NACA is a positive regulator of human erythroid-cell differentiation

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Summary

We have previously identified the transcript encoding NACA (the α chain of the nascent-polypeptide-associated complex) as a cytokine-modulated specific transcript in the human TF-1 erythroleukemic cell line. This protein was already known to be a transcriptional co-activator that acts by potentiating AP-1 activity in osteoblasts, and is known to be involved in the targeting of nascent polypeptides. In this study, we investigate the role of NACA in human hematopoiesis.

Protein distribution analyses indicate that NACA is expressed in undifferentiated TF-1 cells and in human-cord-blood-derived CD34+ progenitor cells. Its expression is maintained during in vitro erythroid differentiation but, in marked contrast, its expression is suppressed during their megakaryocytic or granulocytic differentiation.

Ectopic expression of NACA in CD34+ cells under culture conditions that induce erythroid-lineage differentiation leads to a marked acceleration of erythroid-cell differentiation. Moreover, ectopic expression of NACA induces erythropoietin-independent differentiation of TF-1 cells, whereas downregulation of NACA by RNA interference abolishes the induction of hemoglobin production in these cells and diminishes glycoporphin-A (GPA) expression by CD34+ progenitors cultured under erythroid differentiation conditions. Altogether, these results characterize NACA as a new factor involved in the positive regulation of human erythroid-cell differentiation.

Key words: Erythropoiesis, Cell differentiation, NACA, CD34+ cells, Lentiviral transduction

Introduction

The molecular mechanisms that control determination, self-renewal and differentiation of hematopoietic cells are tightly regulated through both extrinsic and intrinsic signals. One example that involves fine-tuned events concerns the differentiation of myeloid lineages. It has been clearly established that myeloid-cell-specific genes are regulated in part by the combinatorial action of multiple crucial, specific transcription factors present in common hematopoietic progenitor cells as well as by the participation of transcription co-factors (Cantor and Orkin, 2002; Perry and Soreq, 2002; Skalnik, 2002). However, understanding the mechanisms that direct myeloid-cell development and differentiation might require further events linking external stimuli and gene-expression cascades. As an example, in the erythroid-cell lineage (a subclass of the myeloid lineages), in addition to the well-established role of both the erythropoietin/erythropoietin-receptor (Epo/Epo-R) pair and the GATA1 transcription factor, other extrinsic signaling molecules including Wnt/frizzled (Van Den Berg et al., 1998), TGFβ (Zermati et al., 2000), fibroblast growth factors (Huber et al., 1998), growth-factor-independence IB (Osawa et al., 2002), insulin and insulin-like growth factor (Miyagawa et al., 2000), and intrinsically acting factors such as caspases (Zermati et al., 2001) have been reported to be important for erythropoiesis. Further identification of either novel molecules or complex sets of interactions between already-identified crucial transcription factors and co-factors might help to elucidate the molecular mechanisms that control the myeloid differentiation process.

Towards this end, we had previously performed a study in the TF-1 human erythroleukemic cell line to identify novel specific cytokine-induced genes using a gene-trap strategy (Baghdoyan et al., 2000). We thus identified the ubiquitous transcript α chain of nascent-polypeptide-associated complex (NACA) as one of those for which no biological function had been described so far in hematopoiesis. NACA was initially reported in yeast and higher eukaryotes as a heterodimeric complex that binds newly synthesized polypeptides emerging from ribosomes (Lauring et al., 1995; Wiedmann et al., 1994). Further studies revealed that NACA functions as a transcriptional co-activator in osteoblasts (Moreau et al., 1998; Yotov et al., 1998) through an interaction with phosphorylated c-Jun, a member of the activator-protein-1 (AP-1) family. A recent study indicated that NACA also regulates the function of the adaptor protein Fas-associated death domain (FADD) (Stilo et al., 2003). In addition, it is a target for degradation...
mediated by the 26S proteasome GSK3β (Quelo et al., 2004). These data suggest that NACA participates in several protein complexes playing a role in proliferation, apoptosis or degradation, depending on the cellular context and stimuli. Because the expression of NACA was modulated in the presence of granulocytemacrophage colony-stimulating factor (GM-CSF) (Baghdoyan et al., 2000), suggesting a role for NACA in the differentiation process of myeloid lineages, we addressed the question of the potential role of NACA in the development and differentiation of human myeloid-cell subclasses (i.e. erythroid, megakaryocytic and granulocytic cells). To that end, we examined both the expression pattern and the effect of NACA overexpression in such cells generated in vitro from human-cord-blood-derived CD34+ hematopoietic cells. We observed: (i) that NACA is maintained during in vitro erythroid differentiation of these cells but that, in marked contrast, its expression is suppressed during their megakaryocytic or granulocytic differentiation; (ii) that enforced expression of NACA in normal hematopoietic progenitors results in an acceleration of erythroid-cell differentiation; and (iii) that, in the TF-1 cell line, a decrease of hemoglobin expression correlates with silencing of NACA using RNA interference (RNAi). In conclusion, these results suggest that NACA is involved in the positive regulation of human erythroid-cell differentiation.

