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Biochemical and Biophysical Research Communications 305 (2003) 1061–1066

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## Selection of peptides with affinity for the N-terminal domain of GATA-1: identification of a potential interacting protein

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Received 15 April 2003

### Abstract

As most transcription factors, GATA-1 activities are mediated by interactions with multiple proteins. Those identified so far associate with the zinc-finger domain and/or surrounding sequences. In contrast, no proteins interacting with the N-terminal domain have been identified although several evidences suggest its involvement in the control of hematopoiesis. In an attempt to identify proteins that interact with the N-terminal transactivation domain of GATA-1, a random phage peptide library was screened with recombinant GATA-1 protein and the sequence of a selected peptide was used for database protein sequence retrieval. We selected a set of peptides sharing the core sequence  $\phi$ -B<sub>(2-3)</sub>-V<sub>(2-4)</sub> (where  $\phi$ , B, and v represent hydrophobic, basic, and neutral residues, respectively). Using the sequence of the most represented peptide (pep5) as query, we retrieved the HIV accessory protein Nef. We show that Nef binds GATA-1 and GATA-3 in vitro in virtue of its sequence homology with pep5.

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**Keywords:** GATA-1; Transcription factors; Phage display peptide libraries; HIV; Nef

GATA-1 is the founder of a family of transcription factors that bind to the consensus DNA motif (A/T)GATA (A/G) through a zinc-finger domain highly conserved among the family members. Several GATA proteins have been identified in vertebrates (GATA-1–6) as well as in yeast, fungi, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Xenopus leavis*, and *Arabidopsis thaliana* [1,2]. GATA-1 is expressed in erythroid cells, megakaryocytes, eosinophils, and mast cells of the hematopoietic lineages as well as in Sertoli cells of the testis [3–9]. Gene targeting loss-of-function studies in mice have proved that GATA-1 plays an essential role in normal erythropoiesis and in the maturation of megakaryocytes [10,11]. Erythroid cell precursors lacking the X-linked GATA-1 gene arrest at the proerythroblast stage of development and undergo apoptosis [12].

Like most transcription factors, GATA-1 contains discrete functional domains. The DNA-binding domain consists of two C<sub>4</sub> zinc-fingers whose roles have been extensively analyzed either in cell cultures or in vivo [13,14]. The C-terminal finger is essential for the binding to the DNA and mediates the regulation of GATA-1 target genes through the interaction with other transcription factors such as Sp1, EKLF, and PU.1 [15–17]. Sequences surrounding the carboxyl terminal finger are targets of acetylation which stimulates the transcription activity of GATA-1 [18]. The N-terminal finger influences the stability and specificity of DNA binding, mediates the interaction with the coactivator FOG-1 [19], and is directly involved in the GATA-1-dependent maturation of megakaryocytes [20]. The N-terminal region of GATA-1 (NT) encompasses the first 83 residues and functions as a transactivation domain in non-hematopoietic cells [21–23]. Due to an alternative translation initiation site usage, the NT domain is missed in a GATA-1 isoform (GATA-1s) expressed in human and murine erythroid cells [23]. The role played by the NT domain in the hematopoietic cell differentiation is still

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debated. Phenotypic rescue studies conducted in G1E cells, a murine GATA-1-null embryonic stem cell line expressing the human anti-apoptotic bcl-2 protein [24], have shown that the NT domain of the mouse GATA-1 is dispensable for terminal erythroid maturation [25]. In contrast, rescue analyses conducted in vivo show that the NT domain is required for definitive erythropoiesis in mice [26]. Moreover, recent studies have shown that acquired mutations in the GATA-1 gene are associated with acute megakaryoblastic leukemia in children with Down syndrome [27]. The mutations prevent the synthesis of the full-length protein but not of the GATA-1s isoform. These findings suggest that the NT domain is involved in important, though subtle, cellular mechanisms probably mediating the interaction of GATA-1 with yet unknown cellular partners. Since protein–protein interactions often occur between discrete motifs that can be mimicked by simple peptides, we chose to apply the powerful phage display technology to identify novel peptides with affinity for GATA-1. By this means several biologically relevant peptides have been identified [28–31] and their analysis has provided insights into the natural binding partners of target ligands [32].

