Non-histone protein 1 (NHP1) is a member of the Ku protein family which is upregulated in differentiating mouse myoblasts and human promyelocytes

Harald Oderwald, Melya J. Hughes**, Jean-Pierre Jost*

Friedrich Miescher Institute, P.O. Box 2543, CH-4002 Basel, Switzerland

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Abstract **We have previously purified and characterized a ubiquitous non-histone protein (NHP1) which has a high affinity (K_d 10^{-11} M) for different avian vitellogenin gene sequences containing CpGs (Hughes et al. (1989) Biochemistry 28, 9137–9142; Hughes and Jost (1989) Nucleic Acids Res. 17, 8511–8520). Here we show by microsequencing that the peptides derived from the purified p75 and p85 subunits of NHP1 from HeLa cells have between 64 and 100% identity with the human Ku autoantigen. During the differentiation of human HL-60 promyelocytes there is an increase in the amount of p85 subunit protein whereas the level of the p75 subunit is unchanged. In differentiating mouse G8 myoblasts there is, however, an upregulation of both the p75 and p85 subunits and of the p85 mRNA. An inhibition of mouse myoblast differentiation by either cAMP, 3-aminobenzamide or sodium butyrate abolishes the upregulation of the p85 subunit. In G8 myoblasts chemical, or physical stress by UV light or X-rays does not upregulate the level of the p85 subunit. The possible involvement of NHP1 in the active demethylation of bifilarly methylated DNA will be discussed.**

Key words: Ku; Peptide microsequencing; DNA demethylation; Differentiation

1. Introduction

Non-histone protein 1 (NHP1) was first detected as a protein with a very high affinity to the estrogen response element (ERE) of the avian vitellogenin gene [1–3]. The strongest contact points of NHP1 with the ERE were with 5′ AGCG 3′ in the center of the palindrome differing from those of the estradiol receptor (ER) [2]. Of the two polypeptides p75 and p85 forming NHP1 only the p85 subunit can be crosslinked to the bromodeoxyuridine-substituted synthetic ERE by UV irradiation [2]. NHP1 can be separated from the estradiol receptor chromatographically and it neither binds estradiol nor cross reacts with antibodies directed against estradiol receptor. Deletion of the CpG in the center of the dyad symmetry sequence of the ERE decreased the binding of NHP1 by 90% and a conversion of any GC pair to an AT pair diminished the affinity of the binding site for NHP1 [1]. In the purified form, but not in the pure form, NHP1 was shown to produce nicks around the central CpG of the ERE [2] indicating that NHP1 might play a role in the active demethylation of mCpGs. Furthermore, at the onset of active demethylation of the avian vitellogenin gene in the liver there was a transient increase in NHP1 binding activity [4]. It has also been suggested that NHP1 could tag the DNA for the subsequent binding of the estradiol receptor [1–3]. A possible involvement of NHP1 in a general mechanism of active demethylation could account for its apparent ubiquity. Here we show that NHP1 is a member of the Ku autoantigen protein family and that it is upregulated in differentiating mouse myoblasts, a system known to undergo genome-wide demethylation. We hypothesize that NHP1 may be part of an enzyme complex involved in the active demethylation of DNA.

2. Materials and methods

2.1. Protein purification and sequencing of peptides

NHP1 was purified from HeLa cells as described previously [2]. The purified NHP1 subunits were subjected to preparative SDS-polyacrylamide gel electrophoresis and transferred to PCGM glass paper in 50 mM sodium borate containing 0.02% Nonidet P-40 at 60 V and 0.2 A. The single polypeptide bands were cut out and their sequence determined directly from the PCGM paper by Edman degradation (peptides 1 and 2). Alternatively the purified polypeptides were cleaved with cyanogen bromide, digested with trypsin and the derived peptides were separated by HPLC and sequenced by Edman degradation (peptides 3–7).

