Cellular Signalling 20 (2008) 2276–2285

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

NBS1 is required for IGF-1 induced cellular proliferation through the Ras/Raf/MEK/ERK cascade

Arunee Hematulin [a,c,*], Daniel Sagan [a], Friederike Eckardt-Schupp [a], Simone Moertl [a,b]

[a] Institute of Radiation Biology, Helmholtz Center Munich, German Research Center for Environmental Health, Post Box 1129, Ingolstaedter Landstrasse 1, D-85764 Neuherberg, Germany
[b] Department of Biology I, Ludwig-Maximilians-University Munich, Maria-Ward-Strasse 1, D-80638 Munich, Germany
[c] Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, 65000, Thailand

ARTICLE INFO

Article history:
Received 14 August 2008
Accepted 24 August 2008
Available online 28 August 2008

Keywords:
NBS1
Ras/Raf/MEK/ERK
MAPK
IGF-1
Cell cycle progression

ABSTRACT

NBS1 is a member of the Mre11–Rad50–*NBS1* complex, which plays a role in cellular responses to DNA damage and the maintenance of genomic stability. Transgenic mice models and clinical symptoms of *NBS* patients have shown that *NBS1* exerts pleiotropic actions on the growth and development of mammals. The present study showed that after repression of endogenous *NBS1* levels using short interfering RNA, hTERT-RPE cells demonstrated impaired proliferation and a poor response to IGF-1. *NBS1* down-regulated cells displayed disturbances in periodical oscillations of cyclin E and A and delayed cell cycle progression. Remarkably, lower phosphorylation levels of c-Raf and diminished activity of Erk1/2 in response to IGF-1 suggest a link among *NBS1*, IGF-1 signaling and the Ras/Raf/MEK/ERK cascade. The functional relevance of *NBS1* in mitogenic signaling and initiation of cell cycle progression were demonstrated in *NBS1* down-regulated cells where IGF-1 had a limited ability to induce the *FOS* and *CCND1* expressions. In conclusion, our findings provide strong evidence that *NBS1* has a functional role in IGF-1 signaling for the promotion of cell proliferation via the Ras/Raf/MEK/ERK cascade.

1. Introduction

The *NBS1* protein (nibrin, p95) is a component of the trimeric MRN (Mre11–Rad50–*NBS1*) complex, which is implicated in multiple cellular responses related to DNA damage, such as cell cycle control, DNA double strand break repair, maintenance of telomeres and genomic stability [1–4]. The encoding gene *NBS1*, is mutated in the human genetic disorder, Nijmegen breakage syndrome (*NBS*), which is characterized by microcephaly, developmental defects, growth retardation, immunodeficiency, radiation hypersensitivity, and increased cancer risk [4,5].

Based on the clinical symptoms and cellular defects, elucidation of the role of *NBS1* in DNA damage repair processes and cell cycle checkpoint signaling has been of interest to investigators for decades. Recently, several studies described novel functions of *NBS1* in tumorigenesis [6–8]. In this context, Chen et al. showed that over-expression of *NBS1* contributed to cell transformation through the activation of the phosphatidylinositol 3-kinase PI 3-K/Akt signaling pathway [6].

In addition, developmental defects including growth retardation, severe microcephaly, mental retardation, and primary ovarian failure typically have been observed in *NBS* patients [4]. Cells from *NBS* patients usually proliferate poorly and mice with targeted hypomorphic mutations of *NBS1* demonstrated growth retardation and impaired cellular proliferation. Lymphoid and germ cell development were especially disturbed [9]. *NBS1* null mice showed an even more severe phenotype as null mutations are embryonic lethal [10]. Therefore, we hypothesize that *NBS1* has an important role during cell growth and proliferation and that its overexpression induces cell transformation by stimulating these activities.

Cell growth and proliferation are complex intracellular processes that are controlled by a variety of cellular factors which can be initiated by a number of extracellular stimuli. One such stimulus is insulin like growth factor 1 (IGF-1) which plays a critical role in both fetal and postnatal growth [11]. Mutations in the IGF-1 gene result in severe pre-and post-natal growth and developmental defects and mental retardation [12]. Moreover, several studies reported associations between deregulated IGF-1 signaling and cancer [13], where increased IGF-1 signaling stimulates proliferation and promotes metastasis of cancer cells [14,15]. Furthermore, high circulating levels of IGF-1 increase the incidence of colon, breast, and prostate cancer in humans [16–18]. IGF-1-mediated signaling occurs via stimulation of the IGF-1 receptor, leading to activation of numerous downstream networks including the RAF-MAKPs and PI3K-Akt-mTOR systems [19].