Screening a linear 9-residue random phage peptide library against the recombinant N-terminal region of mouse GATA-1 we identified a set of binding peptides that share sequence homology. The deduced amino acid sequence of the most represented selected peptide is homologous to a sequence within the N-terminal flexible anchor domain of the HIV/SIV accessory protein Nef [33,34]. We show that the Nef domain binds GATA-1 in vitro and that the binding is competed by selected peptides in a dose-dependent manner.

## Materials and methods

**Recombinant plasmids and Escherichia coli strains.** Restriction and modification enzymes were from New England Biolab. To construct the expression vector pGST–NTGATA-1, the sequence encoding for the amino-terminal domain of the murine GATA-1 (residues 1–83) was PCR amplified from pGDwt [23] using as primers the oligonucleotides NTfor (5'-CGGAATTCATGGATTTTCCTGGTCTAG-3') and NTrev (5'-CGCTCGAGTACTGTTGAGCAGTGGATA-3'). The amplified DNA fragment was restricted with *EcoRI* and *XhoI* and inserted into pGEX-4T1 vector DNA. To construct the expression vector pGST–Nef<sub>(1–35)</sub>, pLXSN/Nef vector DNA [35], containing the sequence encoding for Nef, BRU allele, was restricted with *BamHI* and *XhoI*. The 116 bp DNA fragment, encoding for residues 1–35, was inserted into pGEX 4T3. The *EcoRI* DNA fragment containing the full-length cDNA of murine GATA-3 (gift from Frank Grosveld) was cloned in pCDNA3 (Invitrogen) to yield the pGATA-3 construct. Plasmids were routinely propagated in *E. coli* cell strain HB101 and DNAs were purified by the alkali lysis method (Qiagen). The construct sequences were confirmed by automated DNA sequencing.

**Protein expression and affinity resins.** GST, GST–NTGATA-1, and GST–Nef<sub>(1–35)</sub> proteins were expressed in *E. coli* cells, strain BL21. Bacteria transformed with pGEX4T1, pGST–NTGATA-1 or pGST–Nef<sub>(1–35)</sub>, respectively, were grown in 100 ml cultures at 37 °C in LB medium (1% Bacto tryptone, 0.5% yeast extract, and 0.5% sodium

chloride) containing 250 µg/ml ampicillin. At OD<sub>600</sub> = 0.5, protein expression was induced for 1 h with 1 mM IPTG. Cells were harvested by centrifugation, suspended in 5 ml lysis buffer (PBS containing 1% Triton X-100, 100 mM EDTA, 1 mM PMSF, and 5 µg/ml leupeptin), and disrupted by five sonication pulses. Cell debris were pelleted by centrifugation and supernatants were incubated with 250 µl of packed G-Sepharose 4B resin (Amersham) for 1 h at room temperature. Resin slurries were transferred into Poly-prep columns (Bio-Rad) and washed three times with 5 ml PBS. Bound proteins were eluted with 1 ml of 1 mM glutathione and extensively dialyzed against PBS. Purified proteins were checked by SDS–PAGE, quantified by Bradford colorimetric assay, and bound to new G-Sepharose 4B to yield affinity resins.

**Phage library screening.** A phagemid library displaying linear 9-mer random peptides as fusion products of the M13 phage pVIII capsid protein was provided by Dr. F. Felici [36]. The initial diversity of the library was of  $4 \times 10^7$  clones. For each selection and amplification round, two Poly-prep columns were packed with 200 µl GST and GST–NTGATA-1 affinity resins, respectively, and washed with MPBS (2% powdered milk in PBS). The phages ( $5 \times 10^{10}$ ) were adsorbed to the GST column and the unbound phages were loaded onto the GST–NTGATA-1 resin. After extensive washes, bound phages were eluted with 300 µl of 100 mM triethylamine and neutralized with 150 µl of 1 M Tris–HCl, pH 7.4. Eluted phages were titrated and amplified in *E. coli* cells, strain TG1, as described [37]. Five rounds of selection and amplification were performed.

