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β-Casein(94-123)-derived peptides differently modulate production of mucins in intestinal goblet cells

Pascale Plaisancié[1]*, Rachel Boutrou[2,3], Monique Estienne[1], Gwénaële Henry[2,3], Julien Jardin[2,3], Armelle Paquet[1] and Joëlle Léonil[2,3]

[1] INRA USC1235, INSERM U1060, CarMeN Laboratory, University Lyon-1, INSA-Lyon, IMBL, Bât. Louis Pasteur, 20 av. Albert Einstein, 69621, F-69100 Villeurbanne, France
[2] INRA, UMR1253 Science et Technologie du Lait et de l'Œuf, F-35000 Rennes, France
[3] AGROCAMPUS OUEST, UMR1253 Science et Technologie du Lait et de l'Œuf, F-35000 Rennes, France

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We recently reported the identification of a peptide from yoghurts with promising potential for intestinal health: the sequence (94-123) of bovine β-casein. This peptide, composed of 30 amino acid residues, maintains intestinal homoeostasis through production of the secreted mucin MUC2 and of the transmembrane-associated mucin MUC4. Our study aimed to search for the minimal sequence responsible for the biological activity of β-CN(94-123) by using several strategies based on (i) known bioactive peptides encrypted in β-CN(94-123), (ii) *in silico* prediction of peptides reactivity and (iii) digestion of β-CN(94-123) by enzymes of intestinal brush border membranes. The revealed sequences were tested *in vitro* on human intestinal mucus-producing HT29-MTX cells. We demonstrated that β-CN(108-113) (an ACE-inhibitory peptide) and β-CN(114-119) (an opioid peptide named neocasomorphin-6) up-regulated MUC4 expression whereas levels of the secreted mucins MUC2 and MUC5AC remained unchanged. The digestion of β-CN(94-123) by intestinal enzymes showed that the peptides β-CN(94-108) and β-CN(117-123) were present throughout 1·5 to 3 h of digestion, respectively. These two peptides raised MUC5AC expression while β-CN(117-123) also induced a decrease in the level of MUC2 mRNA and protein. In addition, this inhibitory effect was reproduced in airway epithelial cells. In conclusion, β-CN(94-123) is a multifunctional molecule but only the sequence of 30 amino acids has a stimulating effect on the production of MUC2, a crucial factor of intestinal protection.

Keywords: Milk bioactive peptides, β-casein, intestinal barrier, intestinal protection, mucus.

Abbreviations: ACE, Angiotensin-converting enzyme; BBMV, brush border membrane vesicles; BSA, bovine serum albumin; CT, controls; DPP IV, dipeptidyl peptidase IV; ELLA, Enzyme-linked lectin assay; FBS, foetal bovine serum; TFA, trifluoroacetic acid; β-CN, β-casein.

In recent years, numerous researches have been devoted to bioactive peptides encrypted in dairy proteins in order to take advantage of such peptides in functional food ingredients for the maintenance of health. In a previous study (Plaisancie et al. 2013), we demonstrated that the peptide β-CN(94-123), that we identified in fermented milks in concentrations ranging between 0·3 and 1·7 μM, has the characteristics of such a bioactive compound. Indeed, this peptide which corresponds to the sequence (94-123) of bovine β-casein, increased expression of the intestinal secreted mucin *MUC2* and of the transmembrane-associated mucin *MUC4* by human intestinal mucus-producing cells. More significantly, it also induced the expression of *Muc2* and *Muc4*, as well as the expansion of mucus cells in rat intestinal mucosa after oral administration.

The intestinal mucus gel covering the mucosal surface is a major component of physiological defence mechanisms. Mucus provides protection from noxious substances (e.g., acidity, proteolytic enzyme activities, or toxins), and constitutes a local physical barrier against microbiota and pathogens (Corfield et al. 2001; Corazziari, 2009). Mucus also regulates epithelial hydration, allows lubrication of the cell surface, and participates indirectly in the immune response due to interactions with secretory