2.2. Cell culture and treatment of cells

Mouse G8 myoblast cells were grown on collagen-coated plates in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 10% heat-inactivated fetal calf serum and 10% horse serum at 37°C and 5% CO_2. Differentiation was induced by changing to DMEM supplemented with 4.5 g/l glucose and 2% heat-inactivated fetal calf serum at a cell confluence of 80–90%. Where indicated, 1 mM 8-bromo-cAMP or 5 mM sodium butyrate dissolved in DMEM were added to the culture medium. 3-Aminobenzamide was dissolved in dimethylsulfoxide (DMSO) and added to the culture at a concentration of 5 mM. The final concentration of DMSO was 0.5%. In UV irradiation experiments the cells were exposed to UV-C radiation (254 nm) using Philips TUV 15 W lamps. X-ray irradiation experiments were carried out in a Müller RT 250 X-ray generator at 15 mA and 250 kV. Human HL-60 promyelocytes were grown in suspension culture in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum. Differentiation was induced at a cell density of 2.5×10^5 cells/ml by the addition of DMSO to a final concentration of 1.25%.

2.3. Antiserum preparation and antibody purification

Antisera were obtained by injecting rabbits with purified histone HI using standard techniques [5]. For NHP1 antisera, the C-terminal peptides KKQELLEALTKHFQD (p75) or AVFEEGGDVDDLLDMI (p85) coupled to keyhole limpet hemocyanin were used for immunization [5]. Peptide antisera were purified over a peptide affinity column containing the p75 or p85 antigenic peptide coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia). Purified antibodies were dialyzed against PBS+50% glycerol and concentrated in a Centriprep-10 (Amicon).

2.4. Western blot analysis and creatine kinase activity determination

Nuclear and cytoplasmic extracts were prepared according to

*Corresponding author. Fax: (41) (61) 721 4091.

**Present address: Cooley Godward Attorneys at Law, 5 Palo Alto Square, Palo Alto, CA 94306-2155, USA.

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Schreiber et al. [6], protein concentration being measured by the Bradford assay [7] using the Bio-Rad protein determination system. Equal amounts of nuclear protein (5 or 30 μg) were separated on 7.5 or 12% SDS-polyacrylamide gels [8]. Proteins were transferred to nitrocellulose BA-S 85 filters (Schleicher and Schuell) in a semi-dry transfer apparatus (Teknomara) at 0.8 mA/cm^2 for 1.5 h. The membranes were treated for 16 h at 4°C in blocking solution (10% non-fat dry milk in PBS, 0.1% Tween 10). NHP1 p75 and p85 antibodies or histone HI antiserum were used at a dilution of 1:500 in blocking solution and incubated for 16 h at 4°C. Secondary antibody (anti-rabbit IgG, Bio-Rad) was diluted 1:5000 and incubated for 1 h at room temperature in the blocking solution. For the detection of the antibody reaction products, membranes were incubated for 1 min in a luminol-based detection solution (ECL, Amersham). Membranes were exposed immediately for various times to Kodak BioMax or X-OMAT films. Creatine kinase activity from 4 μg of cytoplasmic protein from G8 cells was determined colorimetrically by the Voges-Proskauer reaction [9].

2.5. Northern blot analysis

Total RNA from G8 myoblasts was isolated according to the procedure of Chomczynski and Sacchi [10] except that the RNA was dissolved in diethylpyrocarbonate-treated (DEPC) water before storage at −80°C. Poly(A) mRNA was purified from 100 μg total RNA on Dynabeads Oligo (dT)$_{25}$ (Dynal) according to the manufacturer's instructions. 1 μg of poly(A) RNA was separated on a denaturing agarose gel containing 2.2 M formaldehyde as described by Sambrook et al. [11]. Upon capillary transfer to Hybond Nylon membrane (Amersham), the RNA was immobilized by UV-crosslinking [12]. 18S and 28S rRNA were visualized by staining the membrane with 0.3% methylene blue. Prehybridization was performed in 6×SSC, 5×Denhardt's, 0.5% SDS, 0.05% sodium pyrophosphate and 0.1 mg denatured herring sperm DNA at 42°C for up to 2 h. Hybridization was carried out at 42°C for 16 h in 6×SSC, 1×Denhardt's, 0.05% sodium pyrophosphate, 0.1 mg/ml denatured herring sperm DNA containing a kinase-labeled synthetic oligonucleotide complementary to positions 246–300 of the p85 cDNA [13] at 4×10^6 cpm/ml. Washing of the membrane was performed at room temperature in 4×, 2×, 0.5 and finally 0.1×SSC for 10 min each. Blots were exposed to X-OMAT films for 18 h.

