,**B**: They were the results detected by direct GFP fluorescence. DAPI staining of nuclei was shown in the right column. **C**: It shows the results detected by immunoblot. A: GFP protein without any peptide linked showed diffusely through nucleus and cytoplasm; B: GFP fused with BRD7 NLS (65–96) were observed to shift its cytoplasm and nucleus localization to nucleus; C: Immunoblots for cytosolic (C) and nuclear (N) fractions of GFP control protein and GFP fused with BRD7 NLS (65–96) expressed in COS7 cells probed with an antibody against GFP. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

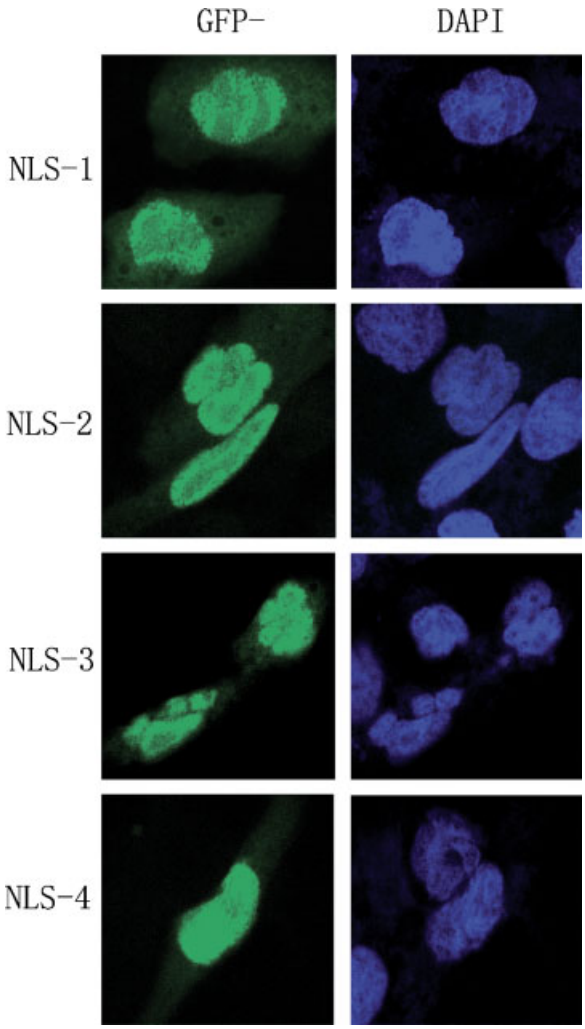


Fig. 4. The four putative bipartite nuclear targeting sequences (NLS-1, NLS-2, NLS-3, and NLS-4) present in the NLS (65–96) region could respectively direct GFP to nucleus in COS7 cells detected by direct GFP fluorescence. DAPI staining of nuclei was shown in the right column. **A:** NLS-1 (65–81); **B:** NLS-2 (66–82); **C:** NLS-3 (67–83); **D:** NLS-4 (80–96).

