Keratinocyte Growth Factor Down-regulates Expression of the Sucrase-Isomaltase Gene in Caco-2 Intestinal Epithelial Cells*

Jie Zhou, Kenneth Wu, Christabel L. Fernandes, Anna L. Cheng, and Paul W. Finch‡

From the Derald H. Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, New York 10029

The molecular mechanisms that regulate the proliferation and differentiation of intestinal mucosal epithelial cells are not well understood. Keratinocyte growth factor (KGF) is an epithelial cell-specific growth factor that may be involved in the maintenance of mucosal epithelial populations and in mediating epithelial repair after injury. The sucrose-isomaltase (SI) gene, which encodes an enterocyte brush border disaccharidase, has served as a model for study of intestinal-specific gene expression and differentiation. KGF down-regulated SI mRNA and protein expression in Caco-2 intestinal epithelial cells but not the expression of other brush border enzymes. The down-regulation was dose- and time-dependent and specifically blocked by anti-KGF antibodies. Transfection experiments using SI promoter constructs demonstrated that KGF decreased SI gene transcription. In contrast, the stability of SI mRNA was not affected by incubation of Caco-2 cells with KGF. Electrophoretic mobility shift analysis demonstrated that binding of nuclear proteins to the SI footprint (SIF) 3 and SIF4 regulatory elements within the SI promoter region was increased in Caco-2 cells that had been incubated with KGF. In transfection experiments using a construct in which tandem copies of the SIF4-binding site were inserted upstream of the SV40 promoter and luciferase gene, incubation with KGF resulted in a significant decrease in luciferase activity. However, transfection with a similar construct containing tandem copies of SIF3 had no significant effect on SV40 promoter activity following KGF treatment. SIF4 may bind E4BP4, a previously identified transcriptional repressor protein. This factor may in part mediate the decrease in SI transcription by KGF in Caco-2 cells.

The mucosal epithelial layer, which forms the interface between the external and internal environments in the gastrointestinal tract, is continuously undergoing renewal. This occurs through proliferation of stem cells in the intestinal crypt (1, 2), migration of daughter cells from the crypts onto the villi, and extrusion of cells into the intestinal lumen at the tips of the villi, possibly as a result of apoptosis or an ordered program of cell senescence (3, 4). Migration of enterocytes from the crypts to the villi is coincident with the acquisition of the differentiated phenotype that enables them to perform specialized digestive, absorptive, and barrier functions. The mechanisms that determine lineage-specific gene expression, or the process of differentiation as cells migrate from the crypts to the villi, are not well understood.

The sucrose-isomaltase (SI) gene encodes a brush border disaccharidase that is expressed exclusively in enterocytes in the adult small intestine (5, 6). SI mRNA is absent from crypt epithelial cells and is initially expressed by enterocytes at the crypt-villus junction. It is highly expressed in the lower half of the villus, but expression is decreased in enterocytes at the villus tip (5–7). For these reasons, the SI gene has been extensively studied as an example of both intestinal- and differentiation-specific gene expression (8). Enterocyte-specific transcription of the SI gene is regulated by an evolutionarily conserved region of the promoter that extends approximately 180 bp upstream of the transcription start point and contains several regulatory elements for transcription in intestinal epithelial cells (8). Cdx-2, an intestinal-specific homeodomain protein (9), and the hepatocyte nuclear factor-1α (HNF-1α) and HNF-1β transcription factors (10) have been shown to bind to sites in the conserved SI promoter region and to positively regulate gene expression. The integration of transcriptional regulatory elements is thought to play a critical role in regulating the complex spatial patterns of SI gene expression in vivo (8).

