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Gene expression pattern

NUCB1, the *Drosophila melanogaster* homolog of the mammalian EF-hand proteins NEFA and nucleobindin

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Abstract

Mammalian NEFA and nucleobindin are calcium-binding proteins containing a signal peptide, two EF-hand motifs, acidic and basic regions and a leucine-zipper motif. Although they have been discussed to be involved in autoimmunity, apoptosis and calcium homeostasis in the Golgi apparatus and bone matrix, their exact role remains unknown. Here we report the cloning of their *Drosophila* homolog, *nucb1*, as well as the analysis of its expression pattern during embryogenesis and the subcellular localization of the NUCB1 protein. The *nucb1* mRNA and the NUCB1 protein were found to be expressed maternally and zygotically, and they accumulate ubiquitously at low levels during all embryonic stages due to a maternal component. From stage 11 onward, high levels of zygotic expression can be detected specifically in the salivary glands and their placodes. In contrast to the known mammalian family members, the NUCB1 protein localizes in a subpattern of cytoplasmic substructures, probably the Golgi apparatus.

Keywords: Protein NEFA; Nucleobindin; EF-hand proteins; Calcium affinity; Signal peptide; Glutamine rich region; *Drosophila* embryogenesis; Salivary glands; Golgi apparatus; Schneider-2 cells; Indirect immunofluorescence

1. Molecular cloning of *nucb1*

Using a degenerate PCR with primers derived from conserved regions of NEFA (Barnikol-Watanabe et al., 1994) and nucleobindin (Miura et al., 1992), a 177 bp fragment was amplified from genomic *Drosophila melanogaster* Oregon-R DNA. The product was cloned, sequenced, and used as a probe to screen an embryonic cDNA library. Several clones were isolated, one of which contained the complete *nucb1* coding sequence (Fig. 1). The 1707 bp open reading frame encodes a 569 amino acid polypeptide with a theoretical molecular weight of 67.4 kDa. After cleavage of the predicted N-terminal signal peptide, the mature protein NUCB1 has a molecular weight of 65.3 kDa. The protein exhibits extensive sequence similarity to NEFA and nucleobindin in the region between amino acid positions 57 and 345, where 50% of its residues are identical and 58% are similar to those of both human NEFA and nucleobindin.

Besides the signal peptide, NUCB1 shares other structural elements with NEFA and nucleobindin, such as one basic and one acidic region, the latter being flanked by two EF-hand motifs numbered 1 and 2 in accordance with those of NEFA and nucleobindin. In contrast to the mammalian proteins, NUCB1 contains a third EF-hand motif but no leucine-zipper structure, which is replaced by a longer, glutamine rich motif, which we have termed the PVQ_5-repeat after its consensus sequence. NUCB1 contains two hypothetical coiled-coil regions as potential multimerization sites and one possible N-glycosylation site.

2. Expression patterns and subcellular localization

The expression pattern of the *nucb1* mRNA during embryonic development was monitored by in situ hybridization of a digoxigenin-labeled antisense RNA probe to whole-mount Oregon-R embryos. Due to a maternal component, *nucb1* RNA can be detected ubiquitously in the preblastoderm embryo, and transcripts accumulate in a ubiquitous pattern throughout the early stages of embryonic development. From early stage 11 onward, a specific zygotic *nucb1* expression pattern is seen by the high levels of transcript in the placodes of the salivary glands, which persists up to the final stages of embryonic development in the differentiated organ (Fig. 2A,C,E). The same expression patterns were observed with antibody staining of

