

The Human Gene AHNAK Encodes a Large Phosphoprotein Located Primarily in the Nucleus

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Abstract. AHNAK is a newly identified human gene notable for the exceptional size (c.a. 700 kD) and structure of its product, and for the repression of its expression in human neuroblastoma cells. Here we report the identification and partial characterization of the protein encoded by AHNAK. The protein is located principally (but not exclusively) in the nucleus and is phosphorylated on both serine and threonine. The abundance of the protein increases appreciably

when cells withdraw from the division cycle, in response to either withdrawal of serum (fibroblasts) or differentiation (neuroblastoma cells). By contrast, the amount of phosphorylation appears to diminish in those settings. The considerable abundance and conjectured fibrous structure of AHNAK protein suggest a role in cytoarchitecture, but no function can yet be discerned.

WE have described previously the identification of a human gene, AHNAK, that appears to encode a protein of exceptional size (c.a. 700 kD) and structure (7). AHNAK was first encountered as one of a group of cDNA clones isolated through subtractive cloning on the basis of differential expression in human tumors of neuroectodermal origin (6). Another cDNA clone from the subtracted cDNA library was identified as CD44, the integral membrane glycoprotein implicated in a variety of specific cell-cell and cell-matrix interactions (6). Both AHNAK and CD44 are expressed in melanoma and pheochromocytoma but are repressed in neuroblastoma cell lines.

Our interest in AHNAK was prompted by the following observations. First, we have documented a pattern of coordinated expression of AHNAK and CD44 in different human cell lineages (6, 7). This may indicate that both genes are subject to the same transcriptional regulation, leading to the suppression of their expression in neuroblastoma cell lines. Analysis of this regulation might reveal factors involved in the pathogenesis of neuroblastoma. Second, our analysis of AHNAK revealed that its predicted protein product has an unprecedented secondary structure, suggesting that it may fulfill a novel function in the cell. The possible importance of this function is reflected in the apparent phylogenetic conservation of AHNAK sequences (7).

AHNAK is expressed as a 17.5-kb RNA in a variety of cells. Among human cell lines examined, we have found diminished or undetectable levels of expression in neuroblastomas, small cell lung carcinomas, and Burkitt lymphomas

(7). AHNAK contains an open reading frame that could be translated into a 700-kD protein product. The salient feature of the predicted protein product is the presence of repeated domains comprising almost 80% of its amino acid sequence. Approximately 4,300 amino acids in the middle portion of the protein are arranged in multiple repeats of a sequence motif of 128 amino acids. Some of the repeats have internal deletions which occur at the same position. The degree of amino acid identity between any two of the repeats is at least 80% and practically all the sequence differences are conservative (7).

Examination of amino acid sequence within the repeated domain has revealed redundancy at a different level: heptad repeats with a proline residue in the seventh position. The character of each heptad could be best represented as $\varphi\pm\varphi P\pm\varphi\pm$ where φ is a hydrophobic residue, \pm is a charged residue, and P is proline. We proposed that AHNAK protein has an unprecedented secondary structure resembling a β -strand and distinct in that it has a periodicity of 2.33, i.e., three turns of the carbon backbone every seven residues (7). This structure segregates the hydrophilic and hydrophobic residues to opposing sides of the polypeptide chain. We have also proposed that seven or eight of these molecules could interact through their hydrophobic surfaces to form an extremely long ($\sim 1.2 \mu$) and thin (9.8 or 10Å) barrel (7). Thus, AHNAK appears to represent a previously unidentified type of fibrous protein. As a first step towards discerning the function of AHNAK we have developed specific antibodies for the AHNAK protein, and then used these to show that the protein resides in the nucleus and is phosphorylated on serine and threonine, particularly in proliferating cells.

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Materials and Methods

Preparation of Antisera

The chemically synthesized peptides CKISMPDVLHLKGPCKM (KIS) and CLKMPKVKMPKFSMPGF (FEN) were used to raise specific anti-AHNAK sera. An additional cysteine group was added to the aminoterminal to allow coupling of the peptides to the tuberculin purified protein derivative with *m*-maleidobenzoyl-*N*-hydroxysuccinimide ether as described (3). Each preparation was injected into two New Zealand rabbits under the auspices of CalTag, Inc. (South San Francisco, CA).