3. Results

3.1. NHP1 is a member of the Ku protein family

Since NHP1 binds the estrogen response element with a very high affinity (K_d of 10^{-11} M) we were interested in

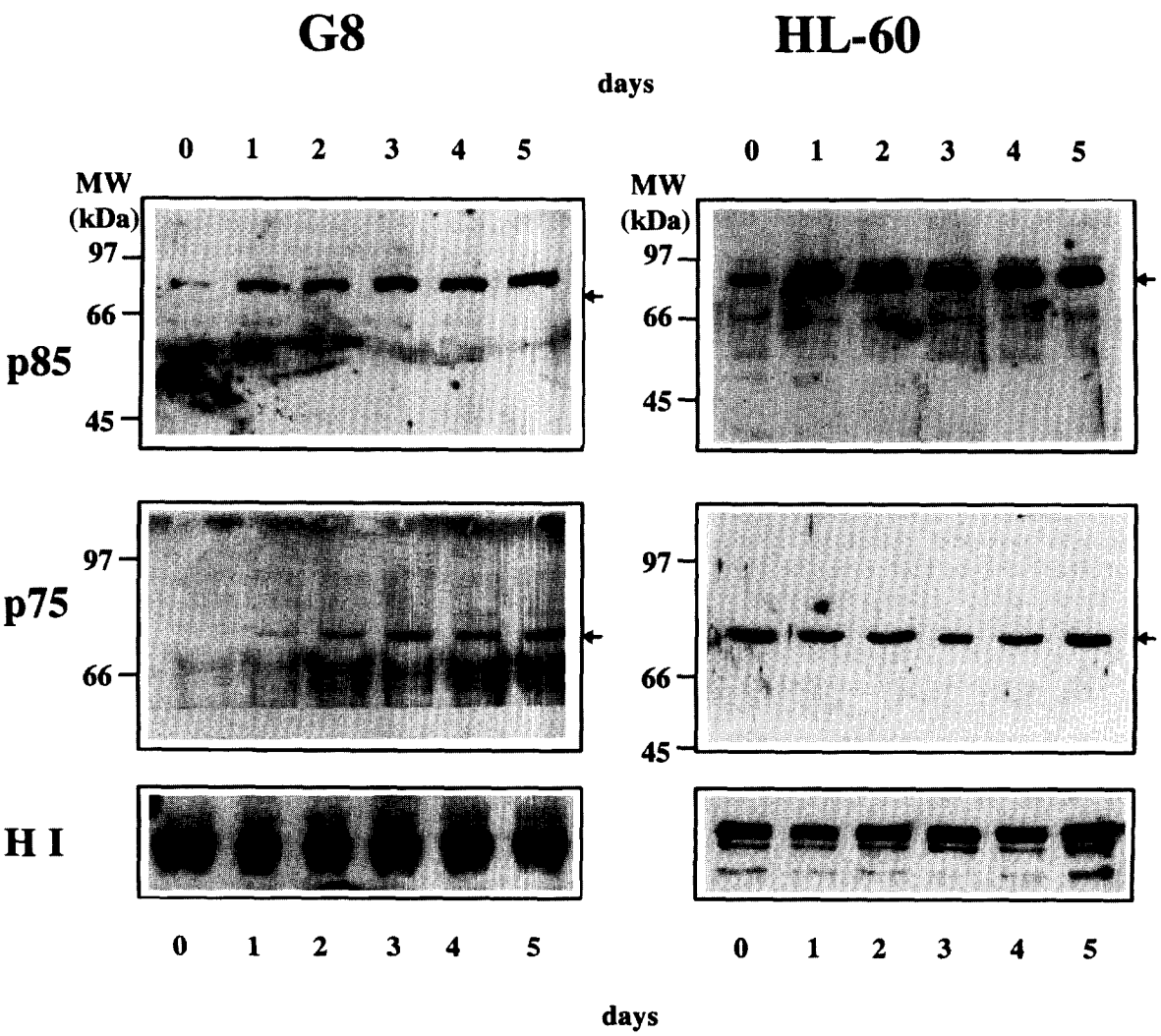

Fig. 1. Western blot showing the upregulation of the NHP1 subunits p75 and p85. Western blots of nuclear extracts (5 μg of protein per lane) were carried out as outlined in section 2. The left-hand panels show the Western blots of p85, p75 and histone HI from differentiating mouse G8 myoblasts whereas those on the right-hand side show Western blots of p85, p75 and histone HI from human HL-60 promyelocytes. Track 0 shows material from the dividing non-differentiating cells and tracks 1–5 are the days following the onset of differentiation.

further characterizing this protein. Table 1 shows the amino acid sequences obtained from peptides derived from the p75 and p85 subunits. Searches in the GenBank and EMBL sequence databases revealed a high degree of homology between the NHP1 p75 and p85 peptides and the p72 and p86 subunits of the human Ku protein. The identity was 70 and 85.7% for the peptides derived from the p75 subunit and between 64.3 and 100% for the p85 subunit.

