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Golgi retention of human protein NEFA is mediated by its N-terminal Leu/Ile-rich region

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Abstract The subcellular localization of the human Ca^{2+}-binding EF-hand/leucine zipper protein NEFA was studied in HeLa cells by immunofluorescence microscopy. Double immunostaining using mouse anti-NEFA monoclonal antibody 1H8D12 and rabbit anti-ERD2 polyclonal antibody proved that NEFA is localized in the Golgi apparatus. The result was confirmed by the expression of NEFA–green fluorescent protein (GFP) fusion protein in the Golgi in the same cell line. Cycloheximide treatment proved NEFA to be a Golgi-resident protein. Seven NEFA deletion mutants were constructed to ascertain the peptide region relevant for Golgi retention. The expression of each NEFA–GFP variant was detected by fluorescence microscopy and immunoblotting. Only the ΔN mutant, lacking the N-terminal Leu/Ile-rich region, failed to be retained in the Golgi after cycloheximide treatment. The other six deletion mutants in which either the basic region, the complete EF-hand pair domain, the two EF-hand motifs separately, the leucine zipper and the leucine zipper plus the C-terminal region is deleted, were localized to the Golgi. The peptide sequence within the Leu/Ile-rich region is discussed as a novel Golgi retention motif.

Key words: NEFA protein; Nucleobindin (NUC, CALNUC); Calcium-binding EF-hand protein; Leu/Ile-rich region; ERD2; Golgi retention

1. Introduction

The human NEFA protein was originally identified in the common acute lymphoblastic leukemia cell line KM3 [1]. The NEFA precursor protein consists of 420 amino acid residues. It is a mosaic protein composed of seven regions. From the N- to the C-terminus the sequences are arranged as follows: a 24-residue signal peptide, a leucine/isoleucine (Leu/Ile)-rich region of 148 residues, a basic putative DNA-binding domain with a potential bipartite nuclear targeting signal, an EF-hand 1 motif, the acidic region, an EF-hand 2 motif and the leucine zipper region (Fig. 3). Mature NEFA protein was isolated from crude membranes and cytosolic fractions of KM3 cells. Its apparent relative molecular mass on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) is 55 kDa [1,2]. NEFA protein binds Ca^{2+}-ions via its EF-hand domain at a ratio of 2 mol Ca^{2+}/mol protein, hereby increasing its α-helical content [3].

Recently, the *Drosophila melanogaster* homolog was analyzed from mRNA of Oregon-R embryos and adult flies and also from the genomic DNA of the fly. Its expression was detected specifically in the larvae salivary glands and in the placodes from embryonal stage 11 onwards [4].

The biological role of NEFA is unknown up to now. NEFA shows strong sequence homology to the other EF-hand/leucine zipper protein nucleobindin (NUC) [1].

Murine NUC was detected and studied as a soluble factor promoting anti-DNA autoimmune responses in MRL/lpr (lupus-prone) mice [5,6], and the exon–intron structure of the human placental NUC gene has been reported [7]. Bovine NUC was studied as an extracellular bone matrix protein, in parallel rat NUC from calvaria was analyzed [8]. It was suggested that NUC may be involved in osteoid mineralization of bone. Rat NUC, synonym CALNUC, derived from GC (GH3) pituitary cells [9], was described as an abundant Golgi-resident protein, being involved in Ca^{2+} homeostasis in the Golgi compartment [10]. Rat CALNUC was found to interact with the G-protein $G_{\alpha i3}$ via its EF-hand domain [11]. Murine NUC had been shown to interact with $G_{\alpha i2}$ [12], and also with the cyclooxygenases COX-1 and COX-2 [13]. Co-overexpression of NUC and COX-1 or COX-2 reduced the NUC secretion level in COS-1 kidney cells. CALNUC from the lepidopteran insect *Spodoptera frugiperda* SF21 ovary cell line was detected in *cis*-Golgi compartments [14].

Recently, the postmitotic growth suppressor Necdin was described as interaction partner of murine NEFA and NUC. The coexpression of Necdin and NEFA was studied in undifferentiated P19 embryonic carcinoma cells and in via retinoic acid induction derived neuronal differentiated P19 cells. Elevated levels of both proteins were detected in the postmitotic neurons. Necdin binds to the NEFA EF-hand domain [15].

Here we present the subcellular localization of human NEFA in HeLa cells.

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Abbreviations: a.p., amino acid position; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; mAb, monoclonal antibody; pAb, polyclonal antibody; TRITC, tetramethylrhodamine isothiocyanate

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2. Materials and methods

2.1. Plasmid construction

The plasmid pEGFP-N1 was obtained from Clontech, restriction enzymes and ligase from Gibco/BRL and Vent polymerase used for all polymerase chain reactions (PCRs) from New England Biolab.