Immunoaffinity purification of antisera was performed on columns containing immunogenic peptide coupled to cyanogen-bromide activated agarose beads as described (3). The antibodies eluted from the column were dialyzed against PBS and used for protein analysis without further concentration. All results reported here were obtained using these preparations of antibodies, which hereafter are named KIS4 and FEN2, and are derived from sera obtained from rabbits injected with the KIS and FEN peptides, respectively.

Immunoprecipitation and Immunoblot Analysis

In vivo labeling of cells for immunoprecipitation analysis was performed by incubation of cells in methionine-free medium containing 250 μ Ci of [³⁵S]methionine per ml or in phosphate-free medium containing 500 μ Ci [³²P]orthophosphate per ml. Protein extracts were prepared from cultured cells by lysis in RIPA buffer (150 mM NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 0.5% NP-40, 0.5% deoxycholate, 0.5% Tween, 0.25% SDS) or by lysis in a buffer containing 150 mM NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, and 0.5% of NP-40. All buffers contained the following protease inhibitors: PMSF at 1 mM, aprotinin at 50 μ g/ml, leupeptin, and pepstatin at 10 μ g/ml each. The lysates were clarified by centrifugation. Immunoprecipitations were carried out in RIPA buffer with 10 to 30 μ l of the immunoaffinity purified antiserum KIS4 for 2 h at 4°C. After the addition of protein A-Sepharose, incubation was continued for another 40 min and the beads were washed four times in RIPA buffer. The immunoprecipitated proteins were analyzed by gel electrophoresis and autoradiography.

Cellular lysates for Western blotting were prepared by boiling pelleted cells in Laemmli sample buffer. Proteins separated on 4% SDS-polyacrylamide gel with 3% upper gel were transferred to nitrocellulose membranes in a semi-dry blotting apparatus (E & K Scientific Products, Inc., Saratoga, CA). The blots were incubated 30 min in a solution containing 150 mM NaCl, 5 mM KCl, 10 mM Tris-HCl, pH 7.0, 2% dry milk, 5% calf serum, and 0.5% Tween. The antibody incubation was for 2 h in the same solution. The antigen-antibody complexes were detected with ¹²⁵I-containing antibody against rabbit IgG.

Subcellular Fractionation

Approximately 10⁷ HeLa cells were washed in hypotonic buffer containing 10 mM KCl, 10 mM Tris HCl, pH 7.4, 1 mM MgCl₂, and protease inhibitors, and suspended in the same buffer containing 0.5% NP-40. After a 10-min incubation on ice, nuclei were separated from the cytoplasmic fraction by centrifugation at 1,000 *g* for 5 min, washed in hypotonic buffer with 0.5% NP-40 and extracted in a buffer containing 150 mM NaCl. The wash fractions were combined with the cytoplasmic supernatant. Total cell lysate, nuclear, and cytoplasmic fractions were analyzed by Western blotting with anti-AHNAK antibodies.

Immunofluorescence

Cells were grown on 12-mm coverslips for at least 24 h before processing. After two washes in PBS, cells were fixed in 3.75% paraformaldehyde for 20 min at room temperature or in methanol for 6 min at -20°C. Both fixation methods produced identical patterns of immunofluorescence. Fixed cells were washed three times with PBS, permeabilized in Ab buffer (PBS, 3% BSA, 0.1% Triton X-100) for 20 min, and incubated with the immunoaffinity purified serum FEN2 for 1 h. The purified antibody and whole serum FEN2 produced identical immunofluorescence patterns in a variety of cells. Coverslips were washed with PBS and incubated for 40 min with the secondary antibodies (affinity-purified rhodamine-conjugated donkey anti-rabbit or fluorescein-conjugated sheep anti-rabbit (Boehringer-Mannheim Biochemicals, Indianapolis, IN) diluted 1:100 in Ab buffer. Staining for DNA was done by incubation with Hoechst 33258 at 5 μ g/ml in PBS. Coverslips were washed in PBS and mounted in Immumount (Shan-

don Southern Instruments Inc., Sewickley, PA). Micrographs were taken with a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) with the X63 oil objective and Kodak T-Max 400 film (Eastman Kodak Co., Rochester, NY).