Materials and Methods

Culture cell lines and transfection of TF-1 cells

The human erythroleukemic TF-1 cell line was kindly provided by H. Gascan (Angers, France). It corresponds to a subclone of the original cell line established by T. Kitamura et al. (Kitamura et al., 1989). Human myelomonocytic U937 and THP1 cell lines were obtained from the ATCC (Rockville, MD).

Cells were cultured in RPMI 1640 containing 10% fetal calf serum (FCS) (Invitrogen, Life Technologies, Meylan, France), 2 mM L-glutamine, 200 IU ml–1 penicillin, and 100 µg ml–1 streptomycin at a mean density of 3×105 cells ml–1 in the presence of 5 ng ml–1 recombinant human GM-CSF (rhuGM-CSF; Sandoz, Rueil-Malmaison, France) for the TF-1 cell line or in the absence of cytokine from the ATCC (Rockville, MD).

Cells were cultured in RPMI 1640 containing 10% fetal calf serum (FCS) (Invitrogen, Life Technologies, Meylan, France), 2 mM L-glutamine, 200 IU ml–1 penicillin, and 100 µg ml–1 streptomycin at a mean density of 3×105 cells ml–1 in the presence of 5 ng ml–1 recombinant human GM-CSF (rhuGM-CSF; Sandoz, Rueil-Malmaison, France) for the TF-1 cell line or in the absence of cytokine for U937 and THP1 cell lines. Cultures were maintained at 37°C in a humidified 5% CO2 atmosphere.

Induction of TF-1-cell monocyte differentiation was performed by a 24-hour exposure to 1 ng ml–1 rhuGM-CSF and 10 nmole l–1 phorbol 12-myristate acetate (PMA) (Sigma, Saint Quentin Fallavier, France).

The presence of hemoglobin in TF-1 cells that had differentiated into erythroid cells was determined by benzidine staining according to the technique described by Graham and Karnovsky (1966).

For transfection experiments, the entire open reading frame of NACA was cloned in the bicistronic retroviral LZRS vector, containing the N-acetyltransferase (N-acetyltransferase) and the EGFP (enhanced green fluorescent protein) expression cassette under the control of the U3 and EF1α transcription units, respectively (Bour et al., 1988; Meylan, France). The vector plasmids were used to transfect the cells using the DMRIE-C reagent according to the manufacturer’s recommendations (Invitrogen).

NACA gene silencing by RNAi

NACA suppression was performed using a small interfering RNA (siRNA) sequence designed as described (Elbashir et al., 2001) (purchased from Dharmacon Research). The oligonucleotide sequence corresponds to residues 116-137 the coding region, relative to the first nucleotide of the start codon, and did match no other genomic sequence except that of the NACA pseudogene identified in a BLAST search from the NCBI website (http://www.ncbi.nlm.nih.gov/). The irrelevant siRNA was a control (non-silencing) siRNA (Qiagen-Xeragon). Duplex RNA was introduced into either TF-1 or CD34+ cells using the transfection reagent Jet-si (Qiogene) according to the manufacturer’s protocol. After 3 days in culture conditions supporting erythroid differentiation, TF-1 cells were harvested for either western-blot analysis or benzidine staining and CD34+ cells for glycoporphin-A (GPA) expression analysis.

Immunofluorescence analysis

Cells were fixed with 3% paraformaldehyde on ice for 30 minutes, permeabilized in 0.1% saponin, stained for 1 hour at room temperature with an anti-NACA antibody (Yotov et al., 1998) (1:1000 dilution), washed and then incubated for 30 minutes with a goat anti-rabbit antibody conjugated to Texas Red (Beckman Coulter, Marseille, France). Stained cells were viewed with a confocal microscope (Zeiss LSM2). Images were processed for presentation using NIH Image and Adobe Photoshop 4.0.

Enrichment of cord-blood CD34+ cells

The human CD34+ cells were obtained from umbilical-cord-blood cells (CB) collected after normal full-term deliveries. CB samples were separated on Ficoll density gradients (lymphocyte separation medium; Eurobio, Les Ulis, France). Low-density cells were recovered and enriched for CD34+ cells using positive immunomagnetic selection with magnetic cell separation (MACS) technology according to the manufacturer’s recommendations (Miltenyi Biotec, Bergish Gladbach, Germany).

