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The N-Terminal Half of NPM Dissociates from Nucleoli of HeLa Cells after Anticancer Drug Treatments

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NPM (nucleophosmin/B23) is a nucleolar phosphoprotein abundant in tumor cells. It dissociates from nucleoli of cells after treatments with various anticancer drugs. To determine the domain of NPM responsible for nucleolar binding, the N- and C-terminal halves of NPM were fused to GFP (green fluorescent protein) and introduced into HeLa cells. The N-terminal half (aa 1–150) of NPM (GFP-NPM_N) was found localized in the nucleoli. A stable transformant of GFP-NPM_N in HeLa cells was prepared and tested for association to nucleoli after anticancer drug treatments. GFP-NPM_N dissociates from nucleoli after treatments with daunomycin, actinomycin D, camptothecin, and toyocamycin. The dissociation is time- and dose-dependent, and correlates with the cytotoxicity induced by the drugs. These results indicate that a stable transformant of GFP-NPM_N in HeLa cells may be useful for the screening of anticancer drugs.

NPM (Nucleophosmin/B23) is a major nucleolar phosphoprotein that is 20 times more abundant in cancer cells than in normal cells (1). The concentration of NPM is directly proportional to cell proliferation. The putative functions of NPM are ribosome assembly and transport. The NPM gene is located on chromosome 5. The breakpoints of the t(2:5), t(3:5), and t(5:17) chromosome translocations of certain lymphomas are located on the NPM gene (2–3). In these cases, the N-terminal portion of NPM (aa 1–117) is fused to the catalytic domain of anaplastic lymphoma kinase (ALK) or the retinoic acid receptor α (RARA). It was reported that the expression of the NPM-ALK fusion protein transforms NIH3T3 cells (4).

In previous studies, we have found by the immunofluorescence technique that NPM shifts from the nucleolus to the nucleoplasm (a phenomenon called NPM-translocation) during exposure to certain anticancer drugs (actinomycin D, doxorubicin, daunomycin, tiazofurin, and camptothecin) (5–9). NPM-translocation correlates with drug-induced apoptosis (10). However, the mechanism of drug-induced NPM-translocation is not well understood. One hypothesis is that anticancer drugs interfere with the binding of NPM to nucleoli. To support this hypothesis, previous studies using the toyocamycin analogs have shown that the 5-position of the pyrrolo[2,3-D]pyrimidine ring and a structure similar to adenine (rather than guanine) effectively dissociate NPM from nucleoli (11). This result indicates that structurally specific analogs could interfere with NPM's binding in nucleoli. To better understand the binding of NPM to nucleoli, we fused either the N-terminal or the C-terminal half of NPM to green fluorescent protein (GFP) and assessed their ability to bind to nucleoli in vivo. We found that the N-terminal, but not the C-terminal, half of NPM binds to nucleoli. We also found that the N-terminal half of NPM, just like the full-length fusion protein or wild-type NPM, dissociates from nucleoli after exposure to anti-cancer drugs. These studies show a direct correlation between NPM translocation and cytotoxicity induced by drugs.

MATERIALS AND METHODS

The full-length cDNA of NPM (a 1311 bp EcoRI fragment in pGEM3 Blue, Ref. 1) was fused in-frame to the C-terminus of GFP. The N- and C-terminal halves of NPM were derived by cleaving the cDNA at the Nla IV restriction site (aa 151, near the midpoint of the molecule). The cleaved fragments (corresponding to aa 1–151 and aa 151–194) were similarly fused in-frame to the C-terminus of GFP. These clones were purified. Their sequences were verified by DNA sequencing. Clones were transfected into HeLa cells by electroporation. Stable transformants of these clones in HeLa cells were selected with G418. These transfomants in HeLa cells have been passed over 45 doubling times without any loss of fluorescence intensity.

To determine the cellular location of the fusion proteins after anticancer drug treatments, cells were typically incubated with drugs for 4 hours before being fixed with 2% formaldehyde in PBS (Phosphate buffered saline) for 20 minutes. The cellular localization of GFP-NPM was then observed with a fluorescence microscope.