Keratinocyte growth factor (KGF) is a fibroblast-derived member of the fibroblast growth factor family that has potent activity for epithelial cells but not fibroblasts, endothelial cells, nor other non-epithelial cell types (11, 12). The demonstration that KGF and KGF receptor (KGFR) mRNAs are expressed throughout the gastrointestinal tract and that systemically administered KGF was able to rapidly induce the proliferation of epithelial cells from the foregut to the colon provides functional evidence for the ability of KGF to activate gastrointestinal epithelial populations in vivo (13). Furthermore, KGF is highly overexpressed in inflammatory bowel disease (14–16), suggesting that it may be involved in mediating epithelial repair after injury caused by inflammatory processes. In this study, we demonstrate that KGF down-regulates SI gene expression in Caco-2 intestinal epithelial cells by decreasing transcription from the SI promoter. This appears to be regulated in part by increased binding of a transcriptional repressor protein to a site in the SI promoter region.

MATERIALS AND METHODS

Cell Culture—A subclone of the human colon adenocarcinoma Caco-2 cell line, designated Caco-2/15, which had undergone a limited number...
of passages and exhibited high levels of surace-isoalactase expression (17), was kindly provided by Dr. A. Quarioni (Cornell University, Ithaca, NY). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin, refed every 48 h, and subcultured serially when ∼80% confluence was reached. Cells to be used in separate, independent treatment groups, were seeded at identical cell densities and were typically used 12–15 days after reaching confluence. However, most transfection experiments were performed on Caco-2 cells that were between 1 and 2 days preconfluence, since previous experience indicated that transfection efficiencies were significantly lower in postcon- fluent cells (18).

Preparation and Analysis of RNA—Total cellular RNA was prepared using the TRIZOL reagent (Life Technologies, Inc.) according to the manufacturer's instructions. The human KGFR cDNA construct used to synthesize antisense transcripts for use in the ribonuclease protection assay (RPA) has been previously described (14). The following human cDNA probes were generated for PCR and cloned into the vector pGEM3Zf(−) (Promega, Madison, WI) for use as hybridization probes for RPA: a 311-bp fragment of SI cDNA (bp 107→418) (19); a 260-bp fragment of the dipetidyl peptide (DPP) IV cDNA (bp 380→640) (20); and a 282-bp fragment of intestinal alkaline phosphatase (IAP) cDNA (bp 138→420) (21). The cloned fragments were sequenced to ensure the correct identity of the cloned fragment and that no errors had been incorporated during the amplification reaction. All fragments were orientated such that transcription with T7 RNA polymerase generated antisense transcripts from HindIII-linearized templates. The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a 316-bp SacI/BamHI fragment (nucleotides 109→427), and the human c-MYC probe was a 250-bp KpnI/EcoRI cDNA fragment (nucleotides 1197→1446). Both fragments were subcloned into the Tripletvector such that transcription with either T3 or T7 polymerases generated the antisense transcript (Ambion Inc., Austin, TX). Transcript levels were determined using RPA using 32P-labeled antisense cRNA transcripts as hybridization probes. Ten μg of total cell RNA was hybridized overnight at 43 °C with 1 × 106 cpm of gel-purified probe. Hybrids were digested with 0.1 unit of RNase A and 20 units of RNase T1 (Ambion Inc., Austin, TX) for 60 min at 37 °C. Protected fragments were resolved on 6% polyacrylamide gels using autoradiography.

KGFR Protein Analysis—Caco-2 cell lysates were prepared in Staph A buffer (10 mM sodium phosphate, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) containing the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) (1 mM) and aprotinin (10 μg/ml) and clarified by centrifugation at 10,000 g at 4 °C. Supernatants were vortexed for 10 s, centrifuged briefly, and then the supernatant was mixed with 2 volumes of assay buffer (without the dithiothreitol, 0.2 mM PMSF). Following 10 min incubation on ice, cellular debris was removed by centrifugation at 10,000 g for 30 min at 4 °C. Lysates were then incubated at 4 °C with 100 ng/ml KGF or vehicle alone. Actinomycin (10 μg/ml) was then added to the medium, and cells were harvested at different time points. Total RNA was prepared, and the level of SI transcripts was determined by RPA.