Construction and production of lentiviral vectors

The entire open reading frame of NACA was cloned in the lentiviral TRIPΔU3-ΕF1α vector plasmid (Sirven et al., 2001) between the BamHI and KpnI restriction sites, under the transcriptional control of the EF1α ubiquitous promoter sequence, to generate the NACA-lentiviral vector. Lentiviral particles were produced as described (Sirven et al., 2001). Briefly, the vector plasmids (either NACA or EGFP control) were introduced into 293-T cells, together with an encapsidation plasmid (p8.91) lacking the Vif, Vpr, Vpu and Nef accessory HIV-1 proteins, and a vesicular stomatitis virus (VSV) protein-envelope-expression plasmid (pHCMV-G) by transient calcium-phosphate co-transfection. The concentrations of lentiviral particles were normalized by measuring the p24 (HIV-1 capsid protein) content of the supernatant.

Transduction of CD34+ cells with lentiviral vectors

106 CD34+ cells ml–1 were plated in 96-well plates in Iscove-modified Dulbecco medium (IMDM; BioWhittaker Europe, Belgium), supplemented with 20% BIT 9500 [mixture of bovine serum albumin (BSA), insulin and transferrin (Stem Cell Technologies, Meylan, France)], 2 mM L-glutamine, 200 IU ml–1 penicillin, and 100 µg ml–1 streptomycin, 80 ng ml–1 recombinant human stem cell factor (rhSCF; Amgen, Thousand Oaks, CA), 10 ng ml–1 thrombopoietin (TPO), pegylated recombinant human megakaryocyte growth and development factor (rhPEG-MGDF) (Amgen), 100 ng ml–1 Flt3 ligand (rhFlt3-L; DNAX, Palo Alto, CA) and 60 ng ml–1 recombinant human interleukin-3 (rhIL-3; R&D Systems Europe, UK). Transduction was performed with either NACA or EGFP lentiviral vectors.
vector particles added at a concentration corresponding to 2500 ng p24 per ml (Amsellem et al., 2002). After 24 hours at 37°C, a second set of particles was added to the cultures. After an additional 48-hour period, cells were harvested by centrifugation, washed and then cultured in conditions that support cell differentiation as described below.

**CD34** + cell differentiation

Erythroid cells were generated from CD34 + cells cultured in either RTM (MABIO-International, Tourcoing, France) or IMDM, supplemented with 20% BIT 9500, 2 mM L-glutamine, 200 IU ml −1 penicillin, 100 µg ml −1 streptomycin, 10 ng ml −1 rhU-L-3, 25 mg ml −1 rhuSCF and 10 ng ml −1 rhuL-6. 2 IU ml −1 recombinant human erythropoietin (rhuEpo) was added at day 7 and cultures were carried on for 7 additional days to achieve terminal red-cell differentiation as already described (Freyssinet et al., 1999).

Megakaryocytic cells were generated from CD34 + cells cultured for up to 14 days in IMDM, 20% BIT 9500, 2 mM L-glutamine, 200 IU ml −1 penicillin, 100 µg ml −1 streptomycin containing TPO at a final concentration of 50 ng ml −1 and 5 ng ml −1 rhuSCF.

Granulocytic cells were generated from CD34 + cells cultured in RTM supplemented with 20% BIT 9500, 2 mM L-glutamine, 200 IU ml −1 penicillin, 100 µg ml −1 streptomycin containing 10 ng ml −1 rhU-L-3, 10 ng ml −1 rhuSCF, 15 ng ml −1 recombinant human granulocyte colony-stimulating factor (rhU-G-CSF; Neupogen, Amgen) and 50 ng ml −1 rhuFlt3-L for 14 days.

**Cytochemistry**

Cell cultures were maintained at 37°C in a humidified 5% CO 2 atmosphere at a mean density of 3 × 10^5 cells ml −1. For microscopy examination, cells were recovered, cytospun onto a slide glass and then subjected to May-Grunwald-Giemsa staining.

**PCR analysis of vector integration**

Integration of the vector into the cellular genome was analysed by PCR on genomic DNA extracted from cells cultured for 10 days under conditions that promote granulocytic differentiation. Amplification of genomic DNA was performed on whole-cell extracts with primers that amplify part of the EF1α sequence (5′-ATCCACCTTGGCTGATACGC-3′) as the forward primer and part of NACA (5′-GTCATCCCTGACTGTAGCTAAGCTTC-3′ and reverse primer 5′-GGTCTGGAACAGAATCT-3′) as the reverse primer. Amplification was performed for 35 cycles at an annealing temperature of 57°C, producing a 1200-bp PCR product.