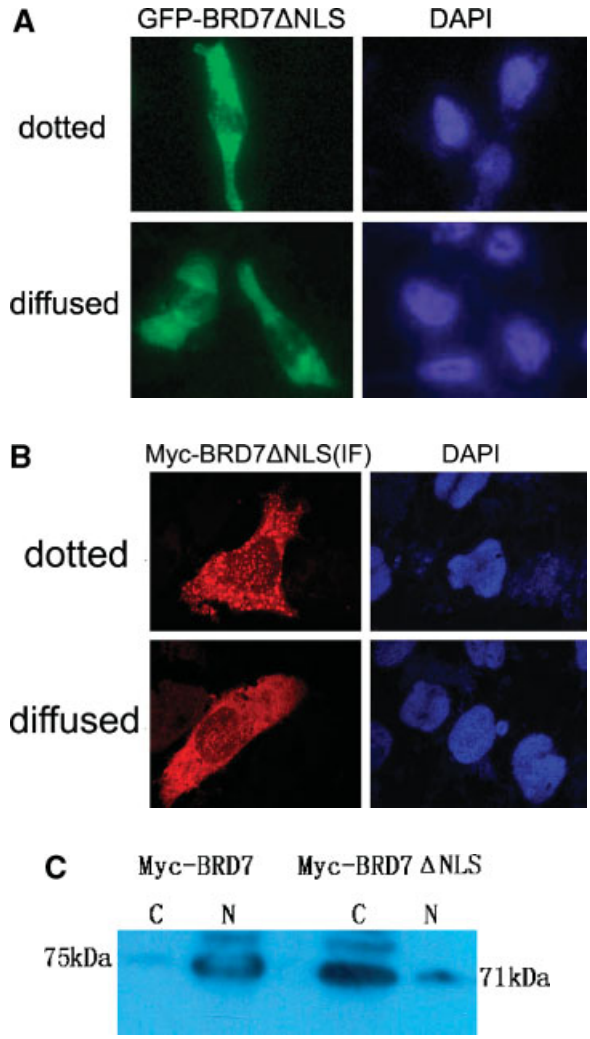


Fig. 5. Effect of the entire NLS (aa 65–96) on the subcellular localization of BRD7 in COS7 cells. Direct GFP fluorescence and indirect immunofluorescence with an antibody against Myc were respectively used to investigate the subcellular localization of NLS-deleted BRD7. It was shown that NLS-deleted BRD7 shifted its nuclear localization to cytoplasmic in two different distribution pattern, dotted and diffused. DAPI staining of nuclei was shown in the right column in (**A**) and (**B**). A: Subcellular localization of NLS-deleted BRD7 tagged with GFP (GFP-BRD7ΔNLS) was detected by direct GFP fluorescence. B: Cellular localization of NLS deleted BRD7 mutant tagged with Myc (Myc-BRD7ΔNLS) was detected by indirect immunofluorescence. **C:** Immunoblots for cytosolic (C) and nuclear (N) fractions of wild-type BRD7 and NLS deleted BRD7 mutant tagged with Myc expressed in COS7 cells and probed with an antibody against Myc.

was identified as functional NLS, which altered the nuclear localization of BRD7 to be cytoplasmic compartment. So NLS deleted BRD7 mutant was used to investigate the effect of BRD7 NLS on the transcription regulation of its target genes in HNE1 cells, we found that BRD7 could downregulate the promoter activity of E2F3 by two magnitudes ($P < 0.01\%$), more predominant than that in COS7 [Zhou et al., 2004], but NLS-deleted BRD7 obviously reduced the negative effect on the promoter activity of E2F3 (Fig. 8). So BRD7 NLS is required for the roles of BRD7 in regulating promoter activity of E2F3, in other words, the distribution ratio of BRD7 in nucleus/cytoplasm has obvious effect on its function of transcriptional regulation.

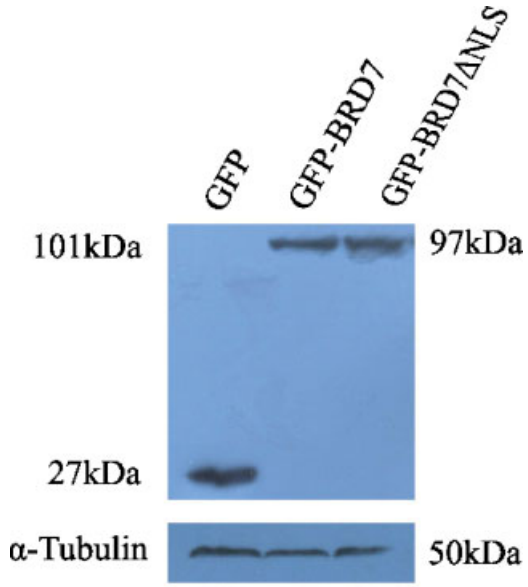


Fig. 6. Western Bloting was performed to detect the expression of GFP, GFP-BRD7 or GFP-BRD7ΔNLS in three clone pools with resistance to G418, and α-Tubulin was used as an internal control.

NLS-Deleted BRD7 Shows no Obvious Effect on the Expression of Cell Cycle Related Molecules, Cyclin D1 and E2F3

BRD7 could downregulate the promoter activity and mRNA expression of E2F3 [Zhou et al., 2004]. Cyclin D1 is an essential cell cycle related molecule which was also examined decreased expression in HNE1 cells transfected with BRD7 by flow cytometry (data not shown). So Western blotting was further performed to examine the protein expression of cyclin D1 and E2F3 in HNE1 clone pools expressing GFP, GFP-BRD7, or GFP-BRD7ΔNLS. As expected, decreased expression of cyclin D1 and E2F3 were found in HNE1 cells transfected with GFP-BRD7, whereas no obvious effect was shown in HNE1 cells transfected with NLS-deleted BRD7 (Fig. 9). These results suggested that NLS is essential for the negative effect of BRD7 on the expression of cyclin D1 and E2F3, which is consistent with the effect on cell cycle progression.

