Secreted Nucleobindin-2 Inhibits 3T3-L1 Adipocyte Differentiation

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Abstract: Nucleobindin-2 is a 420 amino acid EF-hand Ca^{2+} binding protein that can be further processed to generate an 82 amino terminal peptide termed Nesfatin-1. To examine the function of secreted Nucleobindin-2 in adipocyte differentiation, cultured 3T3-L1 cells were incubated with either 0 or 100 nM of GST, GST-Nucleobindin-2, prior to and during the initiation of adipocyte differentiation. Nucleobindin-2 treatment decreased neutral lipid accumulation (Oil-Red O staining) and expression of several marker genes for adipocyte differentiation (PPARγ, aP2, and adipsin). When Nucleobindin-2 was constitutively secreted into cultured medium, cAMP content and insulin stimulated CREB phosphorylation were significantly reduced. On the other hand, intracellularly overexpressed Nucleobindin-2 failed to affect cAMP content and CREB phosphorylation. Taken together, these data indicate that secreted Nucleobindin-2 is a suppressor of adipocyte differentiation through inhibition of cAMP production and insulin signal.

Keywords: Nucleobindin-2, Nesfatin-1, adipogenesis, 3T3-L1, insulin, GST.

INTRODUCTION

Nucleobindins-Nucleobindin-1 (NUCB1 or Calnuc, Nuc) and Nucleobindin-2 (NUCB2 or NEFA) are EF-hand calcium ion binding proteins identified in human with a rather restricted tissue expression pattern [1, 2]. They share 61.65% amino acid identity and a characteristic composition of functional domains – a signal peptide, a Leu/Ile rich region, basic amino acid rich region including putative bipartite nuclear location signal, two calcium ion binding EF-hand domains separated by acidic amino acid rich region and a leucine zipper [2, 3]. Within the cell, both are located in the cytoplasmic and Golgi luminal pools and both are secreted into extracellular space and interact with other proteins in a calcium-dependent manner [4, 5, 6, 7, 8].

Nucleobindin-2 contains 420 amino acids that can be further processed to generate an 82 amino terminal peptide termed Nesfatin-1. However, the physiological action of Nucleobindin-2 and Nesfatin-1 remain poorly defined. It has been reported that Nesfatin-1 but not Nucleobindin-2 is anorexigenic as Nesfatin-1 blocked food intake whereas a Nucleobindin-2 mutant that can not be processed into Nesfatin-1 was without effect [9]. Recently Broberger *et al.* reported that Nucleobindin-2 co-localizes with insulin in rat and human pancreatic β cells [10]. Since islet Nucleobindin-2 content isolated from an animal model of type 2 diabetic rats was lower than that of non-diabetic control animal, Nucleobindin-2 was suggested to play a regulatory role in insulin secretion and as a potential contributor to diabetic pathology [10]. Thus, it appears that both Nesfatin-1 and its precursor Nucleobindin-2 may have distinct physiological functions. Since Nucleobindin-2 contains a signal peptide sequence, Nucleobindin-2 is presented in rat serum and is secreted into conditional medium of 3T3-L1 cells. These facts suggest that these proteins function as endocrine hormones [10, 11]. To examine the potential endocrine function of secreted Nucleobindin-2, we have determined the effect of both Nucleobindin-2 and Nesfatin-1 on 3T3-L1 adipocyte differentiation.

MATERIALS AND METHODS

Plasmid Constructs

The rat Nucleobindin-2 cDNA cloned into the bacterial glutathione S-transferase expression pET41a plasmid system using Sac II and Not I sites (EMD Chemical Inc. Gibbstown, NJ, USA) was kindly provided by Dr. Hiroshi Eguchi (Teijin Pharma Limited, Hino, Japan). For mammalian cell expression, the rat Nucleobindin-2 cDNA also was cloned into the pFLAG-CMV2 plasmid (Sigma-Aldrich Japan, Tokyo, Japan) and pSectag2B plasmid (Life Technologies, Tokyo, Japan) using Hind III and Not I sites. The rat Nesfatin-1 cDNA coding 1-82 amino acids and rat ΔNesfatin-1 cDNA coding 83-420 amino acids were obtained by PCR as pET41a-rat Nucleobindin-2 plasmid was template and cloned into the bacterial glutathione S-transferase expression pET41a plasmid system using Sac II and Not I sites. All of the constructs used in this study were verified by DNA sequencing. GST fusion proteins were isolated from soluble fraction of transformed E.coli according to the manufacture's instructions (GE Healthcare, Tokyo, Japan).