**Detection of NACA transcript by reverse-transcription PCR**

For reverse-transcription PCR (RT-PCR), mRNA from cells cultured under granulocytic differentiation conditions for 10 days was extracted using the 5µMACS mRNA isolation kit (Miltenyi Biotec, Bergish Gladbach, Germany), treated with DNAse I (Boehringer, Mannheim, Germany) and was reverse transcribed with the SuperScript II RNase H-Reverse Transcriptase (Life Technologies, Invitrogen, Cergy Pontoise, France) according to the manufacturer’s instructions, using a random primer poly-d(T)12-18 (Amersham Pharmacia Biotech). For the S-tagged-NACA PCR, 5 µl cDNA were taken from the reverse transcription reaction and samples were submitted to 35 cycles of PCR in a Perkin-Elmer thermal cycler (Perkin-Elmer, Norwalk, CT) using 30 seconds of denaturation at 94°C, 30 seconds of annealing at 57°C and 1 minute of elongation at 72°C. A final elongation step was performed for 7 minutes at 72°C. The primers used were: 5′-AGATCTCGGCTAAGCTACGTTCACATG-3′ (forward) and 5′-CTCGGAGACAGACACTGGATCAGTTATC-3′ (reverse), amplifying a product of 1029 bp from the NACA-encoding gene. To monitor the efficiency of the reactions, β2 microglobulin transcript was assessed using the forward primer 5′-CCACGAGAGAATGGAAAGCT-3′ (contained in the first exon of the gene) and the reverse primer 5′-GATGCTGTTACAGTGTCGG-3′ (contained in the second exon of the gene). The size of the amplified product was 268 bp. The products were electrophoresed on 1.2% agarose gel.

For detection and relative quantification of mRNA levels of endogenous NACA, a LightCycler (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer’s instructions, mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an external control. PCR reactions were performed with a LightCycler FastStart DNA Master SYBR Green PCR Kit (Roche Diagnostics, Mannheim, Germany). PCR was carried out in a final volume of 20 µl, with each primer at 0.5 µM, 3 µM MgCl 2, 2 µl supplied enzyme mix containing the reaction buffer, FastStart Taq DNA polymerase and DNA double-strand-specific SYBR Green I dye in a LightCycler with a 10 minute preincubation at 95°C, followed by 40 cycles of 15 seconds at 95°C, 28 seconds at 60°C and 26 seconds at 72°C. The following primers were used: GAPDH-specific forward primer 5′-GTCACTCCTGAGCTAGAAGG-3′ and reverse primer 5′-GGCTCTTACCTCTGGAGGC-3′; NACA-specific forward primer 5′-GGTCTGGAAACAGAATCTGCAGTTATC-3′ and reverse primer 5′-AGCTCGAGTTACCTCTTGGACACA-3′. Analyses of quantitative real-time PCR curves were performed by absolute quantification at 530 nm by the LightCycler 4.0 software. The GAPDH control reflected the amount of target mRNA in each sample.

**Flow cytometry analysis**

Cells were stained with a fluorescein-isothiocyanate (FITC)-coupled CD34 monoclonal antibody (mAb) (Beckman Coulter, Marseille, France) for the identification of progenitor cells, an anti-glycoporphin-A mAb coupled to phycoerythrin (GPA-PE) (Beckman Coulter, Marseille, France) for the identification of erythroid cells, an anti-CD42b-PE mAb or an anti-CD61 mAb coupled to cyanin-5 (Cy5) for megakaryocytes (BD Biosciences, Heidelberg, Germany) and an anti-CD15-PE mAb for granulocytic cells. Isotypic controls were performed with the same sets of cells. Cells were then subjected to FACS scanning analysis using a FACS Vantage (Becton Dickinson, Mountain View, CA). Control cells were used to set the FACS gains and analysis gates. Cells were analysed according to side scatter, forward scatter, FITC and PE characteristics.

**Western blot analysis**

Cells were harvested and resuspended in lysis buffer 1% NP-40, 50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 2% v/v protease inhibitor cocktail (0.02 mg ml −1 pancreas extract, 0.02 mg ml −1 chymotrypsin, 5 × 10 −4 mg ml −1 thermolysin, 0.02 mg ml −1 trypsin, 0.33 mg ml −1 papain) (Roche Diagnostics, Mannheim, Germany). Protein concentration of lysates was determined with a Bio-Rad protein assay kit (Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer’s recommendations, using BSA as a standard.

