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The C-terminal domain of the nuclear factor I-B2 isoform is glycosylated and transactivates the WAP gene in the JEG-3 cells

Sudit S. Mukhopadhyay [b,1], Jeffrey M. Rosen [a,*]

[a] Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030-3498, USA
[b] Department of Molecular and Cancer Genetics, M.D. Anderson Cancer Center, Houston, TX 77030, USA

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Abstract

The transcription factor nuclear factor I (NFI) has been shown previously both *in vivo* and *in vitro* to be involved in the cooperative regulation of whey acidic protein (WAP) gene transcription along with the glucocorticoid receptor and STAT5. In addition, one of the specific NFI isoforms, NFI-B2, was demonstrated in transient co-transfection experiments in JEG cells, which lack endogenous NFI, to be preferentially involved in the cooperative regulation of WAP gene expression. A comparison of the DNA-binding specificities of the different NFI isoforms only partially explained their differential ability to activate the WAP gene transcription. Here, we analyzed the transactivation regions of two NFI isoforms by making chimeric proteins between the NFI-A and B isoforms. Though, their DNA-binding specificities were not altered as compared to the corresponding wild-type transcription factors, the C-terminal region of the NFI-B isoform was shown to preferentially activate WAP gene transcription in cooperation with GR and STAT5 in transient co-transfection assays in JEG-3 cells. Furthermore, determination of serine and threonine-specific glycosylation (O-linked *N*-acetylglucosamine) of the C-terminus of the NFI-B isoform suggested that the secondary modification by O-GlcNAc might play a role in the cooperative regulation of WAP gene transcription by NFI-B2 and STAT5.

Keywords: Nuclear factor I (NFI); Whey acidic protein (WAP); Glucocorticoid receptor (GR); Signal transducer of activator of transcription 5 (STAT5); Composite response element (CoRE); O-linked *N*-acetylglucosamine (O-GlcNAc)

The study of milk protein gene expression is an attractive system for examining hormonal and developmental regulation of gene expression, and furthermore, has provided insight into mammary gland-specific control of transcription [1]. Studies from several different laboratories have suggested that the spatially and temporally restricted milk protein gene expression patterns result from combinatorial interactions at the protein–protein and protein–DNA level of members of several families of commonly expressed transcription factors [2]. The whey acidic protein (WAP), the major whey protein in rodents, is exclusively expressed at high levels in the mammary gland during late pregnancy and lactation, and has been employed as a model system to study the transcriptional regulation of milk protein gene expression. A distal region of the rat WAP gene, containing a composite response element (CoRE), which confers mammary gland-specific and hormonally-regulated expression, was identified previously in our laboratory [3,4]. In addition, the function of this CoRE was determined by the cooperative interactions among three transcription factors; a specific NFI isoform, the glucocorticoid receptor (GR) and STAT5A [2].

Individual NFI isoforms have been suggested to contribute to different stages of mammary gland development, in particular to gene expression in the terminally differentiated alveolar epithelial cells in late pregnancy and lactation as well as during tissue remodeling during involution. For

* Corresponding author. Fax: +1 713 798 8012.
E-mail addresses: suditmukhopadhy@yahoo.com (S.S. Mukhopadhyay), jrosen@bcm.tmc.edu (J.M. Rosen).
[1] Present address: Advinus Therapeutics Pvt. Ltd., Discovery Biology Group, 2nd Floor, Biotech Park, Zone ABCD, Phase II, Hinjawadi, Pune 411057, India.

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example, NFI has been implicated in regulating the transcription of milk protein genes such as WAP, α-lactalbumin and β-lactoglobulin [5], as well as the lactation-associated transcription of β1,4-galactosyltransferase (β4-GT) and carboxylester lipase (CEL) genes [6,7]. Furthermore, a mammary cell-specific enhancer in the mouse mammary tumor virus (MMTV) promoter includes an active NFI-binding site [8]. In addition, the involution-associated transcription of the TRPM-2/clusterin gene has been shown to be controlled by a unique *N*-glycosylated 74 kDa NFI-C isoform [9].

NFI transcription factors can both activate and repress gene expression [10–12]. Furthermore, the same NFI isoforms can either activate or repress expression from the same promoter in a cell-type dependent manner. Transient transfection studies have shown that the NFI transcription factors are modular, with N-terminal domains mediating DNA binding and dimerization, and C-terminal domains mediating transactivation and repression [13–15]. The individual cell- and tissue-specific isoforms are the result of differential splicing of four NFI genes (A, B, C, and X). NFI family members are highly homologous in their amino-terminal DNA binding and dimerization domains but are divergent in the carboxy-terminal transactivation–repression domains. Further diversity of the transactivation domain is accomplished by alternative splicing that creates regions of variable proline richness. The significance of the proline rich areas is not well understood [5].

