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FEBS Letters 342 (1994) 297–301

FEBS 13837

FEBS LETTERS

Two intestinal specific nuclear factors binding to the lactase-phlorizin hydrolase and sucrase-isomaltase promoters are functionally related oligomeric molecules

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Received 11 February 1994

Abstract

Lactase-phlorizin hydrolase (LPH) and sucrase-isomaltase (SI) are enterocyte-specific gene products. The identification of regulatory cis-elements in the promoter of these two genes has enabled us to carry out comparative studies of the corresponding intestinal-specific nuclear factors (NF-LPH1 and SIF1-BP). Electrophoretic mobility shift assays demonstrated that the two nuclear factors compete for binding on the same cis-elements. The molecular size of the DNA binding polypeptide is estimated to be approximately 50 kDa for both factors. In the native form the factors are found as 250 kDa oligomeric complexes. Based on these results NF-LPH1 and SIF1-BP are suggested to be either identical or closely related molecules.

Key words: NF-LPH1; SIF1 binding protein; Caco2; Transcription factor

1. Introduction

The expression of the two disaccharidases lactase-phlorizin hydrolase (LPH) and sucrase-isomaltase (SI) is limited to the absorptive epithelial cells (enterocytes) of the small intestine [1–4]. These enzymes are differently regulated during the development. Thus in most mammals the LPH level is high in the newborn animal and declines rapidly after weaning [5]. In contrast the level of SI is low at birth but increases significantly shortly after birth [6,7]. However, man is an exception as both LPH and SI are expressed at high levels at birth [8,9]. LPH [10,11] and SI [12] are regulated at the transcriptional level, but post-transcriptional mechanisms modulate the final expression of the gene product. The different developmental regulation of the two genes indicates differences in the complement of transcription factors involved. However the specific small intestinal expression of both SI and LPH could imply that one or several factors are in common or related.

Recently the promoters of LPH and SI were isolated, sequenced and functionally analyzed [13–15]. A 1 kb segment of the LPH promoter carries the information necessary for the specific small intestinal expression and the postweaning down-regulation as demonstrated by the introduction of a promoter–reporter gene construct in the germ line of transgenic mice [11]. A cis-element (CE-LPH1) positioned at −40 to −54 is able to bind a nuclear factor (NF-LPH1) shown to be present exclusively in the intestinal epithelium [13,16]. It has been proposed that NF-LPH1 is involved in the post weaning down-regulation of LPH, thus in man giving rise to the very frequent genetic condition adult-type hypolactasia [13]. The SI promoter has a sequence positioned at −56 to −36 (SIF1) which interacts with a SIF1-binding protein (SIF1-BP), also present exclusively in nuclear extracts of intestinal epithelial origin [15]. The SIF1 element can direct reporter gene expression in the intestinalized colon carcinoma cell line Caco2 [15]. Furthermore the SI promoter is able to direct intestinal specific transcription of a reporter gene in transgenic mice [17]. Differentiated Caco2 cells express both SI and LPH, whereas undifferentiated Caco2 cells only express low levels of the two dissaccharidases. As both NF-LPH1 and SIF1-BP are present in Caco2 cells ([15] and the present paper), we have used Caco2 nuclear extract to study whether CE-LPH1 and SIF1 elements compete for the same nuclear factor(s) and to look for molecular similarities.

2. Materials and methods

2.1. Preparation of nuclear extracts

Nuclear extracts were prepared [18] from postconfluent (12 days) Caco2 cells cultured in minimal essential medium supplemented with 10% fetal calf serum. To the nuclear lysate $(NH_4)_2SO_4$ (0.33 g/ml) was added and allowed to dissolve on ice. Following centrifugation (100,000 × *g*, 25 min, 4°C) the precipitate was dissolved in the dialysis buffer (25 mM HEPES, pH 7.6, 40 mM KCl, 0.1 mM DTT, 10% glyc-

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SSDI 0014-5793(94)00222-H

erol) and dialyzed twice against 50 vols. of the same buffer. The protein concentration of the dialyzed nuclear extract solution was determined [19] using bovine serum albumin as a standard.

