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Role of Hepatocyte Nuclear Factor 1α and 1β in the Transcriptional Regulation of Human Dipeptidyl Peptidase IV during Differentiation of Caco-2 Cells

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Caco-2 cells undergo differentiation to an enterocytic-like cell when maintained in a post-confluent state for 1–2 weeks. During this period Caco-2 cells begin to express high levels brush border membrane associated enzymes such as dipeptidyl peptidase IV. Using the dipeptidyl peptidase IV gene promoter in electrophoretic mobility shift assays, we have shown for the first time that levels of hepatocyte nuclear factor 1α increase three- to fourfold during Caco-2 cell differentiation. Transient cotransfection experiments with 3T3 cells using dipeptidyl peptidase IV promoter constructs and expression vectors containing hepatocyte nuclear factor 1α and β show that the ratio of α and β modulates reporter gene expression. These results suggest that the increase in levels of hepatocyte nuclear factor 1α that occur during intestinal cell differentiation, are important for expression of dipeptidyl peptidase IV and other intestinal proteins.

Small intestinal brush border membrane dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5) functions as an important peptidase in the digestion of proline containing peptides and serves as a marker of cellular differentiation in colon cancer cells such as Caco-2 (1–3). Our laboratory recently identified a 20-bp region of the DPPIV gene promoter 120 bases 5′ from the initiation codon to which hepatocyte nuclear factor 1 (HNF-1) binds and is important for DPPIV expression in Caco-2 and HepG2 cells (4, 5). For a number of years Caco-2 cells have served as a convenient *in vitro* model for the study of intestinal cell differentiation and the processes involved in intestinal gene regulation and the biosynthesis of intestinal proteins. During Caco-2 cell differentiation there is polarization of the cell membrane into an apical (brush border) surface and basolateral membrane (6). Concomitantly there is increased expression of a number of brush border membrane associated enzymes and proteins (7, 8). As interest in brush border membrane enzymes has increased, there has been considerable progress in elucidating their regulation at the gene level. Thus it is known that the gene promoters of several brush border membrane associated proteins such as sucrase–isomaltase (9), lactase (10), dipeptidyl peptidase IV (5), and aminopeptidase N (11) contain *cis*-elements which interact with hepatocyte nuclear factor 1.

In the present study we have found that during Caco-2 cell differentiation there is a rapid fourfold increase in levels of nuclear protein binding to the HNF-1 site of the DPPIV promoter. Using electrophoretic mobility shift assays (EMSA) and supershift assays with specific antibody we found that this increase is primarily due to HNF-1α whereas levels of the β variant are relatively unchanged. We have also shown that HNF-1α stimulates transcription from a minimal DPPIV promoter construct while increasing levels of HNF-1β attenuates transcriptional activity. Thus these studies indicate that the increase in levels of HNF-1α during Caco-2 cell differentiation may be an important determining factor for the concomitant increased expression of DPPIV and other brush border membrane associated proteins observed in this cell line.

EXPERIMENTAL PROCEDURES

Cell culture and transfection. Caco-2 and HepG2 cells were obtained from the American Type Culture Collection. They were maintained in Dulbecco's modified Eagle's medium (DMEM) supple-

Abbreviations used: DPPIV, dipeptidyl peptidase IV; EMSA electrophoretic mobility shift assay; FCS, fetal calf serum; HNF-1, hepatocyte nuclear factor 1.