Phosphoamino Acid Analysis

The AHNAK protein was immunoprecipitated from cells labeled with [³²P]orthophosphate, and then subjected to electrophoresis. The protein was transferred to Immobilon-P (Millipore Continental Water Systems, Bedford, MA), the labeled band was excised, and then hydrolyzed in 6 M HCl at 110°C for 90 min. Phosphoamino acids were separated on cellulose thin layer plates by electrophoresis in two dimensions (2). The migration of unlabeled phosphoamino acids was determined by ninhydrin staining, and radioactive amino acids were detected by autoradiography.

Results

Identification of AHNAK Protein

To identify and characterize the protein product of the AHNAK gene, we have raised anti-AHNAK polyclonal sera in rabbits. Based on the predicted amino acid sequence of AHNAK protein, two synthetic peptides of 17 amino acids each were made and used for immunizations. Both peptides corresponded to regions within the repeated domain of the predicted AHNAK polypeptide. The peptide "KIS" corresponds to the first 16 amino acids of the AHNAK 128 amino acids repeat (7), while the peptide "FEN" corresponds to amino acids 59-74 of the repeat.

The initial characterization of the sera was done using portions of AHNAK protein expressed in *Escherichia coli*. The cDNA fragments of AHNAK were cloned into appropriate pGEX vectors (11) to produce fusion proteins of glutathion-S-transferase (GST)¹ and AHNAK-specific polypeptides. Extracts from bacterial clones induced to express the fusion proteins were analyzed by Western blotting with the antisera raised against AHNAK peptides. We have found that sera from one rabbit injected with the "KIS" peptide (designated KIS4) and one injected with the "FEN" peptide (FEN2) produced antibodies recognizing appropriate GST-AHNAK fusion proteins (data not shown). These two antisera were further examined by Western blot analysis of total protein extracts prepared from cells expressing or lacking AHNAK RNA. Fig. 1 *A* shows a Coomassie-stained gel containing electrophoretically separated high molecular weight proteins from the melanoma cell line C32r (expressing AHNAK RNA) and small cell lung carcinoma line N417 (lacking AHNAK RNA) (7). A very slowly migrating protein species, visible at the top of the gel in the C32r extract but not in the N417 extract, appears to be recognized by various anti-AHNAK antisera (FEN2 and KIS4 and the immunoaffinity-purified antibodies from these sera), but not the preimmune sera (Fig. 1, *B-F*).

Fig. 2 shows that the candidate AHNAK protein was specifically immunoprecipitated from extracts of [³⁵S]methionine-labeled cells with the immunoaffinity-purified antibody KIS4. No protein could be immunoprecipitated from cell line N417, which lacks detectable levels of AHNAK RNA, or when preimmune serum was used. The interaction of antibodies with the protein was blocked by addition of the immunogenic peptide. The molecular weight of AHNAK protein could not be estimated on the basis of its migration