DISCUSSION

BRD7 is a member of bromodomain family. Several lines of evidence have suggested that BRD7, like many other bromodomain proteins, may be involved in transcriptional control of genes [Ada et al., 2000; Kzhyshkowska et al., 2003; Zhou et al., 2004]. Studies have demonstrated that bromodomain is the characteristic of proteins that regulate signal-dependent, but not basal, transcription during active proliferation [Horn and Peterson, 2001; Patrizia et al., 2001]. The mechanism of bromodomain proteins implicated in transcriptional regulation is that bromodomain proteins may modulate chromatin remodeling or modify acetylation of histones, thus facilitating the accession of transcription factors to chromatin [Winston and Allis, 1999]. It was found that BRD7 was mainly localized in nucleus in a thin dotted or striped pattern. Bromodomain-deleted BRD7 was also localized in nucleus, but altered the distribution pattern to be more granular, with no regulation, which was also associated with its biological function in NPC (data not shown). The difference of the nuclear distribution and biological function between wild bromodomain proteins and bromodomain-deleted mutants may not be surprising, considering that bromodomain is an important functional motif and plays important roles in chromatin remodeling [Winston and Allis, 1999; Dey et al., 2003].

The nuclear proteins, including transcription factors, are synthesized in the cytoplasm and require a means of getting to the nucleus [Aplin and Julianob, 2001; Cyert, 2001]. NLS, a defined cis-acting amino acid sequence, has been proved to determine the localization of several important transcription factors. Generally, NLSs are composed of short stretches of highly basic amino acids, some of which are single stretches of amino acids (i.e., monopartite), others of which are comprised of two short stretches separated by a spacer region (i.e., biopartite) [Moreland et al., 1987; Cecilia et al., 2000]. To ascertain the nuclear import mechanism of BRD7, we predicted the region of amino acids 65–96 of BRD7 as a putative NLS in BRD7 by computer program. The putative NLS of BRD7 is consisted of a cluster of tightly linked and overlapped bipartite nuclear targeting sequences (NLS-1: aa 65–80; NLS-2: aa 66–81; NLS-3: aa 67–83; NLS-4: aa 80–96). Our results showed that not only the putative NLS of BRD7 but also any of the four nuclear targeting sequences among the putative NLS could direct the nuclear import of GFP. Moreover, NLS-deleted BRD7 was employed to study the effect of NLS on the subcellular distribution of BRD7. Our results showed that the localiza-

