mal fraction, but no activity was found in the microsomal fraction of the other organs. The reasons for these differences can hardly be discussed on the basis of the present brief study, but a knowledge of its distribution could be useful in further investigations of PAO.

Acknowledgments. We wish to thank Dr Nikolaus Seiler for the stimulus to do these studies and Dr Ignat Minkov for the technical consultation.

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Decrease of mRNA levels and biosynthesis of sucrase-isomaltase but not dipeptidylpeptidase IV in forskolin or monensin-treated Caco-2 cells

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Received 21 January 1991; accepted 15 May 1991

Abstract. Treatment for 48 h of differentiated, confluent Caco-2 cells with $2.5\ 10^{-5}$ M forskolin or 10^{-6} M monensin, which produces a significant decrease of the de novo biosynthesis of sucrase-isomaltase, does not change quantitatively the de novo biosynthesis of dipeptidylpeptidase IV. Western blot analysis and silver nitrate staining indicate that neither drug induces any modification in the steady state expression of these two brush border hydrolases. Northern blot analysis shows that the level of dipeptidylpeptidase IV mRNA does not change in treated as compared to control Caco-2 cells. In contrast, forskolin and monensin dramatically decrease the level of sucrase-isomaltase mRNA. These observations suggest a separate regulation of biosynthesis for sucrase-isomaltase and dipeptidylpeptidase IV in intestinal cells. The mechanisms responsible for such a difference are discussed. Among them, the role of glucose metabolism, which is perturbed by both drugs, appears to be of crucial importance.
Key words. Sucrase-isomaltase; dipeptidylpeptidase IV; glucose metabolism; human colon cancer cells.

Brush border associated hydrolases normally present in human intestine also have a polarized expression in post-confluent cultured Caco-2 cells [1, 2]. This property makes it possible to study the mechanisms by which intestinal hydrolases are synthesized, processed and vectorially transported to the brush border membrane [3, 4]. Several studies have compared the fate of peptidases and disaccharidases and demonstrated asynchronous transport of these proteins, with peptidases being rapidly transported to the cell surface, whereas disaccharidases make the transit much more slowly [5, 6].

Previous reports from this laboratory have also suggested differences in the regulation of the biosynthesis of these brush border enzymes in cultured human colon cancer cells. Most of these studies have focused on the relationships between glucose metabolism and brush border enzyme biosynthesis [7–10], as it is well known that glucose metabolism is severely impaired in cancer cells [11]. Therefore drugs such as forskolin and monensin have been used in order to perturb the intracellular utilization of glucose in Caco-2 cells [2, 7, 8, 9]. For example, forskolin, a potent activator of adenylate cyclase [12], strongly inhibits the biosynthesis of sucrase-isomaltase (EC 3.2.1.10, EC 3.2.1.48.) (SI)[2, 7]. Monensin, an ionophore known to alter distal Golgi functions [13], also decreases the biosynthesis of SI, without affecting the biosynthesis of dipeptidylpeptidase IV (EC 3.4.14.5.) (DPP IV)[8]. The decreased biosynthesis of SI has been correlated with a net reduction in the amount of its specific mRNA [7, 9].

Most of these studies have focused on SI, for which all the necessary tools were available, including antibodies [10] and a cDNA probe [14]. However, DPP IV could be thoroughly investigated in these systems only recently, as both a cDNA for human DPP IV [15] and a monoclonal

antibody that reacts with DPP IV in Western blot analysis have lately been developed. Using these new tools we study here the effects of forskolin and monensin on the steady state expression, biosynthesis and mRNA levels of DPP IV as compared to SI.

Materials and methods

Cell cultures and drug treatments. Caco-2 cells (passages 60 to 70) were cultured as reported[2]. Cells were treated with 1/1000 ethanol (control)[2], 10^{-6} M monensin for 48 h[8] or 2.5 10^{-5} M forskolin[2] for 48 h or for 16 days.