Preparation of Nuclear Extracts—Nuclear extracts were prepared as described by Andrews and Faller (27). Briefly, 106 cells were scraped from a 100-mm dish, pelleted, and then resuspended in cold buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF). Following a 10 min incubation on ice, cells were vortexed for 10 s, centrifuged briefly, and then the supernatant discarded. The pellet was resuspended in cold buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF) and incubated on ice for 20 min. Cellular debris was removed by centrifugation for 2 min at 4 °C, and the supernatant fraction, containing nuclear DNA-binding proteins, was stored in aliquots at −70 °C.

Electrophoretic Mobility Shift Assays—Complementary oligonucleo- tides with overlapping ends were annealed and labeled by filling in the ends with [32P]-labeled dNTPs and Klenow enzyme. Binding reaction mixtures (20 μl) contained 20 μg HEPES, 60 mM KCl, 1 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, 12% glycerol, and 1 μg of poly(dI-dC)-nonspecific competitor DNA (28). Protein (5–7 μg of nuclear extract) was added, and the reaction was incubated at 25 °C for 10 min prior to the addition of labeled probe (1–2 × 105 cpm, 0.1–0.2 ng). Binding reactions were incubated for 20 min at 25 °C. Electrophoresis was carried out at 25 °C in 4% polyacrylamide gels (30:1 acrylamide:bisacryl- amide) in 0.5 × TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, 1.0 mM EDTA). Gels were pre-run at 15 mA for 30 min prior to loading. Electrophoresis of samples was continued for 2–3 h at 15 mA, following which the gels were dried and autoradiographed.

RESULTS

Expression of the KGFR as a Function of Differentiation in Caco-2 Cells—The Caco-2 human colon adenocarcinoma cell line is a commonly used model system for absorptive enterocytes since the cells undergo spontaneous differentiation when they form confluent monolayers (29, 30). To determine whether KGFR is expressed and how expression may change with degree of differentiation, we examined KGFR expression in the corresponding site of the pGL3-Promoter vector (Promega). To verify the presence and orientation of the inserted fragment, representative clones were sequenced. Resulting recombinants were called pSIF1-SV40-LUC, pSIF2-SV40-LUC, and pSIF4-SV40-LUC, respectively. In the pSIF3-SV40-LUC construct, the two SIF3 sites were inserted in the correct orientation and then the insertion had been shown to maximally augment transcription (10). In pSIF1-SV40: LUC and pSIF4-SV40-LUC, the SIF1 and SIF4 sites were oriented in the same direction as the SV40 promoter.

For transfections, cells were plated at a density of 1–2 × 104 cells/cm2 in 60-mm tissue culture dishes and then incubated for 4 h with a medium containing phosphate-DNA (28). After washing and incuba- tion with DMEM, 10% FCS for 24 h, KGF was added to the cells at a concentration of 100 ng/ml. Following a 24 h incubation, cells were twice with ice-cold phosphate-buffered saline (PBS) and then lysed in reporter lysis buffer (Promega, Madison, WI). Lysates were briefly centrifuged in a microcentrifuge to remove insoluble material, and then extracts were stored in aliquots at −70 °C. For luciferase assays, 20 μl of cell extract was mixed with 100 μl of luciferase assay reagent (Promega) at room temperature. After mixing, the reaction was placed in a TD-20 luminometer (Turner Designs Inc., Mt. View, CA), and the light produced was measured for a period of 10–20 s, beginning 10 s after mixing. β-Galactosidase was measured by adding 50 μl of extract to 200 μl of assay buffer (100 mM sodium phosphate buffer, pH 7.3, 1 mM MgCl2, and β-mercaptoethanol). The reaction was linear for 15 min at 37 °C for 2 h or until the color had developed. Results were reported as the fold increase of light units generated per unit of β-galactosidase activity. For these studies, pRSV:LUC, a promoterless luciferase vector was used as negative control for luciferase activity following transfection into Caco-2 cells. pRSV:LUC, in which the Rous sarcoma virus promoter and enhancer are linked to the luciferase gene, or the pGL3-Promoter vector, in which an SV40 promoter drives luciferase expression, was used as positive control.
Caco-2 cells at various times pre- and postconfluence. Both transcript and protein expression levels were lowest in preconfluent cells but increased significantly at the time the cells formed a confluent monolayer (day 4 after plating) (Fig. 1, A and B). After 3 days at this high level, there was a gradual decline in expression with increasing time postconfluence. In order to determine whether functional signaling was possible in postconfluent cells, ligand-stimulated tyrosine phosphorylation of the KGFR was examined in a 12-day postconfluent culture of Caco-2 cells. Following triggering for 5 min with 100 ng/ml KGF, the cell lysate was specifically enriched for the KGFR by immunoprecipitation and then subjected to Western blotting analysis using an anti-phosphotyrosine antibody. As shown in Fig. 1C (middle panel), KGF treatment led to a modest increase in tyrosine phosphorylation of the KGFR. This signal could be competitively inhibited by the inclusion of phenyl phosphate with the immunoprecipitation buffer, thus demonstrating specificity for phosphotyrosine (Fig. 1C, right panel).