N-Acetylglucosamine (O-GlcNAc) is a sugar residue that is used as a frequent post-translational modification of nuclear and cytoplasmic proteins. One molecule of GlcNAc is linked as a single monosaccharide to the hydroxyl group of serines or threonines and is not further elongated. Modification by O-GlcNAc is highly dynamic, and can give rise to functionally distinct protein subsets [16,17]. This reversible post-translational modification is present in a variety of proteins, including numerous chromatin-associated proteins and several transcription factors [18–20]. The ubiquitous transcription factors SP1, members of the AP-1 family, the estrogen receptor (ER), Pax-6, and c-Myc all carry O-GlcNAc residues [20]. A subset of the nuclear RNA polymerase II itself is also O-GlcNAc-modified at the carboxy-terminal moiety (CTD) of the largest subunit [21]. O-GlcNAcylation is highly dynamic, with rapid cycling in response to cellular signals or cellular stages. Although the function of O-GlcNAc has not been precisely determined, recent data suggest it can influence a wide variety of key cellular events, including gene transcription, insulin signaling, glucose metabolism, and cell cycle progression [16,17]. Gewinner et al. have shown that the glycosylated form of STAT5 binds with the coactivator of transcription CBP, which is very important for STAT5-mediated gene transcription [22].

In the present study, we compared the transactivation domains of two specific NFI isoforms expressed in the mammary gland, which are associated with WAP gene transcription. The chimeric protein (N-terminal of NFI-A4 and C-terminal of NFI-B2) with the C-terminal of NFI-B2, but not the C-terminal of NFI-A4 demonstrated cooperativity with GR and STAT5 in the hormonal induction of WAP gene transcription in JEG cells. Preliminary gel shift and co-immunoprecipitation assays suggest the O-GlcNAc modification of the C-terminus of NFI-B2 might play an important role in interaction of this specific NFI isoform with the STAT5 in WAP gene transcription.

Materials and methods

Plasmid construction. pWAPtk-luciferase, STAT5A, GR, prolactin receptor (PrlR), and the NFI constructs have been described previously [2]. To make the chimeric constructs (pCHNFI N-terA-C-terB and pCHNFI N-terB-CterA) between the NFI-A4 and NFI-B2, the pCHNFI-A4 and B2 were digested with EcoNI and NotI and the N-terminal fragment of A ligated with C-terminal fragment of B and vise versa. All the constructs were confirmed by sequencing.

Cell culture and transfection assay. JEG-3 choriocarcinoma cells (American Type Cuture Collection) were cultured in minimal essential medium (MEM: Gibco-BRL) containing 10% fetal bovine serum. Twenty-four hours prior to transfection, cells were plated onto 60-mm dishes. Two hours before transfection, cells were cultured with fresh medium containing MEM, 10% charcoal stripped horse serum (SHS), and insulin (5 μg/ml). Transfection was carried out using 8–10 μg of DNA by the calcium phosphate method. After 24 h of transfection, the cells were washed twice with phosphate-buffered saline (PBS), and then the cells were treated with fresh medium (MEM plus 10% SHS and insulin), with or without ovine prolactin (1 μg/ml) and hydrocortisone (HC) (1 μg/ml). Twenty-four hours after the treatment of hydrocortisone and prolactin, the cells were harvested, lysed with lysis buffer (Boehringer Mannheim), and assayed for luciferase and β-galactosidase activity.

Gel shift assays. Oligonucleotides encompassing the NFI palindromic site of the WAP CoRE (−820 to −720) and the adenovirus NFI consensus binding site were used for gel shift assays (coding strand for WAP NFI, 5′-TTGGGCACAGTGCCCAACAG-3′, and coding strand for adenovirus NFI, 5′-CTAGCTATTTTGGATTGAAGCCAATAT-3′. Equimolar concentrations of each oligonucleotide from both strands were annealed in the presence of 1× React 2 (Promega) buffer at 94 °C for 10 min and then cooled to room temperature for 3–4 h. The double-stranded oligonucleotide was end labeled with [γ-^{32}P]dATP using polynucleotide kinase (Gibco-BRL), and the probe was purified using p-6 Micro Bio-spin columns (Bio-Rad) followed by trichloroacetic acid precipitation to quantify the amount of labeled probe. The chimeric proteins (N-terminal of NFI-A4 fused with C-terminal of NFI-B2, N-terminal of NFI-B2 fused with C-terminal of NFI-A4) were expressed in JEG-3 cells, and nuclear extracts, isolated as described previously [2], were used for the DNA-binding assays. The amount of nuclear extract required for 50% binding of the labeled probe was calculated, and then for each protein that amount was used for the competition assays. Nuclear extracts were isolated from the same passage of JEG-3 cells, and equimolar concentrations of each probe were used in the gel shift assays.

Antibodies. Both the chimera and wild-type NFI constructs contained a hemoagglutinin (HA) epitope at their N-termini. A monoclonal HA antibody (Babco, Berkeley, California) was used in the gel super shift assay to identify the HA-tagged NFI proteins. A monoclonal antibody raised against a synthetic peptide containing Serine-O-GlcNAc (Covance, Berkeley, California) detects Ser-O-GlcNAc and Thr-O-GlcNAc but did not show cross-reactivity with peptide determinants or other closely related carbohydrate antigens. The monoclonal O-GlcNAc antibody was used in the gel super-shift assay to determine the serine and threonine-specific O-GlcNAcylation of the NFI proteins.

Co-immunoprecipitation. Cells were lysed with RIPA buffer and approximately 500 μg of lysate was incubated with 4–5 μl of antibody (HA and PRDX3) for 30 min on ice. 20 μl of protein A plus/protein G agarose beads (Oncogene) were incubated overnight at 4 °C with constant shaking.