Biochemical measurements. Glucose consumption, glycogen content and cAMP intracellular concentrations were determined as described previously[2]. Results are shown in table 1. Enzyme activities of SI and DPP IV were determined according to published techniques[16,17]. Values were obtained from at least three independent experiments and SE were calculated. Results are reported in table 2. As a control, DPP IV enzyme activity was determined in samples prepared from untreated cells in the presence of increasing concentrations of exogenously added monensin.

Table 1. Effect of forskolin and monensin on glucose metabolism and cAMP content in control and treated Caco-2 cells.

	Glucose consumption µg/h/mg prot.	Glycogen content µg/mg prot.	cAMP content pmol/mg prot.
Control (n = 7)	37 ± 2	265 ± 15	1.3 ± 0.1
Monensin (n = 4)	70 ± 3[a]	200 ± 12[a]	1.3 ± 0.1[b]
Forskolin (n = 5)	65 ± 2[a]	87 ± 10[a]	49.6 ± 6.4[a]

Caco-2 cells were harvested on day 16 of culture after a 48-h treatment with 2.5 10^{-5} M forskolin or 10^{-6} M monensin, both diluted in 1/1000 ethanol. Ethanol was shown to be without effect on the measured parameters. Each value represents the mean and SE of 4–7 independent determinations, and n represents the number of determinations in each condition. Data were compared to their respective control using Student's t-test. [a]$p < 0.001$; [b]$p > 0.01$.

Table 2. Effect of forskolin and monensin on SI and DPP IV enzyme activities in Caco-2 cells.

Culture conditions	Monensin added during the assay	Enzyme specific activity	
		SI	DPP IV
Control (n = 7)	0	57 ± 7	57 ± 5
Control (n = 3)	10^{-7} M	nd	70 ± 5[a]
	10^{-6} M	nd	74 ± 6[a]
	10^{-5} M	nd	75 ± 6[a]
Monensin (n = 4)	0	62 ± 5[b]	82 ± 7[a]
Forskolin (n = 5)	0	51 ± 5[b]	62 ± 7[b]

Caco-2 cells were treated as in table 1. Enzyme activities were determined as described. For control purposes, DPP IV enzyme activity was also determined in control cells in the presence of increasing concentrations of monensin directly added in the assay. Each value represents the mean and SE of 3–7 independent determinations, and n represents the number of determinations in each condition. Data were compared to their respective control using Student's t-test. nd: not determined. [a]$p < 0.001$; [b]$p > 0.1$.

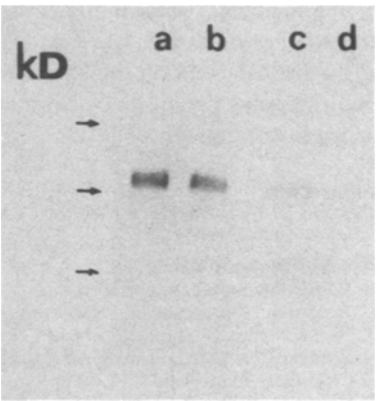

Figure 1. The monoclonal antibody 4H3 is specific for DPP IV. Caco-2 cells were labeled using L-[^{35}S] methionine. Cell homogenates were prepared and subjected to immunoprecipitation with monoclonal antibody HBB 3/775/42 (obtained from H-P. Hauri, Biozentrum, Basel, Switzerland) specific for DPP IV (lane a) or monoclonal antibody 4H3 (to be tested) (lane b). Both antibodies recognize a protein with the same apparent molecular weight. The resulting supernatants were subjected to a second run of immunoprecipitation with the supernatant of the experiment from lane a incubated with 4H3 (lane c), or the supernatant of the experiment from lane b incubated with HBB 3/775/42 (lane d). Arrows indicate the position of molecular weight standards from front to bottom: 200,000, 92,500 and 69,000. No band could be seen in lane c and d, thus demonstrating that both antibodies recognize the same protein, namely DPP IV.