The coding sequence of NEFA was amplified by PCR from NEFA cDNA clone Hir25 adding cleavage site *Xho*I at the 5′-end and *Pst*I at the 3′-end using NeXhoI(f) and NePstI(r) as primers listed below. Seven variants lacking either the N-terminus (ΔN), the basic region (ΔB), the EF-hand 1 plus acidic amino acid-rich region plus EF-hand 2 (ΔEAE), the single EF-hands (ΔEF1 and ΔEF2), the leucine zipper (ΔL) and the leucine zipper plus the C-terminal region (ΔLC) respectively, were constructed. The coding sequence of the ΔLC variant was amplified by PCR using NeXhoI(f) and ΔLCPstI(r) as primers. For the construction of each of the six remaining variants two fragments containing the coding sequence from the 3′-end and the 5′-end to the depletion site, omitting the motif to be deleted, were amplified by PCR. Primers used were NeXhoI(f) and the appropriate Rev1 for each variant for the 5′-end fragment and For1 as well as NePstI(r) for the 3′-end fragment. PCR products of these PCRs were purified and used as templates in a second set of PCRs. Here, overlapping ends were added to the fragments using primers containing a nucleotide sequence homolog to 21 bp of the 5′-end of the 3′-end fragment as well as 21 bp of the 3′-end of the 5′-end fragment. Primers used were NeXhoI(f) and the appropriate Rev2 primer for the 5′-end fragment and For2 as well as NePstI(r) for the 3′-end fragment. The PCR products were again purified. The two fragments of each variant were now subjected to an assembly PCR. The first two cycles were run without any primers to leave time for annealing of the overlapping ends of the two fragments, thus forming the complete coding sequence of the variant. Then the primers NeXhoI(f) and NePstI(r) were added to amplify the sequence.

The PCR products of these six assembly PCRs, the ΔLC and the coding sequences of wild-type NEFA were purified by gel electrophoresis and cloned into the pEGFP-N1 vector after digestion by *Xho*I and *Pst*I. The recombinant plasmids were transformed into *Escherichia coli* DH5α. The open reading frame of the recombinant plasmids was verified by DNA sequencing on ABI Prism 377 (Applied Biosystems).

2.2. Primers

Terminal 5′-end and 3′-end primers:NeXhoI(f), 5′-TTATTCTCGAGCCTGAACATGAGGTGGAGGA-3′; NePstI(r), 5′-GAACCTCTGCAGAATGTGTGGCTCAAACTTCAATT-3′; ΔLCPstI(r), 5′-GAACCTCTGCAGTTTTTTTTCTGTGGCTTTCAA-3′; *Xho*I and *Pst*I cleavage sites are underlined.

Reverse primer 1 (Rev1): ΔNr1, 5′-AGCTTCAAGAGCAGTAAGTAA-3′; ΔBr1, 5′-GTCATAGTGTTCCAGATCACT-3′; ΔEAEr1, 5′-AAAGTCATTAGGATCCAATCC-3′; ΔEF1r1, 5′-AAAGTCATTAGGATCCAATCC-3′; ΔEF2r1, 5′-TCTAAGCCTTTCTTCTTCCAT-3′; ΔLr1, 5′-TTCTTCCTCTGTGAAGAACTG-3′. Forward primer 1 (For1): ΔNf1, 5′-AAGACTCGTCATGAAGAATTT-3′; ΔBf1, 5′-GGAAGCAAAGATCAACTAAAA-3′; ΔEAEf1, 5′-GAATTCTTGGAGCCAGATAGC-3′; ΔEF1f1, 5′-TTGGAGAAAGTATATGACCCT-3′; ΔEF2f1, 5′-GAATTCTTGGAGCCAGATAGC-3′; ΔLf1, 5′-GAGGCTCAGAAGCTGGAATAT-3′. Reverse primer 2 (Rev2): ΔNr2, 5′-AAATTCTTCATGACGAGTCTTAGCTTCAAGAGCAGTAAGTAA-3′; ΔBr2, 5′-TTTTAGTTGATCTTTGCTTCCGTCATAGTGTTCCAGATCACT-3′; ΔEAEr2, 5′-GCTATCTGGCTCCAAGAATTCAAAGTCATTAGGATCCAATCC-3′; ΔEF1r2, 5′-AGGGTCATATACTTTCTCCAAAAAGTCATTAGGATCCAATCC-3′; ΔEF2r2, 5′-GCTATCTGGCTCCAAGAATTCTCTAAGCCTTTCTTCTTCCAT-3′; ΔLr2, 5′-ATATTCCAGCTTCTGAGCCTCTTCTTCCTCTGTGAAGAACTG-3′. Forward primer 2 (For2): ΔNf2, 5′-TTACTTACTGCTCTTGAAGCTAAGACTCGTCATGAAGAATTT-3′; ΔBf2, 5′-AGTGATCTGGAACACTATGACGGAAGCAAAGATCAACTAAAA-3′; ΔEAEf2, 5′-GGATTGGATCCTAATGACTTTGAATTCTTGGAGCCAGATAGC-3′; ΔEF1f2, 5′-GGATTGGATCCTAATGACTTTTTGGAGAAAGTATATGACCCT-3′; ΔEF2f2, 5′-ATGGAAGAAGAAAGGCTTAGAGAATTCTTGGAGCCAGATAGC-3′; ΔLf2, 5′-CAGTTCTTCACAGAGGAAGAAGAGGCTCAGAAGCTGGAATAT-3′.