Reagents

We purchased Nucleobindin-2 and adipsin antibodies from R&D systems (Tokyo, Japan). pY-100 antibody, phospho-Akt1/2/3 antibody, phospho-Erk antibody, phospho-

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1875-5305/12 $58.00+.00

CREB antibody, and CREB antibody were obtained from Cell Signaling Technology (Tokyo, Japan). The horseradish peroxidase (HRP) conjugated anti rabbit IgG antibody was obtained from Thermo Scientific (Tokyo, Japan). Cell culture medium and reagents were from Life Technologies (Tokyo, Japan). All of other chemicals used in this study were purchased from Sigma-Aldrich Japan (Tokyo, Japan). The Shimazu Corporation (Tokyo, Japan) synthesized the rat Nucleobindin-2 peptide as full length (1-420 amino acids). cAMP-Glo Assay kit was obtained from Promega (Tokyo, Japan).

Real-time RT-PCR

Real-time RT-PCR assays were performed using Applied Biosystems 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). Briefly, total RNA was extracted from the 3T3-L1 cells using ISOGEN (Nippon Gene, Tokyo, Japan). The isolated 1 μg of the total RNA was reverse transcribed with random hexamers using a Taqman Reverse Transcription Reagent kit (Applied Biosystems) according to the manufacturer's protocol. In this study PPARγ, aP2, and adipsin genes were chosen as adipocyte genes [12]. Mouse PPARγ gene mRNA was analyzed using a probe and primers (Taqman probe no. Mm0044094, Applied Biosystems). Mouse aP2 and adipsin mRNA levels were analyzed using probes and primers (Taqman probe no. Mm0049557 for aP2 and Taqman probe no. Mm0044266 for adipsin, Applied Biosystems). The CT value of each sample was between 26 and 33 in standard 40 cycles. The PCR results were normalized to mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression using a probe and primers (Taqman probe no. Mm9999991, Applied Biosystems).

Cell Culture

3T3-L1 preadipocytes were cultured in DMEM containing 25 mM glucose, 10% calf serum at 37^0C with 8% CO_2. Confluent cultures were induced to differentiate into adipocytes as previously described [16]. For immunoblotting, cells were washed twice with ice-cold phosphate buffered saline (PBS) and frozen by liquid nitrogen after PBS was removed. Frozen dishes were kept at -80^0C until used.

Immunoblotting

After human blood samples were drawn from five healthy human subjects with normal range of BMI, samples were immediately transferred to child vacuum blood collection tubes containing EDTA-2K, followed by centrifugation at 2000 g for 10 min at 4^0C. The plasma samples were stored at -80^0C until used. The plasma sample was mixed with SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 100 mM dithiothreitol, 0.1% (w/v) bromophenol blue), and heated at 100^0C for 5 min.

Medium was harvested from Day 0 and 12 cells and centrifuged 150g for 10 min at 4^0C to remove floating cells. After protein concentration was estimated, the supernatant was mixed with SDS sample buffer and heated at 100^0C for 5 min.

Scraped frozen cells from frozen culture dishes were rocked for 30 min at 4^0C with NP-40 lysis buffer (25 mM Hepes, pH 7.4, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM sodium chloride, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/μl aprotinin, 1 μg/μl pepstatin, 5 μg/μl leupeptin). Insoluble material was separated from the soluble extract by centrifugation at 15,000 rpm for 30 min at 4^0C, and the total protein amount in the supernatant was determined by BCA method. The samples were resuspended in SDS sample buffer, and heated at 100^0C for 5 min. Samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The samples were immunoblotted with specific antibody as indicated in figure legends.

Chemically synthesized Nucleobindin-2 (1 μg) mixed with SDS sample buffer heated at 100^0C for 5 min was used as a standard for Nucleobindin-2 band size. Samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was subjected to Ponceau S staining to visualize and estimate peptide band.

Adipocyte Differentiation Assay

Two days before the initiation of adipocyte differentiation, either 0 (PBS as vehicle alone) or 100 nM fusion protein was started to add in the tissue culture medium. Each fusion protein was added once every 48h as GST fusion proteins as indicated in figures. On day 8, differentiated adipocytes were stained by Oil-Red O staining kit (DBS, Pleasanton, CA, USA) and quantified by following the manufacturer's instructions. Briefly, the cultured cells were fixed for 30min with 10% formalin in PBS at room temperature, washed and then stained for 1 h with Oil-Red O solution (2:3 vol/vol H_2O: isopropanol containing 0.5% Oil-Red O). After staining, the cells were washed with PBS and extracted with the supplied Dye Extraction solution with placing on a platform rocker for 30 min at room temperature. The extracted dye was transferred into a cuvette and absorbance was read in a spectrophotometer at 520 nm.

cAMP Assay

GloSensor cAMP assay kit was used as detailed by the manufacturer instruction with minor modification. Briefly cells were transiently transfected with pGloSensor plasmid and either pcDNA3.1 or pFALG-CMV2-Nucleobindin-2 (signal sequence for secretion was deleted) or pSectag2B-Nucleobindin-2. After 48 h, transfected cells were serum starved for 6 h and stimulated with 100 nM insulin for 15 min. Cells were lysed with supplied cAMP-Glo lysis buffer and clear lysates were saved. After the protein concentration was determined, lysates were mixed with Kinase-Glo substrate and incubated for 30 min at room temperature with gentle agitation. Then cAMP amount was measured by using a luminescence reader.