Antibodies, immunological techniques and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblots were performed as described elsewhere[18] using either a polyclonal antibody, 459-7, raised against human Caco-2 SI[10], or a monoclonal antibody, 4H3, against human DPP IV (obtained from S. Maroux, Centre de Biologie Moléculaire, Marseille, France). The specificity of this antibody has been tested both in S. Maroux's laboratory (J. P. Gorvel et al., submitted for publication) and ours. Our data (fig. 1) indicate that 4H3 is specific for DPP IV, as shown by cross immunoprecipitation using 4H3 and another monoclonal antibody, HBB 3/775/42 (obtained from H. P. Hauri, Biozentrum der Universität, Basel, Switzerland), which has been shown to be specific for DPP IV[3]. Western blots were quantified by densitometric scanning of the nitrocellulose sheets using a Joyce Loebl Mark III CS apparatus. The preparation of cell homogenates, L-^{35}S-methionine labeling, immunoprecipitation using either the 459-7 anti-SI polyclonal antibody or the HBB 3/775/42 anti-DPP IV monoclonal antibody, SDS-PAGE and fluorography were performed as reported[10]. In some experiments, gels were stained with a silver nitrate reagent using the Biorad kit.

cDNA probes, Northern blot analysis. A partial human SI cDNA, SI2[14], was obtained from D. Swallow (MRC, the Galton Institute, London, UK). A partial human DPP IV cDNA was recently isolated and characterized in our

laboratory[15]. These cDNA probes were labeled with ^{32}P d-CTP using the multiprime labeling system (Amersham Corp., Arlington Height, IL). Total RNA preparation, electrophoresis, transfer to Hybond N and hybridization were as reported[7].

Results and discussion

A 48-h-treatment with monensin or forskolin does not affect the steady state expression of both SI and DPP IV. When Caco-2 cells are grown for 48 h in the presence of 10^{-6} M monensin or 2.5 10^{-5} M forskolin, glucose metabolism is severely impaired. These effects have been previously reported[2,8], and it has been demonstrated that they are not related either to the classical effect of forskolin on adenylate cyclase nor to the monensin-induced Golgi function perturbations. Forskolin and monensin do not induce any significant change in SI enzyme activity (table 2). In contrast, monensin increases DPP IV enzyme activity significantly. This unexpected observation can be explained by the fact that the addition of increasing concentrations of monensin to control samples during the assay results in an increased DPP IV enzyme activity (table 2). The mechanism responsible for this effect of monensin remains to be elucidated; however, this result seems to indicate that the monensin-induced increase of DPP IV enzyme activity is not due to an effect of the drug during the culture of Caco-2 cells. Western blot analysis and densitometric scanning of the nitrocellulose sheets confirm that the two drug treatments do not modify the steady state amounts of SI and DPP IV (fig. 2). This result indicates that the drugs do not induce any degradation of previously synthesized proteins.

When Caco-2 cells are treated with forskolin over the entire period of culture (16 days), a net reduction of the total amount of SI is evidenced by the silver nitrate staining technique (fig. 3, panel A, lane 2). In contrast, DPP IV levels are not modified (fig. 3, panel B, lane 2). The same technique applied to 48 h forskolin-treated Caco-2 cells (fig. 3, panel A and B, lane 3) gives results similar to those obtained by Western blot (fig. 2, lanes 1 and 2). Under the same conditions, there is no effect on the total amount of DPP IV. Long-term (16 days) treatment with monensin is not possible because of cell mortality.

Forskolin and monensin specifically decrease SI biosynthesis and the level of SI mRNA without affecting DPP IV. We have compared the de novo biosynthesis of SI and DPP IV by labeling Caco-2 cells treated for 48 h with forskolin or monensin. Results are shown in figure 4. In the case of SI (fig. 4A), both forskolin and monensin decrease the amount of the radioactive protein, i.e. the quantity of newly synthesized enzyme. This result is in good agreement with previously published data[7,8]. In contrast, the de novo biosynthesis of DPP IV is not modified in treated as compared to control cells (fig. 4B), except that, as previously shown[8], monensin inhibits the conversion of the high mannose form to the complex form of DPP IV.