2.3. Antibodies

The rabbit anti-NEFA polyclonal antibody (pAb) Gu1 and the murine anti-NEFA monoclonal antibody (mAb) 1H8D12 of BALB/c mice, were generated in our laboratory against the recombinant human NEFA protein (rNEFA), heterologously expressed in *Pichia pastoris*. The pAb Gu1 was purified by affinity chromatography on Sepharose column coupled with rNEFA. The rabbit anti-ERD2 pAb was a kind gift of Prof. Dr. H-D. Söling, Department of Neurobiology, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany [16]. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin pAb and tetramethylrhodamine isothiocyanate (TRITC)-conjugated porcine anti-rabbit immunoglobulin pAb were purchased as secondary antibodies from Sigma.

2.4. Double immunolabeling

Adherent growing HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 μg/ml streptomycin and 100 μg/ml penicillin G under 5% CO_2 on slides using silicon coverslips. After 24 h the cells were washed in phosphate-buffered saline (PBS) and fixed with 8% paraformaldehyde. Fixed cells were labeled with mAb 1H8D12 at a dilution of 1:20 and subsequently with FITC-conjugated secondary antibody at a dilution of 1:200. Further cells were labeled with anti-ERD2 pAb at a dilution of 1:100 and subsequently with TRITC-conjugated secondary antibody. For the staining of the nuclei Hoechst H33342 dye was added to the last secondary antibody at 500 μg/ml. The cells were then analyzed with a fluorescence microscope (Axioskop, Zeiss).

2.5. Transfection

Cells cultured for 24 h on slides, as described above, were transfected for 2 h with 1.5 μg recombinant plasmid DNA dissolved in 7.5 μl SuperFect Transfection Reagent (Qiagen) according to the manufactural protocol. Prior to transfection the plasmid DNA was purified by Qiagen's Midi prep kit. The cells were fixed with 8% paraformaldehyde 24 h after beginning of the transfection. Fixed cells were labeled with anti-ERD2 pAb at a dilution of 1:100 and TRITC-conjugated secondary antibody to visualize the *cis*-Golgi compartment. Nuclei were stained by adding Hoechst H33342 dye. 24 h post-transfection the cells were treated with cycloheximide at a concentration of 60 μg/ml to block translation and then incubated at 37°C for up to 4 h [17]. The expression of protein NEFA was detected by fluorescence microscopy and immunoblotting.

2.6. Immunoblotting

5×10^5 cells were transfected in 75 ml culture flasks (Nunc) with the superfection kit using 9 μg plasmid DNA and 30 μl SuperFect reaction mix. 24 h later the cells were lysed with 1 ml Laemmli loading buffer containing 2.3% (w/v) SDS for 10 min at 100°C. After a short centrifugation step the cleared protein extracts were transferred to SDS–PAGE and subsequently electroblotted onto PVDF membrane [18]. The mAb IH8D12 or pAb Gu1 were added at dilutions of 1:200 and 1:1000, respectively, for immunodetection using the ECL system from Amersham Pharmacia Biotech.

3. Results

3.1. The subcellular localization of human NEFA

HeLa cells grown on slides were labeled with anti-human NEFA mAb 1H8D12 and anti-ERD2 pAb. NEFA was detected as epinuclear deposits (Fig. 1A and B), overlapping with the red fluorescence of the KDEL receptor protein ERD2, which marked the Golgi complex (Fig. 1C, yellow; Fig. 1D, red). In some cells also in the cytosol slight staining of NEFA was detectable. The result indicates that NEFA is localized in the Golgi apparatus.