Statistical Analysis

All data are expressed as mean +/- standard deviation in figures. Data were analyzed using 1-factor ANOVA to compare the means of all the groups. The Tukey-Kramer multi-

ple comparisons procedure was used to determine statistical differences between the means with that *P* values less than 0.05 were considered statistical significance by InStat 2.00 program.

RESULTS

Detection of Nucleobindin-2 in Cultured Medium of 3T3-L1 Cells and Estimation of Nucleobindin-2 Effect on Intracellular cAMP Content and Insulin Signal

In order to confirm whether Nucleobindin-2 is secreted into culture medium we overexpressed Nucleobindin-2 construct either from a plasmid that will generate only cytosol localized Nucleobindin-2 intracellulary (pFLAG-CMV2-Nucleobindin-2) or a construct that generates an extracellular secreted Nucleobindin-2 protein and cytosol localized Nucleobindin-2 intracellulary (pSectag2B-Nucleobindin-2). As shown in (Fig. **1A**), pSectag2B-Nucleobindin-2 expression resulted in the presence of Nucleobindin-2 in the cultured medium. In this case, both pFLAG-CMV2-Nucleobindin-2 and pSectag2B-Nucleobindin-2 expressed similar Nucleobindin-2 protein levels in the total cell extracts (Fig. **1B**). We assumed that we could draw a conclusion about secreted Nucleobindin-2 effect alone by comparison of both pFLAG-CMV2-Nucleobindin-2 and pSectag2B-Nucleobindin-2 constructs.

In order to assess the function of secreted Nucleobindin-2, we measured the intra cellular cAMP content and looked at insulin signal pathway. As shown in (Fig. **1C**), not intracellular Nucleobindin-2 but secreted Nucleobindin-2 significantly reduced cAMP content. Interestingly, neither intracellularly overexpressed Nucleobindin-2 nor extracellular Nucleobindin-2 had any significant effect on basal or insulin-stimulated insulin receptor β subunit (upper panel), IRS-1 (upper panel), Akt (second panel) or, Erk1/2 (third panel) phosphorylations (Fig. **1D**). However, insulin induced serine 133 residue phosphorylation of CREB was significantly reduced not by intracellulary overexpressed Nucleobindin-2 but by increased secreted Nucleobindin-2 (forth panel. (Fig. **1D**)). The reduction was estimated as 40% in secreted Nucleobindin-2 sample and it was statistically significant ($p<0.05$ Vs pcDNA3.1). As shown in bottom panel in (Fig. **1D**), neither Nucleobindin-2 nor insulin stimulation did not affect CREB protein expression (bottom panel. (Fig. **1D**). These data suggest that Nucleobindin-2 extracellularly exerts an inhibitory effect on insulin signaling to CREB.

Estimation of Nucleobindin-2 Concentration in Human Plasma Sample

In order to determine the appropriate Nucleobindin-2 concentration for further experiments, we attempted to estimate the Nucleobindin-2 concentration in human plasma. 100 μl/lane of 10 times diluted human plasma was subjected to Nucleobindin-2 immunoblotting (Fig. **2A**, lane 3). As the signal intensity of the 10 times diluted plasma was approximately equal to that of 500 pmol of pure synthesized Nucleobindin-2 (lane 2) in immunoblotting, we estimate that the concentration of Nucleobindin-2 in human plasma is approximately 50 nM based on the mean value of five subjects. As the size control for Nucleobindin-2, the synthesized Nucleobindin-2 was stained as described in materials and methods section (Fig. **2A**, lane 1). Nucleobindin-2 was also detected in the medium of cultured 3T3-L1 cells (Fig. **2B**). The levels were higher in preadipocytes just prior to the initiation of adipocyte differentiation (lane 1) compared to fully differentiated adipocytes (lane 2). As shown in bottom panel in (Fig. **2B**), both samples contained similar amount of albumin derived from serum supplied in cultured medium.

Nucleobindin-2 Reduced Adipocyte Differentiation in 3T3-L1 Cells

As Nucleobindin-2 amount in the culture medium from fully differentiated adipocytes seemed to be smaller than that of preadipocytes (Fig. **2B**), we next examined the potential autocrine or paracine effect of Nucleobindin-2 on 3T3-L1 adipocyte differentiation. Either vehicle (PBS) alone or 100 nM GST or 100 nM GST-Nucleobindin-2 was added to the 3T3-L1 adipocyte tissue culture medium as described in materials and methods section. Nucleobindin-2 as a GST fusion protein added to the tissue culture medium once every 48h was an effective inhibitor of adipocyte differentiation as visually apparent by Oil-Red O staining compared to cells treated with either GST or vehicle alone (Fig. **3A**, **3B**).

