Overexpression of the Linker Histone-binding Protein tNASP Affects Progression through the Cell Cycle*

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H1 histones not bound to DNA appear to be bound to the H1 histone-binding protein NASP, which has been characterized previously in vitro and in vitro (1, 10, 11) as an acidic protein containing functional histone-binding sites, a leucine zipper, ATP/GTP-binding sites, and a functional nuclear localization signal (11, 12). NASP occurs in two major forms as follows: tNASP, found in gametes, embryonic cells, and transformed cells; and sNASP, found in all dividing somatic cells (1). Mouse sNASP (Mr, 45,751) is identical to tNASP (Mr, 83,934), except that it lacks two internal regions of the protein (1, 10). Similarly human tNASP and sNASP occur with identical deletions to those found in the mouse (10, 13).

During the cell cycle, NASP mRNA expression in somatic cells increases and decreases concurrently with histone mRNA changes in expression (1). However, in rapidly dividing cells protein levels of NASP remain fairly constant and only decrease to undetectable levels in non-dividing cells (1). Reports that overexpression of H1 histones can influence progression through the cell cycle (5) prompted us to investigate the effect on progression through the cell cycle by overexpression of NASP and to examine the relationship of NASP to H1 histones in HeLa cells. In this report we demonstrate that 1) the overexpression of tNASP does influence progression through the cycle, 2) tNASP is bound to H1 histones in HeLa cells, 3) in vitro complexes of tNASP-H1 will transfer H1 to DNA, and 4) that NASP and H1 have virtually identical mobilities within the nucleus.

EXPERIMENTAL PROCEDURES

All chemicals and reagents used in this study were molecular biology grade. Restriction enzymes were purchased from Roche Diagnostics. Purification of plasmid DNA and PCR products were carried out using QIAprep Miniprep and QIAquick PCR purification kits (Qiagen, Valencia, CA), and sequencing was performed at the University of North Carolina, Chapel Hill, automated sequencing facility. Affinity-purified goat anti-green fluorescent protein antiserum was purchased from Rockland (Gilbertsville, Pa); rabbit anti-histone H1 (FL-219) polyclonal antiserum was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); and rabbit anti-NASP antiserum was prepared against either N-terminal (nucleotides 96–1099) or C-terminal (nucleotides 1106–2414) recombinant proteins as described previously (1).

Construction of Expression Vectors—The entire coding sequence of mouse tNASP (nucleotides 92–2405, GenBank™ accession number AF034610) was amplified from mouse testis Quick-clone cDNA (Clontech, Palo Alto, CA) using the Expand High Fidelity PCR System (Roche Molecular Biochemicals) and cloned into a pRc/RSV Hi site in the pEGFP-N1 vector, which contains the sequence for expressing green fluorescent protein (GFP); Clontech, Palo Alto, CA). The nucleo-
ization (NLS) deletion mutant (NASP-ΔNLS; nucleotides 92–2192), which lacked the nuclear localization signal (nucleotides 2215–2268), was PCR-amplified and cloned into the same pEGFP-N1 vector. The entire coding region of mouse sNASP (nucleotides 254–1384, GenBank™ accession number AF095722) (1) was also cloned into the pEGFP-N1 vector. The histone-binding site deletion mutant (NASP-ΔH1BS; nucleotides 527–1384), which lacked all histone-binding sites (nucleotides 349–525) (11), was PCR-amplified and cloned into pEGFP-N1.

For cell cycle studies, vectors lacking the GFP sequence were constructed from pEGFP-N1 by excising the GFP by sequential digest by BamHI and NotI. Digested ends were resealed by Klenow enzyme and ligated by T4 DNA ligase. All constructs were sequenced to verify the correct sequence of coding.

In vitro H1-binding studies, full-length tNASP and NASP-ΔHBS (amino acids 567–773) were cloned into His-tag-pE30 vectors. Re-combinant proteins were expressed in BL21 (DE3) pLysS competent E. coli and purified on nickel-nitrilotriacetic acid-agarose columns (Qiagen, Valencia, CA).

Cell Studies—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium-H plus 10% calf serum. Cells were removed from plastic dishes by trypsinization in EDTA. Synchronized populations of HeLa cells were obtained by double thymidine block as described previously (1).