**Colony immunoscreening.** To check the enrichment of positive clones, cells carrying phagemids from each round of selection were infected with the M13 helper phage K07 to induce phage particle synthesis as described [37]. About 100 CFU was plated onto 2× TY–agar containing 35 µg/ml IPTG, 50 µg/ml kanamycin, and 250 µg/ml ampicillin and incubated overnight at 37 °C. Colonies grown on these master plates were replicated on nitrocellulose filters. For immunoscreening, membranes were washed briefly with PBS and incubated for 30 s in 20% acetic acid to inactivate cell phosphatase activities. After blocking with I-buffer (PBS, 0.1% NP-40, and 5% non-fat dried milk) for 2 h at 4 °C, filters were incubated with 2 µg/ml of soluble GST–NTGATA-1 protein overnight at 4 °C. After extensive washes with W-buffer (PBS, 0.1% NP-40), filters were probed with a rabbit anti-GST antibody. The immunocomplexes were revealed by a goat anti-rabbit IgG conjugated to alkaline phosphatase. Positive clones were picked from master plates and grown in 3 ml of 2× TY containing ampicillin. Phagemid DNAs were extracted according to standard procedures and sequenced.

**In vitro binding assays.** <sup>35</sup>S-labelled GATA-1 and GATA-3 proteins were synthesized in reticulocyte lysates according to the manufacturer's instructions (Promega). Briefly, 250 ng pGDwt or pGATA-3 DNA was transcribed and translated in 12.5 µl volume reactions containing 10 µCi [<sup>35</sup>S]methionine and T7 RNA polymerase. Reactions were incubated for 90 min at 30 °C and used immediately or frozen at –80 °C. For binding assays,  $1.5 \times 10^5$  TCA-precipitable cpm of labelled protein was mixed with 18 µg GST or 21 µg GST–Nef<sub>(1–35)</sub> resins (same molar equivalent) in 150 µl of binding buffer (25 mM Hepes, 150 mM NaCl, and 0.5% Triton X-100, pH 7.2). After 2 h at 4 °C, resins were transferred into microspin columns (Bio-Rad) and centrifuged briefly. Resins were washed four times with binding buffer and the bound proteins were eluted with 30 µl Laemmli's buffer and loaded onto a 10% SDS–PAGE. The gel was fixed in 1 M salicylic acid for 1 h, dried, and exposed overnight. For competition assays, the peptides pepNef (RMRAEPAA), pep5 (RMRRSNPTL), and pep5-mut (EM-DASNPTL) were synthesized (R&D). Peptides were reconstituted in binding buffer and the protein concentration was determined. TCA-precipitable cpm ( $10^4$ ) of GATA-1 or GATA-3 was mixed with 425 µg GST–Nef<sub>(1–35)</sub> resins in 160 µl of binding buffer. The larger amount of resin used in the competition with respect to the binding assay was to ensure the quantitative binding of labelled proteins. The binding was challenged with 10- or 100-fold molar excess of each peptide. The

bound complexes were analyzed by SDS-PAGE and fluorography as described.

**Results**

*Identification of peptides binding to the N-terminal domain of the mouse GATA-1*

To identify peptides that bind to the N-terminal domain of GATA-1, we screened a phage library displaying random linear 9-residue peptides by affinity chromatography. The N-terminal domain of the mouse GATA-1 (NTGATA-1) was bacterially expressed as a fusion product of the glutathione S-transferase (GST-NTGATA-1) and affinity purified onto G-Sepharose resin. The GST-NTGATA-1 affinity resin was prepared as described in Materials and methods and used for phage library screening. To subtract for phage-displayed peptides with affinity for the GST protein moiety, the input phages were absorbed onto a GST-Sepharose resin at each round of selection/amplification. Five rounds of selection/amplification were carried out and the enrichment of clones binding to NTGATA-1 was assessed by colony immunoscreening. *E. coli* cells infected with the phagemids eluted from the GST-NTGATA-1 resin were superinfected with the M13 helper phage K07 and plated on agar. Colonies were replicated onto nitrocellulose filters and probed with soluble GST-NTGATA-1

protein. Positive clones were revealed by immunostaining using anti-GST antibodies. As much as 10% and 90% positive clones were obtained from the fourth and fifth rounds, respectively (Fig. 1).