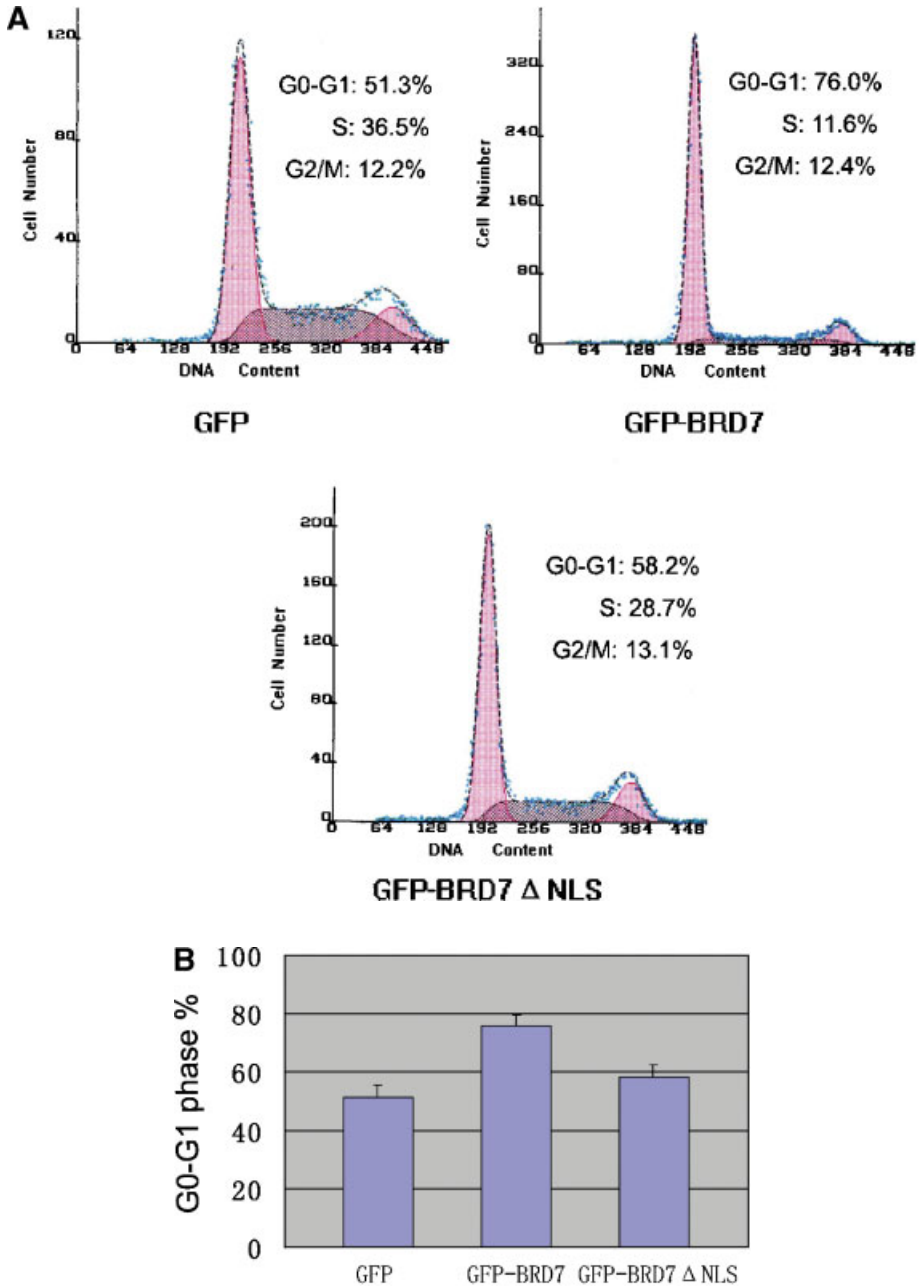


Fig. 7. The effect of BRD7 NLS on the cell cycle progression from G0/G1 to S phase analyzed by flow cytometry. **A:** The cell cycle distribution of clone pools stably expressing GFP, GFP-BRD7, or GFP-BRD7ΔNLS and analyzed with PI staining for the cell cycle phases. **B:** The proportion of cells in G0/G1 phase. Values represent the mean of triplicate experiments plus standard deviations (SD). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tion of NLS deleted BRD7 mutant was mostly shifted to be cytoplasm compartment detected by direct GFP fluorescence and indirect immunofluorescence assay. Therefore, the region of amino acids 65–96 of BRD7 is a strongly functional NLS, which governs the nuclear import of BRD7. NLS of BRD7 is not a typical bipartite nuclear targeting sequence. It spans a cluster of extremely overlapped and functional bipartite nuclear targeting sequences, and the deletion of any NLS sites would change the structure of the other three NLS sites, so the NLS within BRD7 is constitutively undetachable. NLS isn't always constitutively

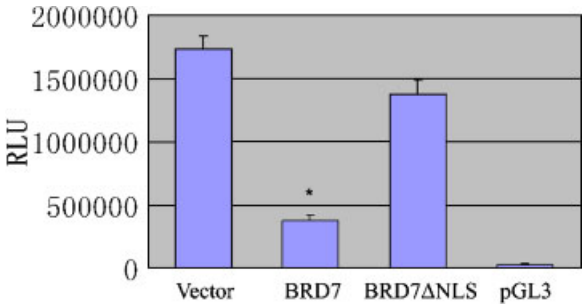


Fig. 8. Luciferase assay of E2F3 promoter activity. Five micrograms DNA containing 2 μg E2F3 reporter plasmid and 3 μg vector plasmid, BRD7, or BRD7ΔNLS was respectively co-transfected into HNE1 cells. The luciferase activity was normalized by the β-galactosidase activity. (1): 2 μg E2F3 reporter plasmid and 3 μg mock vector plasmid; (2) 2 μg E2F3 reporter plasmid and 3 μg BRD7 expression plasmid; (3) 2 μg E2F3 reporter plasmid and 3 μg BRD7ΔNLS expression plasmid; (4) 2 μg pGL3 reporter plasmid. Values represent the mean of three independent experiments plus standard deviations (SD). A single asterisk indicates $P < 0.01$ for BRD7 versus vector.

unchangeable, some atypical NLS were usually found and identified [Maria and Carlo, 2002].