2.2. Electrophoretic mobility shift assay (EMSA)

The EMSAs were carried out essentially as described [20] using synthetic double stranded oligonucleotides (Fig. 1). Before annealing, the upper strands were radioactively labelled by a polynucleotide kinase reaction using [γ-^{32}P]ATP. 25 fmol labelled double stranded oligonucleotides were added to 10 μl gel shift buffer (25 mM Tris, pH 7.8, 5 mM $MgCl_2$, 6 mM KCl, 0.5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 5% Ficoll, 2.5% glycerol, 100 ng/μl dIdC) and incubated with 5 μl nuclear extract (5 μg protein) for 20 min on ice. Non-radioactive oligonucleotides were added in some of the binding reactions as indicated (Fig. 2). 1.6 μl gel shift loading buffer (0.2% Bromophenol blue, 10% glycerol, 0.5 × TBE (45 mM Tris-borate, pH 8.3, 1 mM EDTA)) were added to the samples before loading on a 5% mini slab polyacrylamide gel (Mighty Small, Hoefer Scientific Instruments) cast in 0.5 × TBE, 5% glycerol. The electrophoresis (30 min at 15 mA) was performed using 0.5 × TBE as running buffer with water cooling. Band shifts were visualized by autoradiography after drying the gels.

2.3. Photoaffinity labelling

Some of the thymine bases in the oligonucleotides (Fig. 1) used for the photoaffinity labelling experiments were changed to bromodeoxyuracils (BrdU) by using the corresponding phosphoramidites in the chemical synthesis (Applied Biosystems model 392 DNA-synthesizer).

The photoaffinity labelling was carried out according to the principles earlier described [21]. The nuclear extracts (10 μg protein) were incubated with the BrdU-derivatized and radioactively labelled SIF1 or CE-LPH1 elements as for the EMSAs. The reaction mixtures were then irradiated (30 s, no cooling) by focussing the light-beam directly on the reaction vessel. The light-source was a mercury lamp (Osram HBO) equipped with a filter eliminating light below 305 nm and the beam was focussed by a quartz lens. Gel-shift loading buffer was added and the samples were run on an EMSA gel. The wet gel was autoradiographed for 16 h at 4°C. The DNA–protein complexes were identified and cut out of the gel. The corresponding areas in the competition experiments were also cut out and analyzed. The gel slices were crushed and the radioactive complexes were eluted by incubation for 16 h at 20°C in 1 ml 0.1% SDS, 10 mM Tris (pH 8.0). The gel material was removed by centrifugation and DNA-protein complexes were precipitated by trichloroacetic acid to 10% using 15 μg bovine serum albumin. The pellets were resuspended in 10 μl 0.2 M Tris-HCl, pH 7.5. The samples were made to 2% SDS, 40 mM DTT, 7% glycerol, 0.01% Bromophenol blue. After boiling (5 min) the samples were analyzed together with a ^{14}C-labelled molecular weight marker from Amersham by SDS-polyacrylamide gel (15%) electrophoresis [22].

2.4. Gel filtration

The nuclear extract (120 μg protein) was size-fractionated on a Superose S12 3.2/30 column (Pharmacia, Uppsala, Sweden) equilibrated in and eluted (10 μl/min, 10°C) by 20 mM HEPES, pH 7.9 containing 0.1 M KCl and 0.2 mM EDTA using the SMART chromatography system (Pharmacia). Fractions of 40 μl were collected and 5 μl of each fraction were analyzed by EMSA. Apoferritin (443 kDa), amylase (200 kDa), alcohol dehydrogenase (150 kDa), serum albumin (66 kDa) and carboanhydrase (29 kDa) were used as molecular weight markers.