MAPKs are a family of serine/threonine protein kinases that regulate many cellular activities including cell proliferation, cell differentiation, cell movement, and cell death. One major subgroup of MAPKs is the

* Corresponding author. Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, 65000, Thailand. Tel.: +66 5526 1936; fax: +66 5526 1935.
E-mail address: hematulin_a@yahoo.com (A. Hematulin).

0898-6568/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2008.08.017

extracellular signal-regulated kinase (ERK) family, which plays a crucial role in the regulation of cell growth and proliferation. In response to IGF-1 stimulation, the ERK cascade is induced via Ras/Raf/MEK and activates cell cycle progression from G1 to S phase by increasing the cyclin D1 expression [20–22]. In addition, cyclin D1 translation and stabilization is influenced by the PI3-K/Akt pathway [23–25].

Cyclin D proteins are essential regulators of cell cycle transition and cell proliferation. In early G1, cyclin-dependent kinase 4 and 6 (Cdk4 and Cdk6) are activated by D-type cyclins and initiate phosphorylation of the retinoblastoma (Rb) protein. This is followed by expression of E-type cyclins that activate Cdk2 during mid- to late G1 phase. The Cdk2/cyclinE complex completes the phosphorylation of Rb leading to the activation of E2F mediated transcription, which drives the cell into S phase [26]. During S phase, transcription of the cyclin A gene is activated leading to its accumulation during S and G2 phase [27]. The expression of cyclin D1 is largely dependent on extracellular signaling pathways and its function is tightly regulated at several levels, including transcription, translation, and influences protein stability, complex formation, and the abundance of CDK inhibitors. Thus, D-type cyclin represents a fundamental link between mitogens, nutrient stimulation, and cell cycle machinery [28]. This report describes a link between *NBS1*, mitogenic signaling pathways and cell cycle regulation. These results contribute to the functional elucidation of the observed phenotypes in cell lines with *NBS1* mutations and of the clinical symptoms of *NBS* patients on the molecular level.

2. Experimental procedures

2.1. Cell culture and transient cell transfection with short interfering RNAs

The telomerase-positive, immortalized human retinal pigment epithelial cell line, (hTERT-RPE) was cultured in Dulbecco's Modified Eagle Medium (DMEM/F12) containing 2.5 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 0.25% sodium bicarbonate, 40 units/ml penicillin G, and 40 μg/ml streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

The day before transfection, approximately 8×10^4 cells were seeded into a 60 mm culture plate containing 5 ml DMEM/F12 medium. 24 h later, 20 nM of each *NBS1* siRNA or scrambled control siRNA was transfected into each well using silentFect™ Lipid Reagent (Bio-Rad) according to the manufacturer's protocol. The transfection mixture was removed 8 h after transfection, and cells were washed with PBS before being incubated in fresh media for further experiments. Two siRNA duplexes targeting *NBS1* mRNA were used in this study. The first siRNA duplex (si*NBS1*#1) consists of two unmodified oligonucleotides: sense; AUGAUGUGGCCAUAGAAGATT, antisense; UCUUCUAUGGCCACAUCAUCC (Ambion). The second siRNA duplex (si*NBS1*#2) consists of a chemically modified oligonucleotide duplex;sense: GCUAUAUGCAACUUGGAGGAUUUA, antisense: UAAAUCCUCCAAGUUGCAAUAUAGC (Stealth™, Invitrogen). The scrambled siRNA duplexes designated by the manufacturer to not target any human genes: scram#1; sense: UUCUCCGAACGUGUCACGUUdTdT, antisense: ACGUGACACGUUCGGAGAAdTdT (QIAGEN) were used as a negative control for si*NBS1*#1 and scram#2; sense: UAAUAAUCCUAACCGUUGUAACAGC, antisense: GCUGUUACAACGGUUAGGAUUAUUA (Stealth™, Invitrogen) were used as a negative control for si*NBS1*#2.