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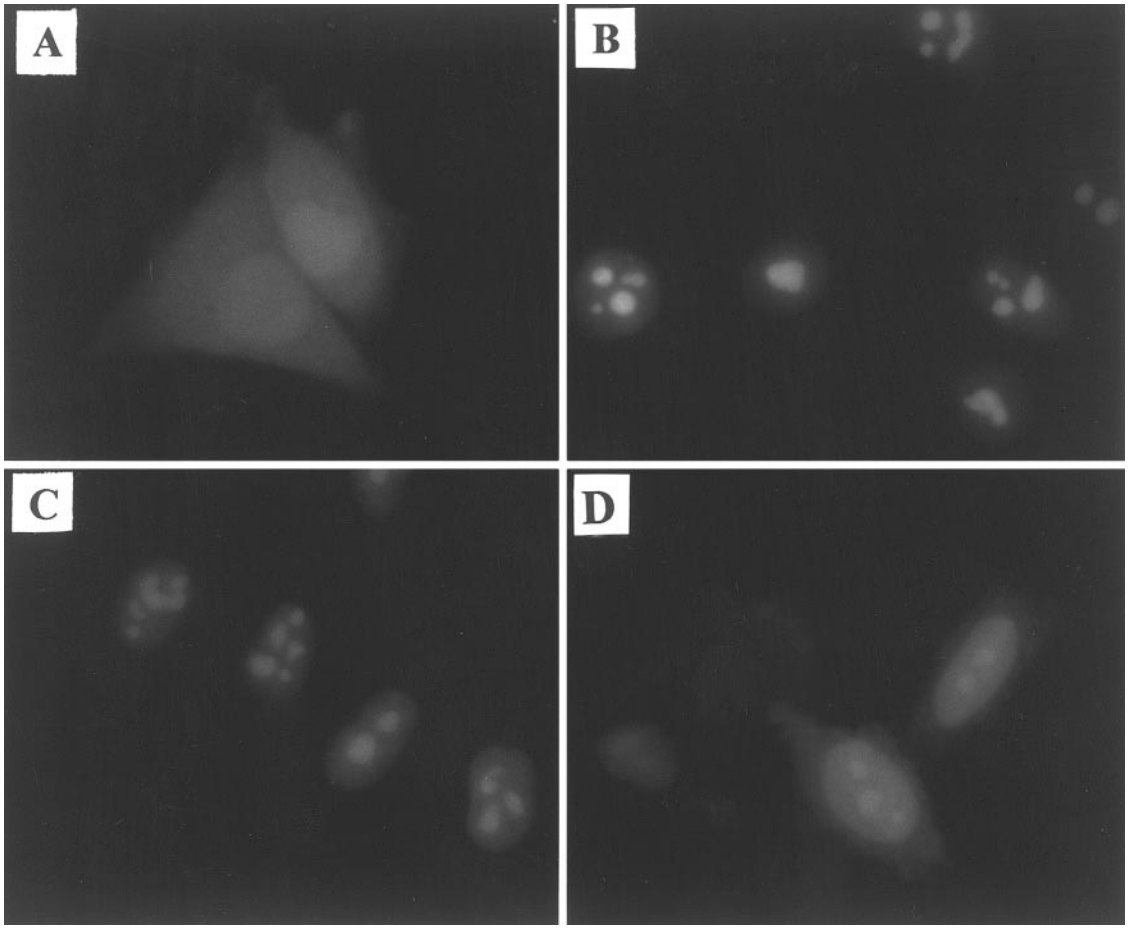

FIG. 1. Localization of GFP-NPM fusion proteins in HeLa cells. (A) GFP; (B) fusion protein of GFP and full-length NPM (GFP-NPM_F); (C) fusion protein of GFP and the N-terminal half of NPM (GFP-NPM_N); (D) fusion protein of GFP and the C-terminal half of NPM (GFP-NPM_C). Nucleolar fluorescence was observed in cells with either the full-length or the N-terminal half of NPM.