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TATTTGCACAGTTGTTGGCCTGCATTGCGCACAAGTGGGAAGTACACGACAGAAACACACACTCGCAGCCATGGTGCAGAACGTGGCCCTGCTGGGATTGGCACTGATTGCGATTTCCGC  120
                                                    M  V  Q  N  V  A  L  L  G  L  A  L  I  A  I  S  A                    17
CTCGATTGTCGCCCTGCCCGTGACACAGAATAAGAAGGATCACAAGGAGGCGGCGGAGTCCTCCACTCCGGCCACCGCCGACGTGGAAACGGCCCTGGAGTACGAGCGCTACCTGCGGGA  240
 S  I  V  A  L  P  V  T  Q  N  K  K  D  H  K  E  A  A  E  S  S  T  P  A  T  A  D  V  E  T  A  L  E  Y  E  R  Y  L  R  E    57
GGTTGTCGAGGCCCTTGAGGCGGACCCTGAGTTCCGTAAGAAGCTGGACAAGGCGCCCGAGGCCGACATTCGGAGTGGCAAGATCGCACAGGAGCTGGACTACGTGAACCACCATGTGCG  360
 V  V  E  A  L  E  A  D  P  E  F  R  K  K  L  D  K  A  P  E  A  D  I  R  S  G  K  I  A  Q  E  L  D  Y  V  N  H  H  V  R    97
GACCAAGCTGGACGAGATCAAGCGCCGTGAAGTGGAGCGCCTGCGGGAGCTGGCGAATCAAGCATACGAGCTGTCCAACGACATTGACCGGAAGCACCTGAAGGTGTCTCAGCATCTGGA  480
 T  K  L  D  E  I  K  R  R  E  V  E  R  L  R  E  L  A  N  Q  A  Y  E  L  S  N  D  I  D  R  K  H  L  K  V  S  Q  H  L  D   137
CCACGACAACGAGCATACCTTCGAGATCGAAGATCTGCGAAAGCTCATTCAGAAGACCTCCGACGACCTGGCCGAGGCGGACCGCAAGCGACGTGGCGAGTTCAAGGAGTACGAAATGCA  600
 H  D  N  E  H  T  F  E  I  E  D  L  R  K  L  I  Q  K  T  S  D  D  L  A  E  A  D  R  K  R  R  G  E  F  K  E  Y  E  M  Q   177
GAAAGAGTTTGAGCGTGAGGCGCAGAAAAAGGAAATGGATGAGGAGTCGCGGAAGAAGTTTGAGACCGAGCTCAAGGAGAAGGAGGAAAAGCATAAGGACCACGAGAAGCTGCACCACCC  720
 K  E  F  E  R  E  A  Q  K  K  E  M  D  E  E  S  R  K  K  F  E  T  E  L  K  E  K  E  E  K  H  K  D  H  E  K  L  H  H  P   217
TGGCAACAAGGCCCAACTAGAGGATGTGTGGGAGAAGCAGGACCACATGGACAAGAACGACTTTGATCCGAAGACATTCTTCTCCATCCACGACGTCGACAGCAACGGCTACTGGGACGA  840
 G  N  K  A  Q  L  E  D  V  W  E  K  Q  D  H  M  D  K  N  D  F  D  P  K  T  F  F  S  I  H  D  V  D  S  N  G  Y  W  D  E   257
GGCTGAGGTCAAAGCTCTGTTTGTCAAGGAACTGGACAAGGTCTATCAGAGTGATCTTCCCGAGGACGACATGAGGGAGCGAGCAGAGGAAATGGAACGTATGCGCGAGCACTACTTTCA  960
 A  E  V  K  A  L  F  V  K  E  L  D  K  V  Y  Q  S  D  L  P  E  D  D  M  R  E  R  A  E  E  M  E  R  M  R  E  H  Y  F  Q   297
GGAGACGGACATGAACCACGACGGCTTAATCAGCATCGACGAGTTCATGGTGCAGACTAACAAGGAAGAATTTCAAAAGGACCCCGAATGGGAGACCATCGACCGACAGCAGCAGTATAC 1080
 E  T  D  M  N  H  D  G  L  I  S  I  D  E  F  M  V  Q  T  N  K  E  E  F  Q  K  D  P  E  W  E  T  I  D  R  Q  Q  Q  Y  T   337