2.2. Cell proliferation and viability assays

To determine the effect of *NBS1* down-regulation on cell proliferation rate, RPE cells were transfected with si*NBS1*#1, si*NBS* #2 or scram#1, scram#2. At 1 to 4 days after siRNA transfection, cells were harvested by trypsinization, and counted with a cell counting chamber (Neubauer improved, Hecht) and a particle counter (Coulter Z1, Beckman).

The colorimetric cell proliferation kit I (MTT) (Roche) was used for the measurement of cell proliferation after stimulation with growth factor IGF-1 (Sigma-Aldrich). 24 h after transfection with siRNA, cells were seeded into 24-well plates and incubated for 4 h. After starvation for 24 h, cells were pre-treated with vehicle alone or 10 μM U0126 (Cell Signaling) and subsequently stimulated with 300 μl medium containing 10% FCS and 100 nM IGF-1 per well. 48 h after stimulation, 30 μl MTT labeling reagent was added, and cells were incubated for another 4 h. Following the addition of 300 μl solubilization solution, the samples were incubated for an additional 12 h and absorbance was determined against a background control at 595 nm.

2.3. Cell treatments

24 h after siRNA transfection, the medium was aspirated from the dishes and replaced with 5 ml of fresh medium containing the following reagents. For aphidicolin treatment, cells were treated for 24 h with 10 μg/ml of aphidicolin diluted in DMSO (Sigma-Aldrich). Subsequently, cells were released from blockade by washing twice with PBS before adding 5 ml fresh medium. For IGF-1 and U0126+ IGF-1 treatment, cells were cultured in serum-free medium for 48 h, pretreated with 10 μM U0126 for 1 h and then stimulated with 100 ng/ml IGF-1. The treated cells were collected at different time points for cell cycle analysis, real time PCR, and western blot analysis.

2.4. Cell cycle analysis by flow cytometry

DNA staining of isolated nuclei for cell cycle analysis was performed using a modified method according to Nüsse et al. [29]. At each indicated time point, the treated cells were detached with trypsin, collected by centrifugation at 3000 ×*g* for 5 min, and the supernatant was carefully removed. The cell pellet was gently resuspended in 500 μl of solution containing 584 μg/ml NaCl, 1000 μg/ml Na-citrate, 10 μg/ml RNAase, 0.3 μg/ml Nonidet P-40, and 50 μg/ml propidiumiodide (PI), and vortexed for a short time interval. The cell suspensions were incubated for 30 min at room temperature, followed by the addition of 500 μl of solution containing 15 mg/ml citric acid, 0.25 mM sucrose, and 50 μg/ml PI. The cell suspensions were mixed and stored at 4 °C before flow cytometric measurement. Cell cycle distributions were analyzed on a FACScan (Becton-Dickinson).

2.5. Real-time PCR (RT-PCR)

Total RNA was prepared with Trizol reagent according to the manufacturer's instruction (Invitrogen). First strand cDNA synthesis was carried out with the Quantitect reverse transcription kit (Qiagen), according to the manufacturer's instruction. Real time PCR was performed using the LightCycler FastStart Reaction Mix SYBR Green I (Roche). The primer pair for *NBS1* amplification was 5′ - CAG ACC TTA ATT CCT GAC TGT C - 3′ as the forward primer and 5′ - TTT ACA GTG GGT GCA TCT TGT G - 3′ as the reverse primer. The primers for *CCND1* (QT00495285), *FOS* (QT00007070), and *B2 M* (QT00088935) amplification were purchased from Qiagen (QuantiTect Primer Assay).

2.6. Western blot analysis and antibodies

Total cell lysates were prepared by treating RPE cells in lysis buffer (150 mM NaCl, 10 mM *Tris*–HCl pH 7.2, 0.1% SDS, 1% Triton X-100, 1% Deoxycholate, and 5 mM EDTA) for 10 min on ice. Cell lysates were collected after centrifugation at 16,000 ×*g* for 10 min. Protein concentration was determined using the Bradford reagent (Sigma-Aldrich). Cells lysates were mixed with Laemmli's buffer (100 mM *Tris*–HCl pH 6.8, 4% SDS, 0.2% Bromophenol blue, 20% Glycerol, and 200 mM DTT) and then boiled at 95 °C for 5 min. 30 μg of protein from each sample were separated by electrophoresis in 8% or 10%