20 µg proteins from extracts (total lysate) were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membrane (Amersham, Arlington Heights, IL). The membranes were probed with a rabbit anti-NACA immune serum (Yotov et al., 1998) followed by an anti-rabbit immunoglobulin (lg)-coupled to horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories, Baltimore, MD). Antigen detection was visualized by using the super signal west pico chemiluminescence system (Perbio Science, Pierce, France).

After signal extinction, membranes were probed again with a mouse anti-Grb2 or a rabbit anti-phosphosidostide-3-kinase-p85 immune serum (Santa Cruz Biototechnology, Heidelberg, Germany).

For ectopic expression of S-tagged-NACA, 3 × 10^5 cells were lysed.
Lysates were incubated for 2 hours with 10 µl protein-S-coated beads and retained proteins were resolved by 10% SDS-PAGE and then western blotted.

Results
Expression of NACA in TF-1 cells cultured under either erythroid or monocytic differentiation conditions
We had previously identified the NACA-encoding gene as a cytokine-modulated gene in the erythroleukemic TF-1 cell line. In order to investigate the regulation of NACA in hematopoietic cells, we examined the expression of NACA in the TF-1 cell line under various culture conditions. This cell line offers the advantage that it proliferates when cultured in the presence of GM-CSF and differentiates into hemoglobin-positive cells when cultured in the presence of Epo for several days. It also undergoes monocyte/macrophage differentiation when exposed to PMA (Kitamura et al., 1989; Hoang et al., 1996). We observed that, when exposed to PMA, TF-1 cells displayed a marked underexpression of NACA (Fig. 1) compared with the same cells cultured without PMA (basal expression). In addition, similar NACA expression could be detected in other monocyte/macrophage cell lines such as THP1 and U937 (Fig. 1). By contrast, when TF-1 cells were triggered to erythroid differentiation in the presence of Epo, NACA expression was maintained (or slightly enhanced) in the long term during the erythroid differentiation course (Fig. 1). These data indicate that NACA is preferentially maintained during erythrocytic differentiation of the TF-1 cells, whereas it is downregulated during monocyte/macrophage differentiation.

Enforced expression of NACA in TF-1 cells induces Epo-independent erythroid differentiation
The effect of NACA in the TF-1 cells was also examined using ectopic expression of the molecule. Cells cultured with GM-CSF were transfected with a retroviral vector that drives the expression of both NACA and EGFP from a single bicistronic transcript (Michiels et al., 2000). The efficiency of this transfection was about 2% (Fig. 2A). EGFP-positive (+) cells (about 10^5 cells per transfection experiment) were selected by cell sorting then cultured in the presence of GM-CSF for 24 hours. Among such sorted NACA-transfected cells, 80% were benzidine stained, indicating hemoglobin production in an Epo-independent manner (Fig. 2B). These data strongly argue in favor of a role for NACA in the erythroid differentiation of TF-1 cells.

Fig. 2. Enforced expression of NACA induces erythroid differentiation of TF-1 cells in the absence of Epo. TF-1 cells were transfected with NACA-IRES-EGFP vector plasmid and cultured for 48 hours in the presence of GM-CSF. Cells were then sorted by FACS (10^5 EGFP-positive and EGFP-negative cells were collected) and benzidine stained. (A) Fluorescence imaging showing 2% of the EGFP-positive cells obtained after transfection with the NACA-IRES-EGFP vector plasmid. (B) Benzidine staining of sorted cells (EGFP-positive and EGFP-negative) performed 24 hours after EGFP sorting. Hemoglobinized cells stain dark blue. The proportion of benzidine was quantified after counting 200 cells. 80% of the sorted cells were benzidine stained (mean from three different experiments).

Silencing of NACA by RNAi results in a reduction of the Epo-induced hemoglobin expression in TF-1 cells
In order to examine whether silencing the NACA-encoding
gene would have an effect on erythroid-cell differentiation, we performed gene-silencing experiments on TF-1 cells cultured in the presence of Epo. Confocal analysis (Fig. 3A) showed that NACA was no longer detectable within the cells transfected with the siRNA duplexes chosen to selectively inhibit NACA expression. In addition, the proportion of TF-1 cells targeted with the siRNA duplexes was high as assessed by the number of cells where NACA was no more detected. Western-blotting analyses (Fig. 3B) clearly showed that a 3-day treatment of these cells with a specific siRNA for NACA caused a profound decrease in NACA protein production. In control groups, consisting of mock-transfected cells or cells transfected with an irrelevant siRNA, the NACA protein level remained unchanged. The reduction of the level of NACA was accompanied by a dramatic drop in the number of TF-1 cells that synthesized hemoglobin compared with the number of cells that displayed unchanged level of NACA, when cultured in the presence of Epo (3.8% vs 12%, respectively) (Fig. 3C). After additional days of culture in the presence of Epo, the NACA siRNA effect was abolished and hemoglobin synthesis was restored in the treated cells. A maximum of 50% hemoglobin-positive cells was reached at day 7 in treated and untreated cells (Fig. 3C, right). These results indicate that the downregulation of NACA impairs hemoglobin production by TF1 cells.