3.2. Regulation of NHP1 during the differentiation of mouse G8 myoblasts and human HL-60 promyelocytes

Since it has been reported that NHP1 DNA binding activity was upregulated by estradiol during the active demethylation of the avian vitellogenin gene [4] we decided to follow the amount of NHP1 during the genome-wide demethylation of mouse myoblasts. As shown in the left-hand panel of Fig. 1 (G8) the level of the p75 and p85 subunits increased progressively during the differentiation of myoblasts while histone HI, which was used as an internal standard, remained constant. Fig. 3A shows the time course of mouse myoblast differentiation was followed by using muscle creatine kinase activity as a marker.

Table 1
Comparison of the amino acid sequences of peptides derived from NHP1 and the Ku autoantigen

	p85
Peptide 1	V R S G N K A A V V L X Y
	| | | | | | | | | | |
Ku(p86) 2	V R S G N K A A V V L C M 14
% identity	84.6
Peptide 2	C S G N X A A V V L C X S Y
	| | | | | | | | |
Ku(p86) 3	R S G N K A A V V L C M D V 16
% identity	64.3
Peptide 3	I Q P G S Q Q A D F L D A L I V
	| | | | | | | | | | | | | | |
Ku(p86) 98	I Q P G S Q Q A D F L D A L I V 113
% identity	100.0
Peptide 4	K X A P T E A Q L N A V D A L I D X R
	| | | | | | | | | | | | | | |
Ku(p86) 443	K Y A P T E A Q L N A V D A L I D S M 461
% identity	84.2
Peptide 5	L E D L F P G
	| | | | | |
Ku(p86) 473	L E D L F P T 479
% identity	85.7
	p75
Peptide 6	K I L V L Q E L D N P G A K
	| | | | | | | | | | | |
Ku(p72) 101	N I Y V L Q E L D N P G A K 114
% identity	85.7
Peptide 7	S L E L D Q F K X S
	| | | | | | | |
Ku(p72) 117	I L E L D Q F K G Q 126
% identity	70.0

Peptides 1–5 are from the p85 subunit whereas peptides 6 and 7 are from the p75 subunit of NHP1. The amino acid sequences of the subunits are aligned with the corresponding sequence of the Ku protein. Identical residues are indicated by vertical bars. The numbers on the lower sequence indicate the amino acid position in the human Ku protein [13,43–46].

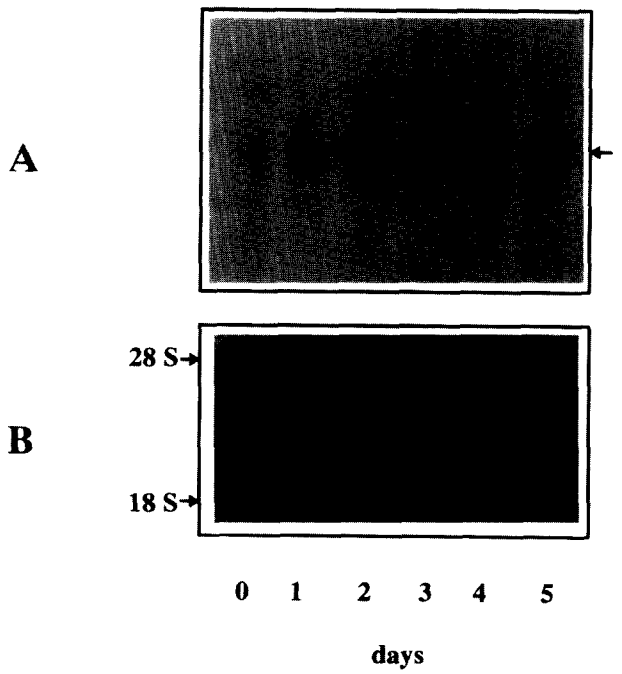

Fig. 2. Northern blot analysis of poly(A) mRNA from untreated (day 0) and differentiating mouse G8 myoblasts (days 1 to 5). Experiments were carried out as outlined in section 2. (A) Hybridization product of the labeled p85 oligonucleotide probe to poly-(A)mRNA. (B) Methylene blue staining of the above membrane prior to hybridization.