KGF Down-regulates SI Expression in Postconfluent Caco-2 Cells—Having demonstrated functional KGFR signaling in postconfluent Caco-2 cells, we then sought to examine KGF effects on the enterocytic phenotype. Differentiation is indicated by increased expression of a number of brush border enzymes, including SI, IAP, and DPP IV, which are typical components of mature small intestine enterocytes. Following treatment of postconfluent cells with various concentrations of KGF for 24 h, there was a specific down-regulation of SI mRNA expression, in a dose-dependent fashion (Fig. 2A). In contrast, there was only a slight decrease in IAP expression, and while there were some fluctuations in DPP IV transcript levels, overall there appeared to be no significant change. The down-regulation of SI mRNA by KGF was not transient and was sustained over a period of at least 48 h (Fig. 2B). To rule out the possibility that a contaminant such as endotoxin was responsible for the down-regulation of SI RNA, we preincubated KGF with the neutralizing monoclonal anti-human KGF antibody, 1G4 (31), for 2 h and then incubated this mixture with Caco-2 cells. No decrease in SI RNA was observed when compared with cells stimulated by KGF incubated with a nonimmune immunoglobulin, thus providing evidence for the KGF specificity (data not shown).

To determine that KGF treatment also resulted in decreased SI protein synthesis, Caco-2 monolayers were pulse-labeled with [35S]methionine and -cysteine for 30 min. Monolayers were then lysed and subjected to immunoprecipitation with an anti-SI monoclonal antibody. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. A single radiolabeled polypeptide of ~200-kDa

![Figure 1](image1.png)

**Fig. 1.** KGFR expression during differentiation of Caco-2 cells. A, time course of KGFR mRNA expression. RNA was isolated from Caco-2 cells at various times after plating, and the level of KGFR mRNA was determined by RPA (top panel). Cells reached confluence at day 4 after plating (denoted by the *). The same RNAs were also examined for SI (middle panel) and GAPDH mRNA expression (bottom panel). B, time course of KGF protein expression. Lysates of Caco-2 cells were immunoprecipitated with the C-17 polyclonal antibody and then subjected to immunoblotting using the same antibody. Cells reached confluence at day 4 after plating. C, ligand-stimulated tyrosine phosphorylation of KGFRs in 12-day postconfluent Caco-2 cells. Lysates from either untreated cells or cells exposed to 100 ng/ml KGF for 5 min were immunoprecipitated with the C-17 antibody and then subjected to immunoblotting with C-17 (left panel), anti-phosphotyrosine (middle panel), or anti-phosphotyrosine in the presence of phenyl phosphate (right panel). Molecular mass markers (myosin, 200 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 68 kDa) are indicated.