3.2. The expression of NEFA–green fluorescent protein (GFP) fusion protein and its deletion mutants

The complete open reading frame of NEFA cDNA except the stop codon was fused to the 5′-end of the GFP gene in the

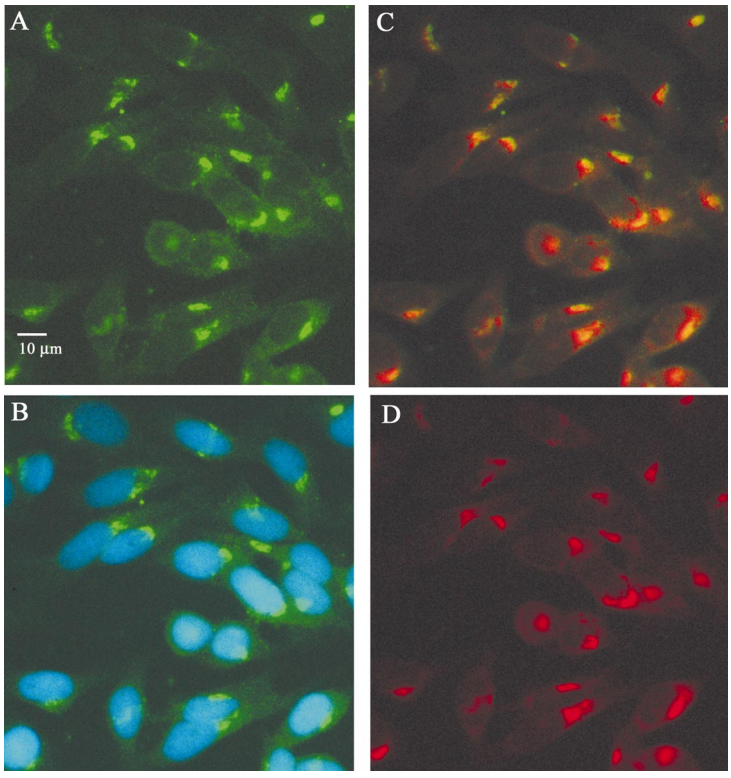

Fig. 1. Immunofluorescence staining of NEFA in HeLa cells. Fixed cells were labeled with anti-human NEFA mAb 1H8D12 stained by FITC (green), with anti-ERD2 pAb stained by TRITC (red) and with nuclear stain Hoechst H33342 (blue). A: NEFA (green). B: NEFA (green) and nuclei (blue). C: Overlapped staining NEFA plus ERD2 (yellow interference). D: ERD2 (red).

vector pEGFP-N1. The recombinant plasmid was transfected in HeLa cells and its expression was detected by fluorescence microscopy 24 h post-transfection directly in vital as well as in paraformaldehyde-fixed cells. Additionally, the fixed HeLa cells were labeled with the anti-ERD2 pAb and the Hoechst H33342 dye. The NEFA–GFP fusion protein accumulated epinuclear and overlapped with the ERD2 protein (Fig. 2a–d). This coincides with immunodetection of the endogenous