Addition of 1.25% DMSO to human HL-60 promyelocytes triggers their differentiation along the granulocyte pathway [14]. The progress of differentiation was monitored by morphological and biochemical changes in the cell (not shown). The immunoblots in the right-hand panels of Fig. 1 (HL-60) demonstrate that the two subunits p75 and p85 are regulated quite differently. An increase in the level of p85 was observed after the first day of differentiation whereas the level of the p75 subunit showed no change during differentiation.

The level of expression from the p85 gene during the course of G8 myoblast differentiation, was assayed by the hybridization of a radiolabeled antisense oligonucleotide to poly(A) mRNA. Fig. 2 shows that the non-differentiating cells had a very low level of p85 mRNA while differentiation resulted in a substantial increase in p85 mRNA.

3.3. Effect of inhibition of G8 myoblast differentiation on the expression of NHP1

It is known that myogenesis can be inhibited by the addition of either cyclic AMP [15], 3-aminobenzamide [16] or sodium butyrate [17,18] to the differentiation medium. Recently, we have also shown that the inhibition of differentiation by 3-aminobenzamide resulted in the suppression of the genome-wide demethylation [19]. Fig. 3A shows that the presence of 1 mM 8-bromo-cAMP in the tissue culture medium inhibited the increase in creatine kinase activity and the upregulation of p85 (Fig. 3B). 3-Aminobenzamide is another inhibitor of mouse myoblast differentiation which inactivates ADP-ribosyltransferase, an enzyme essential in DNA repair [20]. 5 mM 3-aminobenzamide also inhibited the upregulation of muscle creatine kinase (Fig. 3A) and of NHP1 p85 subunit (Fig. 3C). Finally, 5 mM sodium butyrate, another inhibitor of myogen-