To determine the relative quantity of GFP-NPM in nucleoli, fluorescent images of cells were captured with a CCD camera. The fluorescent intensity in the nucleoli and the nucleoplasm were then digitized (12). The identification of nucleoli was guided by the phase-contrast images. The percentage of fluorescence localized in nucleoli was determined via the following formula:

$$\%\ \text{nucleolar fluorescence} = \frac{\text{nucleolar fluorescence intensity} \times \text{nucleolar area}}{\text{nucleoplasm fluorescence intensity} \times \text{nucleoplasm area}}$$

Data from at least 100 cells was acquired.

A cell survival assay was used to determine the toxic effects of the anticancer agents. Cells were treated with drugs for 4 hours. After rinsing twice with PBS, the cells were trypsinized, re-suspended in a fixed volume, counted with a hemacytometer, and appropriate numbers of cells (25–10,000) were plated in petri dishes (three concentrations in triplicates). Cells were cultured for 14 days and stained with crystal violet (0.5% in 95% ethanol). The number of colonies (which have more than 40 cells per colony) was determined and used as an indicator of cytotoxicity. The average plating efficiency (# of colonies per # cells plated) for the control cells was approximately 80%. The percentage of cell survival was determined as the % plating efficiency of drug-treated cells over the % plating efficiency of control cells.

RESULTS

Figure 1A (control) shows that GFP without NPM distributes uniformly throughout a cell. The fusion proteins with either the full-length or the N-terminal half of NPM (GFP-NPM_F, GFP-NPM_N) localized into nucleoli (Figs. 1B and 1C). However, the C-terminal half of NPM (GFP-NPM_C) did not efficiently localize into nucleoli (Fig. 1D). These results indicate that the N-terminal half of NPM contains the binding domain for nucleolar localization.

To study whether the binding of GFP-NPM_N to nucleoli is affected by anticancer drugs, cells were exposed to either actinomycin D (0.01 μg/ml), daunomycin (1 μg/ml), camptothecin (10 μM), toyocamycin (10 μM) or mycophenolic acid (10 μM) for 4 hours. We observed an increase in nucleoplasmic fluorescence after these drug treatments, indicating the dissociation