*Sequences of peptides binding to NTGATA-1 and genomic database search*

The DNA inserts of 20 individual positive clones from the fifth round of selection/amplification were analyzed and their deduced peptide sequences are shown in Table 1. The alignment of peptide sequences reveals that they share the core motif  $\phi$ -B<sub>(2-3)</sub>-v<sub>(2-4)</sub>, where  $\phi$ , B, and v represent hydrophobic, basic, and neutral residues, respectively. The pep5 sequence, the most represented peptide binding to NTGATA-1, was used to search genomic databases for proteins containing sequence homology. All non-redundant coding sequences available at the GenBank database were searched with the program BLASTP [38]. The first 43 sequences scored by homology degree corresponded to the Nef protein, an accessory viral protein expressed by the HIV-1, HIV-2, and SIV viruses. The homology spans residues 19–26 of the amino-terminal region of Nef and ranges from 75% to 87% depending on Nef alleles (Fig. 2).

Table 1  
9-mer peptides binding NTGATA-1

Name of phage clones	Peptide sequences	Number of time selected
pep1	L R T R K S S L S	4
pep2	G L R Q R K F S S	1
pep3	L M H R R T N P P	2
pep4	A N Y R R P Q A V	1
pep5	R M R R S N P T L	5
pep6	Y R A H R A R A V	1
pep7	L R A K R T S I T	1
pep8	Y R L I K R N S P	1
pep9	R L R R H Q L V L	1
pep10	Y K R H S P H A P	1
pep11	Y K R R I V E P L	1
pep12	Y K R H E A R I V	1
Core motif <sup>a</sup>	$\phi$ B <sub>(2-3)</sub> v <sub>(2-4)</sub>	

<sup>a</sup>  $\phi$ , B, and v represent hydrophobic, basic, and neutral residues, respectively.

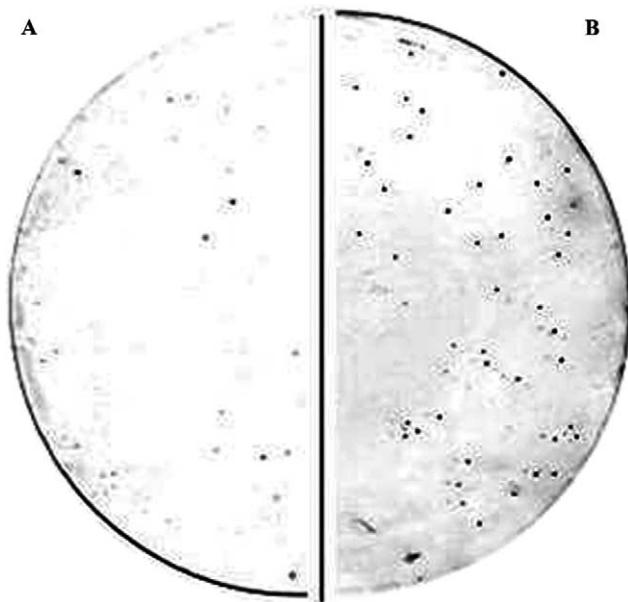


Fig. 1. Immunodetection of phage-displayed peptides that bind NTGATA-1. The expressing phagemids ( $\cong 100$  CFU) eluted from the fourth (A) and fifth (B) rounds of selection were replicated onto nitrocellulose filters and probed with soluble GST-NTGATA-1. Protein complexes were detected with rabbit anti-GST serum and immunostained with a secondary antibody conjugated to alkaline phosphatase.

pep5 RMRRSNPTL  
 Nef MGGKWSKSSVVGWPTVRERMRAEPAADGVGAASRDLEKHGA  
 ITSSNTAATTNAACAWLEQEEEEVGFVTPQVPLRPMTYKAA  
 VDLSHFLKEKGGLEGLIHSQRQDILDLWIYHTQGYFPDWQN  
 YTPGPGVRYPLTFGWCYKLVPEPDKVEEANKGENTSLHPV  
 SLHGMDPPEREVERFDSRLAFHHVARELHPEYFKNC

Fig. 2. Sequence of the Nef protein. The sequence of the Nef protein, BRU allele, is shown. The homology with pep5 is underlined.

