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Isolation and characterization of *nudC* from mouse macrophages, a gene implicated in the inflammatory response through the regulation of PAF-AH(I) activity

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Abstract **We report the characterization of a cDNA induced in mouse macrophages that encodes a 332-amino acid protein with extensive sequence identity with members of the mammalian *nudC*-like genes. The interaction between mNUDC and the regulatory β subunit of platelet activating factor acetylhydrolase I (PAF-AH(I)) shown in this article indicates a new function of NUDC. Thus, we show that NUDC increases the catalytic activity of PAF-AH(I) and that this regulatory activity is located in the carboxyl terminal half of the protein which is highly conserved. This suggests a novel function for mammalian *nudC*-like genes as anti-inflammatory proteins.**

Keywords: Monocytes/macrophages; Inflammation; Platelet activating factor acetylhydrolase; nudC

1. Introduction

The phagocytosis of particles and fibers by alveolar macrophages induces a state of cellular activation characterized by the increased synthesis of different inflammatory and fibrotic mediators [1–3]. In vitro models that mimic the initial development of fibrosed lung diseases have been studied [4,5]. Using mouse RAW 264.7 macrophages, we constructed a subtracted cDNA library enriched in sequences overexpressed in silica-treated cells. The differential screening of the library led to the isolation of nine cDNAs corresponding to genes induced by the inflammatory stimuli [6]. One of them was expressed as a 1.5 kb transcript [6] and revealed a significant similarity to r*nudC* [7] a rat orthologs of the *nudC* gene of *Emericella nidulans* and was therefore named murine *nudC*.

Nud genes, first identified in *E. nidulans*, have been implicated in Nuclear distribution [8] encode various cytoplasmatic subunits as cytoplasmatic dynein heavy chain (CDHC, *nudA*), cytoplasmic dynein intermediate chain (CDIC; *nudI*), cytoplasmic dynein light chain (CDLC, *nudK*), and proteins implicated in dynein regulation as *nudF* and *nudC* [7,9]. The cytoplasmic dynein/dynactin complex powers various aspects of mitosis, including establishment of bipolarity, organization of spindle poles, alignment and segregation of chromosomes, as well as regulation of microtubule dynamics [10,11]. These functions have been probably conserved during evolution, as indicated by the fact that they have been found in species such as *E. nidulans*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Homo sapiens* [12–14].

Mammalian *nudC* and *nudF* have been implicated in processes regulated by cytoplasmic dynein such as proliferation or neuronal migration [7]. It has also been shown that murine *nudC* has a role in eukaryotic cell proliferation [15] and in spindle formation, a process dependent of the phosphorylation by polo-like kinases [16]. The different in size between *E. nidulans nudC* and its orthologous in mammalians organisms suggest that extra functions may reside in the mammalian proteins.

NudF is a homologue of the regulatory β subunit of human platelet activating factor acetylhydrolase type I (PAF-AH(I)) [17], a member of the family of platelet activating factor (PAF) acetylhydrolases [18]. These enzymes inactivate the potent pro-inflammatory phospholipid PAF by removing the acetyl group at its sn-2 position [19]. In mammalian cells one extracellular and three intracellular PAF-AH are known [19,20]. PAF-AH(I) is an intracellular oligomeric enzyme consisting of two catalytic subunits (α_1 and/or α_2) and a regulatory β subunit, which upon binding to the catalytic homodimer upregulates the enzyme activity [21]. These intracellular enzymes inactivate PAF during its formation thus controlling the secretion of active PAF. Alternatively, they could also act by inhibiting intracellular functions of PAF [22].

We have compared the sequences of *nudC* from various species and the results indicate a high conservation of the NUDC domain in all organisms studied. In the case of mammalian genes the homology is conserved along the entire sequence. Two novel motifs suggest new functions of *nudC* that are not shared by non-mammalian genes. Our results indicate that one of these novel functions is the regulation of PAF-AH through the interaction with its regulatory β subunit (NUDF). The interaction between these two proteins induce the activation of PAF-AH(I) thus indicating that murine nudC has a role as regulator of the inflammatory response.

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Abbreviations: GST, glutathione-*S*-transferase; LIS-1, lisoencephaly gene 1; NudC, nuclear distribution clone C; NudF, nuclear distribution clone F; PAF, platelet activating factor; PAF-AH(I), platelet activating factor acetylhydrolase type I

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2. Materials and methods

2.1. Materials

Restriction endonucleases and DNA modifying enzymes, [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{35}S]dATP (1000 Ci/mmol) were purchased from Amersham International. The GeneAmp PCR reagent kit was from Perkin–Elmer. All other chemicals were reagent or molecular biology grade and were obtained from Boehringer Mannheim, Merck, or Sigma.