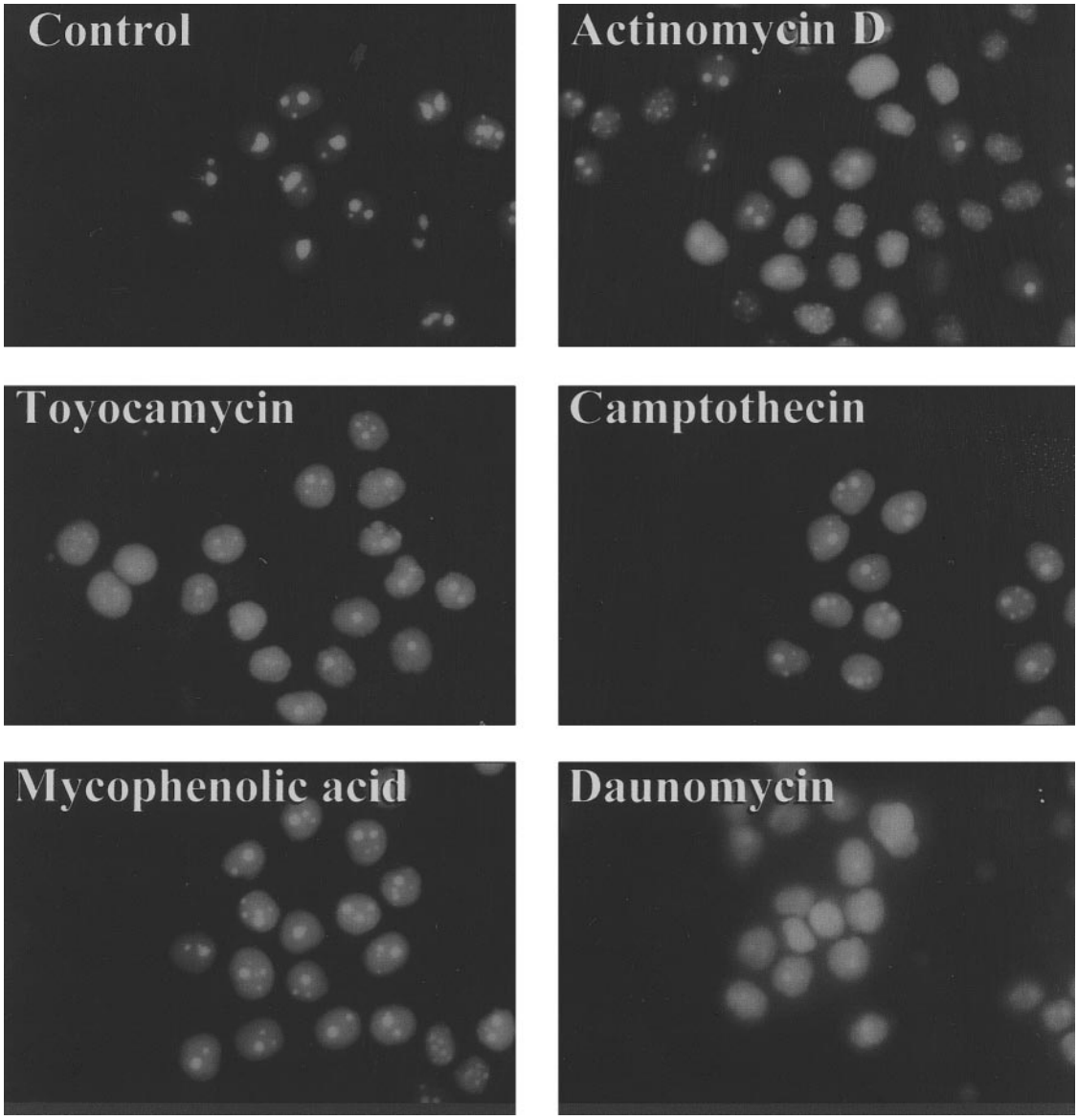

FIG. 2. Localization of GFP-NPM_N after various drug treatments. Cells were incubated with drugs for 4 hours. Cells were then fixed with 2% formaldehyde in PBS for 20 minutes. The cellular localization of GFP-NPM_N was captured with fluorescence microscopy.

of GFP-NPM_N to the nucleoplasm (Fig. 2). At the same time, the nucleoli became smaller and more circular shaped.

To determine the amount of NPM dissociated from nucleoli to the nucleoplasm, a percentage of GFP-NPM_N fluorescence in the nucleoli was calculated from the total GFP-NPM_N fluorescence (see Materials and Methods).

Table 1 shows the average percentage of GFP-NPM_N that remains in nucleoli after each drug treatment. We found that nucleoli of control cells have, on average, 49% of total nuclear GFP-NPM_N. A substantial reduc-

TABLE 1

NPM Remains (% of Total) in Nucleoli after Anticancer Drug Treatment

Drugs	% ± S.D.
Control (no drug)	49.30 ± 8.80
Actinomycin D (0.01 μg/ml)	16.21 ± 3.72
Daunomycin (1 μg/ml)	13.28 ± 4.20
Camptothecin (10 μM)	19.61 ± 3.68
Toyocamycin (10 μM)	15.12 ± 3.79
Mycophenolic acid (10 μM)	26.53 ± 4.93