Indirect Immunofluorescence—HeLa cells were grown on chamber slides with polyornithine wells (Falcon® CultureSlide, BD Biosciences), washed twice with cold (4 °C) PBS (phosphate-buffered saline, pH 7.0), fixed with chilled (−20 °C) methanol (20 min), washed twice with cold PBS, and incubated in rabbit anti-NASP (N-terminal) antisera or preimmune serum (1:500) in PBS. Cells were incubated for 45 min, washed in PBS (3 × 5 min each), and incubated in fluorescein-conju-gated, affinity-purified goat anti-rabbit IgG Fc fragment (1:1000 in PBS; Cappel, West Chester, PA) for 20 min. Washed cells were viewed with a Zeiss fluorescence microscope.

Chemical Transfection—Plasmid-DNA complexes were transiently transfected into HeLa cells using Effectene Transfection Reagent according to the manufacturer's instructions (Qiagen, Valencia, CA). This method is based on a non-liposomal lipid formulation and resulted in >90% transfection efficiency. Cells were transfected into HeLa cells using Effectene Transfection Reagent according to the manufacturer's instructions (Qiagen, Valencia, CA).

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and anti-GFP antiserum confirmed the expression of the constructs in these cells (Fig. 2A). Western blots of nuclear and cytoplasmic fractions from non-transfected HeLa cells confirmed the presence of both s and t forms of NASP in the nucleus and cytoplasm (Fig. 2B).

Association of H1 Histones with NASP—In myeloma 66-2 cells H1 histones were found to co-purify bound to native NASP (1). Similarly in HeLa cells, isolation of NASP by HPLC size chromatography or affinity chromatography with anti-NASP antibodies resulted in the co-purification of H1 histones (Fig. 2, C and D). Histone analysis by reverse phase HPLC of H1 histones extracted from H1-NASP complexes identified subtype H1s (H1.2) as the H1 histone (data not shown). Histone H1.2 is the most abundant subtype in HeLa cells (15); however, other histone H1 subtypes may not have been detected because of limited amounts of sample.

Effect of the Overexpression of NASP and NASP-ΔNLS on the Cell Cycle—In experiments overexpressing NASP and NASP deletion mutants in synchronized HeLa cells, we used constructs without the GFP sequence. Chemical transfection of cells with the vectors expressing NASP and NASP deletion mutants was done 24 h before the cells were released from the double thymidine block to ensure complete expression of the construct. Fig. 3 shows a typical experiment in which cell cycle progression was analyzed by FACS analysis up to 8 h after release from the double thymidine block. Overexpression of tNASP clearly affects the progression of cells through the cell cycle; compare Fig. 3, A and B. Similarly overexpression of NASP-ΔNLS affects the progression of cells through the cell cycle; compare Fig. 3, A and C. Fig. 3, D and E, shows that cells transfected with NASP-ΔHBS, the mutant lacking histone-binding sites, or sNASP do not delay progression through the cell cycle.

Table I presents the data collected from 6 independent experiments at 4 and 6 h after release from the double thymidine block. At 4 and 6 h after release there were significantly fewer HeLa cells, which were overexpressing tNASP, in the G2 and S phases than there were non-overexpressing HeLa cells (71.45 versus 50.8%; p = 0.01 at 4 h, and 79.85 versus 60%; p = 0.01 at 6 h; Fig. 4 and Table I). Similarly there were significantly fewer overexpressing tNASP-ΔNLS HeLa cells than non-overexpressing cells in the G2 and S phases (71.45 versus 53.17%; p < 0.01 at 4 h, and 79.85 versus 59.27%; p = 0.01 at 6 h; Fig. 4 and Table I). There was, however, no significant difference between tNASP and NASP-ΔNLS (Fig. 4). Data from 2, 8, and 24 h showed no significant differences between any of the cells tested. There was no difference in the progression of cells through the cell cycle between untreated cells and cells treated with delivery reagents only (Table I). At 4 and 6 h, cells transfected with NASP-ΔHBS (p = 0.37 (4 h) and p = 0.48 (6 h)) or sNASP (p = 0.34 (4 h) and p = 0.34 (6 h)) did not significantly delay progression through the cell cycle (Fig. 3, D and E; Table I). These data indicate that the overexpression of tNASP is different from the overexpression of sNASP in its effect on cell cycle progression and that the histone-binding sites are necessary for the delay in cell cycle progression. However, the presence of a NLS is not required for this effect.