1. Abbreviation used in this paper: GST, glutathion-S-transferase.

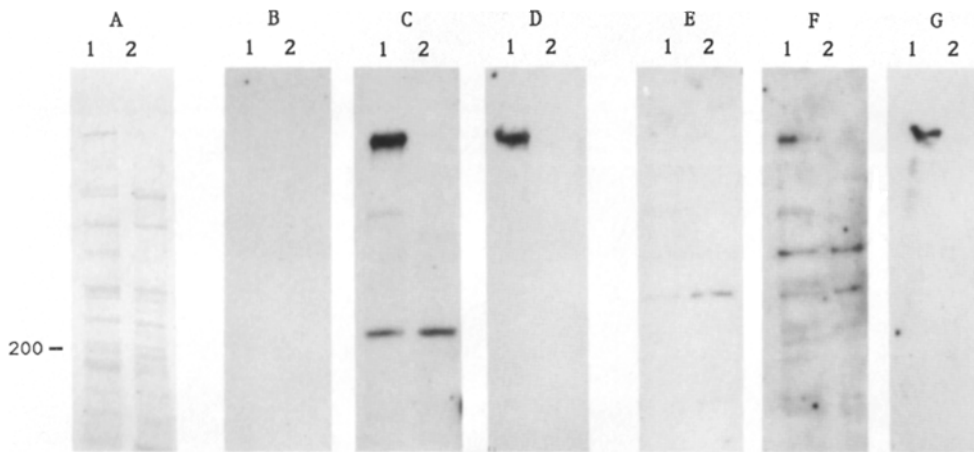


Figure 1. Western blot analysis of AHNAK protein with polyclonal immune sera. Total protein extracts (150 μ g) from the melanoma cell line C32r (lanes 1) and small cell lung carcinoma cell line N417 (lanes 2) were separated on a 4% acrylamide gel. (A) Coomassie blue stain gel. (B-G) Identical Western blot strips containing proteins shown in A probed with: preimmune serum KIS4 diluted 1:200 (B); immune serum KIS4 diluted 1:200 (C); immunopurified antibodies from the serum KIS4 diluted 1:100 (D); preimmune serum FEN2 diluted 1:100 (E); immune serum FEN2 diluted 1:100 (F); and immunopurified antibodies from serum FEN2 diluted 1:10 (G).

in the gel: the protein band appeared much higher on the gel than the largest molecular weight marker of 200 kD. Our estimate of the molecular weight of AHNAK protein, based on the length of the open reading frame of AHNAK RNA, is \sim 700 kD (7).

In further characterization of the antisera, we found that KIS4 recognized only the denatured form of AHNAK protein and gave no signal in immunofluorescence. In contrast, FEN2 reacted with both native and denatured protein, and could be used in immunofluorescence. Since immunopurified KIS4 had a significantly higher titer than FEN2, we used KIS4 for all analyses except immunofluorescence. All immunopurified preparations of the two antisera appeared to have the same specificity when tested accordingly (see above).

Subcellular Localization of AHNAK Protein

The antibodies raised against AHNAK protein were used to address the question of its subcellular localization. For convenience we analyzed HeLa cells, which are readily fractionated into subcellular organelles and which produce abundant AHNAK protein (see below). HeLa cells were fractionated into crude nuclear and cytoplasmic fractions by lysis in hypotonic buffer in the presence of nonionic detergent. Proteins were extracted from nuclei by 150 mM NaCl in the presence of 0.5% NP-40. Fig. 3 shows Western blot analysis of AHNAK in the nuclear and cytoplasmic fractions of HeLa cells. The major portion of AHNAK protein was detected in the nuclear rather than the cytoplasmic fraction.

Next, we used indirect immunofluorescence to examine the subcellular localization of AHNAK. Fig. 4 shows the

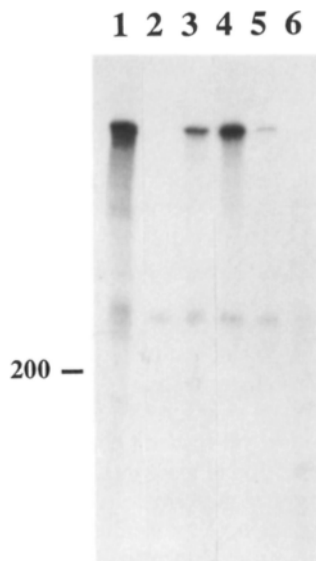


Figure 2. Immunoprecipitation of AHNAK protein. Cells were labeled with [35 S]methionine and lysed in RIPA buffer. Equal amounts of protein extracts (200 μ g) were analyzed by immunoprecipitation with 20 μ l of the immunopurified antibodies KIS4. The following cell lines were analyzed: melanoma C32r (1); small cell lung carcinoma N417 (2); neuroblastoma NGP (3); mouse fibroblasts NIH 3T3 (4); NIH 3T3 with addition of 2 μ g of the immunogenic peptide KIS (5); and NIH 3T3 extract immunoprecipitated with the preimmune serum (6).



Figure 3. Subcellular localization of AHNAK protein in HeLa cells. Subcellular fractions were prepared as described in Materials and Methods. Proteins extracted from the equivalent of 10^5 cells were analyzed by Western blotting with the immunopurified antiserum KIS4. T, total cell lysate; N, nuclear fraction; and C, cytoplasmic fraction.