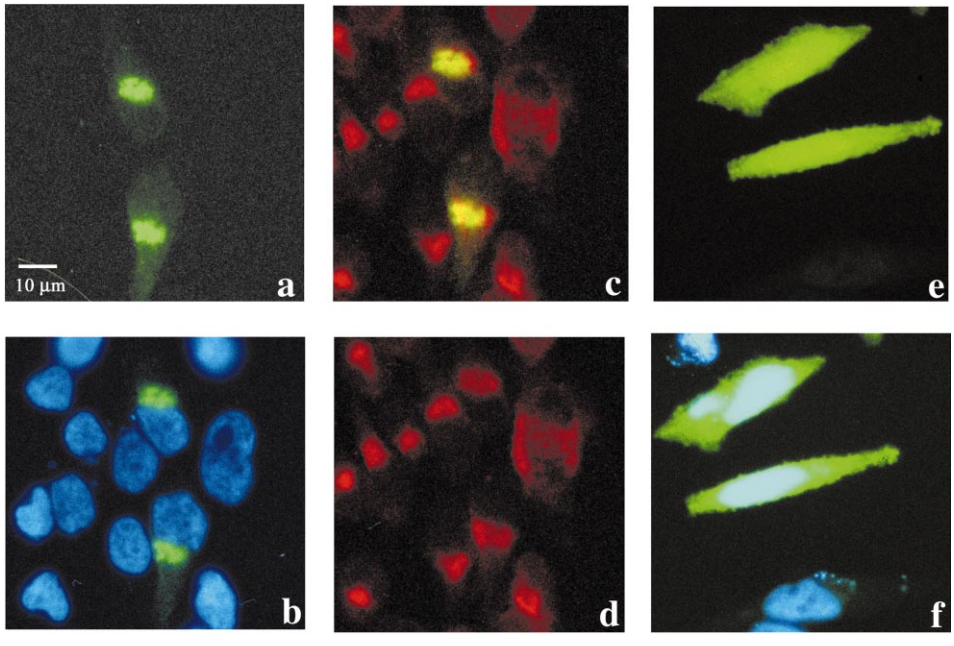

Fig. 2. Fluorescence microscopy analyses of the expression of NEFA–GFP fusion protein in HeLa cells. At 24 h after transfection the cells were fixed and labeled with anti-ERD2 pAb and TRITC, and with Hoechst H33342. a: Green fluorescing NEFA–GFP fusion protein in the transfected HeLa cells. b: NEFA–GFP (green) and nuclei, stained by Hoechst H33342 (blue). c: Overlapped staining NEFA–GFP plus ERD2 (yellow interference). e: GFP (green). f: GFP (green) and Hoechst H33342 (blue).

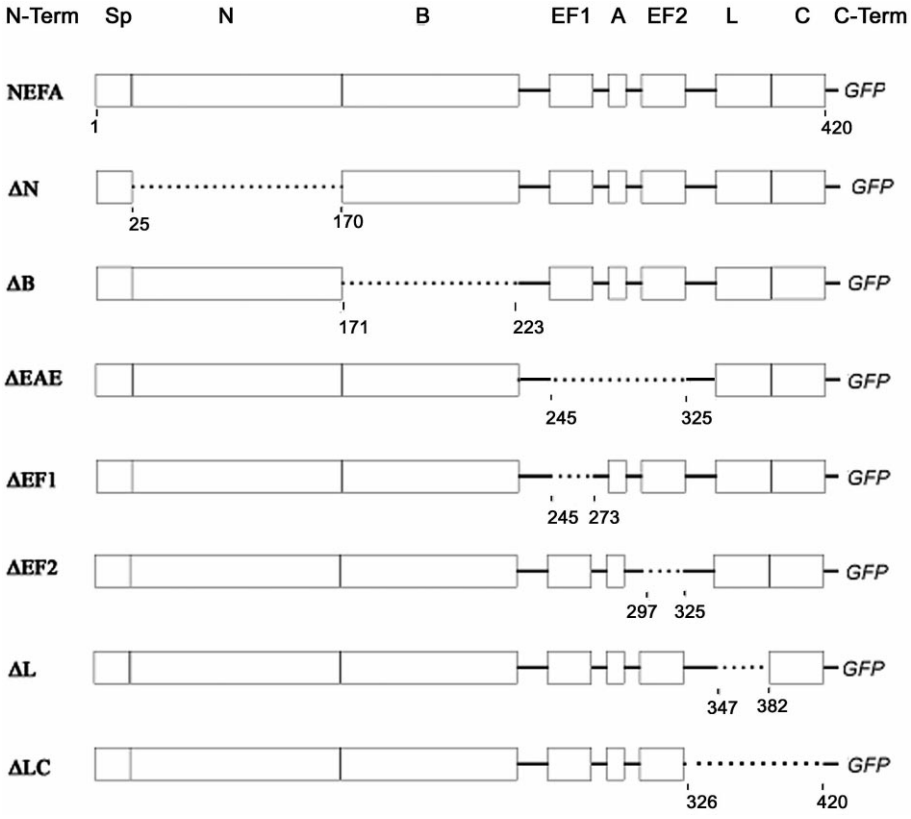

Fig. 3. Deletion mutants of the NEFA–GFP fusion protein. Numbers indicate the amino acid positions. Boxes signify the sequence motifs, solid lines the intervals. Dotted lines indicate the deleted regions, defined by the deficient first and last amino acids, which are denoted. Sp, signal peptide; N, Leu/Ile-rich region; B, basic region; EF1, EF-hand 1; A, acidic region; EF2, EF-hand 2; EAE, EF-hand 1+acidic region+EF-hand 2; L, leucine zipper region; LC, leucine zipper region+C-terminal region.

native NEFA, shown in Fig. 1. Thus, NEFA is localized in the Golgi. The expressed fusion protein was verified by immunoblotting of whole cell lysates 24 h post-transfection (see Fig. 4). The transfection rate of the recombinant plasmid pEGFP-N1-NEFA was 15–30%. The cells could be held in cell culture for maximal 3 days, showing morphological alterations like vacuolization and rounded cell shapes. For control experiments HeLa cells were transfected only by the vector pEGFP-N1. The transfection rate also was 15–30%, and as expected, GFP was detected all-over the cell (Fig. 2e). The GFP-transfected HeLa cells remained in culture longer than 3 days, showing no remarkable morphological alterations.