2.2. NudC cDNA cloning

Isolation of murine *nud*C cDNA by differential screening of a subtracted cDNA library from the murine macrophage cell line RAW 264.7 constructed in the λZAPII vector (Stratagene) has been described previously [6]. Independent *nud*C-containing clones were isolated after three rounds of screening of 10^6 clones of a RAW 264.7 cDNA library in λZAPII following standard procedures. The cDNA insert of *nud*C was labeled by random priming using the ''RediPrime'' (Amersham) labeling system in the presence of [α-^{32}P]dCTP to be used as a probe. pBluescript SK phagemids containing *nud*C cDNAs were excised in vivo from the λZAPII vector by coinfection of *Escherichia coli* XL1-Blue cells with VCS M13 helper phage (Stratagene), according to the supplier's instructions.

2.3. DNA sequencing and analysis

Nucleotide sequences were determined by the dideoxy chain termination method on single and double-stranded template DNA using Sequenase 2.0 (USB Amersham) with custom-designed oligonucleotide primers Sequence analysis and comparison were performed with the University of Wisconsin's Genetics Computer Group version 8.0 package of programs. The *nudC* sequences (human, fungus, fish, or rat) were downloaded from the GenBankTM/EMBL database. Amino acid sequences were aligned using the CLUSTALW program.

2.4. SDS–PAGE and Western blot analysis

Proteins were resolved by SDS–PAGE with Tris/Glicine buffer system. Electrophoreses were performed at 100 V, and proteins were transferred from gels to Hybond polyvinyldene difluoride membranes (Amersham) in 48 mM Tris, 39 mM glycine, 20% methanol, 0.0375% SDS, using a Bio-Rad Transblot semidry transfer cell at 20 V for 1 h.

For Western blot analysis, membranes were blocked with 5% nonfat dry milk in Tris-buffered saline at 4 °C overnight, then incubated with the appropriate primary antibody (1:500) in blocking buffer for 2 h, and washed twice with Tris-buffered saline containing 0.1% Tween 20 for 30 min. Membranes were then incubated with a 1:10 000 dilution of a peroxidase-linked goat anti-rabbit IgG (Sigma) in blocking buffer for 1 h, washed twice, and developed with the ECL system (Boehringer Mannheim).

2.5. PAF-AH activity

PAF-AH activity was determined by using a commercially available assay kit (Cayman Chemical). The assay uses 2-thio PAF (synthetic analog of PAF which contains an acetyl group attached by a thioester bond at the sn-2 position) which serves as a substrate for PAF-AH. Upon hydrolysis of the acetyl thioester bond at the sn-2 position by PAF-AH, free thiols are detected using 5,5-dithiobis (2-nitrobenzoic acid) (DTNB; Ellman's reagent). The detection range of the assay is from 0.02 to 0.2 μmol/min ml of PAF-AH activity.

2.6. Immunoprecipitation

RAW 264.7 cells were mixed with 1 ml of buffer A (50 mM Tris, 5 mM EDTA, 150 mM sodium chloride, 10 mM sodium fluoride, 1 mM pervanadate, 1% Triton X-100, pH 7.4) containing 1 mM PMSF, and were incubated on ice for 1 h with occasional vortexing. Cell lysates were prepared by centrifuging the cells at 8000 rpm at 4 °C for 20 min. One milliliter of the cell lysate was incubated with 20 μl of protein G-Sepharose beads suspension overnight at 4 °C under continuous rotation. Immune complexes bound to protein G were sedimented by rapid centrifugation and beads were washed three times with 1 ml of buffer A. The pellets were resuspended in 60 μl of sample loading buffer containing 0.1 M β-mercaptoethanol and boiled for 5 min. Proteins were resolved by SDS–PAGE on 12% gels and subjected to Western blotting as described.

2.7. GST pull-down assay

GST-NUDC or GST-ΔNUDC generated in *E. coli* XL1-Blue was incubated with the lysates of RAW 264.7 cells at 4 °C overnight. Proteins were adsorbed to glutathione-agarose beads for an additional 1 h. The bound proteins were resolved by SDS–PAGE and transferred to Hybond N membranes. The blots were then probed with anti NUDF antibody (sc7577; Santa Cruz Biotechnology).