Expression of NACA in human cord-blood cells is restricted to CD34+ cells and to the erythroid-cell lineage

In order to examine the role of NACA in cord-blood-derived
normal cells, we studied the cell specificity of NACA expression in human primary CD34+ progenitor cells expanded ex vivo and the same cells induced to differentiate along the erythroid, the megakaryocytic or the granulocytic lineage (Fig. 4A). Growth and maturation of the cells were examined by studying the cell-surface markers CD34 (hematopoietic progenitors), GPA (erythroid cells), CD42b and CD61 (megakaryocytic cells), and CD15 (granulocytic cells) (Fig. 4B). Cell extracts were analysed for the presence of NACA protein by western blotting. Under these conditions, we observed NACA production in the primary human hematopoietic CD34+ progenitors and throughout the successive stages of erythroid-cell differentiation, from progenitors (days 7 to 7+2) to mature erythroblasts and erythrocytes (day 7+7). By contrast, NACA production was abolished early during megakaryocytic and granulocytic differentiation, whereas NACA-encoding transcript was still detected in these differentiated cells (Fig. 4C) after 10 days of culture. These results indicate that NACA production is tightly regulated during hematopoiesis. Its high production level,
NACA influences erythropoiesis present in hematopoietic progenitor cells, persists during erythroid differentiation.

NACA overproduction in cord-blood CD34+ cells induces acceleration of erythroid differentiation in vitro

In order to explore further the putative role of NACA in the behavior of normal progenitor cells, we looked at the effect of NACA overproduction in human CD34+ cells. For that purpose, we engineered the lentiviral TRIPΔU3-EF1α-S-tagged-NACA vector (Sirven et al., 2001; Amsellem et al., 2002), which contains the coding sequence of NACA tagged at its N-terminus with an S-tag sequence. CD34+ cells were transduced by this lentiviral vector or the EGFP control vector and then cultured under conditions that trigger either erythroid- or granulocytic-cell differentiation. These two cell lineages were chosen both for their distinct NACA expression and for the high number of cells that can be obtained in liquid culture (by comparison with megakaryocyte culture). Transduction of the cells by the S-tagged-NACA lentiviral vector was assessed by western-blot detection of NACA after enrichment of the ectopic protein (Fig. 5A). Surprisingly, although the expression of EGFP was maintained along the granulocytic differentiation process (Fig. 5A, middle, B), no ectopic S-tagged-NACA
protein was detected in the granulocytic cells. Nevertheless, the integrated S-tagged NACA gene was present in the genome (Fig. 5C) and was transcribed as shown in Fig. 5D using RT-PCR analysis of the mRNA of these cells. These results indicate that the ectopic expression of NACA cannot be maintained in the granulocytic cells, a cell lineage in which downregulation of the endogenous NACA protein is also observed (Fig. 4). By contrast, the ectopic form of the protein was detected in cells grown under erythroid differentiation conditions (Fig. 5A, top). Transduction of NACA in CD34+ cord-blood cells further cultured under erythroid differentiation conditions did not change their ability to proliferate because the absolute number is similar by either NACA or EGFP transduction (result not shown).

Interestingly, an early effect of NACA was revealed by the number of cells that expressed the erythroid-cell-specific cell-surface marker GPA (Fig. 6A,B). Indeed, 5 days after transduction, the total number of GPA-positive cells was strongly enhanced (when compared with similar cultures initiated with control EGFP-transduced cells; 71.5% vs 30%) (Fig. 6B). The mean value of fluorescence intensity detected in these cells was twice as high, indicating an increased number of GPA molecules at the surface of the NACA-transduced cells (data not shown). In addition, cytological analysis revealed that NACA-transduced cells are characterized by a higher number of mature erythroblasts (Fig. 7) in comparison with the GFP-transduced cells, which contain more immature erythroblasts (i.e. 45% acidophilic erythroblasts versus 4%, respectively) (Fig. 7). These results demonstrate that the transduction of NACA both accelerates erythroid maturation of cells and increases the number of erythroid-cell-specific GPA molecules.

In the absence of Epo, transduction of NACA in CD34+ cultures had no effect on the expression of the GPA marker. The cells expressed similar level of GPA when transduced with either NACA or GFP and died after 7 days of culture (data not shown).

