FEBS Letters 581 (2007) 821–825

p14^{ARF} interacts with N-Myc and inhibits its transcriptional activity

Stefano Amente[a,1], Barbara Gargano[a,1], Daniel Diolaiti[b], Giuliano Della Valle[b], Luigi Lania[a], Barbara Majello[a,*]

[a] *Department of Structural and Functional Biology, University of Naples 'Federico II', Naples, Italy*
[b] *Department of Biology, University of Bologna, Bologna, Italy*

Received 6 December 2006; revised 8 January 2007; accepted 23 January 2007

Available online 2 February 2007

Edited by Varda Rotter

Abstract In this study, we report that the human p14^{ARF} associates in vivo with the N-Myc and inhibits N-Myc mediated transcriptional activation. We have determined that the region (aa 140–300) encompassing the N-Myc BoxIII is required for efficient interaction in vivo. Furthermore, we demonstrate that in the SK-N-BE neuroblastoma cell line p14^{ARF} over-expression delocalized N-Myc from the nucleoplasm into nucleoli and that N-Myc regions required for interaction with p14^{ARF} are also important for nucleoli co-localization. Finally, we determine that the N-terminal region of the p14^{ARF} protein is involved in binding to c-Myc and N-Myc proteins.

Keywords: p14^{ARF}; N-Myc; C-Myc; Protein interactions; Transcription; Tumor suppressor

1. Introduction

Genes of the Myc family contribute to the genesis of several types of human tumors. In all cases, Myc expression is increased in the tumors indicating that Myc elevated expression contributes to tumorigenesis [1,2]. N-Myc and c-Myc have different patterns of gene expression; whereas c-Myc is expressed during embryonic development and in adult tissues, N-Myc is found in undifferentiated types of cells in the lung, heart, nervous system, kidney, limbs, etc. [3,4].

N-Myc oncogene is implicated in the pathogenesis of neural crest derived tumors including neuroblastoma, the most frequent solid malignancy in infants. Amplification of N-Myc gene is the major negative prognostic marker in human neuroblastomas [5,6]. Although N-Myc and c-Myc share large regions of sequence conservation, their physiological role in the cell does not seem redundant since gene inactivation studies have shown that functional loss of either c-Myc or N-Myc leads to embryonic lethality [4,7–9].

Myc proteins act as transcription factors, although c-Myc transcriptional activity has been well characterized and several co-activators have been found, much less is known about N-Myc co-factors mainly because of the assumption that c-Myc and N-Myc have redundant functions [1,10].

Among c-Myc partners, the tumor suppressor protein ARF (alternative reading frame) has been found to directly bind the Myc protein and dramatically block c-Myc's ability to activate transcription and induce hyperproliferation and transformation [11–13].

Although ARF is not detectably expressed in most normal tissues, increased levels of mitogenic signals or aberrant expression of oncogenes induce the transcription of ARF. In particular, c-Myc was the first oncogene to be found to activate ARF that in turns, in order to induce cell cycle arrest, must functionally inhibit cell cycle regulated gene expression [14,15]. The p14^{ARF} protein has both p53-dependent and p53-independent tumor-suppressive activities. In a p53 background, ARF antagonizing the E3 ubiquitin ligase activity of MDM2 determines the stabilization of p53 incrementing its transcriptional activity; the result is the induction of cell-cycle arrest or apoptosis [15]. More recently, several ARF p53 independent functions have been documented. In particular, ARF has been shown to associate with transcription factors as Foxm1b, E2F, DP1, c-Myc, all of them directly involved in the regulation of cell cycle genes expression [11,15–17].

Given the similarity between N-Myc and c-Myc proteins but also the striking divergence among their expression pattern, we were interested to determine whether p14^{ARF} interacts and regulates N-Myc. We found that p14^{ARF} binds N-Myc in vivo and inhibits N-Myc transcriptional activity. We also found that ARF over-expression in neuroblastoma cells delocalizes the N-Myc protein from the nucleoplasm to the nucleoli where it co-localizes with ARF. We have mapped the N-Myc region involved in ARF binding in the central region of the protein (aa 140–300) and we have demonstrated that N-Myc mutants that do not interact with p14^{ARF} are not recruited into nucleoli upon ARF co-expression. Finally, we mapped in the N-terminal region of the p14^{ARF} protein the domain involved in c-Myc and N-Myc binding, respectively.