DNA Supercoiling by H1-NASP Complexes—To test the ability of NASP to transfer H1 histones to DNA, calf thymus H1 histones were biotinylated and bound to tNASP in vitro, and the H1-NASP complexes were subsequently separated by chro-
matography on Bio-Rad Micro Bio-Spin 30 (MBS30) columns. MBS30 columns separate H1-NASP complexes from unbound histones. Fig. 5 demonstrates that biotinylated H1 histones are retained on the MBS30 column (lanes 1 and 2); however, when H1 is complexed with NASP, both are eluted from the column (lanes 4 and 6). When NASP-ΔHBS is substituted for tNASP, it elutes from the column (lane 5) but does not bind H1 histones and does not non-specifically carry H1 histones through the column (lane 3).

The separated H1-NASP complexes were incubated with relaxed SV40 DNA to test the ability of H1 histones to supercoil the SV40 DNA (Fig. 6). Supercoiled and relaxed SV40 DNA are shown in Fig. 6, lanes 1 and 2. The addition of H1 histones to relaxed SV40 DNA causes supercoiling (lane 3), whereas the addition of tNASP does not (lane 4). As shown in Fig. 6, lanes 6 and 7, the addition of H1-NASP complexes, which have passed through the MBS30 column, to relaxed SV40 DNA results in supercoiling the DNA. If H1 only is chromatographed and the resulting eluate incubated with relaxed SV40 DNA, no supercoiling occurs because the H1 histones are retained by the column (lane 5). If increasing amounts of tNASP are added at the time of the addition of H1-NASP complexes to relaxed SV40 DNA, then there is a reduction in the intensity of the supercoiled DNA bands (Fig. 7). This indicates that tNASP competes with DNA for H1 binding.

Mobility of NASP in the Nucleus—Given our results that NASP and H1 histones co-purify from HeLa cells and that NASP can transfer H1 histones to DNA in vitro, it is possible that H1 histones not bound to DNA are bound to NASP. If NASP-H1 complexes were present in the nucleus, then we would expect that the mobility properties of NASP would be similar to those of H1. Consequently, we used FRAP to determine the time necessary for NASP to move into a bleached area of the nucleus. Bleaching ~50% of a nucleus containing tNASP-GFP (see also Fig. 1B) resulted in a recovery of fluorescence to a plateau level of 92.5 ± 4.6% in 220–225 s (n = 3 experi-

**Fig. 3.** Cell cycle changes from overexpression of NASP in HeLa cells. FACS analysis demonstrating double thymidine-blocked HeLa cells allowed to progress in synchrony through the cell cycle. A, normal progression through the cell cycle. B, overexpression of tNASP affects progression through the cell cycle. C, overexpression of NASP-ΔNLS (lacking nuclear localization signal) affects progression through the cell cycle. D, overexpression of NASP-ΔHBS (lacking histone binding sites) does not delay progression through the cell cycle. E, overexpression of sNASP (somatic NASP) does not delay progression through the cell cycle.
ments). Fig. 8 shows a typical experiment in which 50% of the nucleus was bleached (Fig. 8A), and the fluorescence recovery was 96.5 ± 1.2% in 225 s (Fig. 8B). This compares favorably to the 200–250 s necessary for recovery of H1-GFP fluorescence in the nucleus (4). As fluorescence intensity increases in the bleached area, there is a concomitant decrease in intensity in the unbleached area (Fig. 8A), indicating that NASP is redistributed within the nucleus. Because HeLa cells transfected with tNASP-GFP have very little tNASP-GFP in the cytoplasm (Fig. 1B), photobleaching the entire nucleus resulted in no recovery of fluorescence in 300 s (n = 3 experiments), indicating that essentially no tNASP-GFP entered the nucleus from the cytoplasm in these experiments.

**DISCUSSION**

In this study we have demonstrated that the H1 histone-binding protein NASP is present in the nucleus and cytoplasm of HeLa cells and can be directed to the nucleus by its NLS.