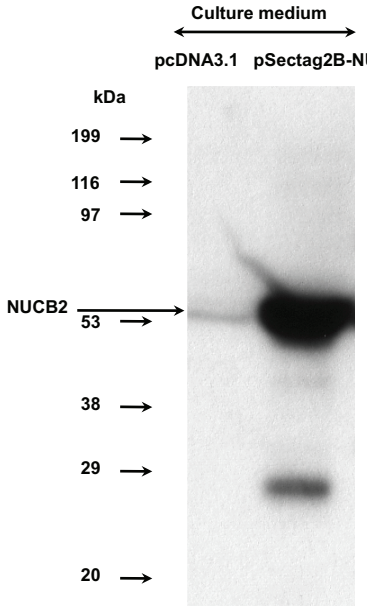
The morphological reduction in adipocyte differentiation detected by reduced accumulation of Oil-Red O staining cells induced by GST-Nucelobindin-2 was confirmed by RT-PCR, which represented the reduction in PPARγ, aP2, and adipsin expression at the transcription level (Fig. **3C**). These data are consistent with a role of Nucleobindin-2 in inhibiting/limiting adipocyte differentiation (the data has been submitted for publication).

The Nesfatin-1 Portion is Required for the Inhibition of Adipocyte Differentiation by Nucleobindin-2

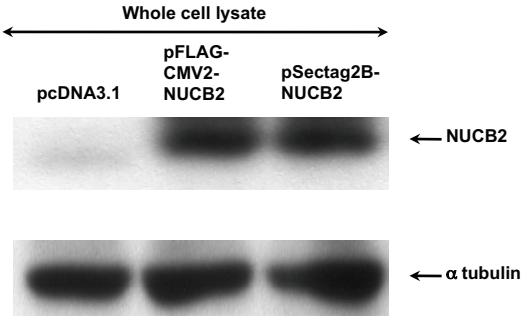
Since Nesfatin-1 is proteolytically cleaved from Nucleobindin-2 by protein converting enzyme 1 and/or 2 [9], we determined whether the full- length Nucleobindin-2 protein or the Nesfatin-1 domain was required for the inhibition of adipocyte differentiation. We generated a Nucleobindin-2 protein that had the 82 amino terminal domain of Nucleobindin-2 deleted (termed as ΔNesfatin-1: 83-420 amino acids) or the Nesfatin-1 portion itself (25-106 amino acids). As shown in (Fig. **4A**), GST-ΔNesfatin-1 had no significant effect whereas Nesfatin-1 itself was capable of inhibiting 3T3-L1 adipocyte differentiation (Fig. **4A**). The ability of Nesfatin-1 to inhibit adipocyte differentiation was confirmed by RT-PCR, which represented the reduction in PPARγ, aP2, and adipsin expression at the transcription level (Fig. **4B**). These data indicate that the Nesfatin-1 domain is responsible for the inhibition of adipocyte differentiation at physiological condition.

DISCUSSION

In this study we discovered another new physiological function of Nucleobindin-2 to inhibit adipocyte differentiation. We have confirmed that Nucleobindin-2 is expressed in 3T3-L1 preadipocytes and secreted in conditional medium as previously reported [11]. This Nucleobindin-2 bioactivity required the Nesfatin-1 domain because Nesfatin-1 by itself