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CE-LPH1:          5'-CTAGTATTTTACAACCTCAGTTGT-3'
                  3'-GATCATAAAATGTTGGAGTCAACT-5'

BrdU CE-LPH1:     5'-TUTUACAACCUCAGUT-3'
                  3'-AAAAUGTUGGAGUCAA-5'

mut1 CE-LPH1      5'-GATCTTTgcCAACCTCAGTTG-3'
                      3'-AAAcgGTTGGAGTCAACCTAG-5'

mut2 CE-LPH1      5'-GATCTTTTACAACCTgcGTTG-3'
                      3'-AAAATGTTGGAcgCAACCTAG-5'

mut3 CE-LPH1      5'-GATCTTTTACAAgCTCAGTTG-3'
                      3'-AAAATGTTcGAGTCAACCTAG-5'

SIF1              5'-GGCTGGTGAGGGTGCAATAAAACTTTATGAGTA-3'
                  3'-CCGACCACTCCCACGTTATTTTGAAATACTCAT-5'

BrdU SIF1         5'-GTGCAAUAAAACUTUAUGAGUA-3'
                  3'-CACGTUATUTUGAAAUACUCAT-5'

Unspec.           5'-GGTACTACTTCTAGCTTCGGAACT-3'
                  3'-CCATGATGAAGATCGAAGCCTTGA-5'
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Fig. 1. Sequences of the synthetic oligonucleotides used in the electrophoretic mobility shift assays and photoaffinity labelling experiments. 'U' indicates positions changed to BrdU. Mut1-, mut2- and mut3-CELPH1 are mutated CE-LPH1 oligonucleotides where the lower-case letters indicate the new bases. Two sequences in SIF1 which are inverted repeats are underlined. A homologous sequence in CE-LPH1 is also underlined.

3. Results

3.1. CE-LPH1 and SIF1 elements compete for the same nuclear factors

EMSA carried out using the radioactive CE-LPH1 as a probe shows one dominating broad band with low mobility (Fig. 2A, lane 1). The band disappears successively when the non-radioactive double stranded CE-LPH1 is added in increasing concentrations and is very weak at 100 times excess (Fig. 2A, lanes 2–4). This weakening in band intensity is not seen when similar amounts of a non-specific oligonucleotide are added (Fig. 2A, lane 8). Thus the band is a specific CE-LPH1–protein complex. In order to investigate whether CE-LPH1 and SIF1 are competing for the same nuclear factor(s) increasing amounts of the non-radioactive SIF1 were added. This causes a progressive weakening of protein/CE-LPH1 band and at 100 times excess the band is absent (Fig. 2A, lanes 5–7) indicating that CE-LPH1 and the SIF1 element are competing for binding to the same factor(s). SIF1 seems to have similar affinity to the factor binding the CE-LPH1 as addition of the same amount of unlabelled probe (CE-LPH1 or SIF1) causes similar degree of weakening of the retarded band.

EMSA carried out using a radioactive SIF1 oligonucleotide as a probe shows one dominant retarded band. In addition some minor bands with a higher mobility are seen as well (Fig. 2B, lane 1). The dominant band becomes successively fainter when increasing amounts of unlabelled double stranded SIF1 are added. (Fig. 2B, lanes 5–7). The band is left unaffected when 100 times excess of a non-specific oligonucleotide is added (Fig. 2B, lane 8). These results demonstrate that the band represents specific protein–SIF1 complexes and our results thus confirm those earlier reported [15]. In order to further investigate whether the CE-LPH1 and SIF1 element are competing for the same protein(s) increasing amounts of non-labelled double stranded CE-LPH1 was added to the reaction mixture using the SIF1 probe. The