2. Materials and methods

2.1. Plasmids

pcDNA3-N-Myc, N-Myc d(1–300), N-Myc d(1–134), N-Myc d(20–90), and N-Myc d(96–140), N-Myc d(350–464), N-Myc d(350–464) were kindly provided by Dr. T. Fotsis [18]. pCMV14-N-Myc and GFP-p14^{ARF} have been previously described [19,13]. F:p14^{ARF}, F:p14^{ARF}(1–65), and F:p14^{ARF}(65–132) were obtained by subcloning the corresponding cDNA ARF coding sequences into the p3XFLAG-CMV10 vector (Sigma).

*Corresponding author. Fax: +39 081 679233.
E-mail address: majello@unina.it (B. Majello).

[1]These authors contributed equally to this work.

0014-5793/$32.00
doi:10.1016/j.febslet.2007.01.062

2.2. Cell culture, luciferase assay and immunofluorescence

Human 293 T, SK-N-BE and Tet-21/N cell lines were grown in DMEM supplemented with 10% fetal calf serum. Tet-21/N cells [19] were maintained in the presence of tetracycline (1 μg/ml) for at least one week before transfection. 1×10^5 cells/well were plated and grown for an additional 24 h in 6 well plates in the presence of tetracycline. The cells were washed with 1× PBS and then transfected with the appropriate constructs by the polyethylenimine (PEI 25 K) method as described [20]. After transfections, the cells were incubated for 48 h in presence or absence of tetracycline as required. The activity of firefly was measured with the dual luciferase assay kit (Promega) according to the manufacturer's instructions by using a T20/20 luminometer (Turner Design). pRL-CMV (Promega) was co-transfected for normalization. The amounts of transfected plasmids DNAs are indicated in the legend to figures. For immunofluorescence analysis, SK-N-BE cells were transfected with lipofectamine with 600 ng each of GFP-p14^{ARF} and/or pcDNA3-N-Myc or pcDNA3-N-Myc d(1–300) plasmids and the cells were processed for immunofluorescence as described [13] using the N-Myc (C2, Santa Cruz Biotechnology) antibody.

2.3. Antibodies and co-immunoprecipitations

The following antibodies were used for the immunological techniques: anti-Myc (N262 for IP and 9E10 for WB, Santa Cruz Biotechnology, Inc.), anti-Max (C17, Santa Cruz Biotechnology, Inc.), anti-ARF (C-18, Santa Cruz Biotechnology, Inc.), and anti-N-Myc (C2, Santa Cruz Biotechnology). Co-immunoprecipitations from transiently transfected cells were carried out as previously described [13]. All interactions were carried out overnight at 4 °C. The beads were washed at least five times using buffer F (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 30 mM $Na_4O_7P_2$, 50 mM NaF, 5 μM $ZnCl_2$, 0.1 mM Na_3VO_4, 1% Triton and 0.1 mM PMSF) before loading on SDS–PAGE.