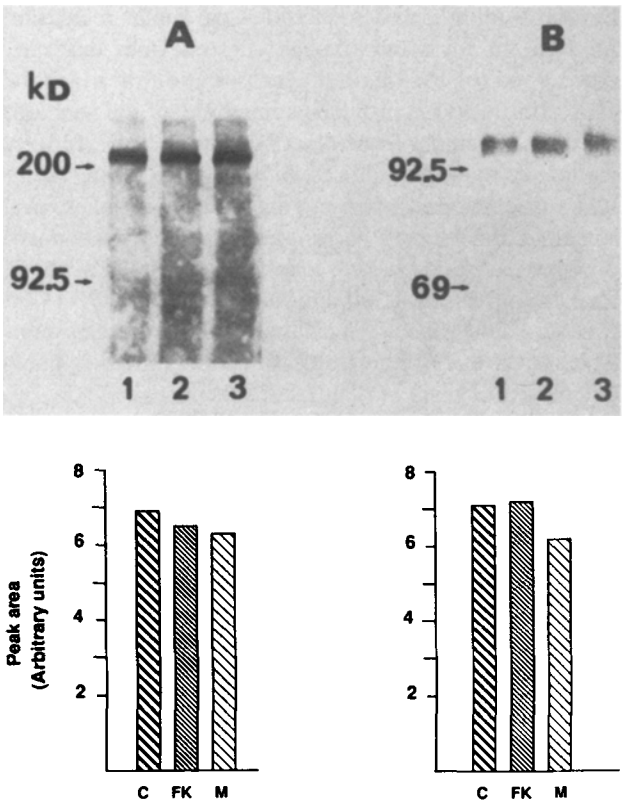

Figure 2. Western blot analysis of SI and DPP IV after a 48-h treatment of Caco-2 cells with forskolin or monensin. Caco-2 cell homogenates (day 16 of culture, without or with a 48-h drug exposure) were run on a 7.5% polyacrylamide gel. After transfer to nitrocellulose sheets, SI was detected using polyclonal antibody 459-7 (panel A) and DPP IV was detected using monoclonal antibody 4H3 (panel B). 100 µg of protein was loaded on each lane. Lane 1: control; lane 2: forskolin; lane 3: monensin-treated cells. The nitrocellulose sheets were quantified using a densitometric scanner, and the area corresponding to each spot was reported on the lower panel. No significant difference in band intensity could be seen. This gel is representative of 3 independent experiments.

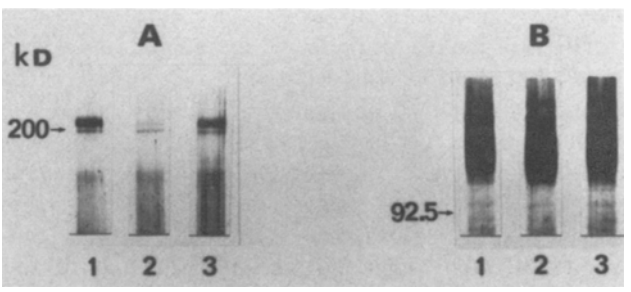

Figure 3. Immunoprecipitation of SI and DPP IV from unlabeled Caco-2 cells, after a 48-h or a 16-day treatment with 2.5 10^{-5} M forskolin. The same amount (500 µg) of cell homogenate proteins was subjected to immunoprecipitation using polyclonal antibody 459-7 for SI (panel A) or monoclonal antibody HBB 3/775/42 for DPP IV (panel B). Gels were stained with silver nitrate. Lane 1: control cells; lane 2: 16-day-treated cells; lane 3: 48-h-treated cells. The total amount of immunoisolated SI was strongly reduced in the 16-day-treated cells only. There is no detectable change in the total amount of DPP IV. This experiment was repeated 2 times for SI and 3 times for DPP-IV with no significant differences between the observed results.