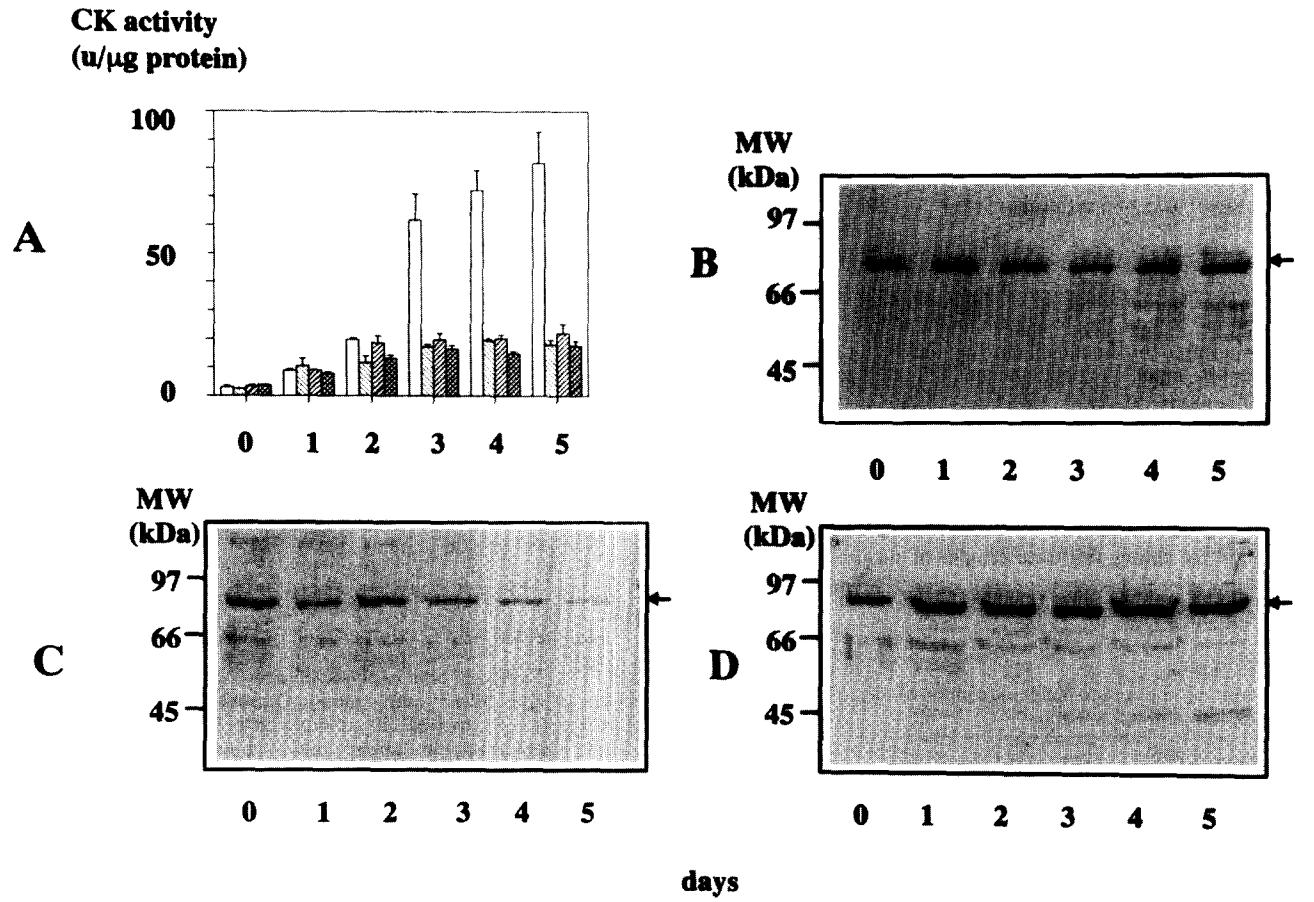

Fig. 3. Effect of inhibitors of myoblast differentiation on the upregulation of NHP1. (A) Creatine kinase (CK) activity in G8 mouse myoblasts. (Open bars) Denote the activity of creatine kinase in differentiating myoblasts in the absence of inhibitors, whereas (descending hatched bars) contained 1 mM cAMP, (ascending hatched bars) 5 mM 3-aminobenzamide or (cross-hatched bars) 5 mM sodium butyrate. The data are the means of three independent experiments ± S.D. (B–D) Western blots of mouse G8 myoblasts grown in the presence of cAMP (B), 3-aminobenzamide (C) and sodium butyrate (D). Each lane contained 30 μg of nuclear extract.

esis [17,18], also inhibited the increase in immunoreactive p85 (Fig. 3D). When the results of Figs. 1, 3 and 4 are compared it is noticeable that there was a much stronger signal for the zero time points of Figs. 3 and 4 than for Fig. 1. This was due to the overexposure of the Western blot in Figs. 3 and 4. In addition, in Figs. 3 and 4 we had 30 μg of nuclear extract per lane instead of the 5 μg used in Fig. 1. This was necessary for the visualization of the extremely low concentrations of p85 in this series of experiments.

3.4. Physical and chemical stress do not lead to upregulation of NHP1

It has been proposed that Ku is involved in DNA recombination and repair [21–32] and it is known that some of the proteins involved in DNA repair are upregulated by DNA damage [33–39]. Therefore, with NHP1 being a member of the Ku protein family, it was of interest to see whether chemical stress or DNA damage could upregulate NHP1. Dividing G8 myoblasts were treated with 1% DMSO or various doses of UV light and X-rays. DMSO treatment of G8 myoblasts did not lead to any increase in NHP1 levels (Fig. 4A). Furthermore, 1% of DMSO had no effect on the differentiation or the activity of creatine kinase either (data not shown). Similarly, various doses of X-rays or UV light had no detectable effect on the level of the p85 protein (Fig. 4B and C). Testing of even earlier time points did not reveal any transient upregulation of p85 (data not shown).

4. Discussion

We have shown previously that NHP1 binds in a sequence-independent manner and with a high binding affinity to DNA containing CpG base pairs [1,2]. The binding activity of NHP1 increases more than 2-fold in the liver of estradiol treated immature roosters where it coincides with the active demethylation of CpGs situated within the promoter region of the vitellogenin gene [4]. Similarly, we show here that there is an increase in the NHP1 p75 and p85 subunits during the differentiation of mouse G8 myoblasts. In previous experiments we have shown that during mouse G8 myoblast differentiation there is a genome-wide demethylation [19]. Moreover, in G8 myoblasts the kinetics of the increase in NHP1 ran in parallel with an increase in 5-methyldeoxycytidine excision repair activity [19], where one of the key enzymes responsible for the active demethylation is 5-methylcytosine-DNA glycosylase [40]. In sharp contrast, in teratocarcinoma cells where passive DNA demethylation (non-enzymatic demethylation) occurs during differentiation, there was no increase in either NHP1 or 5-methylcytosine-DNA glycosylase activity (data not shown). To our surprise, the size of the