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mented with fetal calf serum (FCS), 100 units/ml each penicillin, and streptomycin in 5% CO_2 at 37°C. Caco-2 cell cultures were maintained with 20% FCS whereas 3T3 cells were maintained in 10% FCS. For transient transfection, adherent confluent Caco-2 cells were seeded in 12-well plates at 2.5×10^5 cells per well and used for transfection 24 h later. Plasmids for transfection were prepared either by alkaline lysis, cesium chloride banding, and purification over columns of Sepharose CL-4B or by using plasmid DNA isolation kits (Qiagen). Liposome-mediated transfection was carried out using Lipofectamine reagent (Gibco-BRL). Lipofectamine (8 μg) was mixed with luciferase reporter plasmid (pGL2 vector, Promega) (3 μg) and a plasmid (0.15 μg) encoding sea pansy (*Renilla reniformis*) luciferase (Promega) as a transfection efficiency control in serum free medium (100 μl). After incubation at room temperature for 15 min additional medium (300 μl) was added and the entire solution was carefully layered onto the cells. Twenty-four h later the transfection medium was removed and replaced with normal growth medium. Cells were washed with phosphate-buffered saline and harvested 48 h after the start of transfection. Soluble lysates were prepared by sonication in 100 μl of Reporter Lysis Buffer (Promega) followed by centrifugation (2 min) in a microfuge. In other experiments expression vectors for HNF-1α and β (2 μg) were cotransfected with the DPPIV (3 μg) and *Rinella* luciferase (0.15 μg) plasmids. The pBJ5 expression vector harboring the coding sequence for HNF-1α and β have been previously used by our laboratory (5) and others (9) and were the generous gift of Dr. Gerald Crabtree, Stanford, CA.

Supernatants were assayed for luciferase activity using a dual-luciferase reporter assay system (Promega) and the photons released were measured for 10 s in a Monolight 2010 luminometer (Analytical Luminescent Laboratory). The reporter luciferase activities were divided by the respective *Rinella* luciferase activity in order to correct for transfection efficiency and preparation of the cell extracts. These results were then normalized to a percentage of the value obtained using a positive control vector (pGL2-control, Promega) which directs the synthesis of luciferase.

Analysis of DNA-binding proteins. Nuclear extracts were prepared from confluent cells (12) and 5 μg protein was used in electrophoretic mobility shift assays (EMSA). Oligonucleotide probes were synthesized by the UCSF Biomolecular Resource Center and end-labeled using T4 polynucleotide kinase [γ-^{32}P]ATP to a specific activity of approximately 7.5×10^5 cpm/ng. EMSA binding reactions (20 μl) contained 1 ng of double-stranded [^{32}P]-labeled oligonucleotide, and reagents from a BandShift kit (Pharmacia Biotech) consisting of 40 mM Tris/HCl, pH 7.5, 200 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 2 mM dithiothreitol, 0.05% Nonidet P-40, and 1 μg poly(dI–dC). In competition studies, a 100-fold molar excess of the indicated unlabeled oligonucleotide was used. Specific antibody to either the HNF-1α or HNF-1β transcription factors were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

RESULTS

As seen in the EMSA in Fig. 1, during growth of Caco-2 cells there was a significant increase in nuclear protein binding to the DPPIV promoter HNF-1 site encompassing bases −147/−135 in the −150/−131 oligonucleotide. The increase was most rapid during the preconfluent phase so that by the time the cells were confluent (0 day) the amount of nuclear protein binding as quantified by densitometry was some 3.5-fold higher than the 15% confluent cells. After confluency, the increase was considerably slower, reaching a peak (4-fold) in cells which were maintained for 7 days in the post-confluent state. After 14 days the amount of nuclear protein binding to the DPPIV promoter was somewhat reduced (3.3-fold) from the 7 day peak value. The data in Fig. 1 is representative of several individual Caco-2 cell differentiation experiments that were performed.