To elucidate the stability of the binding of NEFA in the Golgi apparatus the NEFA–GFP-transfected cells were treated with cycloheximide 24 h post-transfection. After incubation for 2 h and 4 h at 37°C the cells were fixed and the expression of the NEFA–GFP fusion protein was ascertained by fluorescence microscopy. The experiments were carried out in parallel with the seven different deletion mutants of NEFA in order to characterize the region which is engaged in the location of NEFA in the Golgi. The deletion mutant constructs are summarized in Fig. 3.

All seven mutant NEFA–GFP fusion proteins were expressed in HeLa cells and the expressed proteins were checked by immunoblotting of the cell lysates 24 h post-transfection, as shown in Fig. 4. The transfection rates of the pEGFP-N1-NEFA mutants were similar to that of the wild type. The transfected HeLa cells generally survived for at least 3 days in culture.

The expression of wild-type NEFA–GFP fusion protein and the deletion mutants, ΔN lacking the N-terminal Leu/Ile-rich region, ΔEAE lacking the complete EF-hand domain and ΔL lacking the leucine zipper, before and after cycloheximide treatment, are presented in Fig. 5. Only the ΔN mutant disappeared from the cells after cycloheximide treatment. In contrast, wild-type NEFA, ΔL and ΔEAE were retained in the Golgi longer than 4 h following cycloheximide treatment.

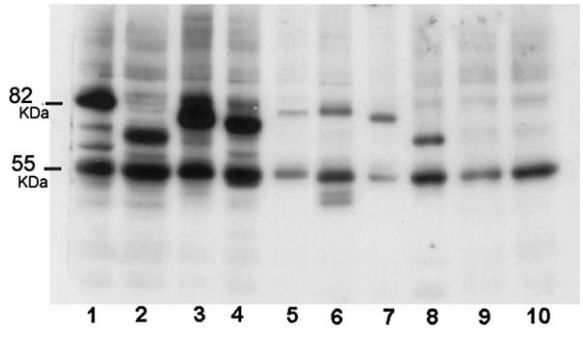

Fig. 4. Immunoblot of the expressed NEFA–GFP fusion protein and the deletion mutant–GFP fusion proteins. At 24 h post-transfection the HeLa cell lysates were supplied on 10% SDS–PAGE and blotted on PVDF membrane. The proteins were detected with the rabbit anti-NEFA pAb Gu1 and HRP-conjugated secondary antibodies using ECL system. Lanes 1, NEFA; 2, ΔN; 3, ΔB; 4, ΔEAE; 5, ΔEF1; 6, ΔEF2; 7, ΔL; 8, ΔLC; 9, GFP; 10, untransfected HeLa cells. The protein band at 55 kDa is the endogenous NEFA.