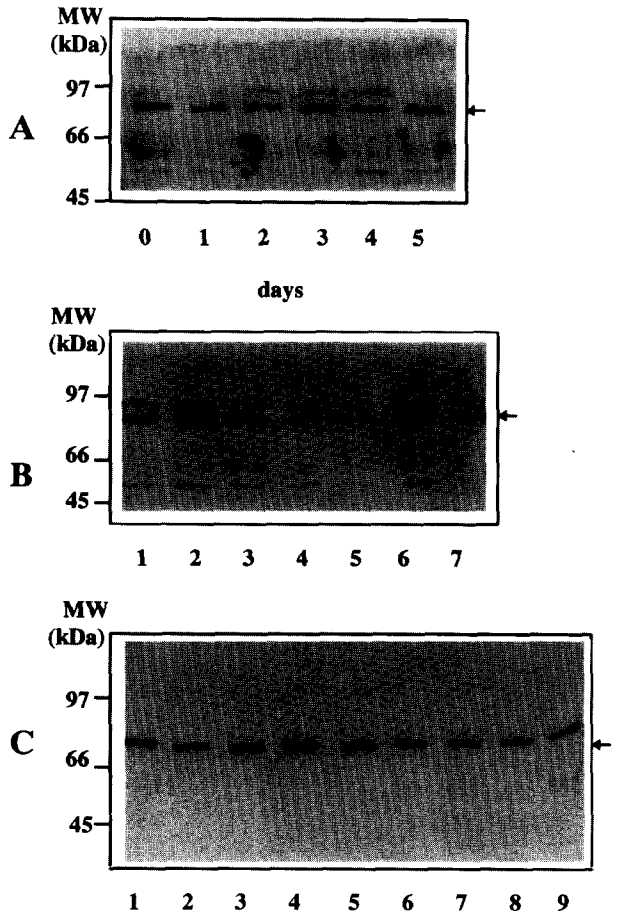

Fig. 4. Chemical or physical stress to G8 mouse myoblasts did not upregulate the p85 subunit of NHP1. Western blots were carried out as above with 30 μg of nuclear extracts. (A) Dividing G8 myoblasts (day 0) and cells treated with 1% DMSO for between 1 and 5 days. (B) Effect of UV light (254 nm) on the level of p85. G8 myoblasts were grown in medium without irradiation (lane 1) or the cells were irradiated with 10 J/m^2 (lanes 2,5), 50 J/m^2 (lanes 3,6) or 200 J/m^2 (lanes 4,7). (C) Effect of X-ray irradiation on the level of the p85 protein. G8 myoblasts were grown in medium without irradiation (lanes 1,2,6) or after irradiation with 1 Gray (Gy) (lanes 3,7), 10 Gy (lanes 4,8) or 20 Gy (lanes 5,9). The cells were harvested 4 h (lanes 2–5) or 1 day after irradiation (lanes 6–9).

NHP1 subunits and the amino acid sequence of the peptides derived from both subunits have a high identity with the heterodimeric Ku autoimmune antigen. As already shown by other groups the Ku protein is involved in DNA repair and DNA recombination [21–32]. For these reasons we thought that NHP1 may be related to the active demethylation of DNA. In the present case, how could NHP1 or Ku participate in the process of active DNA demethylation? We have shown that the active DNA demethylation of specific sites in the vitellogenin promoter occurred in non-dividing mature hepatocytes on both DNA strands simultaneously [3,41]. Even in cases where one strand is demethylated before its complementary DNA the reaction proceeds to completion on both strands simultaneously [42]. Should DNA demethylation occur through the mechanism of base excision repair, it is clear that the simultaneous reaction on both strands would lead to DNA double strand breaks and the gene would be cut into two or more pieces. In order to avoid double strand breaks on a symmetrical CpG it is conceivable that NHP1 could act like the Ku protein by binding to the extremities of broken strands, holding them together during the process of base excision repair. If this hypothesis is correct then the inhibition or the knocking-out of the NHP1 genes in G8 myoblasts should generate numerous double strand breaks in the DNA during differentiation.

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