### The amino-terminal domain of Nef binds GATA-1 in vitro

Nef is a protein encoded by primate immunodeficiency viruses [39]. Although Nef is important in the pathogenesis of lentiviruses (reviewed in [40]), the molecular mechanisms underlying its functions are partly understood. Nef structure consists of two domains: a N-terminal flexible membrane anchor domain (residues 1–57) and a well-folded major core domain (residues 58–206). The core domain contains several sequence motifs that mediate the interaction of Nef with proteins involved in cellular signalling or trafficking pathways (reviewed in [41]). The N-terminal domain contains the sequence for myristoylation that mediates the membrane anchoring of the protein [42]. More recently, it has been shown that Nef binds p53 via its N-terminal domain and protects cells against p53-mediated apoptosis [43]. Since the Nef amino-terminal domain includes the sequence homologous to pep5, we decided to verify whether this domain binds to GATA-1. The sequence encompassing residues 1–35 of Nef, allele BRU, was expressed in bacteria as a GST fusion protein (GST–Nef<sub>(1–35)</sub>) and used to prepare an affinity resin suitable for in vitro binding assays. As shown in Fig. 3, <sup>35</sup>S-labelled GATA-1 is specifically bound by the GST–Nef<sub>(1–35)</sub> affinity resin.

### Synthetic peptides compete for GST–Nef<sub>(1–35)</sub> binding to GATA-1

To assess whether Nef<sub>(1–35)</sub> binds to GATA-1 through the residues homologous to pep5, we performed in vitro

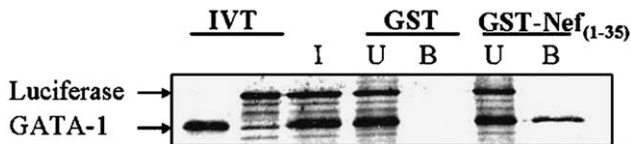


Fig. 3. The N-terminal domain of Nef binds GATA-1 in vitro. In vitro translated (IVT) <sup>35</sup>S-labelled GATA-1 or luciferase was mixed (I) and loaded onto GST or GST–Nef<sub>(1–35)</sub> resins. Unbound (U) and bound (B) proteins were analyzed by SDS–PAGE and fluorography.

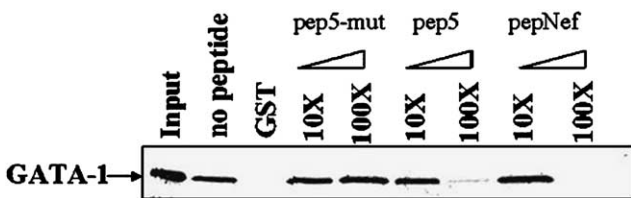


Fig. 4. The binding of the N-terminal domain of Nef to GATA-1 is competed by peptides pep5 and pepNef. In vitro translated <sup>35</sup>S-labelled GATA-1 (Input) was loaded onto the GST resin (GST) or the GST–Nef<sub>(1–35)</sub> resin and the binding was unchallenged (no peptide) or challenged with a 10- (10×) or 100-fold (100×) molar excess of the indicated competitor peptides. Bound protein was analyzed by SDS–PAGE and fluorography.

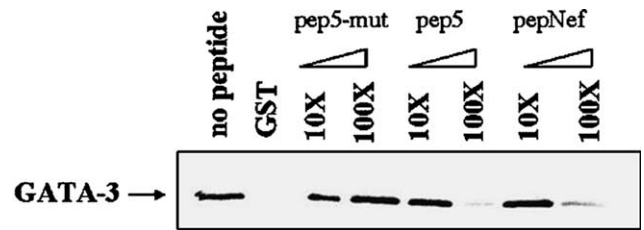


Fig. 5. The N-terminal domain of Nef binds GATA-3. In vitro translated <sup>35</sup>S-labelled GATA-3 (Input) was loaded onto the GST resin (GST) or the GST–Nef<sub>(1–35)</sub> resin and the binding was unchallenged (no peptide) or challenged with a 10- (10×) or 100-fold (100×) molar excess of the indicated competitor peptides. Bound protein was analyzed by SDS–PAGE and fluorography.