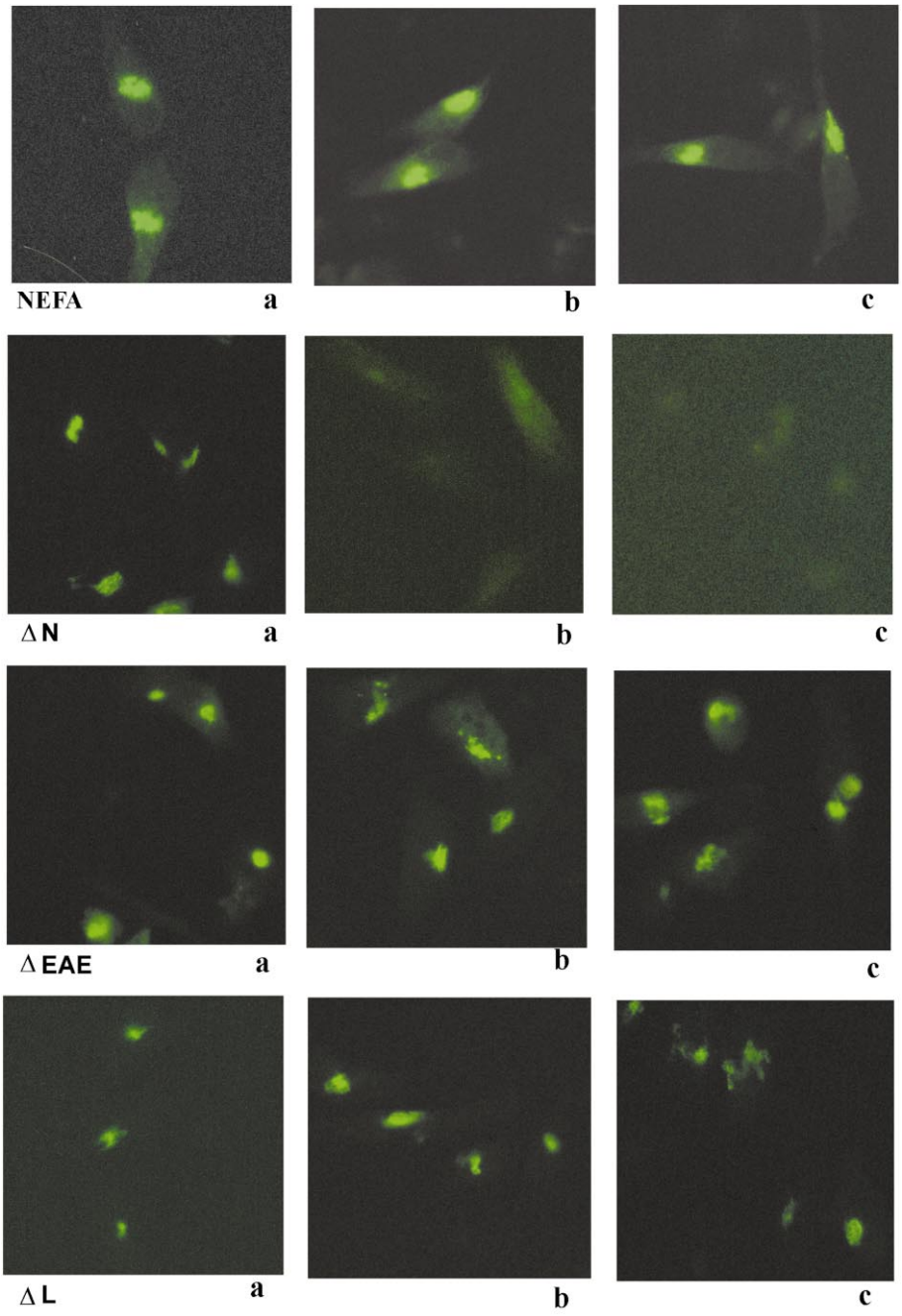

Fig. 5. The expression of NEFA–GFP fusion protein and the deletion mutants in HeLa cells before and after cycloheximide treatment, detected by fluorescence microscopy. At 24 h post-transfection the cells were treated with cycloheximide and incubated continuously at 37°C. a: untreated, b: 2 h and c: 4 h after cycloheximide treatment.

Other mutants as ΔB, ΔEF1, ΔEF2 and ΔLC also persisted in the Golgi (data not shown). The results indicate that NEFA is a Golgi-resident protein and that its Golgi retention signal is located in the N-terminal Leu/Ile-rich region.

4. Discussion

The NEFA homologs rat NUC, named CALNUC [9] and insect CALNUC [14] have been identified as Golgi-resident proteins, however their retention mechanisms are unknown. Both proteins, NEFA and NUC (CALNUC), contain neither transmembrane segments, nor a C-terminal KDEL tetrapeptide, which could mediate binding to ERD2, the KDEL receptor in the Golgi complex [16,19,20].

Recently, the soluble Ca^{2+}-binding EF-hand proteins reticulocalbin, calumenin, ERC-55 (synonym TCBP-49 or E6BP), Cab45 and CBP-50, were described as luminal proteins of the Golgi and the ER of mammalian cells [21]. These proteins are characterized by C-terminal HEEF, HDEF or HDEL tetrapeptides, which are defined as retention signals in the secretory pathway. Cab45 is strictly localized in the Golgi compartments and it contains a C-terminal HEEF motif [22]. However, both NEFA and NUC lack any of these C-terminal tetrapeptides. It is also excluded that NEFA via palmitoyla-