### Table 1

|                | Day 5 | Day 7 | Day 9 |
|----------------|-------|-------|-------|
| EGFP Control   | 30    | 49.7  | 81.7  |
| NACA           | 71.5  | 76.8  | 87.8  |

### Table 2

| Cell type              | Day 7 | Day 12 |
|------------------------|-------|--------|
| proerythroblasts       | 18    | 1      |
| basophilic erythroblasts| 71    | 18     |
| polychromatophilic erythroblasts | 7      | 36     |
| acidophilic erythroblasts | 4      | 45     |

### Fig. 6
Flow-cytometry analysis of GPA surface expression in NACA- and EGFP-transduced CD34+ cells along differentiation of the erythroid lineage. 72 hours after the initiation of transduction, CD34+ cells were cultured under conditions optimized for erythroid-cell growth and maturation. Cell suspensions were stained at the indicated days of culture (day 5 (D5), day 7 (D7) and day 9 (D9)) with an anti-GPA-PE mAb and analysed by flow cytometry for cell-surface expression of GPA. (A) Unfilled profiles represent GPA expression by EGFP-transduced cells and filled profiles represent GPA expression by NACA-transduced cells. Isotype-matched control mAb was used in all experiments to set the parameters for analysis. Positive GPA fluorescence (gate M1) is set according to the isotype-matched control mAb. One representative experiment out of three performed is shown. (B) Results are expressed as the proportion of GPA cell-surface-marker-positive cells present in the cell culture.

### Fig. 7
Morphological characterization of either NACA- or EGFP-transduced CD34+ cells cultured in erythroid differentiation conditions. Pictures represent May-Grunwald-Giemsa staining of either EGFP- or NACA-transduced cells cultured for either 7 days or 12 days under erythroid differentiation conditions. The table indicates the proportion of proerythroblasts and basophilic, polychromatophilic and acidophilic erythroblasts in these transduced cells. Different cell types were quantified after counting a total of 200 cells per experiment (mean of three different experiments).
Effect of NACA-specific RNAi on CD34+ cells cultured in conditions that support erythroid differentiation

The efficiency of siRNA transfection is low in CD34+ cells, making it difficult to analyse either the protein-extinction or the cytological characteristics of the cells after transfection. However, CD34+ cells cultured under conditions that support erythroid differentiation with an siRNA specific for NACA caused a significant threefold decrease in the number of cells expressing the erythroid marker GPA (6.35% vs 20.95%) (Fig. 8) in comparison with untreated cells. These results suggest a functional correlation between the expression of NACA and the erythroid marker GPA on CD34+ cells.

Discussion

The rationale of this study was to investigate the putative role of NACA in hematopoietic-cell differentiation. NACA was initially identified as being encoded by a cytokine-responsive gene (Baghdoyan et al., 2000). In this study, we propose a new role for NACA as a regulator of erythroid differentiation. First, we showed that the NACA production level is selectively maintained during erythroid-cell differentiation, whereas it is downregulated during cell differentiation towards other lineages. This is observed both in the human TF-1 leukemic cells during erythroid versus granulocytic differentiation and in primary CD34+ human progenitor cells during erythroid versus megakaryocytic or granulocytic differentiation. In addition, the production of NACA is also maintained in MEL cells (a mouse erythroleukemic cell line) when cultured in erythroid conditions (result not shown) and downregulated in monocyte/macrophage cell lines such as THP1 and U937. More importantly, functional studies showed that enforced expression of NACA is sufficient to induce the Epo-independent hemoglobinization of TF-1 cells, as well as to accelerate the Epo-dependent erythroid differentiation of CD34+ progenitors. Finally, we found that the use of RNAi reduces the extent of Epo-dependent hemoglobinization of TF-1 cells and the number of CD34+ progenitors that produce the Epo-dependent marker GPA. Altogether, we thus conclude that NACA is a crucial positive regulator of erythroid-cell differentiation.

Whether NACA is acting at the level of erythroid-cell-lineage determination or the level of maturation of already committed cells remains an important question. The enforced production of NACA did not affect the number of either erythroid cells or BFU-E (burst-forming unit-erythroid) generated by NACA- or EGFP-transduced CD34+ progenitor cells grown (respectively) under erythroid or semi-solid-culture conditions. These results do not support a role for NACA in erythroid-cell-lineage determination of already committed cells but, by contrast, NACA-transduced cells in culture undergo accelerated erythroid differentiation processes. This acceleration is evidenced by the higher GPA expression levels as well as by the higher proportion of matured cells observed at early times of culture and a clear early hemoglobinization of the colonies derived from the NACA-transduced cells (data not shown). However, once differentiated, mature erythroid cells generated in the presence or absence of enforced NACA production are morphologically indistinguishable. Besides, although the enforced expression of NACA appears to be sufficient to induce the Epo-independent differentiation of TF-1 cells, this is not the case for native CD34+ multipotent progenitor cells, which still require the presence of Epo to differentiate towards the erythroid lineage. This result favors the hypothesis that NACA is acting at the level of erythroid-cell maturation rather than at the level of erythroid-cell-lineage determination of already committed cells. The Epo-dependence or -independence could be explained by the fact that Epo-independent differentiation of TF-1 cells might be a consequence of the activation of an alternative pathway because of a higher level of NACA produced via transfection of TF-1 cells than the level of NACA.