1A



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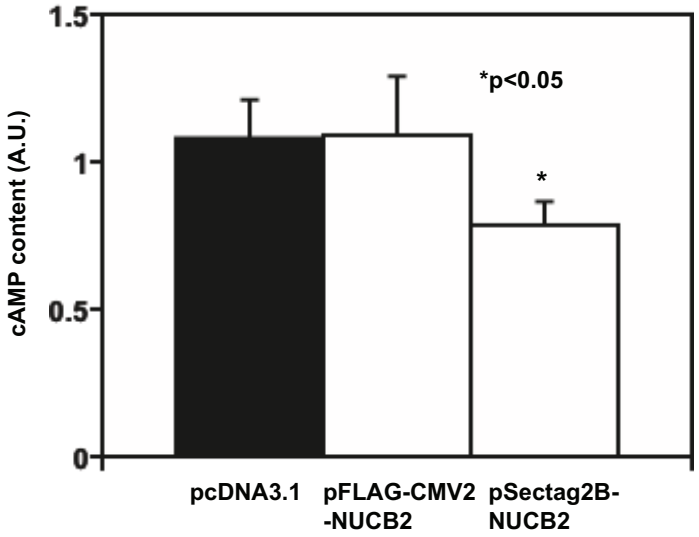
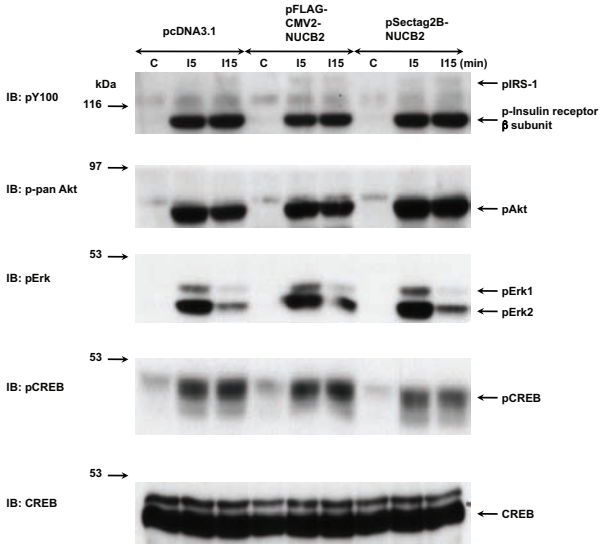


1C



1D

Figure 1. Detection of Nucleobindin-2 in cultured medium of 3T3-L1 cells and estimation of Nucleobindin-2 effect on intracellular cAMP content and insulin signal

(1A) Either pcDNA3.1 or pSectag2B-Nucleobindin-2 was expressed in 3T3-L1 cells and cultured medium was subjected to Nucleobindin-2 immunoblot as described in materials and methods section. These blots are one of the representative blots independently performed four times.

(1B) Either pcDNA3.1 or pFLAG-CMV2-Nucleobindin-2 or pSectag2B-Nucleobindin-2 was expressed in 3T3-L1 cells and cell extracts were prepared and immunoblotted by anti-rabbit polyclonal Nucleobindin-2 antibody to pick up Nucleobindin-2. As a loading control, a-tubulin immunoblot was performed and there were no loading problem among samples. These blots are one of the representative blots independently performed four times.

(1C) Either pcDNA3.1 or pFLAG-CMV2-Nucleobindin-2 or pSectag2B-Nucleobindin-2 was transiently transfected with pGloSensor plasmid. After 48hrs, cAMP production was estimated as described in materials and methods section. Results are expressed as means $\pm$ S.D. of four independent experiments. $P < 0.05$ *vs*. pcDNA3.1 control plasmid transfected cells.

(1D) Either pcDNA3.1 or pFLAG-CMV2-Nucleobindin-2 or pSectag2B-Nucleobindin-2 was transiently transfected. After 48 h, cells were stimulated with 100 nM insulin for 5 or 15 min. Then whole cell lysate samples were made and subjected to pY-100, phospho-Akt1/2/3, phospho-Erk, phospho-CREB, and CREB immunoblot as described in materials and methods section. This blot is one of the representative blots independently performed four times. Please note that gel was smiled in the case of CREB immunoblot.