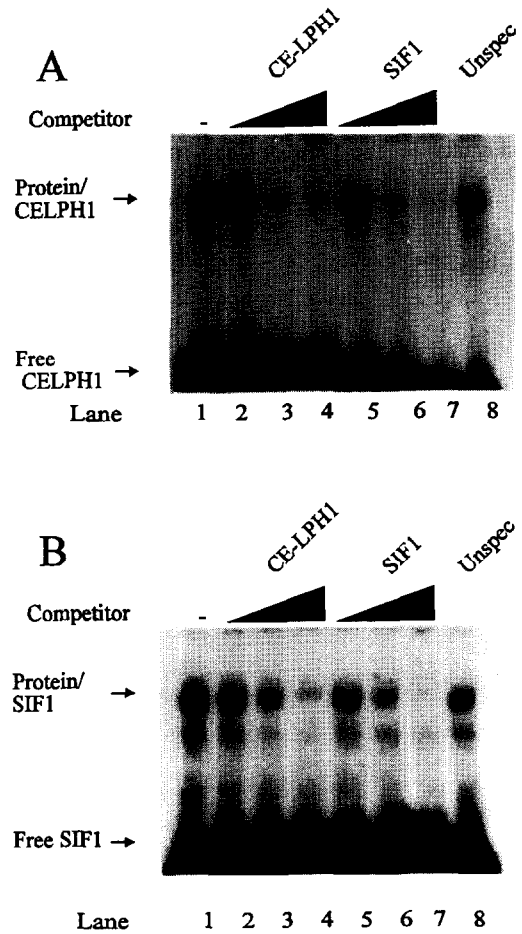

Fig. 2. (A) Electrophoretic mobility shift assays (EMSA) using end-labelled double stranded CE-LPH1 as probe (25 fmol). Competition with unlabelled double stranded oligonucleotides was performed as follows: lane 1 no competitor; lane 2 CE-LPH1 (25 fmol); lane 3 CE-LPH1 (250 fmol); lane 4 CE-LPH1 (2.5 pmol); lane 5 SIF1 (25 fmol); lane 6 SIF1 (250 fmol); lane 7 SIF1 (2.5 pmol); lane 8 Unspec (2.5 pmol). (B) EMSA using end-labelled double stranded SIF1 (25 fmol) as probes. Competitions with unlabelled double stranded oligonucleotides were performed as in (A).

addition of increasing amounts of CE-LPH1 causes progressive loss of the dominating band (Fig. 2B, lane 2–4) thus adding further evidence that the same factor(s) are able to bind to the SIF1 element and CE-LPH1.

Three different mutated double stranded CE-LPH1 oligonucleotides were synthesized (Fig. 1) and used as competitors in EMSAs (Fig. 3A,B). Mut1 CE-LPH1 was not able to compete with CE-LPH1 and SIF1 for protein binding, whereas the mutations introduced in mut2 CE-LPH1 and mut3 CE-LPH1 did not affect the ability of the oligonucleotides to compete with CE-LPH1 and SIF1.

3.2. NF-LPH1 and SIF1-BP are proteins of similar size

Fig. 4 shows the result of photo-labelling of the Caco2 nuclear extract using the BrdU-derivatized CE-LPH1 and SIF1 as probes and subsequently analyzed by 15% SDS-PAGE. Both BrdU-CE-LPH1 and BrdU-SIF1 cross-link to a polypeptide with an approximate size of 50 kDa. The photo-labelling of the peptide by BrdU-CE-LPH1 and BrdU-SIF1 can be competed out by excess of both unlabelled BrdU-CE-LPH1 or BrdU-SIF1 in the reaction mixture, thus demonstrating the specificity of the labelling reaction.

Fig. 5 shows the result of the size fractionation of the Caco2 cell nuclear extract using a gel filtration chromatography. The collected fractions were individually analyzed by EMSA. The dominating proteins binding to CE-LPH1 and SIF1 were eluted at the same positions showing that they have similar size. The estimated molecular weight of both factors is about 250 kDa.