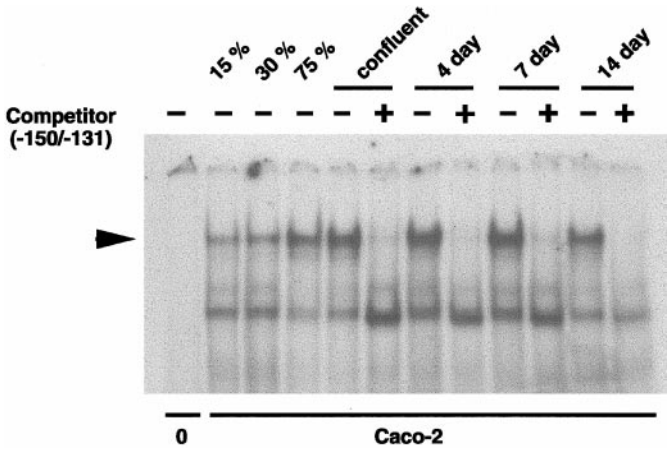

FIG. 1. Effect of Caco-2 cell differentiation on levels of HNF-1. Actively dividing cells were isolated at different stages of confluency (15, 30, and 75%) and also maintained in the postconfluent state (4, 7, and 14 days) to induce enterocytic-like differentiation. Nuclear proteins were isolated from Caco-2 cells at these different stages and used in EMSA with a 20-bp oligonucleotide (−150/−131) containing the DPPIV promoter HNF-1 *cis*-element. The arrow head indicates the position of HNF-1. No added nuclear protein; (0).

It is known that the HNF-1 transcription factor consists of α and β forms that are the products of related but separate genes. Since HNF-1 can interact with its *cis*-element as either homo- or heterodimers of the two, it was of interest to determine if the increase in transcription factor binding was due to α, β or both. This was accomplished by using specific antibody to HNF-1α and β in EMSA supershift experiments shown in Fig. 2. In the early (15%) preconfluent cells, antibody to HNF-1α shifted the majority of the nuclear protein binding to the DPPIV HNF-1 site to a slower migrating species. In contrast, antibody to HNF-1β resulted in comparatively little or no supershifting. A similar situation was apparent when nuclear proteins from 14 day Caco-2 cells were used. Thus these results indicate that the observed changes in HNF-1 during Caco-2 cell differentiation is primarily due to an increase in the HNF-1α variant.

We next looked to see the effect of increasing amounts of HNF-1α and β on transcription from the DPPIV promoter using transient transfection assays. The pBJ5 expression vector containing the coding sequence for either HNF-1α or β was cotransfected with a minimal DPPIV promoter construct (−150/−41) in the pGL2 luciferase vector. For these studies we used mouse 3T3 fibroblasts which do not normally express the HNF-1 transcription factor (5, 13). As shown in Fig.

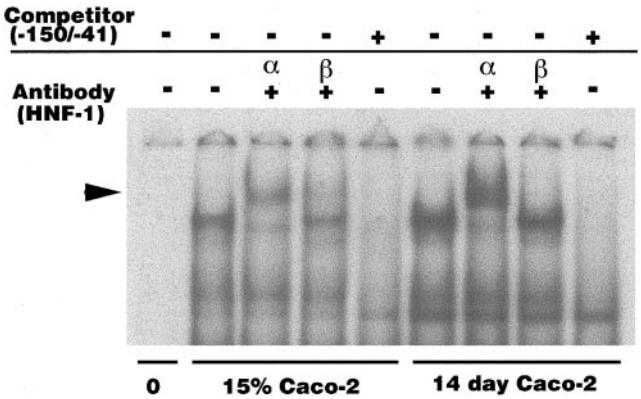

FIG. 2. EMSA supershift analysis of Caco-2 cell nuclear proteins with HNF-1 antibodies. Binding reactions were carried out in the presence (+) or absence (−) of antibody (1 μg) specific to either HNF-1α or HNF-1β for 30 min. prior to electrophoresis. Labeled −150/−131 oligonucleotide was used in the reactions. The arrowhead indicates the position of the supershifted complex. No added nuclear protein; (0).