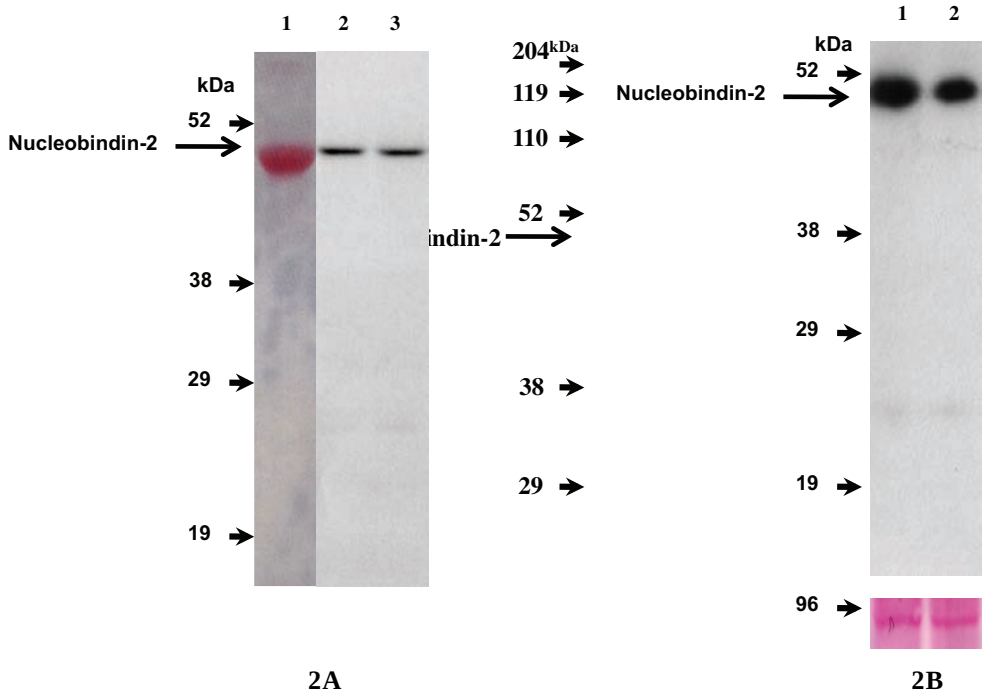


Figure 2. Estimation of Nucleobindin-2 concentration in human plasma sample

(2A) Representative immunoblot for estimation of Nucleobindin-2 protein concentration in human plasma sample. Sample was prepared and immunoblotted by anti-rabbit polyclonal antibody to Nucleobindin-2 antibody as described in materials and methods section. After a couple of preliminary experiments, in order to estimate the Nucleobindin-2 concentration in human plasma, 500 pM of synthesized Nucleobindin-2 (Lane2) and 100 µl/lane of 10 times diluted human serum sample (Lane3) were subjected to Nucleobindin-2 immunoblot as described in materials and methods section. We also ran 1 µg of synthesized Nucleobindin-2 and the transferred membrane was subjected to Ponceau S staining to confirm the Nucleobindin-2 band size (Lane1). These are representative immunoblots independently performed three times.

(2B) Representative immunoblot analysis for Nucleobindin-2 protein presence in tissue culture medium in 3T3-L1 cells (day 0 as preadipocytes for lane 1 and day 12 as fully differentiated adipocytes for lane 2). Sample was prepared from tissue culture medium and immunoblotted by anti-rabbit polyclonal antibody to Nucleobindin-2 antibody as described in materials and methods section. Ponceau S stain for albumin showed loading volume was essentially identical. These are representative immunoblots independently performed three times.

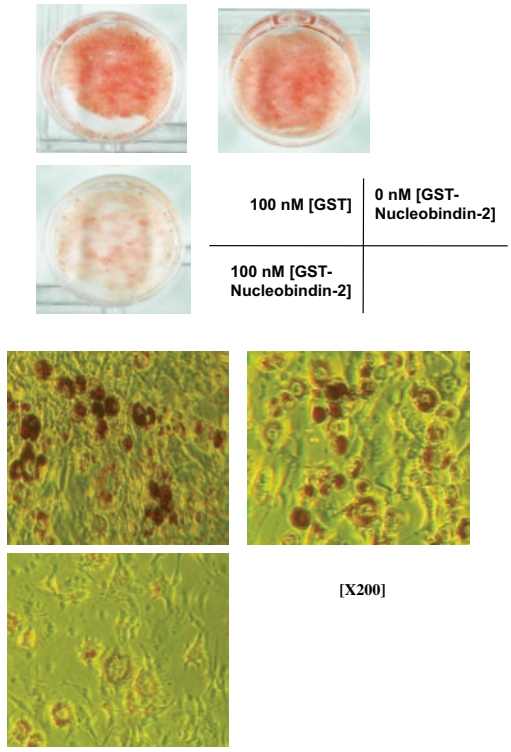
was able to inhibit adipocyte differentiation at the same dose that Nucleobindin-2 did and deletion of Nesfatin-1 portion from Nucleobinding-2 resulted in an inactive protein. Thus, we concluded that biological active portion of Nucleobindin-2 to inhibit adipocyte differentiation is the 82 amino terminals Nesfatin-1 domain.

Nucleobinding-2 was also reported to function as a precursor for Nesfatin-2 [9] and Nesfatin-3 [9] peptides. Although Δ Nesfatin-1 contains the Nesfatin-2 and Nesfatin-3 subdomains, ΔNesfatin-1 was biologically inactive and our data suggested that the Nesfatin-2 and Nesfatin-3 domains were not involved.

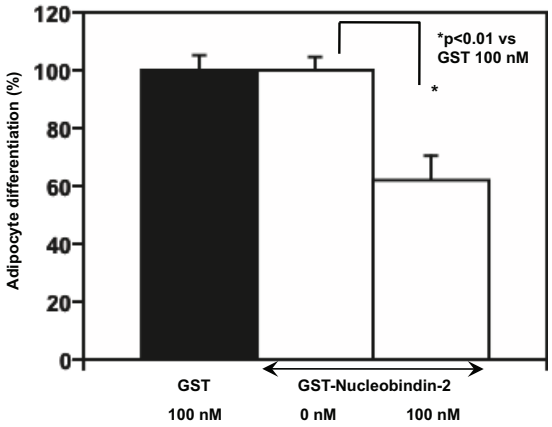
When Nucleobindin-2 is overexpressed in the cytosol intracellularly, Nucleobindin-2 did not affect cAMP production. On the other hand secreted Nucleobindin-2 reduced cAMP content. Furthermore insulin induced serine 133 residue phosphorylation of CREB was significantly reduced by secreted but not by intracellular Nucleobindin-2.