![Figure 2](image2.png)

**Fig. 2.** KGF down-regulation of SI mRNA in Caco-2 cells. A, dose-response analysis of SI expression in response to treatment with KGF. Twelve-day postconfluent cells were treated for 24 h with various concentrations of KGF. Following harvesting of cells, RNA was isolated, and transcript levels were determined by RPA. For comparison, the levels of the IAP, DPP IV, and GAPDH genes were also determined. B, time course of down-regulation of SI expression by KGF. Twelve-day postconfluent Caco-2 cells were treated with 100 ng/ml KGF for the times indicated. Cells were harvested, RNA isolated, and SI and GAPDH expression determined by RPA.
was present in control Caco-2 cells (Fig. 3), corresponding to the single chain SI precursor (23). There was a dose-dependent decrease in this SI protein species following incubation of post-confluent cells with increasing amounts of KGF (Fig. 3). Thus, incubation of Caco-2 cells with KGF results in down-regulation expression of both SI transcript and protein synthesis.

**Mechanism of KGF Down-regulation of SI Gene Expression** —To determine the mechanism by which KGF inhibits SI synthesis, we examined its effects on SI gene transcription and mRNA stability. To examine effects on SI transcription, the −324 HS-LUC and −3424 HS-LUC SI promoter plasmids, which contain 324 and 3424 bp, respectively, of the human SI gene 5′-flanking region (18), were transfected into Caco-2 cells. Following washing, cells were treated with either 100 ng/ml KGF or vehicle for 24 h, and luciferase activity was determined. As shown in Table I, luciferase activity directed by the −324 HS-LUC construct was decreased 39% when cells were incubated with KGF for 24 h in the absence or presence of KGF, and cell lysates analyzed for the expression of luciferase and β-galactosidase. pA3-LUC (Rous sarcoma virus promoter and enhancer) was used as negative and positive controls, respectively. Values for luciferase activity are expressed as light units/unit of pRSV-LUC (Rous sarcoma virus promoter and enhancer) were used as negative and positive controls, respectively. Values for luciferase activity are expressed as light units/unit of pRSV-LUC (Rous sarcoma virus promoter and enhancer) were used as negative and positive controls, respectively. Values for luciferase activity are expressed as light units/unit of pRSV-LUC

### TABLE I

**Effect of KGF on SI promoter activity**

The −324 HS-LUC and −3424 HS-LUC SI promoter constructs were co-transfected into Caco-2 cells along with pSV-β-galactosidase, treated for 24 h in the absence or presence of KGF, and cell lysates analyzed for expression of luciferase and β-galactosidase. pA3-LUC (promoterless) and pRSV-LUC (Rous sarcoma virus promoter and enhancer) were used as negative and positive controls, respectively. Values for luciferase activity are expressed as light units/unit of β-galactosidase activity. Results represent mean values ± S.D. from seven independent experiments.

| Plasmid          | −KGF | +KGF | p*  |
|------------------|------|------|-----|
| pA3-LUC          | 396 ± 120 | 431 ± 109 | 0.718 |
| pRSV-LUC         | 1.52 × 10⁶ ± 273883 | 1.62 × 10⁶ ± 303388 | 0.606 |
| −324 HS-LUC      | 18966 ± 4308 | 11546 ± 4880 | 0.037 |
| −3424 HS-LUC     | 52102 ± 12327 | 28412 ± 15710 | 0.032 |

* Paired Students t test; p < 0.05 was considered statistically significant.