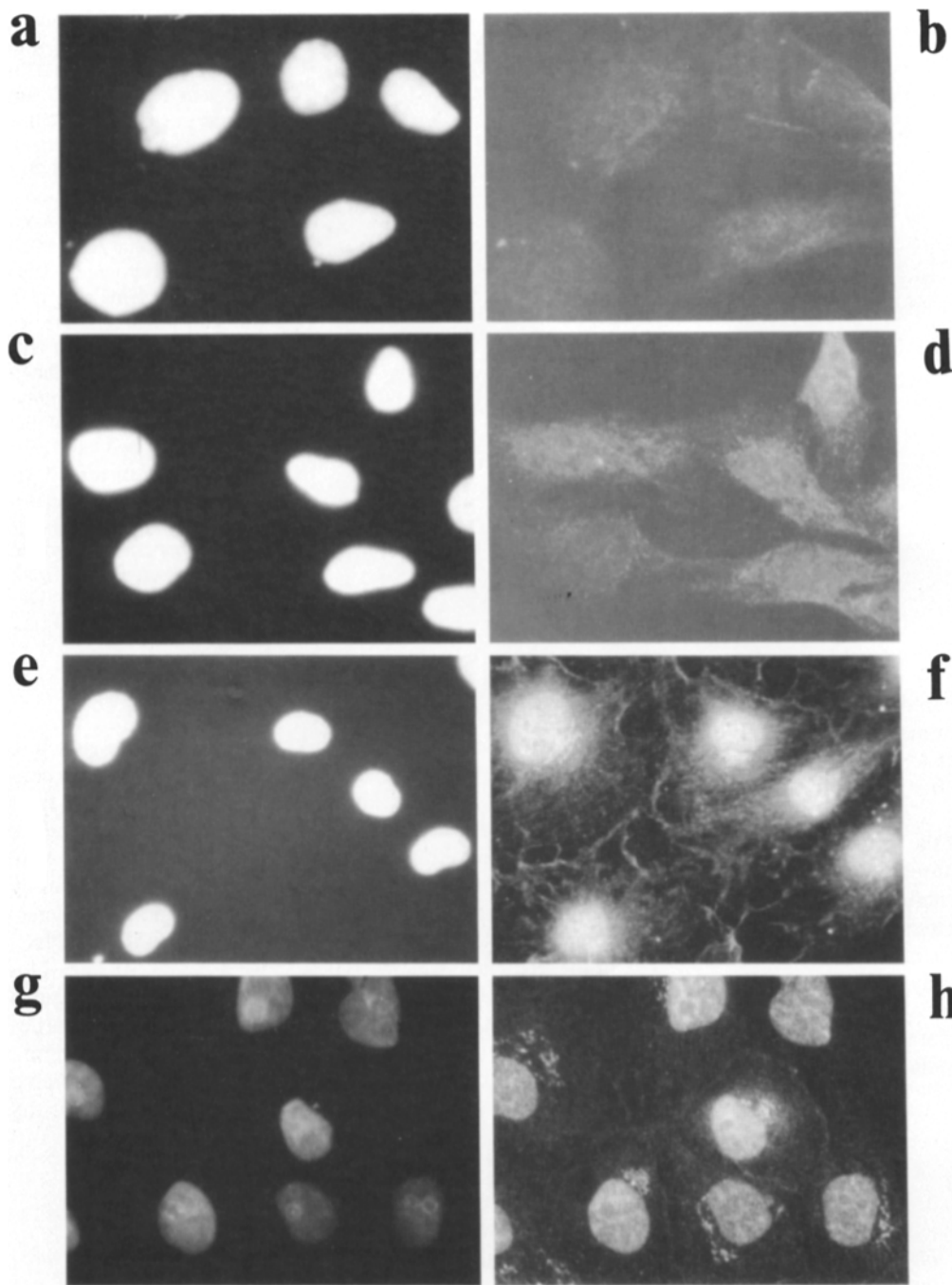


Figure 4. Localization of AHNAK protein in cultured cells by indirect immunofluorescence. NIH 3T3 cells (*a-f*) or Ptk₁ (*g* and *h*) cells were stained with Hoechst 33258 (*a*, *c*, *e*, and *g*), preimmune serum (*b*), anti-AHNAK immunoaffinity-purified serum FEN2 in presence (*d*), or absence (*f* and *h*) of the immune peptide followed by secondary antibody conjugated to rhodamine (*b*, *d*, and *f*) or fluorescein (*h*).

pattern of staining observed with NIH 3T3 cells. Specific staining of proteins in the nuclei and in an asymmetric perinuclear structure was evident with the immune antibody, but not with the preimmune serum or when the antibodies were preincubated with the immunogenic peptide (Fig. 4). In addition, we consistently observed a weak, diffuse cytoplasmic staining. The predominantly nuclear localization of AHNAK protein by immunofluorescence is in agreement with the results of the cell fractionations.