### TABLE I

**Percent of cells in S+G2 phase**

|       | Normal | tNASP full-length | NLS-mutant | HBS-mutant | sNASP full-length | Delivery reagent | p value |
|-------|--------|-------------------|------------|------------|------------------|-----------------|---------|
| 4 h   |        |                   |            |            |                  |                 |         |
| Exp. 1| 60.70  | 42.90             | 49.30      |            |                  |                 |         |
| Exp. 2| 62.90  | 52.20             | 52.80      |            |                  |                 |         |
| Exp. 3| 71.30  | 51.90             | 57.30      |            |                  |                 |         |
| Exp. 4| 86.70  | 88.10             | 73.60      |            |                  |                 |         |
| Exp. 5| 70.10  | 53.10             | 46.40      |            |                  |                 |         |
| Exp. 6| 77.00  | 86.90             | 79.40      |            |                  |                 |         |
| Mean  | 71.45  | 50.80             | 53.17      | 76.03      | 66.47            |                 |         |
| S.D.  | 9.53   | 7.30              | 4.06       | 19.87      | 17.62            |                 |         |
| t test, N vs. tN | 0.01 |                   |            |            |                  |                 |         |
| t test, N vs. NLS- | 0.003 |                   |            |            |                  |                 | p = 0.01 |
| t test, N vs. HBS- | 0.37 |                   |            |            |                  |                 | p < 0.01 |
| t test, N vs. sN   | 0.34 |                   |            |            |                  |                 | NS      |
| 6 h   |        |                   |            |            |                  |                 |         |
| Exp. 1| 72.90  | 51.20             | 50.10      |            |                  |                 |         |
| Exp. 2| 71.60  | 61.50             | 63.70      |            |                  |                 |         |
| Exp. 3| 78.80  | 67.30             | 64.00      |            |                  |                 |         |
| Exp. 4| 88.50  | 88.60             | 81.00      |            |                  |                 |         |
| Exp. 5| 78.50  | 61.30             | 56.40      |            |                  |                 |         |
| Exp. 6| 88.80  | 87.90             | 88.00      |            |                  |                 | 88.80   |
| Mean  | 79.85  | 60.00             | 59.27      | 79.27      | 75.13            |                 |         |
| S.D.  | 7.41   | 8.15              | 7.94       | 15.56      | 16.60            |                 |         |
| t test, N vs. tN   | 0.01 |                   |            |            |                  |                 |         |
| t test, N vs. NLS- | 0.01 |                   |            |            |                  |                 |         |
| t test, N vs. HBS- | 0.48 |                   |            |            |                  |                 | NS      |
| t test, N vs. sN   | 0.34 |                   |            |            |                  |                 | NS      |

**FIG. 4.** Comparison of cells in S + G2 at 4 and 6 h after overexpression of tNASP or tNASP-ΔNLS in HeLa cells. Error bars represent ± S.D. Cells overexpressing either tNASP or tNASP-ΔNLS are significantly different from the non-overexpressing HeLa cells but not from each other, see Table I.

**FIG. 5.** Western blot showing binding of H1 histones to tNASP but not NASP-ΔHBS. Lanes 1–4, biotinylated H1 histones stained with alkaline phosphatase (AP)-conjugated avidin. Lanes 5 and 6, NASP stained with rabbit anti-recombinant NASP and an alkaline phosphatase-conjugated secondary antibody. Lane 1, control. Biotinylated H1 (1 μg) not chromatographed on a Micro Bio-Spin 30 column. Lane 2, negative. Biotinylated H1 (1 μg) chromatographed on and retained by a Micro Bio-Spin 30 column. Lane 3, negative. Biotinylated H1 (1 μg) + NASP-ΔHBS (3 μg) chromatographed on a Micro Bio-Spin 30 column with the biotinylated H1 retained. Lane 4, biotinylated H1 (1 μg) + tNASP (3 μg) chromatographed on a Micro Bio-Spin 30 column with the biotinylated H1 retained. Lane 5, biotinylated H1 (1 μg) + NASP-ΔHBS (3 μg) chromatographed on a Micro Bio-Spin 30 column with biotinylated H1 not bound by the NASP-ΔHBS, anti-NASP probed. Lane 6, biotinylated H1 (1 μg) + tNASP (3 μg) chromatographed on a Micro Bio-Spin 30 column with the biotinylated H1 carried by the tNASP, anti-NASP probed.
Even though transfection of cells with tNASP-GFP resulted in almost complete nuclear localization of the construct, endogenous NASP is clearly not exclusively in the nucleus (Figs. 1 and 2). From Western blotting (Fig. 2) it would appear that there is less tNASP in the nucleus than in the cytoplasm, whereas sNASP is more evenly distributed between the two compartments. Because tNASP occurs in rapidly dividing cell populations (gametes, embryonic cells, and transformed cells), its presence in the cytoplasm may serve as a storage site for excess linker (H1) histones. Alternatively, the majority of NASP in the cytoplasm could simply reflect a lagging degradation mechanism in rapidly dividing cells because in normal somatic cells there is little or no NASP in the cytoplasm (1).