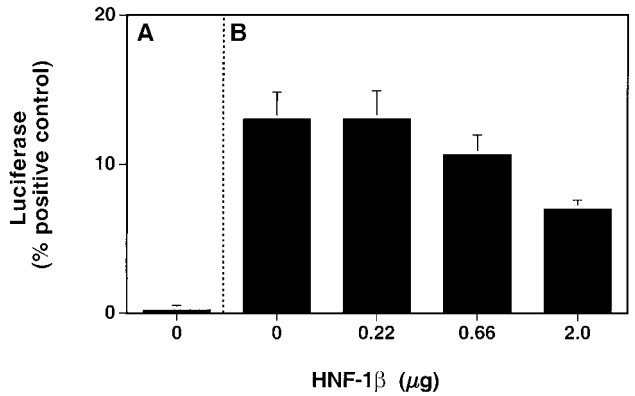

FIG. 4. Effect of HNF-1β on activation of the DPPIV promoter by HNF-1α. Mouse 3T3 cells were cotransfected with the −150/−41 region of the DPPIV promoter (3 μg in the pGL2 vector) and either the pBJ5 null expression vector (A) or pBJ5 plasmids containing the coding region for HNF-1α or HNF-1β (B). The amount of HNF-1α in Panel B was kept constant at 0.66 μg and titrated with increasing amounts of HNF-1β. The total amount of plasmid used in the HNF-1 transfections was maintained at 2 μg by addition of the pBJ5 null vector. Luciferase activity was measured in cell extracts and normalized as described under Experimental Procedures. Results are means ± SD of four individual experiments.

3, increasing amounts of HNF-1α plasmid resulted in increased transcription from the DPPIV promoter construct, thus representing an 18-fold stimulation in activity at the highest level tested when compared to the null pBJ5 vector. In contrast, HNF-1β was much less effective in stimulating reporter gene expression. Figure 4 shows the effect of titrating a fixed amount of HNF-1α plasmid (3 μg) with increasing amounts of HNF-1β in a series of cotransfection experiments with the DPPIV (−150/−41) pGL2 construct. As shown, with increasing amounts of the HNF-1β, there was a progressive decrease in reporter gene expression indicating that HNF-1β acts as a repressor of HNF-1α mediated enhancer activity.

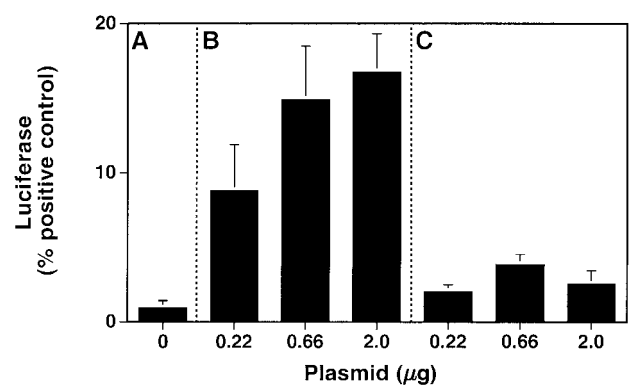

FIG. 3. Activation of the DPPIV promoter by HNF-1α in mouse 3T3 cells. Mouse 3T3 cells were cotransfected with the −150/−41 region of the DPPIV promoter (3 μg in the pGL2 vector) and either the pBJ5 null expression vector (A) or pBJ5 plasmids containing the coding region for HNF-1α (B) or HNF-1β (C). The total amount of plasmid used in the HNF-1 transfections was maintained at 2 μg by addition of the pBJ5 null vector. Luciferase activity was measured in cell extracts and normalized as described in Experimental Procedures. Results are means ± SD of four individual experiments.

DISCUSSION

Caco-2 cells spontaneously differentiate in tissue culture to a cell type exhibiting characteristics typical of small intestinal epithelial cells in the absence of any exogenous inducing agents. Electron microscopic studies have shown that differentiated Caco-2 cells are highly polarized with a typical brush-border membrane and have tight junctions between individual cells (6). In addition, this cell phenotype is characterized by high levels of enzymes typically associated with the brush border membrane and by the formation of domes in culture which are a characteristic marker of polarized epithelial cells (6–8). Thus Caco-2 cells have been used as a convenient cell model for examining the processes of intestinal cellular differentiation *in vitro*. Furthermore, immunofluorescence studies of tumors derived from the Caco-2 cell line in nude mice show that brush border membrane enzymes are expressed *in vivo* and may also be found in tumors from patients with colorectal carcinomas (3). Thus expression of brush border membrane enzymes in tissues such as the colon where these enzymes are normally absent may be an important diagnostic tool for the detection of colonic malignancies.