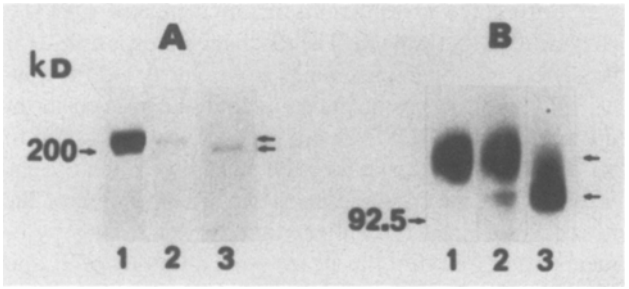

Figure 4. Immunoprecipitation of SI and DPP IV from L-[^{35}S] methionine labeled Caco-2 cells (day 16 in culture) after a 48-h treatment with forskolin and monensin. The same amount of radioactive cell homogenate was subjected to immunoprecipitation using polyclonal antibody 459-7 for SI (panel A) or monoclonal antibody HBB 3/775/42 for DPP IV (panel B). Arrows indicate the complex (upper) and the high mannose (lower) forms of the proteins. Lane 1: control; lane 2: forskolin; lane 3: monensin-treated cells. Both drugs strongly inhibit SI biosynthesis. The amount of labeled DPP IV is not modified. Monensin displays its well-known inhibitory effect on the maturation of both hydrolases. Results presented in this figure are representative of 4 distinct experiments for SI and DPP-IV.

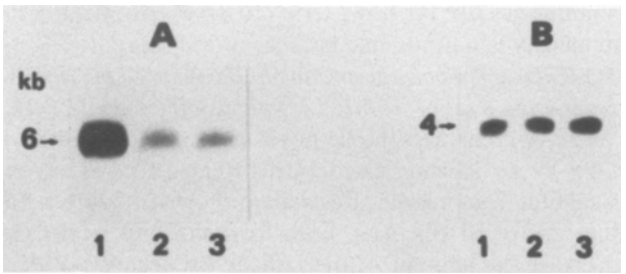

Figure 5. Northern blot analysis of SI and DPP IV mRNAs from Caco-2 cells (day 16 in culture) after a 48-h treatment with forskolin or monensin. The same amount of total RNA (10 µg) was loaded on each lane of 1% agarose gel. Specific RNAs were detected by hybridization with ^{32}p labeled pSI2 probe for SI (panel A) or ^{32}p labeled DP I 101 probe for DPP IV (panel B). Lane 1: control; lane 2: forskolin; lane 3: monensin-treated cells. Both drugs similarly decrease the quantity of SI mRNA without modification of the amount of DPP IV mRNA. Three distinct mRNA preparations of control and treated Caco-2 cells were analyzed by Northern blot and gave results similar to those presented in this figure.

Analysis of the results presented in figures 2 and 4 confirms that the apparent decrease in biosynthesis of SI cannot be explained by rapid degradation. Indeed, the total amount of this protein does not change when Caco-2 cells are treated for 48 h with either forskolin or monensin. The level at which these drugs may exert their inhibitory effect on SI biosynthesis was further documented here by comparing the mRNA level of SI and DPP IV in treated Caco-2 cells, using Northern blot analysis. Results presented in figure 5 show an absence of effect of forskolin and monensin on DPP IV mRNA (fig. 5B), whereas, as previously shown[7,9], treatment with both drugs dramatically decreases the level of SI mRNA (fig. 5A).

It should be pointed out that both drugs always display a similar effect on SI expression, whatever the regulation level studied. Data from table I clearly show that the only effect common to both drugs is on glucose metabolism, leading to an increase of glucose utilization. Therefore, we can conclude that it is glucose metabolism, and not cAMP changes or a blockade of distal Golgi functions, that is directly involved in the regulation of SI expression. This particular regulation of SI is probably related to its biological function, as glucose is one of the products of the reaction catalyzed by this enzyme. More surprisingly, preliminary experiments indicate that biosynthesis of aminopeptidase N, another brush border hydrolase which is not directly linked to glucose metabolism, may also be inhibited in forskolin-treated Caco-2 cells (data not shown). On the other hand, an involvement of carbohydrate metabolism in the regulation of mature aminopeptidase N and sucrase isomaltase has been suggested in pig intestinal explants, where relatively high concentrations of fructose or sucrose may decrease or suppress the expression of these two enzymes[19]. DPP IV regulation differs: like aminopeptidase N, this enzyme is not directly involved with glucose metabolism, but, unlike that of aminopeptidase N and SI, the biosynthesis of DPP IV appears to be totally independent of glucose metabolism changes.