Fig. 8. Effect of siRNA on the GPA cell-surface expression of CD34+ cells cultured in erythroid differentiation conditions. CD34+ progenitor cells were cultured for 3 days under conditions optimized for erythroid-cell growth and maturation in the presence of either an siRNA specific for NACA or an irrelevant siRNA. The GPA cell-surface marker was analysed by flow cytometry in the absence of siRNA duplexes (left), in the presence of an siRNA specific for NACA (center) and in the presence of an irrelevant siRNA (right). The numbers (insert) correspond to the proportion of GPA-positive cells.
produced via lentiviral infection of normal CD34+ cells. However, a reasonable hypothesis would also take into account the fact that TF-1 is a leukemia-derived cell line predisposed to the erythroid-cell lineage and that has probably already undergone erythroid-specific differentiation genetic events during the leukemic process. By contrast, in normal cells, NACA might be involved at a step that requires Epo for differentiation and/or maturation. Based on all these data, we conclude that NACA is a crucial limiting factor that is required for erythroid-cell maturation.

Although the NACA-encoding transcript appeared to be slightly downregulated in the differentiated cells, they were detected in the three studied cell lineages, whereas NACA protein was only detected in erythroid cells. Unexpectedly, whereas exogenous NACA transcripts could be detected in both erythroid and granulocytic differentiated infected cells, exogenous NACA protein could only be detected in erythroid differentiated cells. Thus, in addition to the discovery of a new function of NACA in erythroid-cell maturation, this study also demonstrates a strikingly different regulation of the NACA protein in erythroid versus granulocytic or megakaryocytic differentiated cells. The mechanism of this regulation might involve protein-protein interactions and/or protein modifications. Given the recent evidence for the involvement of GSK3β kinase in the regulation of proteasomal degradation of NACA (Quelo et al., 2004), it would be interesting to determine the importance of the proteasome-mediated downregulation of NACA in granulocytic and megakaryocytic cells, and of its maintenance in erythroid cells.

In addition to this post-translational modification as a possible molecular mechanism for the contribution of NACA in erythroid-cell differentiation, several possibilities arise from the previously reported activities of NACA. As a subunit of the nascent-polypeptide-associated complex (NAC) (Lauring et al., 1995; Wiedmann et al., 1994), NACA influences the targeting of polypeptides devoid of signal peptide. As a consequence of the association of NACA at the surface of the ribosomes, these proteins do not undergo rough-endothelial-reticulum processing and therefore retain a cytosolic localization. Both Epo and Epo-R that exhibit a signal peptide should not be influenced by the presence of NACA. However, the possibility cannot be excluded that this mechanism is involved in the cytosolic retention of other factors important for erythroid cell maturation. NACA is also known as a co-activator of c-JUN-mediated transcription in developing bone during embryogenesis (Moreau et al., 1998; Quelo et al., 2002). However, because c-JUN behaves as a negative regulator of erythropoiesis (Elagib et al., 2004), the positive effect of NACA on erythroid-cell maturation cannot be explained by its c-JUN-mediated positive contribution. Intriguingly, a recent study reports the positive effect of the c-JUN co-activator RNA helicase RHI/Guô through its association with MafK and other transcription factors that are essential for erythroid-cell-specific transcription (Brand et al., 2004). Another interesting possibility is related to the recently described function of NACA as a negative regulator of apoptosis, through its interaction with FADD (Stilo et al., 2003). It is notable that a deregulation of the FADD-dependent pathway is reported to result in an ineffective erythropoiesis in some patients with myelodysplastic syndromes (Fontenay-Roupie et al., 1999). Further experiments are obviously needed to distinguish between these and other possible mechanisms to understand the function of NACA in erythroid-cell maturation. We believe that the identification of protein partners of NACA in erythroid cells will be a definitive step towards this goal.

On the whole, this study demonstrates the unexpected crucial role of NACA in erythroid-cell maturation. New challenges are now to identify the upstream and downstream pathways that are involved in this new function of NACA.

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