2.8. PAF-AH(I) purification procedure

Isolation and partial purification of PAH-AH(I) was carried out using fresh murine brains following a protocol previously used to isolate the bovine complex [18]. In short, cell-free extracts were fractionated with 40% and 65% saturated $(NH_4)_2SO_4$. The precipitate of the latter was resuspended in 10 mM Tris–HCl pH 7.4 containing 1 mM EDTA and 10% glycerol and subjected to chromatography on a DEAE. Sepharose CL-6B column (2.5 × 20 cm) equilibrated with the same buffer. Adsorbed proteins were eluted with a 35 ml linear gradient from 0 to 400 mM NaCl.

3. Results

3.1. Identification and characterization of murine nudC as an orthologous gene of nudC from E. nidulans

Nuclear distribution clone C (NudC) is one of the nine cDNAs isolated by differential screening of a subtracted RAW 264.7 macrophage cDNA [6]. The cDNA spans 1345 bp followed by a poly(A) tail (GenBank Accession Number X8443, see supplementary material). The ATG at base 81 is probably the genuine initiator since it is located within a favorable environment for translation initiation. Following the TAG end codon at position 1076, there is a 190 bp 3′-UTR with a consensus polyadenylation site located at position 1271. Comparison of the sequence against the GenBankTM/EMBL nucleotide data bases using the FASTA sequence alignment algorithm showed a high similarity to r*nud*C (92.1% identity). We therefore named it murine *nudC*. *NudC* orthologs from diverse species share a high conservation in their amino acid sequence (Fig. 1). The searching for protein motifs in the amino acid sequence revealed pattern matches for three domains. One of them located in the carboxyl terminal region is the NUDC domain. The NUDC domain is also present in the *nudC* gene of *E. nidulans*. The other two domains have been identified only in mammalian nudC genes. One of them located between Lys 68 and Arg 76 contains a basic stretch similar to the nuclear localization signal (NLS).The putative NLS sequence KARREKRAR is very similar to the steroid hormone receptor NLS consensus sequence, RKWKR/K**R/K [23]. Murine NUDC also contains an acidic region between Asp 147 and Lys 160, DAEEEEDEEDEKDK, where 11 out of 14 residues are negatively charged. Although the amino acid sequences in the carboxyl-terminal region are very similar in mammalian NUDC and *E. nidulans* proteins, the DNA sequences are quite different due to the high number of silent mutations that exist (data not shown). It is conceivable that this region of NUDC has been conserved through evolution for an important function. The amino acid sequence shared by mammalian proteins but not with NUDC of *E. nidulans* suggest new specific functions of the former.

3.2. NUDC protein

The translation of the open reading frame in the mouse *nudC* cDNA results in a 332 amino acid protein with a calculated molecular mass of 36 630 Da. We made two Glutathione-*S*-