Several studies have demonstrated particular regulation mechanisms for some brush border enzymes that may correspond to physiopathological situations in which the expression of these proteins is impaired[19–21]. Such abnormalities remain to be described for DPP IV. Indeed, its metabolic function is still unknown and, until recently, tools for its study were not available. Using these new tools, we have recently shown that the growth-dependent differentiation of Caco-2 cells was associated with an increase of mRNA level and biosynthesis of DPP IV[22]. Further studies should elucidate the mechanisms responsible for this modulation, and which nutritional or pharmacological agents may influence them.

Acknowlegments. This work was supported by the Fondation pour la Recherche Médicale, The Association pour la Recherche sur le Cancer (A.R.C.) and the Groupement des Entreprises Françaises pour la Lutte contre le Cancer (G.E.F.L.U.C.).

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0014-4754/91/11-12/1211-05$1.50 + 0.20/0

Neuronal degeneration in the striatum of the groggy rat: A new mutant with a movement disorder

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Received 15 January 1991; accepted 21 March 1991

Abstract. A new mutation displaying abnormal movement was obtained in the progeny of a female Wistar rat which had been given 10 mg/kg methylnitrosourea at an early stage of the gestational period. Genetic studies revealed that the character is inherited by an autosomal single recessive gene, and we designated this mutation *groggy* (gene symbol *gr*). The abnormal movement of the groggy rat was first apparent around postnatal day 15, while the histological studies revealed the appearance of numerous necrotic neurons in the striatum of the groggy rat on postnatal days 60 and 120.
Key words. Neurological mutant; movement disorder; striatum; neuronal degeneration; Wistar rat.

Numerous mutations with neurological abnormalities have been reported in mice. In rats, however, very few neurological mutants have yet been described. In the course of studying the effects of methylnitrosourea administration to pregnant rats in terms of the development of the progeny, a new mutation was obtained displaying movement disorder. In the present study, the clinical and genetical features of this mutant rat are first described; thereafter, results of histological examination of the brain are reported.

Materials and methods

Origin of animals. A female Slc:Wistar rat was given 10 mg/kg methylnitrosourea intraperitoneally on gestational day 4 (the onset of pregnancy was designated gestational day 0). This mother bore 5 normal-appearing offspring (2 males and 3 females). These animals were randomly mated and one female gave birth to 7 offspring among which 3 animals (2 males and 1 female) displayed the same pattern of movement disorder. Then, one of the abnormal males was paired with the abnormal female, and all the offspring obtained from this pair displayed the same pattern of movement disorder. Further breeding is performed in 2 ways. Pedigreed stock is maintained by brother-sister-mating. The mutants used for the experiments are produced by mating homozygous males with heterozygous females, since the offspring of the homozygous females mated with homozygous males become dirty owing to the creeping movement of the mother during forstering.

Histological study. On various postnatal days males of the mutant rats were deeply anesthetized by ethyl ether, and killed by a cardiac perfusion with 10% formalin after the blood vessels had been flushed out with physiological saline. After perfusion, the brains were quickly removed, bisected coronally, and fixed with Bodian II solution for 24 h. They were then dehydrated with an ethanol series and embedded in paraffin wax. For the control, normal rats of the Slc:Wistar strain were sacrificed and their brains were embedded in paraffin wax by the same methods. Coronal sections 8–10 μm thick were stained with cresyl violet before light microscopic examination.

Count of necrotic neurons in the striatum. The number of necrotic neurons appearing in the striatum of the Slc:Wistar and the mutant rats during postnatal growth was counted in the coronal section of the caudate nucleus cut at the plane approximately corresponding to FIG. A29 of Craigie's Neuroanatomy of the Rat [1]. Five males were examined on each selected postnatal day, and statis-