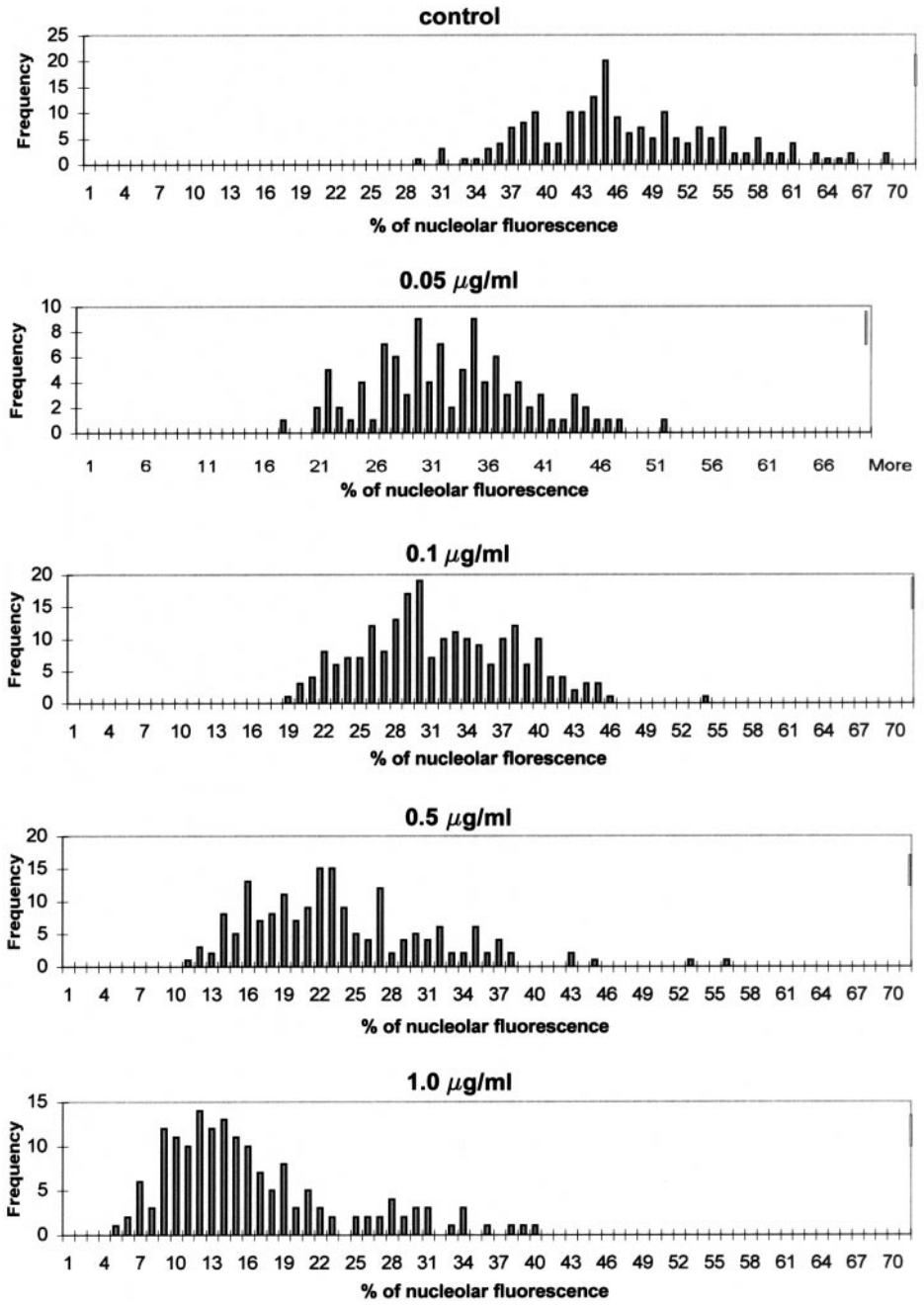

FIG. 3. Histograms of cells after treatments with various concentrations of daunomycin. Abscissa: % of nucleolar fluorescence in cells; Ordinate: frequency of cells.

tion of GFP-NPM_N in nucleoli (13–26% of the total nuclear GFP-NPM_N) was observed after a maximum dosage of drug treatment: actinomycin D (16.2%), daunomycin (13.2%), camptothecin (19.6%), toyocamycin (15.1%) and mycophenolic acid (26.5%). The dissociation of nucleolar GFP-NPM_N to the nucleoplasm is dose-dependent. Figure 3 (histogram) shows that the amount of nucleolar GFP-NPM_N in HeLa cells decreases with increasing concentrations (0.05 to 1 μg/ml) of daunomycin.