In our laboratory we have observed that although levels of DPPIV in undifferentiated Caco-2 cells are low, differentiated cells express DPPIV at levels equiv-

alent to that found associated with the brush border membrane of small intestinal enterocytes (8). Therefore, it is of considerable interest to delineate the biochemical mechanisms involved in the induction of DPPIV during Caco-2 cell differentiation. In the present study, we have conclusively shown that levels of HNF-1 binding to the DPPIV promoter increase significantly during growth and differentiation of Caco-2 cells. Previous studies (11) with the aminopeptidase N promoter did not find differentiation dependent changes in HNF-1 levels in Caco-2 cells possibly because preconfluent cells were not examined. We have also shown in a series of transient cotransfection experiments that HNF-1α stimulates reporter gene expression in a dose dependent fashion when transfected into 3T3 cells which normally do not express HNF-1. Our study clearly demonstrates that it is primarily the α form of HNF-1 that increases during Caco-2 cell differentiation and that HNF-1α is responsible for stimulating reporter gene expression with the DPPIV promoter in 3T3 cells. Conversely, the β variant of HNF-1 does not does not stimulate reporter gene expression from the HNF-1 site of the DPPIV promoter instead acting as a repressor of the stimulatory effect by HNF-1α. Thus these results show that different levels and ratios of HNF-1α and β are important in modulating DPPIV gene expression.

At present, little is known of the factors which control expression of HNF-1. It is known that both the α and β forms are present in similar tissues such as kidney, liver, intestine, and pancreas although their levels vary (14–17). During development and organogenesis, levels of HNF-1β tend to be comparatively higher suggesting that it participates in early stages of development while HNF-1α appears later and is thought to be important in maintaining the differentiated phenotype of some cells (17). Along the crypt–villus axis of the small intestine studies have shown that HNF-1α and β are both primarily expressed in cells of the crypt (18), although RNA transcripts for both types have been noted in the differentiated cells of the villus (19). It has also been documented that the ratio of HNF-1α and β vary along the longitudinal axis of the small intestine, which our results indicate may be important for the observed differential expression of a number of intestinal proteins along the length of the small intestine (9, 18). In the normal colon levels of HNF-1β are highest whereas levels of HNF-1α are low (18), which coincidentally is where small intestinal brush border membrane proteins are not normally expressed. It is well documented that some colorectal tumors do express brush border membrane proteins such as DPPIV and sucrase–isomaltase (3, 6). Recently the overexpression of DCoH in human colon cancers has been described (20). DCoH is a protein dimerization cofactor that is important in facilitating dimerization of HNF-1 subunits and their interaction with DNA (21). It is thought that overexpression of this protein could alter the transactivation potential of HNF-1 which might lead to the aberrant expression of HNF-1 dependent proteins such as DPPIV in these tumors. Thus our study is of potential significance in elucidating the patterns of HNF-1 related DPPIV expression in not only the normal small intestine but also colon cancer.

In summary, it is becoming apparent that a common feature of a number of intestinal brush border membrane proteins which have been examined to date is that they have HNF-1 *cis*-elements in their gene promoters which bind HNF-1. In addition to DPPIV they include sucrase–isomaltase (9, 22), lactase (10), aminopeptidase N (11), and other intestinal genes such as guanylin (13) and α_1-anti-trypsin inhibitor (15, 16). Therefore the findings in this study are important in elucidating the regulation of other intestinal genes by HNF-1 and provide insight into the regulatory processes involved in the differentiation of intestinal absorptive cells and the development of certain colon cancers.

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