competition assays. We synthesized the nonapeptides RMRRSNPTL, corresponding to the peptide pep5, the mutant EMDASNPTL (pep5-mut), and RMRRAE-PAA (pepNef), corresponding to the Nef residues 19–27 of the BRU allele product. The binding of GST–Nef<sub>(1–35)</sub> to <sup>35</sup>S-labelled GATA-1 was challenged with 10- or 100-fold molar excess of each peptide and the bound GATA-1 was analyzed by SDS–PAGE. As shown in Fig. 4, either pep5 or pepNef efficiently competes for GATA-1 binding while the mutant peptide does not. This result indicates that the Nef<sub>(1–35)</sub>/GATA-1 interaction is specific and involves the arginine residues 19, 21, and 22 of the Nef N-terminal flexible anchor domain.

### The N-terminal flexible anchor domain of Nef binds another GATA member in vitro

Nef plays key functions in the HIV-1 infection of T lymphocytes. In these cells GATA-3 is the predominantly expressed GATA factor. As all GATA members, the sequence homology between GATA-1 and GATA-3 is restricted to the zinc-finger domain. However, the transactivation domain of GATA-3 resides within the N-terminal region of the protein [44]. Thus, we decided to verify whether Nef<sub>(1–35)</sub> binds GATA-3 as well in vitro. As shown in Fig. 5, GST–Nef<sub>(1–35)</sub> binds GATA-3 and the binding is competed by peptides pep5 or pepNef while it is not by the mutant peptide pep5-mut.

## Discussion

GATA-1 is a zinc-finger transcription factor that plays a pivotal role in erythroid and megakaryocytic cell maturation. A growing body of evidence indicates that GATA-1 activities are mediated by the assembling of multiprotein complexes on discrete GATA-1 functional domains. To date, several proteins have been identified on the basis of their ability to interact physically with the two zinc-finger domains of GATA-1 ([45] and references therein). In contrast, very little is known about the proteins that interact with other GATA-1 domains.

In particular, no cellular partners have been yet identified to bind the N-terminal domain of GATA-1, though several experimental and genetic data strongly suggest its involvement in important processes. In this regard, the studies have been mainly hampered by the impossibility to count on conventional methods for screening. For instance, the yeast two-hybrid system, the leading method used to identify interacting partners, is not applicable to the GATA-1 N-terminal domain since it efficiently transactivates yeast gene promoters (unpublished data). As described here, we have taken an alternative approach to identify proteins potentially capable of interacting with GATA-1. Screening a phage random peptide library, we have identified a set of phages that bind the N-terminal transactivation domain of GATA-1 (residues 1–83). The phage-displayed peptides share the sequence core motif  $\phi$ -B<sub>(2-3)</sub>-V<sub>(2-4)</sub> (where  $\phi$ , B, and v represent hydrophobic, basic, and neutral residues, respectively) which mediates the physical interaction with GATA-1 as proved by competition binding assays. Searching databases for proteins containing sequence homology to the most represented peptide (pep5), we retrieved the HIV accessory protein Nef. The homology spans residues 19–26 of the amino-terminal domain (numeration referred to the BRU allele product) and ranges from 75% to 87% depending on Nef alleles. We show that in virtue of this homology the amino-terminal domain of Nef binds GATA-1 and GATA-3 *in vitro*. The competition data indicate that the interaction is mediated by the Nef residues R19, R21, and R22. These data, together with the recent finding showing that the same sequence is involved in the physical association with the tumor-suppressor protein p53 [43], suggest that Nef may act as a nuclear regulatory factor.

## Acknowledgments

We thank Dr. F. Felici for providing the phage peptide library and Prof. F. Grosveld for the GATA-3 cDNA construct. This work was supported by C.N.R. Grant 97.01213.PF/49 to C.S.

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