4. Discussion

There is mutual competition between CE-LPH1 and the SIF1 elements for the nuclear proteins which bind to

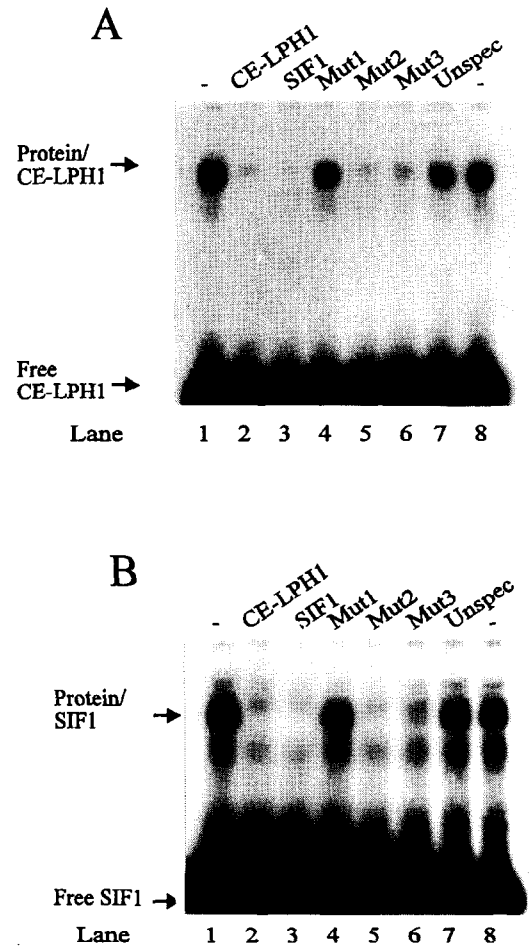

Fig. 3. (A) Electrophoretic mobility shift assays (EMSA) using end-labelled double stranded CE-LPH1 (25 fmol) as probes. Competitions with 2.5 pmol unlabelled double stranded oligonucleotides were performed as follows: lane 1 no competitor; lane 2 CE-LPH1; lane 3 SIF1; lane 4 mut1 CE-LPH1; lane 5 mut2 CE-LPH1; lane 6 mut3 CE-LPH1; lane 7 Unspec; lane 8 no competitor. (B) EMSA using end-labelled double stranded SIF1 (25 fmol) as probes. Competitions with unlabelled double stranded oligonucleotides were performed as in (A).

them. This shows a functional relationship between these factors and their promoter elements. This is reflected in sequence similarities between CE-LPH1 and SIF1 (Fig. 1), which both contain a TTTA sequence. This sequence is important for the formation of a protein/DNA complex as a mutation in this sequence (mut1 CE-LPH1) abolishes the protein binding ability of CE-LPH1 (Fig. 3). Mutations outside this region (mut2 CE-LPH1 and mut3 CE-LPH1) do not affect the protein binding (Fig. 3).

Furthermore the ability of CE-LPH1 to compete with SIF1 is abolished by the mutation in the TTTA sequence. This indicates that a homologous sequence in SIF1 is involved in the protein binding. Inspection of the sequences of CE-LPH1 and SIF1 shows that this is the case as the latter has an inverted repeat structure (TTTAT). Based on these results we suggest that the TTTAC sequence of CE-LPH1 and the TTTAT sequence of SIF1 bind nuclear factors that are either identical or closely related.

The determination of the molecular weight of native and denatured NF-LPH1 and SIF1-BP demonstrate further the similarities between the two factors. Thus they both have molecular weights around 250 kDa. However