Not all the transcription factors constitutively localize in the nucleus. Two subcellular distribution forms, nuclear form and latent cytoplasm form, had been found in some transcription factors, such as NF-κB [Baeuerle and Baltimore, 1988]. BRD7 localized in nucleus, while NLS deleted BRD7 localized in cytoplasm. We were prompted to know whether the cytoplasm form of BRD7 still functions. We found that NLS-deleted BRD7 revealed obviously decreasing negative effect on the promoter activity of E2F3, and could not down-regulate the protein expression of cyclin D1 and E2F3, as well as could not inhibit the cell cycle progression from G1 to S phase when compared with wild-type BRD7.

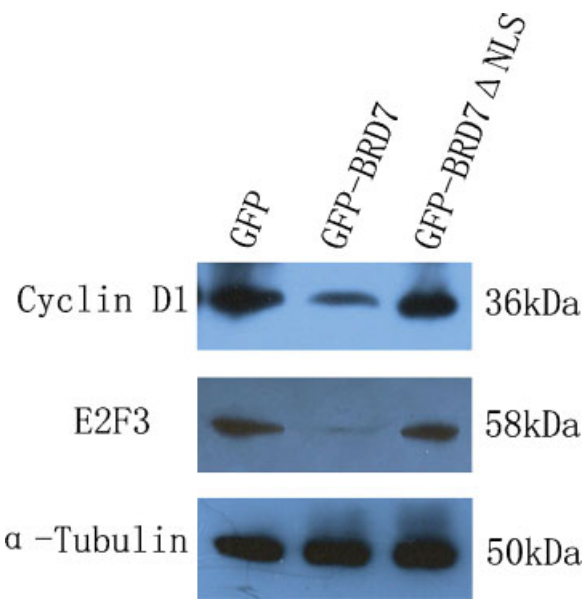


Fig. 9. Western Bloting was performed to detect the protein expression of cyclin D1 and E2F3 in three clone pools of HNE1 stably expressing GFP, GFP-BRD7ΔNLS, or GFP-BRD7. α-Tubulin was used as an internal control.

E2F3 and cyclin D1 are essential cell cycle related molecules. The regulating pathway controlling G1-S is Rb/E2F pathway. E2F3 is an activator of cell cycle forms heterdimers with DP2 or DP1 [Zheng et al., 1999]. Rb binding turns E2Fs into repressors or inactive transactivators [Stevaux and Dyson, 2002]. Passage through the restriction point and transition to S phase is triggered by the activation of the cyclin D/cdk complex, which phosphorylates Rb. Phosphorylated Rb dissociates from E2F, which is then free to initiate DNA replication [Morgan, 1997; Sherr and Roberts, 1999]. So the inhibition of cell cycle progression from G1 to S phase could be explained by BRD7 working through cell cycle related molecules, such as cyclin D1 and E2F3. The NLS of BRD7, maybe not its active domain, governs the nucleus import of BRD7 and results in rapid functional change of BRD7 when it was deleted. It was also thought that the transcriptional regulation of BRD7 involved in the inhibition of cell cycle progression from G1 to S phase and cell growth in NPC. BRD2 (RING3), another bromodomain protein, is thought to participate in transcriptional regulation. It is known that BRD2 could transactivate the promoters of several cell cycle regulatory genes, including dihydrofolate reductase, Cyclin D1, Cyclin A, and Cyclin E [Denis et al., 2000], but that with a NLS mutant failed to translocate to the nucleus and transactivate the promoter of dihydrofolate reductase [Guo et al., 2000]. Therefore, the NLS motifs of some proteins play important roles in transcription regulation.

ACKNOWLEDGMENTS

We gratefully acknowledge the guidance and the assistance of Prof. Jiahui Xia and Prof. Kun Xia in the performance of confocal microscopy.

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