ACACGAGGAGTATCTGGAGTACGAACGCCGGCGGCAGGAGGAAGTGCAGCGCTTGATTGCTCAGGGCCAGCTGCCACCGCACCCGAACATGCCACAGGGATACTATGCTGCTCCACCACC 1200
 H  E  E  Y  L  E  Y  E  R  R  R  Q  E  E  V  Q  R  L  I  A  Q  G  Q  L  P  P  H  P  N  M  P  Q  G  Y  Y  A  A  P  P  P   377
AGGAGGCGTGGCCTACCAACAGGCACCACCGGGCGCCCAATTGCACTACCAGCATCCTGACCAAGTACACGCCCAGCAGCAACAGCAATACGCGCAACAGCAACAGCAATATGCCCAGCA 1320
 G  G  V  A  Y  Q  Q  A  P  P  G  A  Q  L  H  Y  Q  H  P  D  Q  V  H  A  Q  Q  Q  Q  Q  Q  Y  A  Q  Q  Q  Q  Q  Q  Y  A  Q  Q  Q   417
ATACCAACAGCAGCAGTACGGAAACGGACAGCAGCCTGTGCAGCTGCAACCCAACCAGGTTTACCAGCACGCTGGACAGATTCCGCAGCAACAACAACCGGTATACCAAAATCAACCTGT 1440
 Y  Q  Q  Q  Q  Y  G  N  G  Q  Q  P  V  Q  L  Q  P  N  Q  V  Y  Q  H  A  G  Q  I  P  Q  Q  Q  Q  P  V  Y  Q  N  Q  P  V   457
GTATCAGCAACAGCAGCCAGTCTATCAGCAGCAACAGCCAGTGCAGCAACAGCAAAAGCCTGTGCAACAGCCGGTGCAACAGCAGCAACAGCCTGTGCAACAGCAGCAGCATCCTGTGCA 1560
 Y  Q  Q  Q  Q  P  V  Y  Q  Q  Q  Q  Q  P  V  Q  Q  Q  Q  K  P  V  Q  Q  P  V  Q  Q  Q  Q  Q  Q  P  V  Q  Q  Q  Q  Q  H  P  V  Q   497
GCAGCAGCAGCAAACTGTGCAGCAACAGCAACCAGTACAGCAGCAGCAAACTGTGCAGCAACAGCAACCAGTACAGCAGCAGCAGCAAACTGCCCAACAGCAACCCGTAGCACAACA 1680
 Q  Q  Q  Q  T  V  Q  Q  Q  Q  P  V  Q  Q  Q  Q  Q  T  V  Q  Q  Q  Q  P  V  Q  Q  Q  Q  Q  T  A  Q  Q  Q  P  V  A  Q  Q   537
ACAGATCCACAATCAGAGTCCTCCGCCCGTTCTGAATCAACAGGTGCCAGTGCAGCAGCAACAGAAACAGCATCAAGAATCATTAAATCAACAACACTAAGCATTCCCTTGCTAACGCAT 1800
 Q  I  H  N  Q  S  P  P  P  V  L  N  Q  Q  V  P  V  Q  Q  Q  Q  K  Q  H  Q  E  S  L  N  Q  Q  H  Stop                      569
TTCTTT  1806
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Fig. 1. *nucb1* cDNA and deduced NUCB1 amino acid sequences. The arrow indicates the predicted signal peptide cleavage site. The EF-hand motifs numbered 1 (amino acid positions 239–267), 2 (291–319) and 3 (128–156) are underlined and their presumptive helical regions additionally printed in boldface. The basic region is identified by a broken line and the acidic region by a double line. The PVQ_5-repeat is printed in italics. A potential N-glycosylation site is located at amino acid position 541. Coiled-coils are predicted from amino acid positions 140 to 206 and 100 to 125. This sequence has been deposited in GenBank (accession no. AF044203).