The perinuclear structure stained by the antibodies resembled morphologically the Golgi apparatus. To examine this possibility we performed double staining of cells with FEN2 and wheat germ agglutinin, which predominantly binds to the elements of the Golgi network. We observed that the

wheat germ agglutinin and the anti-AHNAK antibody bound to the same perinuclear structure. Thus, it remains possible that AHNAK protein might be associated with the Golgi network (data not shown), but that possibility requires further assessment (see Discussion).

We have performed immunofluorescent localization of AHNAK protein in several human cells lines, including epithelial cells, melanoma and neuroblastoma cells. Independent of the cell line we have observed staining of nuclei and a perinuclear asymmetric structure resembling the Golgi network (data not shown). We have also examined the rat kangaroo kidney cell line Ptk₁ for the localization of AHNAK protein after confirming that the same high molecular weight protein could be immunoprecipitated from extracts of these

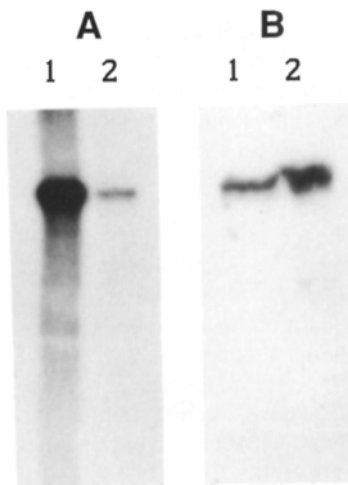


Figure 5. Analysis of AHNAK protein in growing versus quiescent NIH 3T3 cells. (A) 2×10^5 cells from actively proliferating (lane 1) or quiescent (lane 2) culture were labeled with inorganic [32 P] for 2.5 h, lysed in RIPA buffer, and analyzed by immunoprecipitation with the immunoaffinity-purified KIS4 antibodies. The smear in lane 1 is due to the presence of radioactive DNA in the cell lysate. (B) 10^5 cells from parallel cultures were analyzed by Western blotting with antibodies KIS4.

cells (data not shown). Again, we have observed immunofluorescence in the nuclei and the structure resembling the Golgi apparatus (Fig. 4 H).

AHNAK Is a Phosphoprotein

We have reported previously that the carboxyl-terminal portion of the AHNAK sequence contains several repeats of the sequence "lysine-serine-proline-lysine," which is identical to the "DNA finger" sequences found in histone proteins (4). The "DNA fingers" found in histones are the sites of mitosis-specific phosphorylation on serine residues (4). We conducted experiments to determine if AHNAK protein is phosphorylated and, if so, whether the modification is related to the cell cycle. To this end, AHNAK protein was immunoprecipitated from NIH 3T3 cells after metabolic labeling with radioactive inorganic phosphate. We examined actively growing and quiescent cells deprived of serum for 2 d. Fig. 5 A shows that AHNAK protein was phosphorylated in growing cells and that this phosphorylation was greatly diminished in quiescent NIH 3T3 cells. In contrast, Western blot analysis demonstrated that quiescent cells contain higher levels of AHNAK protein (Fig. 5 B). We conclude that AHNAK is a phosphoprotein and that the transition of fibroblasts into G₀ is accompanied by both accumulation and apparent dephosphorylation of AHNAK protein.

We have performed phosphoamino acid analysis of AHNAK protein in actively growing NIH 3T3 cells and have determined that it is phosphorylated on serine and, to a much lower extent, on threonine (data not shown). Serine residues are found in four conserved positions within every repeat unit of AHNAK (7) with occasional substitutions by threonine residues. The carboxy-terminal unique portion of AHNAK is relatively serine rich (7), while threonine residues are infrequent. Tyrosine residues are found at a conserved position in some of the repeats of AHNAK, but no phosphorylation of tyrosine was observed (data not shown). Identical results were obtained when phosphoamino acid analysis was performed with AHNAK protein immunoprecipitated from HeLa cells (data not shown).