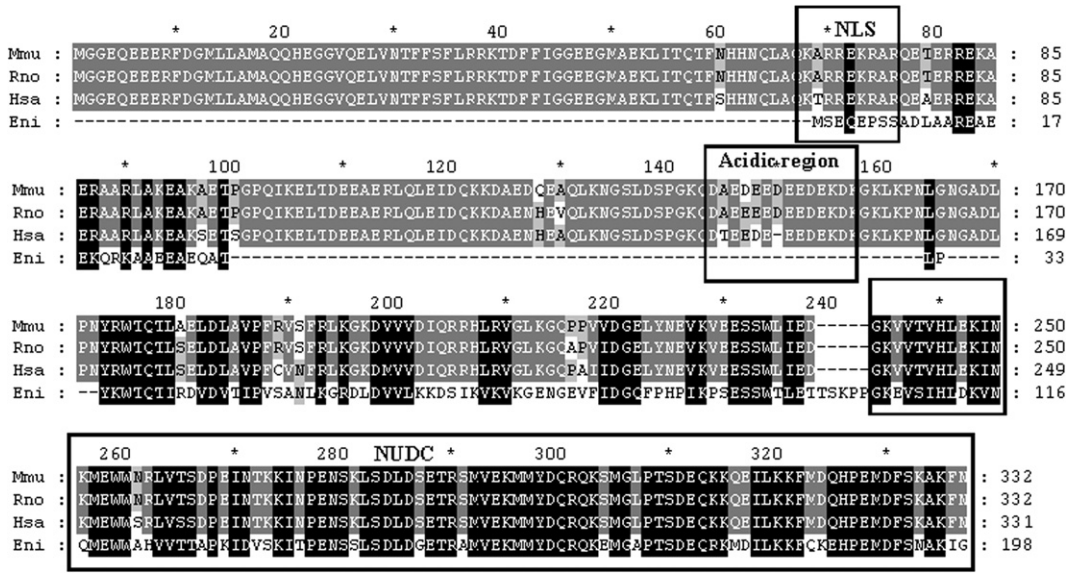

Fig. 1. Conservation of NUDC domain between *E. nidulans* and mammalian genes. The comparison of amino acid sequences of *E. nidulans* and orthologs mammalian NUDC is shown. Black background represents 90–100% identity; grey backgroung with white letters represents 80–90% identity and grey background with black letters 70–80% identity. The motifs found in mammalian amino acid sequences are boxed. Eni: Emericella nidullans; Has: Homo sapiens; Mmu: Mus musculus; Rno: Ratus norvegicus.

transferase (GST) constructs in the bacterial vector pGEX, one containing the complete cDNA (GST-NUDC) and another containing the first 300 bp from the 5′ end (GST-ΔNUDC). Plasmids were transformed in *E. coli* XL-Blue which were induced with IPTG. Both the complete and the truncated proteins were effectively induced by IPTG (Fig. 2A) and purified to homogeneity by affinity chromatography on glutathione-sepharose (Fig. 2B). To test if murine *nud*C is translated in mouse cells, rabbit polyclonal antibodies were raised against the GST-ΔNUDC fusion protein produced in *E. coli*. Western blot analysis of cell extracts from RAW 264.7 cells, (Fig. 2C) showed a major band with an apparent molecular mass of 43 kDa, which should correspond to the protein product coded for by murine *nudC*. The observed size is higher than the 37 kDa calculated from the *nudC* cDNA sequence, suggesting that the protein may be susceptible of post-transcriptional processing or regulatory events.

3.3. Interaction between NUDC and the regulatory subunit of PAF-AH(I)

All functions attributed to NUDC are thought to be due to its interaction with NUDF and directed to the molecular motor dynein. However, NUDF is also the regulatory β subunit of PAF-AH(I), a well known anti-inflammatory enzyme To study the interaction between NUDC and the regulatory subunit, control and PMA treated RAW 264.7 cells were homogeneized and the intracellular NUDC was immunoprecipitated using the specific antibody raised in the laboratory. NUDF was analyzed in the immunoprecipitate by Western blot using anti murine NUDF antibodies. The presence of the regulatory

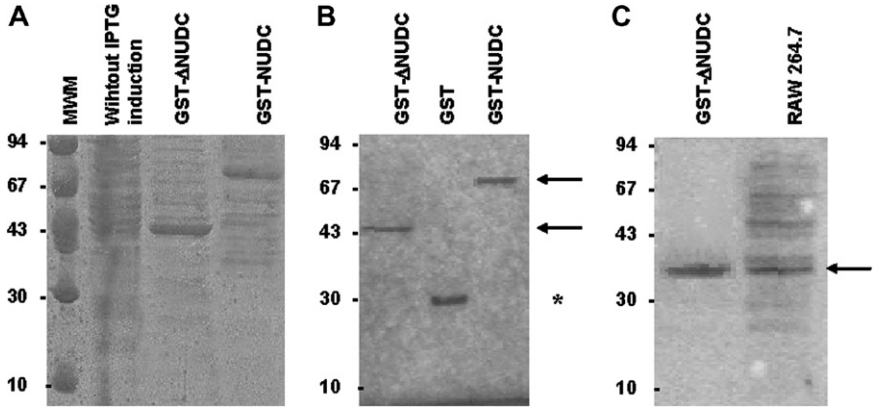

Fig. 2. Purification of recombinant NUDC proteins produced in *E. coli*. Twenty microliter aliquots of bacterial extracts (A) induced with IPTG (GST-ΔNUDC and GST-NUDC), and the corresponding purified fusion proteins (B) were analyzed by SDS–PAGE and stained with Coomasie blue. Arrows indicate NUDC fusion proteins and an asterisk indicates GST protein. (C) Protein extracts from RAW 264.7 cells were resolved by SDS–PAGE and transferred to a PVDF filter. Western blot analysis was performed using the antibody raised in rabbit against mNUDC. The arrow indicates the endogenous mNUDC protein.