### TABLE II

**KGF effect on SI promoter activity as a function of degree of confluence of Caco-2 cells**

The −3424 HS-LUC SI promoter construct and pSV-β-galactosidase were co-transfected into Caco-2 cells at various times after plating, treated for 24 h in the absence or presence of KGF, and cell lysates analyzed for expression of luciferase and β-galactosidase. pA3-LUC (promoterless) and pRSV-LUC (Rous sarcoma virus promoter and enhancer) were used as negative and positive controls, respectively. Values for luciferase activity are expressed as light units/unit of β-galactosidase activity. Results represent mean values of duplicate determinations.

| Plasmid       | −2   | 0 | +2  | +4  | +6  |
|---------------|------|---|-----|-----|-----|
| pA3           | 160  | 52| 76  | ND  | 19  |
| pA3 + KGF     | 376  | 58| 47  | ND  | 74  |
| pA3 RSV       | 2.38 × 10⁶ | 0.58 × 10⁶ | 3.45 × 10⁶ | 3.1 × 10⁵ | 50,200 |
| pA3 RSV + KGF | 2.64 × 10⁶ | 1.08 × 10⁶ | 4.65 × 10⁶ | 5.4 × 10⁵ | 47,200 |
| −3424 HS-LUC  | 54,286 | 24,645 | 10,097 | 6,032 | 3,922 |
| −3424 HS-LUC + KGF | 31,798 | 10,690 | 4,651 | 2,478 | 1,108 |

* ND, not determined.
(SIF)-1, SIF2, and SIF3. SIF1 binds the intestine-specific homeodomain protein Cdx2 (9), and SIF2 and SIF3 bind HNF-1α and HNF-1β (10). In addition, there is a negative cis-acting element, SIF4, that may be a binding site for the E4BP4 transcriptional repressor protein (8, 25). We performed EMSA to determine whether binding of any of these transcription factors was affected by KGF treatment. Since HNF-1 binds SIF3 with greater affinity than SIF2 (10), these experiments were only performed using the SIF3-binding element. As shown in Fig. 5, binding of nuclear proteins to SIF1 was not affected by using extracts prepared from KGF-treated cells. In contrast, binding of nuclear proteins to the SIF3 and SIF4 oligonucleotides was increased with extracts prepared from KGF-treated cultures (Fig. 4).

Again, we were concerned that there might be differences in the levels of these transcription factors between Caco-2 cells at various levels of confluence or that the magnitude of the KGF-mediated effect might change along with altered levels of KGFR expression. Therefore, we examined binding using nuclear extracts prepared from 1-day and 12-day postconfluent cells that had either been untreated or treated with KGF. No significant changes were detected either in the levels of SIF1, SIF3, or SIF4 binding between the two extracts (data not shown). These results are in agreement with those described by Traber et al. (24) that demonstrated that no qualitative changes in the patterns of footprints on the SI promoter region were obtained using nuclear proteins prepared from Caco-2 cells at either 4 or 19 days after plating. Furthermore, no significant differences were detected in the magnitude of the changes observed in response to KGF treatment as detected by EMSA (data not shown).

To determine how these changes in binding might affect SI gene transcription, the pSIF1-SV40:LUC, pSIF3-SV40:LUC, and pSIF4-SV40:LUC plasmids, which contain tandem copies of each of these sites cloned upstream of an SV40 promoter and luciferase reporter gene, were transfected into Caco-2 cells, and luciferase activity was determined. As shown in Table III, insertion of each of these promoter elements upstream of the SV40 promoter led to increased luciferase activity compared with that directed from the SV40 promoter alone in control, untreated cells. However, a significant decrease in luciferase activity observed was only observed in cells transfected with pSIF4-SV40:LUC (81% activity present in untreated cells; p = 0.009) following KGF treatment. These results suggest that modulation of transcription factor binding to SIF4 may play a role in KGF-mediated down-regulation of SI gene transcription.

**DISCUSSION**

We have demonstrated that KGF down-regulates expression of the intestinal and enterocytic specific SI gene in Caco-2 intestinal epithelial cells. SI is first expressed as enterocytes reach the crypt-villus junction, is abundant in these cells from the base of the villi to the mid-villus region, and then decreases as they move from the mid-villus toward the tip, resulting in lower levels in villus tip cells (5–7). In the small intestine KGF mRNA expression was localized predominantly to mesenchymal cells at the tip of each villus (14), directly beneath the differentiated enterocytes that express lower levels of SI mRNA than are found in mid-villous enterocytes (5–7). Interestingly, there is also an inverse correlation between KGF and SI expression in the small intestine. The highest levels of SI mRNA are present in the proximal and distal jejunum, with expression decreasing to approximately 50 and 20% of this level in the proximal and distal ileum, respectively (33). However, KGF expression is three times higher in the ileum than in the jejunum (13). Therefore, the highest levels of KGF expression are found where SI expression is lowest. The patterns of expression of these two genes, along with the observation that KGF down-regulates SI in vivo, suggest that this may also be an endogenous regulator of SI expression in vivo.