AHNAK Protein in Differentiating Neuroblastoma Cells

AHNAK was first identified as a gene whose expression is

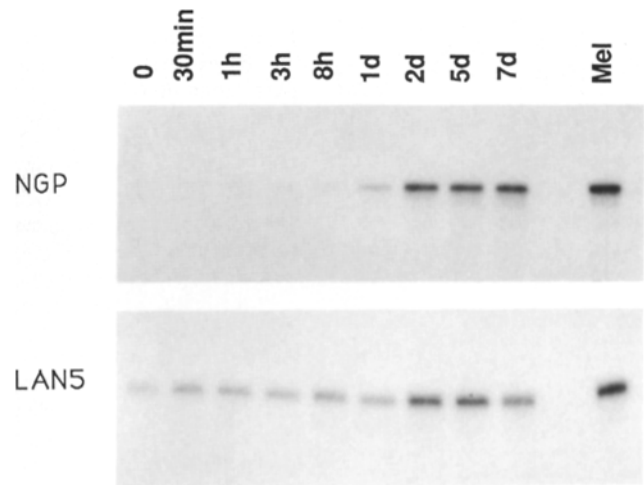


Figure 6. Effect of differentiation on expression of AHNAK RNA. Cells were treated for the periods of time indicated and total cellular RNAs (20 μ g) were analyzed for the levels of AHNAK RNA by RNase protection assay of a specific riboprobe prepared with a 5' end fragment of AHNAK cDNA (7). The length of the protected fragment is 330 nucleotides. The lane labeled Mel contains 20 μ g of total RNA from the melanoma cell line HT144.

reduced in neuroblastoma cell lines in comparison to other, more differentiated cells of neuroectodermal origin (6, 7). We examined if any changes in the expression of AHNAK occur in differentiating neuroblastoma cells. Neuroblastoma cell lines NGP and LAN5 were treated with retinoic acid to induce neuronal differentiation (8) and analyzed for the levels of AHNAK RNA over the course of treatment. In both cell lines we observed an increase in the levels of AHNAK RNA after 1–2 d of exposure to retinoic acid, after which the levels of AHNAK RNA remained unchanged (Fig. 6). The long interval between application of retinoic acid and the induction of AHNAK RNA most likely indicates that the latter is an indirect consequence of more immediate changes induced by retinoic acid. The timing of AHNAK RNA induction in neuroblastoma cells coincides with the cessation of cellular division and the appearance of morphological changes in the transformed neuroblasts (8, 9).

Next we analyzed the levels and phosphorylation of AHNAK protein in untreated versus differentiating neuroblastoma cells (Fig. 7). AHNAK protein labeled with radioactive phosphate was precipitated from untreated cells of neuroblastoma NGP and after treatment with retinoic acid for 2 d. Fig. 7 (A and B) shows that the phosphate content of AHNAK protein in differentiating cells was diminished, whereas the levels of the protein increased. Similar results were obtained with the neuroblastoma cell line LAN5 (data not shown). The levels of AHNAK remained elevated for up to 7 d after the beginning of the treatment (Fig. 7 C).

Discussion

The Size of AHNAK Protein

As predicted from nucleotide sequence (7), AHNAK protein isolated from cells appears to be exceptionally large. The data presented here do not permit a reliable assessment of size because large proteins may migrate anomalously in