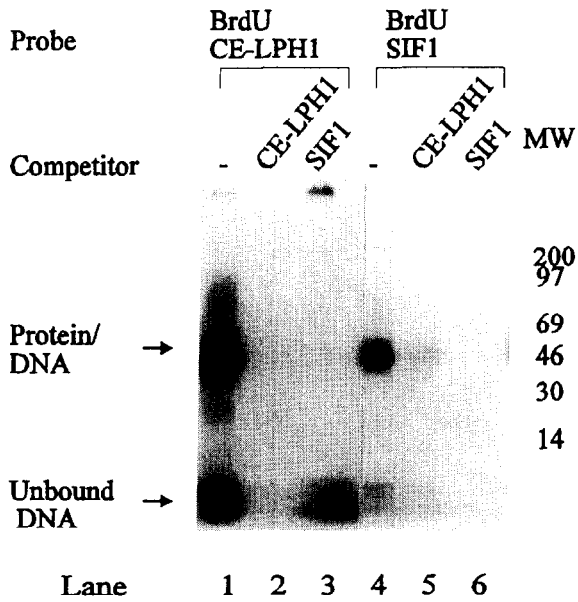

Fig. 4. Molecular size determination of the denatured forms of NF-LPH1 and SIF1-BP. Nuclear extracts from Caco2 cells were incubated with the BrdU-derivatized end-labelled CE-LPH1 or SIF1 and photoactivated. Radioactively labelled BrdU CE-LPH1 was crosslinked without competitor (lane 1), or with 100 times excess of unlabelled BrdU CE-LPH1 (lane 2), or with 100 times excess of unlabelled BrdU-SIF1 (lane 3). Radioactively labelled BrdU SIF1 was crosslinked without competitor (lane 4), or with 100 times excess of unlabelled BrdU CE-LPH1 (lane 5), or with 100 times excess of unlabelled BrdU SIF1 (lane 6). The protein/DNA complexes and the free DNA were first separated by native polyacrylamide gel electrophoresis. The protein/DNA complexes and the corresponding areas in the competition experiments were cut out of the gel, eluted and then analyzed by sodium dodecyl sulphate polyacrylamide gel (15%) electrophoresis. The position of the molecular weight (MW) standards are indicated.

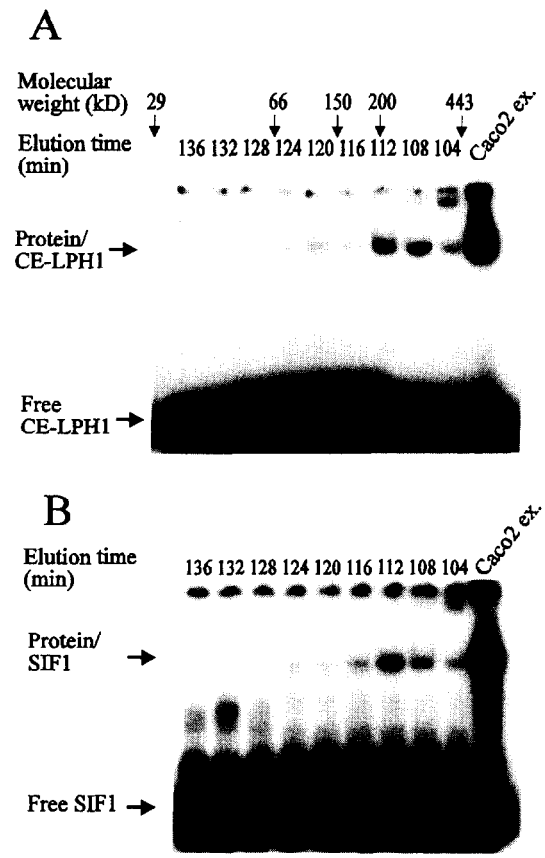

Fig. 5. Molecular size determination of the native forms of NF-LPH1 (A) and SIF1-BP (B). Gelfiltration of a nuclear extract from Caco2 cells was carried out on a Superose S12 column and 40 μl fractions were collected. The elution time for the fractions is indicated. The presence of NF-LPH1 and SIF1-BP in the collected fractions was analyzed by electrophoretic mobility shift assays. The arrows indicate the elution times of the molecular weight standards.

in both cases the photoaffinity labelling demonstrates that a polypeptide of 50 kDa has been active in binding to the DNA. On this basis we suggest that the 250 kDa complex is a homo- or hetero-oligomer. It might be speculated that the CE-LPH1/SIF1-binding polypeptide is associated with one or more polypeptides, which act as co-activators. Such complexes could enable the cell to differentially regulate the expression of the two disaccharidases after birth using the same or a closely related DNA-binding protein.

Acknowledgements: We thank Mona Nilsson, Anette M. Kennebo and Jette Møller for excellent technical assistance, and we wish to thank Henrik Clausen for his help using the SMART chromatography system for the gel filtration. This work was supported by grants from the Danish Medical Research Council and the Lundbeck Foundation. This project was a part of a program under Biomembrane Research Center, Aarhus University.

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