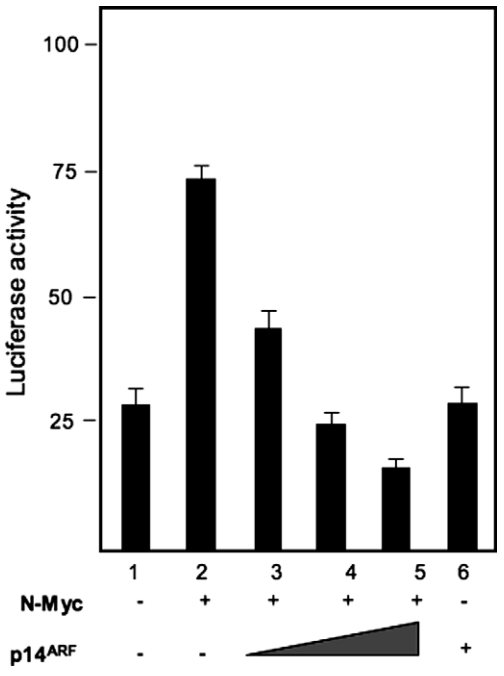

Fig. 1. ARF expression inhibits N-Myc activated transcription. Tet21Ncells were cotransfected with 100 ng of hTERT-Luc and different amounts (0.1; 0.5 and 1 μg, respectively) of F:p14^{ARF}, as indicated. N-Myc was activated by tetracycline withdrawal. Each histogram bar represents the mean of three independent transfections made in duplicate with a standard deviation less than 10%.

3. Results and discussion

3.1. p14^{ARF} over-expression inhibits N-Myc transcriptional activity

It has been recently shown that ARF could inhibit c-Myc's ability to activate transcription [11–13]. We sought to determine whether p14^{ARF} might affect the transcription activity of N-Myc. To this end, we used a well characterized inducible cell line Tet-21/N, a human neuroblastoma cell line in which N-Myc expression is controlled through a Tet-off inducible promoter [19]. Tet-21/N cells were co-transfected with the hTERT-Luc construct and an expression vector for p14^{ARF}. N-Myc induction was obtained by tetracycline withdrawal and the cell extracts were analyzed for the human Telomerase promoter (hTERT) driven luciferase expression. As reported in Fig. 1, p14^{ARF} inhibits in a dose-dependent manner the N-Myc mediated activation of the human TERT promoter [21], while no effect was observed on basal expression of the hTERT promoter. Moreover, co-transfection experiments performed in the p53 null H1299 cell line gave similar results indicating that the decrease of N-Myc mediated transcription by ARF does not involve p53 (data not shown).

3.2. N-Myc associates with p14^{ARF} in vivo

We have previously demonstrated that p14^{ARF} directly associates with the c-Myc protein. Even if it is a general assumption that the interaction partners of c-Myc are also N-Myc partners, the failure of expression of either of the two Myc family members is not redundant and it cannot be excluded that they can form different complexes with their interactors.

To investigate the physical interaction between p14^{ARF} and N-Myc, Co-immunoprecipitation assays were performed with protein extracts from 293T transiently transfected cells. The cells were co-transfected with pcDNA3-N-Myc along with F:p14^{ARF} (F:ARF) and nuclear extracts were prepared and subjected to CoIP as reported in Fig. 2. IPs were carried out using either the N-Myc or the ARF antibodies, and the immunoprecipitated materials were analyzed by Western blotting with anti-N-Myc and anti-FLAG antibodies, respectively. The results reported in Fig. 2A showed that p14^{ARF} co-immunoprecipitated with N-Myc. In the converse experiment (Fig. 2B), using the ARF antibody for immunoprecipitation, N-Myc co-precipitated with ARF, whereas control IgG did not.

To identify N-Myc sequences involved in N-Myc-ARF interaction, we carried Co-IP analysis using protein extracts from 293T cells transiently co-transfected with F:ARF expression vector and several N-Myc deletion mutants as indicated in Fig. 3B. The protein extracts were immunoprecipitated with the ARF antibody and the Co-IP proteins analyzed by WB with N-Myc and FLAG antibodies, respectively. As shown in Fig. 3A, the N-Myc deletion mutant d(1–300) did not associate with p14^{ARF} (lane 10), suggesting a crucial role of the N-terminus portion of the protein in ARF-binding. However, deletions of either BoxI d(20–90) or BoxII d(96–140) retained ARF-binding. The strength of interaction of the d(20–90) and d(1–134) mutants was reproducibly higher than that of the other N-Myc deletions; we do not have an explanation for this behavior but we can speculate that these deletions could better expose the N-Myc domain for ARF binding. From these data we conclude that the N-Myc region involved in p14^{ARF} interaction resides in the region from aa 140 to 300.

3.3. p14^{ARF} induces N-Myc nucleolar co-localization

It has been shown that c-Myc and ARF co-localize in the same cellular compartment upon ARF over-expression (11–