Cytotoxicity of HeLa cells after the daunomycin-treatment was determined by a cell survival assay. Cells were exposed to 0.05–1 μg/ml daunomycin for 4 hours before plating onto drug-free media. Cells were cultured for 14 days, and the number of viable colonies was then counted. Figure 4 shows the percentage of cell

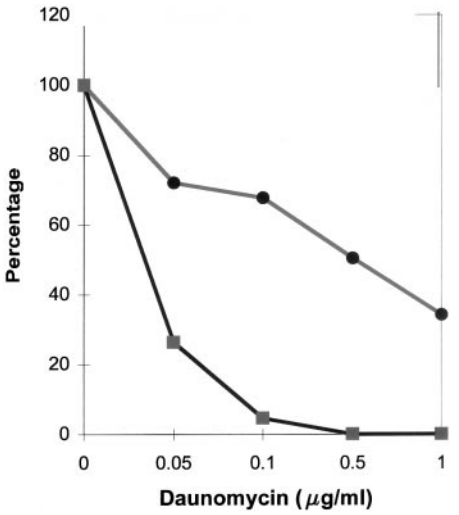

FIG. 4. Correlation of the loss of nucleolar NPM with cell survival. Concentration-response curves of % nucleolar NPM and % cells survival of Jurkat cells treated with daunomycin. ● % nucleolar fluorescence; ■ % survival. Abscissa: Percentage; Ordinate: daunomycin concentrations.

survival and nucleolar NPM after the treatment of various concentrations of daunomycin. We observed a direct correlation between the loss of nucleolar NPM and decrease of cell viability. At a concentration of 0.05 μg/ml, there approximately 25% of nucleolar NPM is lost, which corresponds to approximately 75% of the cell death.

DISCUSSION

Our studies show that the N-terminal of NPM binds to nucleoli. However, to which nucleolar components it binds to are not known. The amino acid sequence at the N-terminal portion (aa 1–130) has 50% sequence identity with the chromatin assembly factor, nucleophosmin (1, 13), suggesting that this portion could bind to nucleoproteins. In a preliminary experiment, NPM was removed from formaldehyde fixed HeLa cells by a treatment with DNase I but not with RNase A (data not shown). These results indicate that NPM could bind to DNA. If so, it is likely that the action(s) of topoisomerase inhibitors (daunomycin and camptothecin) alter the DNA conformation, which then affects NPM's binding site(s) in the nucleoli. The precise mechanism remains to be elucidated.

In spite of a good correlation between dissociation of NPM and cytotoxicity (shown in Fig. 4 and Ref. 10), the cause-effect relationship between them has not yet been established. It is possible that the dissociated nucleolar proteins (such as NPM) leak to the cytoplasm and cause an apoptotic reaction. To support this hypothesis, we recently found that nucleolar proteins translocate to the cytoplasm of Jurkat cells (within one hour) after a camptothecin-treatment (data not shown). The translocation occurs prior to the activation of the caspase-3 activity and the cleavage of poly-ADP-ribosyl polymerase (PARP), a hallmark of apoptosis.

Our data shows that a loss of 20–25% of nucleolar NPM during initial drug-treatment corresponds to the eventual death of over 95% of the cells (Fig. 4). Whether the loss of NPM is directly related to cell-death remains to be investigated. However, the phenomenon of losing 25% of NPM from nucleoli may be a useful early indicator for cell death.

In conclusion, we found that: (1) The nucleolar binding domain of NPM is in the N-terminal half of the molecule; (2) The localization of GFP-NPM_N in nucleoli is affected by anticancer drug treatments. Loss of nucleolar localization was observed with actinomycin D, daunomycin, toyocamycin, camptothecin and mycophenolic acid; and (3) A HeLa cell clone expressing GFP-NPM_N may provide a useful tool for screening anticancer drugs. Measuring NPM translocation is fast, convenient, and does not require the use of antibodies.

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