How could insulin phosphorylate CREB? Insulin decreases cAMP production by activating PI3 kinase/Akt/PDE3B pathway leading to cAMP hydrolysis and leads to reduce PKA activity and CREB phosphorylation [13]. However insulin also can phosphorylate CREB through activated Erk [14]. As shown in (Fig. **1D**) (the third panel), insulin actually could stimulate Erk. Therefore it is still possible that insulin can phosphorylate CREB if insulin activated Erk activity could overcome the inhibition of PKA activity.

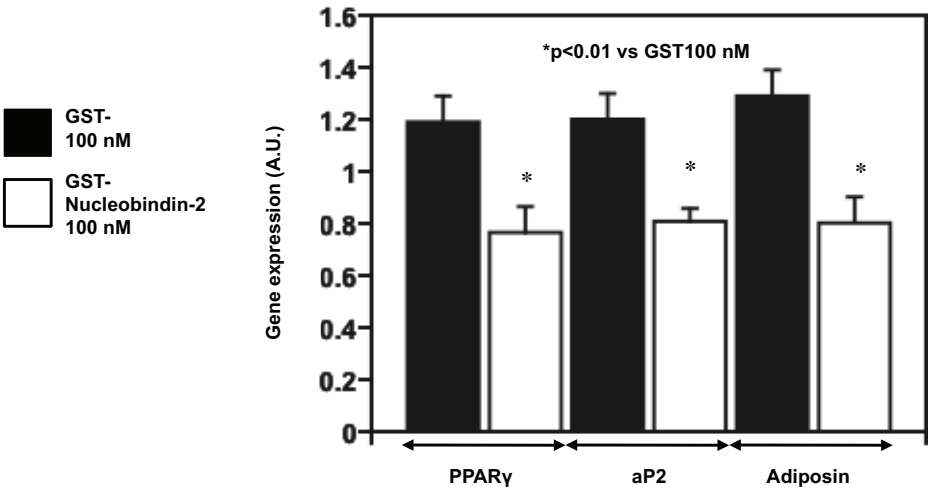
How can we account for the mechanism of Nucleobindin-2 to decrease CREB phosphorylation? Since extracellularly added Nesfatin-1 was reported to increase intracellular calcium level in insulin secreting cells [15], Nesfatin-1 could increase PDE3B activity and enhance cAMP hydration leading to decrease intracellular cAMP level. If that is the case, PKA activity will be decreased and also CREB phosphorylation will be decreased. In our data Nucleobindin-2 to inhibit adipogenesis required Nesfatin-1 portion. Therefore Nucleobindin-2 is also possible to increase intracellular calcium level and PDE3B activity inducing cAMP hydrolysis and



3A



3B



3C

Figure 3. Effect of GST-Nucleobindin-2 on adipocyte differentiation of 3T3-L1 cells

(3A) As described in materials and methods section, the cells were treated with indicated concentration either vehicle alone or GST or 100 nM GST-Nucleobindin-2 protein. The differentiated adipocytes were stained with Oil-Red O. Red stained cells represent differentiated adipocytes. These are representative stain independently performed five times.

(3B) The Oil-Red stained cells were incubated with extraction buffer and absorbance of extracted samples were measured by spectrophotometer at 520 nm. Results are expressed as means ± S.D. of five independent experiments. *P < 0.01 *vs*. vehicle.

(3C) The confluent preadipocytes were started to treat with either 100 nM GST or 100 nM GST-Nucleobindin-2. After cells were fully differentiated to adipocytes, total RNA was extracted and real-time RT-PCR was performed for PPARγ, aP2, and adipsin using GAPDH as internal control. Results are expressed as means ± S.D. of four independent experiments. *P < 0.01 *vs*. 100 nM of GST.

PKA deactivation. This will explain for Nucleobindin-2 to reduce CREB phosphorylation even after insulin stimulation.