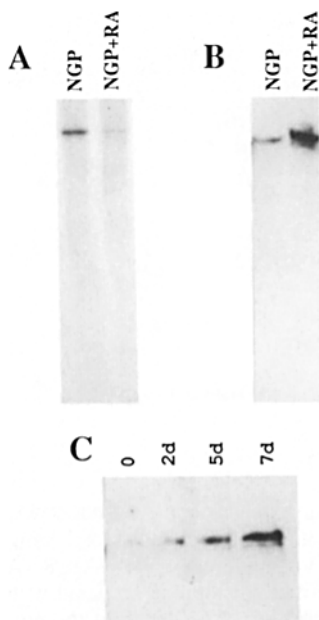


Figure 7. Effect of differentiation on expression and phosphorylation of AHNAK protein. (A) NGP cells were treated with 5 μ M of retinoic acid for 48 h. 2×10^5 cells from untreated (lane NGP) and treated with retinoic acid culture (lane NGP + RA) were labeled with inorganic [32 P] for 2.5 h, lysed in RIPA buffer, and analyzed by immunoprecipitation with the immunoaffinity purified serum KIS4. (B) 2×10^5 cells from parallel cultures were analyzed by Western blotting with antibodies KIS4. (C) Western blot analysis of protein lysates (100 μ g of protein per lane) prepared from NGP cells treated with retinoic acid for the periods of time indicated.

polyacrylamide gels. Thus, we continue to use the estimate of 700 kD for the mass of the protein, based on the deduced amino acid sequence. The representation of AHNAK in cDNAs still suffers from two apparently small gaps, but the corresponding regions of the genomic DNA have been sequenced and found to contain open reading frames that are continuous with the open reading frame represented in the adjoining cDNAs (7). It seems likely that we have the entire coding domain for AHNAK in view. We consider it unlikely that the gene produces a second protein by means of alternative splicing, since no introns are apparent in the nucleotide sequence of AHNAK (7). Therefore, we conclude that the protein identified here with two distinctive antisera and in diverse cell lines is the principal or sole product of AHNAK.

The Subcellular Location of AHNAK Protein

Our results indicate that the bulk of AHNAK protein is located in the nucleus. How can a protein with a mass of 700 kD gain access to the nucleus? Two properties of the AHNAK proteins may provide an explanation. First, the rodlike structure conjectured for the protein may facilitate passage through the confining diameter of nuclear pores. Second, the carboxy-terminal domain of the protein contains several possible nuclear localization signals that might facilitate nuclear transport by increasing the efficiency of interaction with proteins that bind the nuclear localization signals (10).

Several features of the AHNAK protein suggest that it may have a role in the architecture of the nucleus. First, the protein is both relatively stable (data not shown) and abundant (Fig. 1 A). Second, molecular modeling predicts a remarkably elongated and rigid tertiary structure (7). And third, our preliminary results indicate that the protein might bind DNA in a nonspecific manner (data not shown). These properties cause one to think of the nuclear matrix or scaffolding (1, 5), but the AHNAK protein is too readily extractable from the nucleus to be considered a component of these structures. Instead, the decidedly hydrophilic surface of the projected structure suggests that the protein could easily reside in the nucleoplasm. Moreover, it is possible that the two terminal domains of the protein (which are not part of the fibrous structure) could have functions of their own that are not of an architectural nature.

The nuclear staining of AHNAK protein was mainly punctate, an observation that seems at odds with the predicted structure for the protein. Given the denaturation that accompanies fixation, however, it seems unreasonable to expect the pattern of staining to necessarily reflect the native structure of the protein. Examination by immunoelectron microscopy might be more revealing.

Analysis by immunofluorescence indicated that a minor fraction of AHNAK protein may be associated with the Golgi network. The protein displays no structural feature that would either predict or explain sequestration in a membranous organelle. Our preliminary data from subcellular fractionations confirm that the cytoplasmic AHNAK protein is confined to the membrane-containing fraction, but further work will be required to determine whether the protein does in reality reside in the Golgi apparatus.

Modifications of the AHNAK Protein During Changes in Cellular Proliferation and Differentiation

The product of AHNAK is phosphorylated on serine and threonine. Both amino acids are abundant throughout the protein, but the particular residues subject to phosphorylation and the responsible kinase(s) are not known. The phosphorylation appears to decrease substantially when cells are rendered quiescent. It is conceivable that this decrease is due to limitations of isotopic labeling in quiescent cells, but we note that the decrease was observed with cells rendered quiescent by either deprivation of serum or induction of differentiation in rich growth medium. By contrast, the abundance of AHNAK protein increases appreciably in the G_0 stage of the cell cycle, as if the protein might have an especially prominent function in quiescent cells. Perhaps dephosphorylation is a correlate of that function.

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