β subunit in the immunoprecipitate demonstrates the interaction of both proteins (Fig. 3A). To confirm the association of NUDC and the regulatory β subunit in vitro, we used a GST pull-down assay. Purified GST-NUDC and GST-ΔNUDC proteins were incubated with cell lysates from RAW 264.7 cells, the complexes purified by chromatography on glutathione-sepharose and analyzed for NUDF (Fig. 3B). NUDF was pulled down when the complete GST-NUDC protein was used (Fig. 3B, lane 1) but not when GST-ΔNUDC was used (Fig. 3B, lane 3). This indicates that the region of NUDC interacting with NUDF should be localized in the region of NUDC where the acidic region and the NUDC domain are located.

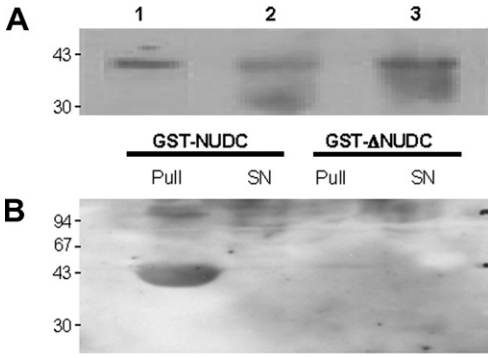

Fig. 3. Interaction between NUDC and NUDF in vitro. (A) Co-immunoprecipitation of NUDF and mNUDC. Lysates from control RAW 264.7 cells (lane 2) or PMA treated (lane 3) were immunoprecipitated with anti NUDC. The immunorecipitate was resolved by SDS-PAGE and immunoblotted with anti NUDF. Five percent of total input is shown in lane 1. (B) Bacterially expressed GST-NUDC and GST-ΔNUDC were incubated with lysates of RAW 264.7 cells at 4 °C overnight. Complexes were adsorbed to glutathione-agarose beads and processed as indicated in Section 2. NUDF protein that asociates to GST-NUDC was detected by Western blot. Pull represents the adsorbed protein and SN the supernatant of the pull-down assay.

3.4. Functional interaction of mNUDC and PAF-AH(I)

The interaction between murine NUDC and the regulatory β subunit of PAF-AH(I) (NUDF) prompted us to investigate whether there is a functional relationship between the two proteins. For this purpose we isolated PAF-AH(I) from mouse brains and analyzed the catalytic activity. The last step of purification consisted in a DEAE-Sepharose chromatography. When fractions from this chromatography were analyzed, two peaks containing PAF-AH activity were separated. The first one eluted in the range of 150–200 mM NaCl and the second one eluted at about 300 mM NaCl (Fig. 4). Both peaks were analyzed by Western blot for NUDF. This was only present in peak 2 (Fig. 4, lower panels). We concluded that peak 1 contained only PAF-AH catalytic subunits while peak 2 contained both catalytic and regulatory β subunits of PAF-AH and we therefore named it holoenzyme. This agrees with previous studies on PAF-AH(I) purification [18]. To demonstrate further the functional interaction of mNUDC and PAF-AH(I) we incubated the recombinant protein GST-NUDC with peak 1 or peak 2 and measured its effect on enzymatic activity. In peak 2 (which contained the regulatory β subunit) the enzymatic activity increased by 60% while no change in activity was observed in peak 1, which did not contain the regulatory β subunit (Fig. 5A). Thus, NUDC appears to be able to interact with the regulatory β subunit of PAF-AH(I) rendering an enzyme with increased catalytic activity. Finally, we used recombinant GST-NUDC and GST-ΔNUDC to study the specificity of the effect of NUDC on PAF-AH(I) activity. For this purpose we incubated increasing amounts of each recombinant protein with either purified PAF-AH(I) catalytic subunits or PAF-AH(I) holoenzyme and determined their effect on PAF-AH catalytic activity. As shown in Fig. 5B, GST-NUDC showed no effect on PAF-AH(I) catalytic subunits while inducing the upregulation of PAF-AH(I) holoenzyme in a dose-dependent fashion. On the other hand, GST-ΔNUDC had no effect in either form of the enzyme. This again suggest that the c-terminal region of NUDC should be respon-