whole-mount embryos using a polyclonal antibody generated against a synthetic NUCB1 peptide (Fig. 2B,D,F). The only notable difference in the mRNA and protein patterns was that the time point of zygotic protein expression in salivary glands was slightly delayed in comparison to the RNA expression. NUCB1 does not localize to the nuclei but rather to perinuclear cytoplasmic structures in the cellular blastoderm stage embryo (Fig. 2G). This location is seen more clearly in the cells of the salivary glands, where the protein is enriched between the nucleus and the lumen (Fig. 2H). In *Drosophila* Schneider tissue culture cells, the subcellular location of NUCB1 was assessed by indirect immunofluorescence studies (Fig. 3). The protein is localized in small clusters in the cytoplasm, which strongly resemble the Golgi apparatus, whose structures are spread over the whole cytoplasm in Schneider cells (Stanley et al., 1997). Neither the nuclei nor the cell surfaces were stained. Thus, these results are consistent with the localization of the NUCB1 protein in the embryo.

The subcellular localization of *Drosophila* NUCB1 is therefore in accordance with that of rat nucleobindin, which has been identified as a Golgi resident protein and termed CALNUC (Lin et al., 1998). However, it is in contrast to the observation of human nucleobindin in the nuclei of tumor cells (Wang et al., 1994), and the additional localization of human NEFA on cell surfaces (Barnikol-Watanabe et al., 1994). Furthermore, mouse nucleobindin was found in sera (Kanai et al., 1993) and, like human NEFA (Barnikol-Watanabe et al., 1994), in culture super-

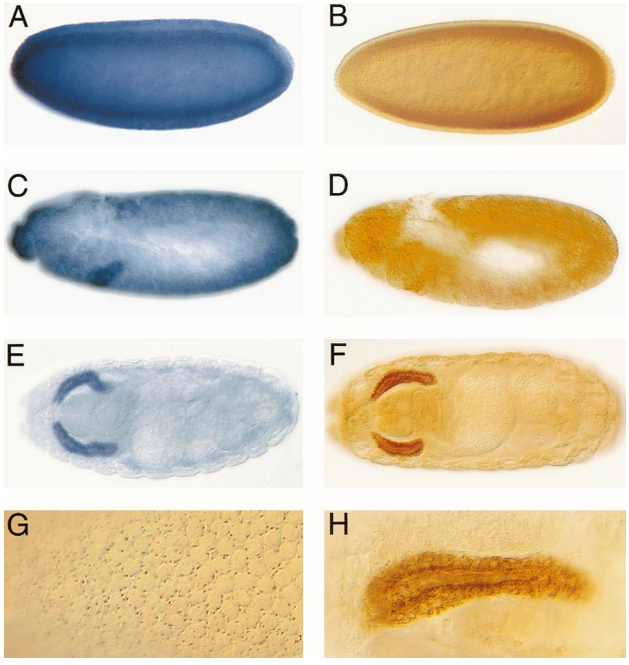

Fig. 2. Expression pattern of *nucb1* during embryonic development of *Drosophila*. (A,C,E) RNA in situ hybridizations with a *nucb1* antisense transcript. (B,D,F,G,H) anti-NUCB1 antibody staining experiments. (A,B) blastoderm stage; (C,D) late stage 11 in lateral view; (E,F) stages 16–17 in dorsal view; (G) enlargement of the blastoderm surface; (H) enlargement of a salivary gland at stage 17 in dorsal view. All embryos are oriented with their anterior to the left and dorsal up, except (E), (F), and (H) which show a dorsal view with anterior to the left.