Since Nesfatin-1 domain is necessary for Nucleobindin-2 inhibitory function of adipogenesis, Nucleobindin-2 could work through unidentified Nesfatin-1 receptor. Alternatively, Nucleobindin-2 could be an orphan ligand having own unknown receptor, which is different from Nesfatin-1 receptor. In fact, our data that not intracellular Nucleobindin-2 but secreted form of Nucleobindin-2 clearly reduced intracellular

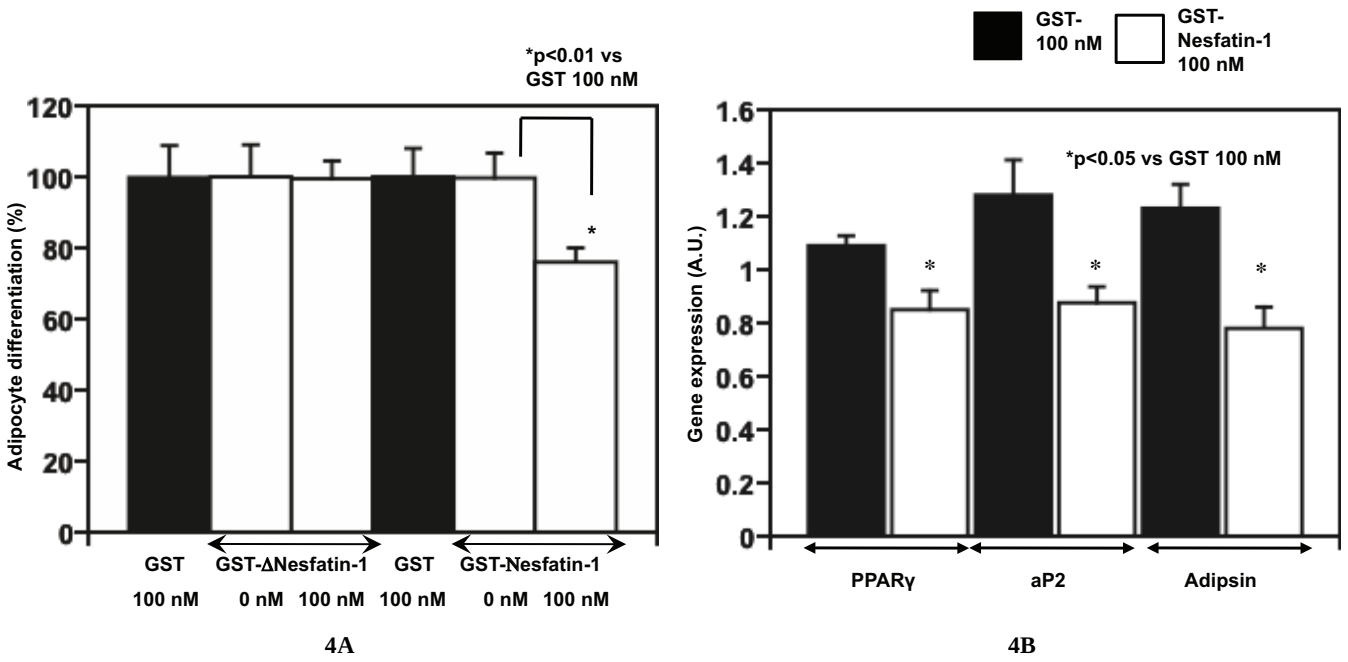


Figure 4. Effect of GST-Δ Nesfatin-1 or GST-Nesfatin-1 on adipocyte differentiation on 3T-3L1 cells

(4A) The cells were treated with indicated concentration either GST or GST-ΔNesfatin-1 or GST-Nesfatin-1 during differentiation induction periods as described in materials and methods section. The differentiated adipocytes were stained with Oil-Red O and then, cells were incubated with extraction buffer and absorbance of extracted samples was measured by spectrophotometer at 520 nm. Results are expressed as means ± S.D. of five independent experiments. *P < 0.01, *vs.* vehicle.

(4B) The cells were treated with indicated concentration either GST or GST-Nesfatin-1 during differentiation induction periods. After cells were fully differentiated to adipocytes, total RNA was extracted and real-time RT-PCR was performed for PPARγ, aP2, and adipsin using GAPDH as internal control. Results are expressed as means ± S.D. of four independent experiments. *P < 0.05 *vs.* 100 nM of GST.

cAMP production support an idea that Nucleobindin-2 works extracellularly perhaps through receptor causing reduction of cAMP production and at least in part interferes insulin signal leading partial inhibition of adipocyte differentiation.

We estimated that the concentration of Nucleobindin-2 in human serum is approximately 50 nM by the design of immunoblot analysis using recombinant Nucleobindin-2 as a standard, whereas the experimental concentration to inhibit adipocyte differentiation in culture was 100 nM as a design of GST tagged fusion proteins. Thus, our *in vitro* designed experiments might suggest some physiological information. Based upon these data, we propose that Nucleobindin-2 is an adipocyte-secreted factor (adipokine) that inhibits adipocyte differentiation.

ABBREVIATIONS

GST	=	Glutathione-S-transferase
S.D.	=	Standard deviation
NUCB2	=	Nucleobindin-2
aP2	=	Adipocyte fatty acid-binding protein-2
CREB	=	cAMP response element binding protein
BMI	=	Body Mass Index
PDE3B	=	Phosphodiesterase 3B

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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