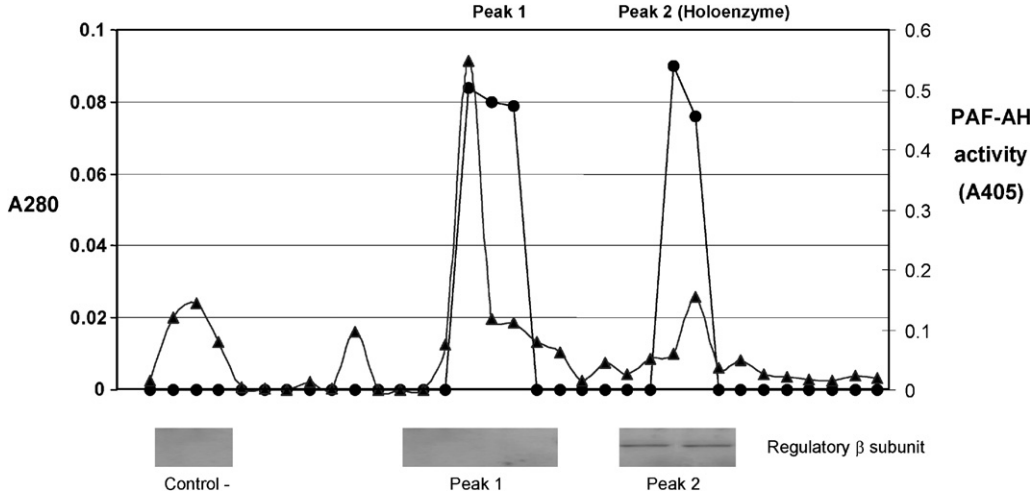

Fig. 4. Purification of PAF-AH(I) and identification of the catalytic and holoenzyme complexes. Murine brains were processed as indicated in Section 2. One hundred microliter of the 65% ammonium sulfate precipitate resuspended in chromatography buffer was loaded onto a DEAE-Sepharose CL-GB column. The adsorbed proteins were eluted with a 35-ml linear NaCl gradient from 0 to 400 mM. Protein concentration of the eluates was determined by measuring the A280 (triangles). PAF-AH activity was determined as described in Section 2 and represented as A405 (circles). Fractions displaying catalytic activity were concentrated, subjected to SDS–PAGE and NUDF determined by Western blot (lower panels).

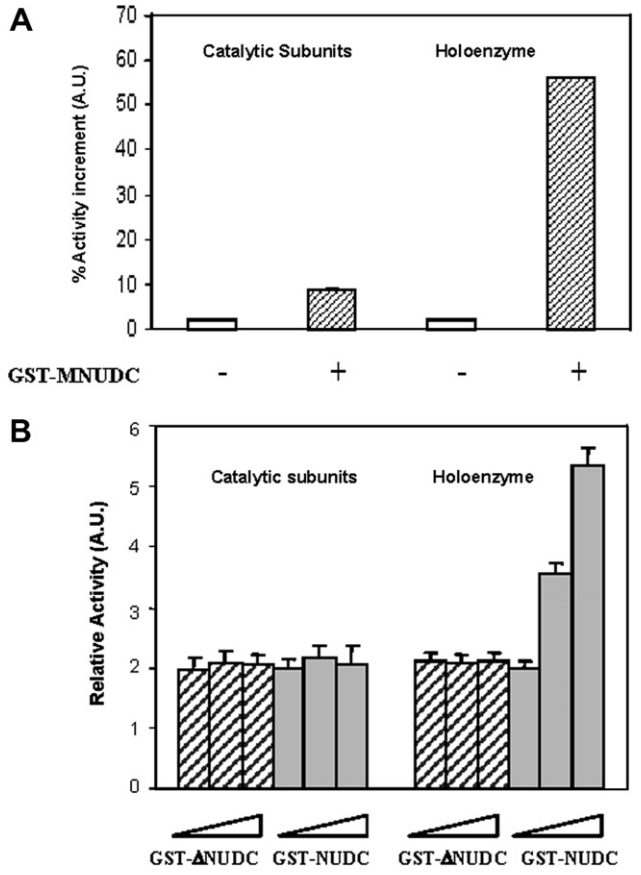

Fig. 5. Interaction of mNUDC and the regulatory subunit of PAF-AH(I). Effect on the activity of catalytic subunits and holoenzyme. (A) PAF-AH activity was measured in pooled fractions containing the catalytic subunit and the holoenzyme of PAF-AH(I) in the absence (empty bars) and in the presence (dashed bars) of GST-NUDC as indicated ecombinant GST-NUDC (50 μg) was incubated for 60 min on ice with the indicated fractions prior to determining enzymatic activity. The values are the means ± S.E. of three determinations. (B) Catalytic subunits and holoenzyme of PAF-AH(I) were incubated as above with increasing amounts (20, 40, and 60 μg) of the GST-ΔNUDC or GST-NUDC. PAF-AH activity was determined as in A. The values are the means ± S.E. of three determinations.

sible of the interaction with the regulatory β subunit of PAF-AH(I). As mentioned above, this region contains an acidic motif and the NUDC domain which could be good candidates for being responsible of the interaction.