SI expression has also been reported to be down-regulated by a number of other soluble factors including epidermal growth factor (EGF) (34), interleukin 6 (34), and interferon γ (35).

**FIG. 4.** Stability of SI and c-myc transcripts following treatment of Caco-2 cells with KGF. Confluent monolayers of Caco-2 cells were cultured for 24 h in the presence of 100 ng/ml KGF. Cycloheximide was added at 10 μg/ml, and cells were harvested at the time indicated. Total cellular RNA was isolated, and the expression of the SI and c-myc transcripts was analyzed by RPA, as described under “Materials and Methods.”

**FIG. 5.** EMSA of SIF1-, SIF3-, and SIF4-binding proteins in extracts prepared from control and KGF-treated Caco-2 cells. Binding of nuclear proteins to the SIF1, SIF3, and SIF4 elements was assessed in Caco-2 cells that had either been incubated in medium alone (−) or medium containing 100 ng/ml KGF for 24 h. The competitors used consisted of a 100-fold molar excess of the corresponding unlabeled double-stranded oligonucleotide.

**TABLE III**

Effect of KGF on transcription directed by SI promoter regulatory elements

| Plasmid          | −KGF (μluc) | +KGF (μluc) | p*          |
|------------------|------------|------------|-------------|
| pA3-LUC          | 350 ± 111  | 400 ± 100  | 0.517       |
| pSV40-LUC        | 776,000 ± 96,000 | 867,000 ± 205,000 | 0.468       |
| pSIF1-SV40-LUC   | 1.4 × 106 ± 195,000 | 1.29 × 106 ± 223,000 | 0.362 |
| pSIF2-SV40-LUC   | 937,000 ± 285,000 | 947,000 ± 262,000 | 0.910       |
| pSIF4-SV40-LUC   | 909,000 ± 232,000 | 739,000 ± 133,000 | 0.009       |

*Paired Students t test; p < 0.05 was considered statistically significant.
Although there is no endogenous EGF within the gastrointestinal tract, the structurally related transforming growth factor α, which also acts through the EGF receptor, is synthesized by intestinal epithelial cells with the highest expression present in differentiated villus cells (36, 37). Therefore, autocrine-acting transforming growth factor α and paracrine KGF may cooperate to down-regulate SI expression in mature enterocytes.

In keeping with the observation that SI was down-regulated by interleukin 6 and interferon γ, SI expression was decreased in enterocytes along the entire villus length during the local inflammatory response of Crohn’s disease (35) in which there is increased expression of both of these cytokines (38). KGF is highly expressed throughout the lamina propria in Crohn’s disease (14–16). Thus, KGF may also cooperate with these cytokines to down-regulate SI expression throughout the villus during chronic inflammation.