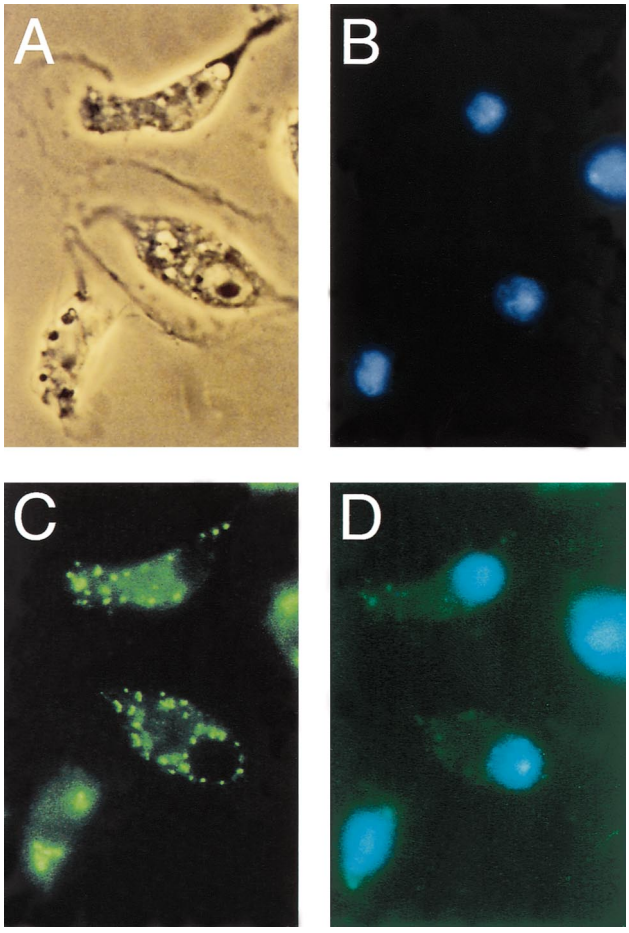

Fig. 3. Indirect immunofluorescence experiments. Adherent S2 cells were fixed, permeabilized and labeled with the affinity purified polyclonal anti-NUCB1-antibody. (A) Phase contrast; (B) DNA staining with Hoechst-33342; (C) fluorescence anti-NUCB1-antibody; (D) fluorescence anti-NUCB1-antibody and Hoechst-33342.

natants (Kanai and Tanuma, 1992). Bovine nucleobindin was isolated independently from bone matrix (Wendel et al., 1995). Thus, in addition to having identified the first insect member of the NEFA-nucleobindin class of calcium-binding EF-hand proteins, we show that it is ubiquitously expressed during embryogenesis and localized in a cytoplasmic structure likely to be the Golgi apparatus. The function of the protein has to await further analysis since a mutant for the gene, which maps to 75A3-75A7, is not yet available.

3. Experimental procedures

3.1. Cloning and sequence analysis of the nucb1 cDNA

A fragment of the *nucb1* gene was amplified by PCR using the primers 5′–GARGARTTYAARAARTAYGAR-ATG–3′ (forward) and 5′–CCANACYTCYTGNAR-YTGRTCYTT–3′ (reverse) and ligated into the pCR2.1 vector (Invitrogen). Three clones were sequenced and one of them was used as a template to prepare a digoxigenin-labeled DNA probe, which was used to screen an embryonic Canton-S cDNA library in the Lambda gt10 vector (Clontech). Out of 2×10^6 clones, 18 tested positive. One of them was sequenced and contained the complete open reading frame, which was analyzed using the GCG Wisconsin package 9.1 and the programs SignalP 1.1 (Nielsen et al., 1997), Coils 2.1 (Lupas, 1996) and Paircoil (Berger et al., 1995).

3.2. RNA in situ hybridization

Antisense and sense RNA probes were transcribed in vitro from the *nucb1* cDNA subcloned into the vector pBluescript SK (Stratagene) with the Digoxigenin RNA Labeling Kit (Boehringer), hybridized in situ to whole-mount embryos (Lehmann and Tautz, 1994) and detected using a pre-absorbed anti-digoxigenin-F_{ab} antibody fragment and NBT and BCIP (Boehringer) as alkaline phosphatase substrates.

3.3. Antibody staining

Polyclonal antisera against the peptide LPVTQNKKCHKEAAESC, coupled to KLH (Pierce), were raised in rabbits, preabsorbed against embryos and used for in situ labeling experiments at a final dilution of 1:3000. For immunoflurorescence studies, the antisera were affinity purified against the immobilized peptide and used at a dilution of 1:400. Whole-mount embryos were prepared and labeled as described (Patel, 1994). Bound antibodies were detected using the ABC Vectastain Elite Kit (Vector) and diaminobenzidine as substrate. S2 cells (Schneider, 1972; Invitrogen) were grown on coverslips, fixed with 8% formaldehyde, permeabilized with 0.3% Triton X-100 and blocked with SuperBlock (Pierce). The primary antibody was detected with a FITC-conjugated goat-anti-rabbit antibody (Sigma). Nuclei were stained with Hoechst-33342 (Molecular Probes).

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