4. Discussion

We report here the isolation and characterization of the cDNA encoding murine *nudC*, an inducible gene in macrophages activated by inflammatory stimuli whose product is closely related to that of *E. nidulans nudC* gene over the last third of its protein sequence [8]. Eventhough there is a high number of silent mutations in this region, murine *nudC* gene is highly conserved and its amino acid sequence shows 92% identity to rat *RnudC* [7] and 94% identity to human *HNUDC* [16] suggesting important conserved functions.

NUDC translated in RAW 264.7 cells showed an apparent molecular mass of 43 kDa, which is larger than the 37 kDa expected from its DNA sequence, suggesting that post-translational modifications may occur [16]. Murine NUDC is much larger than the 22-kDa *E. nidulans* NUDC protein. The different size of these proteins may be the reason of additional functions in the case of mammalian genes. Through its conserved NUDC region, NUDC may be part of the large cytoplasmic dynein complex involved in vesicle transport, nuclear movement, and/or protein trafficking [10,24]. In addition to the conserved c-terminal region, mNUDC contains other domains, which suggest potentially distinct functions. Firstly, NUDC contains a potential NLS, a motif not found in *E. nidulans* NUDC. Interestingly, HNUDC and rNUDC also have a potential NLS, but the function and localization of the protein should await for further [25,26]. Secondly, mNUDC contains an acidic region in amino acids 147–160. This highly charged region may be involved in protein/protein interactions. The acidic domain is reminiscent of a repeated sequence found in an NLS-binding protein, Nopp140 [27]. Nopp140 can move from the cytoplasm to the nucleus, potentially shuttling other proteins with it.

The fact that murine *nud*C have been found in activated macrophages suggests a possible function in inflammation. Thus, mNUDC is able to interact with NUDF, as is the case of HNUDC and Lisoencephaly gene 1 (LIS-1) [7]. We have demonstrated this interaction by using GST-hybrid proteins in a pull-down assay and by immunoprecipitation. This interaction appears to be very important in the regulation of dynein complex. In addition, *nudF* is a causative gene for MLIS-1 and it is also known as [PAF-AH(I)] regulatory β subunit [28,29]. This enzyme is highly specific and transforms the acetylated PAF in the inactive form lyso-PAF. As PAF is an important pro-inflammatory secondary lipidic messenger, PAF-AH(I) acts regulating these pro-inflammatory functions. The interaction of NUDC and NUDF suggests that NUDC has a function in the activity of PAF-AH(I). The interaction depends of the carboxyl terminal half of NUDC which contains an acidic region and the NUDC domain.The acidic region has been proposed as a site of interaction with other proteins and the NUDC domain is the only motif found conserved in all organisms studied. In this regard, we have shown that the recombinant protein GST-NUDC increases by 60% the activity of PAF-AH(I) only when the regulatory β subunit is present suggesting an implication of NUDC in the regulation of the pro-inflammatory actions mediated by PAF. It is also interesting that we isolated *nudC* cDNA from the monocyte-macrophage cell line RAW264.7 stimulated with inflammatory stimuli since PAF has been suggested to play a role in the differentiation of monocytes [30]. It has been reported that PAF is able to mediate the signal transduction of inflammatory stimuli as LPS [31] or TNF-α. The depletion of PAF would inhibit the activation of NFκB activation by these[32]. The possible role of NUDC regulating the levels of intracellular PAF and therefore regulating the signalling of pro-inflammatory factors as LPS or TNF-α is very interesting. In summary then, our findings suggest the interesting possibility that murine NUD*C*, a dynein and microtubule-associated protein has an anti-inflammatory action by increasing the activity of PAF-AH(I) thus regulating the pro-inflammatory actions of PAF [33]. This also makes NUDC a possible target to prevent autoimmune diseases induced by PAF [20].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.05.065.

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