Our results indicate that KGF-mediated down-regulation of SI expression occurs primarily at the level of transcription and not through SI transcript destabilization. The mechanisms that control transcription from the SI promoter have been studied in some detail (8) making it possible to examine which transcription factor(s) might be regulated by KGF. Specifically, we found that binding of nuclear proteins to the SIF4 regulatory element in the SI promoter region was up-regulated in KGF-treated cells, as determined by EMSA. Insertion of a tandem repeat of the SIF4 element upstream of the SV40 promoter initially increased its activity relative to the control promoter alone. However, KGF treatment was able to significantly decrease the activity of the pSIF4-SV40:LUC construct but not that of the control (Table III). We interpret these results as indicating that the introduction of the tandem SIF4 sites into the pSV40:LUC promoter vector results in some non-specific transcriptional activation. However, following KGF treatment, the specific binding of a transcriptional repressor mediates down-regulation of promoter activity. SIF4 was only weakly protected in footprinting experiments using Caco-2 nuclear extracts but was protected to a greater extent using liver nuclear extracts (25). Screening of a liver cDNA library with an SIF4 oligonucleotide identified E4BP4, a transcription factor of the basic leucine zipper class, as being able to bind this sequence (8, 25). This protein has been shown to be a transcriptional repressor (39), which would agree with our observations that the binding of nuclear proteins to the SIF4 oligonucleotide was increased in extracts prepared from KGF-treated cells, but also that this regulatory element was able to mediate down-regulation of SV40 promoter activity in Caco-2 cells following KGF treatment. Based upon our data, we would predict that SIF4 would be protected to a greater extent in footprinting experiments using nuclear extracts prepared from KGF-treated Caco-2 cells.

KGF was also shown to increase the binding of nuclear proteins to the SIF3 regulatory element (Fig. 5), which has previously been identified as a binding site for HNF-1 proteins (10). However, we were unable to demonstrate any significant effect of the SIF3 regulatory element on SV40 promoter activity following KGF treatment (Table III). SIF3 has been reported to be a positive regulator of SI gene expression (10). However, since none of the transcriptional proteins identified to date as modulators of the SI promoter are sufficient to direct the intestine-specific pattern of expression of this gene alone, SI gene transcription is likely to be modulated by the combinatorial effects of multiple factors. Therefore, it is perhaps not surprising that SIIF promoter elements might not be able to mediate the expected alterations in gene transcription, based upon EMSA data, when placed out of their usual molecular context upstream of another promoter. A more specific way of further determining the roles of these regulatory sites in mediating the effects of KGF on SI promoter activity would be to specifically mutate them within the conserved promoter region and then determine the effects using transient transfection assays.

We found that the levels of SIF-binding proteins did not change appreciably between 1- and 12-day postconfluent cells. Furthermore, there appeared to be no significant change in the magnitude of alterations triggered by KGF treatment between these different sets of nuclear extracts. However, we also found from transfection analysis of the −3424 HS-LUC SI promoter construct that KGF mediates the greatest fold decreases in SI transcription in cells that are at progressively greater periods of postconfluence. This suggests that there may be as yet uncharacterized SIF elements that might not be readily detectable in untreated cells, that are involved in mediating the more pronounced KGF effects in postconfluent cells, or, alternatively, that these changes are mediated by proteins involved in protein-protein interactions, as opposed to protein-DNA interactions, and would not be readily detectable by the assays used in the current study.

Although the mechanism through which KGF treatment leads to altered binding of these transcription factors to their respective binding sites has not been investigated, there are a number of potential possibilities. KGF might, for example, stimulate increased transcription factor biosynthesis. Alternatively, both HNF and E4BP4 proteins exist as phosphoproteins, with the levels of phosphorylation being important for DNA binding activity (40, 41). Other mechanisms could include heterodimerization with related transcription factors. HNF-1α and HNF-1β are known to heterodimerize (42–44). HNF-1β is a weaker inducer of transcription than HNF-1α (10, 44). Therefore, regulation of binding is likely to be complex.

In conclusion, we have shown that KGF is able to specifically down-regulate intestine-specific SI expression. We have further demonstrated that this is due to decreased transcription as opposed to KGF-mediated destabilization of SI transcripts. In an effort to define which transcription factors may be mediating these changes, we have identified the SIF4 regulatory element as a potential mediator of SI down-regulation. However, further work will be required to fully appreciate the molecular mechanisms involved in controlling this process and whether they are commonly used in KGF regulation of other intestinal genes.

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Jie Zhou, Kenneth Wu, Christabel L. Fernandes, Anna L. Cheng and Paul W. Finch

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