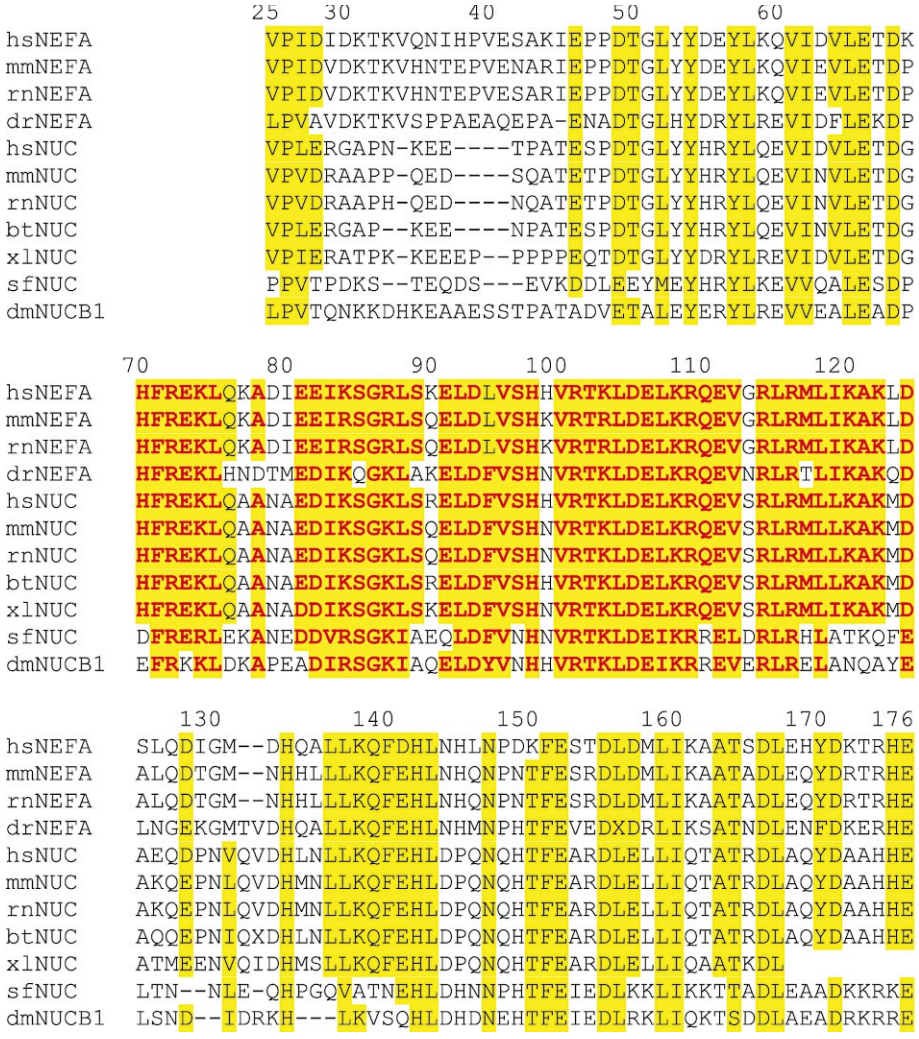

Fig. 6. Homology amino acid sequence alignment of the Leu/Ile-rich region from vertebrate and invertebrate members of the NEFA/NUC protein family. Consensus peptide clusters and common identical residues, including the equivalent acidic D/E, the basic K/R, the hydrophobic L/I/V and F/Y residues, are shaded yellow. The peptide stretches, discussed as putative Golgi retention relevant sequence motif, are typed in red. Dashes indicate gaps, introduced to optimize the homology alignment. Amino acid numbering according to human NEFA. GenBank/EMBL/DDBJ database entries: *Homo sapiens* hsNEFA: X76732; hsNUC: M96823, U1336–1340. *Mus musculus* mmNEFA: AJ2225; mmNUC: M96823. *Rattus norwegicus* rnNEFA: AF240142; rnNUC: Z36277. *Bos taurus* btNUC: E479146, BE589748. *Xenopus laevis* xlNUC: BG579082. *Danio rerio* drNEFA: AW018668. *S. frugiperda* sfNUC: AF170072. *D. melanogaster* dmNUCB1: AF044203, AF034856.

tion or myristoylation may be attached to Golgi membranes, like eNOS [23], SCG10 [24] and $G_{\alpha i3}$ [25], because NEFA is a cysteine-free protein and also a myristoylation motif is absent from its N-terminus.

NEFA and NUC proteins are highly conserved proteins. The strong amino acid sequence homology is not restricted to vertebrates, but includes also invertebrates, spanning about 830 million years of evolutionary distance of *Drosophila* and humans from their common ancestor [26].

Fig. 6 shows the alignment of the NEFA and NUC sequences corresponding to the N-terminal Leu/Ile-rich region of human NEFA, amino acid position (a.p.) 25–170, which by the above described experiments was defined as relevant for Golgi retention. The amino acid sequences homologous to a.p. 70–123 are predominantly consensus in these proteins. The segments, as shown in Fig. 6, by their alternating hydrophobic and charged amino acid residues may share similar structural characteristics. The peptide stretch typed in red is proposed as the putative Golgi retention motif of human NEFA. This would constitute a novel Golgi retention motif, preserved in the NEFA/NUC protein family members since early metazoan evolution.

Finally, we find that NEFA is ubiquitously expressed in cells from early embryonal stages as well as in adult tissues and in the various cell lines we studied.

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