Overexpression of tNASP significantly delays the progression of cells through the cell cycle, and surprisingly, overexpression of NASP-H9004 also significantly delays progression through the cell cycle (Fig. 3 and Table I). However, overexpression of sNASP does not appear to have a significant effect.

The most obvious difference between tNASP (Mr 83,934) and sNASP (Mr 45,751) is the presence of an additional histone-binding site (1, 11). The identical C-terminal sequences of tNASP and sNASP contain a leucine zipper flanked by coiled coil regions downstream and possible DNA-binding sites upstream; these structural features are often indicative of protein dimers that bind DNA (17). If tNASP dimers form in the cytoplasm and bind H1 histones, then the overexpression of tNASP may result in insufficient linker histones for DNA rep-
that excess tNASP can compete with DNA for H1. Klein
DNA by transferring H1 from NASP to DNA and importantly
NASP complexes can cause the supercoiling of relaxed SV40
DNA from the Xenopus laevis form of NASP (N1/N2). Conse-
quentially, our results are consistent with the hypothesis that
an equilibrium exists between NASP-H1 histone complexes and
H1 histones bound to DNA. This equilibrium influences the
availability of H1 histones to DNA and affects the progression
of the cell through the G1/S phase transition. It can be upset by
overexpression of tNASP with its extra histone-binding site
and is consistent with the observation that H1 histone overex-
pression has been shown to slow G1/S phase progression (5) and
in some cases inhibit transcription initiation (6).
Recent reports of the mobility of H1 histones in the nucleus
(3, 4) concluded that an intermediate (4) or modulating protein
(3) might be present to regulate the movement of H1. By using
FRAP experiments on HeLa cells, this study has demonstrated
that the mobility of NASP in the nucleus is essentially the
same as that of H1 histones (Fig. 8). Although it could be
fortuitous that the recovery times are similar, one interpreta-
tion of this result is that NASP and H1 have identical mobility
characteristics because they are in a complex together in the
nucleus. This interpretation is supported by the results from the
co-precipitation experiments (Fig. 2). The recovery times of
NASP and H1 (~225 s) differ markedly from other nuclear
proteins. For example, GFP-HMG-17 and GFP-SF2/ASF have
recovery times on the order of 30 s, whereas histone H2B has
no recovery over 90 s (16). The less mobile (immobile) fraction
determined in a FRAP experiment is a reflection of the resi-
dence time of the molecule in a compartment or complex and
and can be modified by various treatments such as acetylation (4).
Small immobile fractions (~10%, e.g. GFP-SF2/ASF (16)) indi-
cate that most of the molecules are replaced within the meas-
ured time, while large immobile fractions indicate that very
few of the molecules are replaced. Histone H2B would appear
to be replaced in the nucleosome very infrequently (16), whereas
H1 histones are replaced every 200–250 s (4). The immobile fractions of NASP and H1 vary (~3.5% NASP versus
~9–26% for H1), but their recovery times (~225 s) are similar,
which may indicate populations of H1 and NASP that are not
complexed with each other or are in different phosphoryla-
tion or acetylation states. Phosphorylation of linker histones
is known to inhibit chromatin remodeling (2, 18), and NASP may
serve to mediate such interactions. Taken together, the results
presented in this study indicate that NASP is most likely the
H1-regulating protein in the nucleus and that a dynamic equi-
librium exists between NASP-H1 complexes and DNA.

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