*For correspondence; e-mail: pascale.plaisancie@inserm.fr

immunoglobulins (Pelaseyed et al. 2014). The intestinal mucus gel owes its properties to secreted mucins, which are high-molecular-weight glycoproteins produced by goblet cells of the epithelium. To date, up to 20 different mucins (MUC) have been identified in humans and divided into two main classes: secreted and cell surface-associated (membrane-associated) mucins (Corfield, 2014). Secreted gel-forming mucins include MUC2, MUC5AC, MUC5B, MUC6 and MUC19. Each of these mucins has a characteristic organ and cell type-specific distribution. Normal stomach mucosa is characterised by the production of MUC5AC, primarily by surface epithelial mucus cells, and by the production of MUC6 by the gastric glands. The epithelium of the small and large intestine contains characteristic goblet cells that produce MUC2. The protective role of MUC2 is definitively proven by the development of spontaneous colitis and intestinal tumours in *Muc2* knockout mice (Velcich et al. 2002; Van der Sluis et al. 2006). In human, many studies support the hypothesis that alterations in MUC2 synthesis, secretion, and/or degradation are involved in the initiation or maintenance of intestinal disorders such as duodenal ulcer, ulcerative colitis, necrotising enterocolitis and colon carcinomas (Pugh et al. 1996; Mizoshita et al. 2007; Strugala et al. 2008; Mudter, 2011). The membrane-associated mucins have also received increasing attention for their role in the protection of epithelia. In the gut, prominent membrane-associated mucins are MUC1, MUC3, MUC4, MUC12, MUC13 and MUC17 which are detectable both in goblet cells and in enterocytes, providing a static external barrier that can limit direct access of pathogens (Corfield et al. 2000; Corfield, 2014).

The secreted mucin MUC2 in association with the membrane-associated mucin MUC4 acting as a physico-chemical barrier for the protection of the epithelial cell surface, it can be speculated that fermented milks containing peptide β-CN(94-123) could offer specific health benefits to consumers for preventing or treating many intestinal diseases. However, some issues still need to be clarified before the use of this peptide. In particular, the question arises about the minimal active sequence. β-CN (94-123), a 30 amino acid residues peptide, could be considered as a multifunctional molecule. It contains encrypted in its sequence, an opioid peptide named neocasomorphin-6 (β-CN f114-119), a peptide with anti-oxidant activity (β-CN f98-105) and a peptide inhibitor of angiotensin converting enzyme (ACE-I) (β-CN f108-113), which could affect production of mucins (Philanto-Leppälä et al. 1998; Jinsmaa & Yoshikawa, 1999; Gupta et al. 2010). The enzymatic hydrolysis of β-CN(94-123) during intestinal digestion may also generate other small sequences carrying biological activities. All these points require experiments. The identification of bioactive sequences smaller than β-CN (94-123) will help to clarify the mechanisms of action involved in the activation of goblet cells, will allow designing synthetic peptide more resistant to enzymatic digestion and will make easier the development of novel regulators of intestinal host defence. To achieve this objective, we tested *in vitro*, on intestinal HT29-MTX cells, the activity of peptides chosen using several strategies based on (Fig. 1) (i) the known bioactive peptides which are encrypted in the sequence of β-CN(94-123) (ii) *in silico* prediction of opioid peptide reactivity and (iii) an approach based on the digestion of β-CN(94-123) by enzymes of the brush border membrane of intestinal epithelial cells, knowing that the active sequences must escape the action of brush border enzymes to reach the goblet cells and exert their physiological effects.

Materials and methods

In vitro studies

Cell culture. HT29-MTX, a human colon carcinoma derived mucin-secreting goblet cell line, was grown in 25-cm^2 plastic culture flasks in DMEM supplemented with 10% feotal bovine serum (FBS) and 100 mg penicillin-streptomycin/ml at 37 °C in a 5% CO_2 atmosphere in a humidified incubator as previously described (Zoghbi et al. 2006). HT29-MTX cell line is a well-established model which produces mucin in response to various stimulants (Zoghbi et al. 2006; El Homsi et al. 2007). These cells also exhibit immunopositivity for μ-opioid receptors on their cell membranes (Zoghbi et al. 2006). The NCI-H292 epithelial cells (American Type Culture Collection, Manassas, VA, USA), a human airway epithelial cell line that produces mucins, were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml and 10% FBS. The cells were grown at 37 °C in 5% CO_2 fully humidified air and were subcultured twice weekly (Borchers et al. 1999).

To study the effect of peptides, cells were seeded in 12-well culture plates. Experiments were performed when cells reached confluency (NCI-H292) or 21 d after cells reached confluency (HT29-MTX), as previously described (Plaisancie et al. 2013). All experiments were performed at least three times in triplicate. Peptides were synthesised from GENOSPHERE Biotechnologies (Paris, France).

Enzyme-linked lectin assay for mucins from cell culture media. An enzyme-linked lectin assay (ELLA) was used to measure mucin-like glycoprotein secretion as previously described (Zoghbi et al. 2006). The amount of glycoprotein secreted in the incubation medium was expressed as nanograms of mucin-like glycoprotein per 10^6 cells, and results are given as per cent of controls.

Quantitative real time PCR analysis. Measures were performed with the real-time fluorescence detection method using the Mastercycler® ep realplex (Eppendorf, Hambourg, Germany) with Maxima™ SYBR Green qPCR Master Mix (Fermentas, St. Leon-Rot, Germany) in PCR plates,

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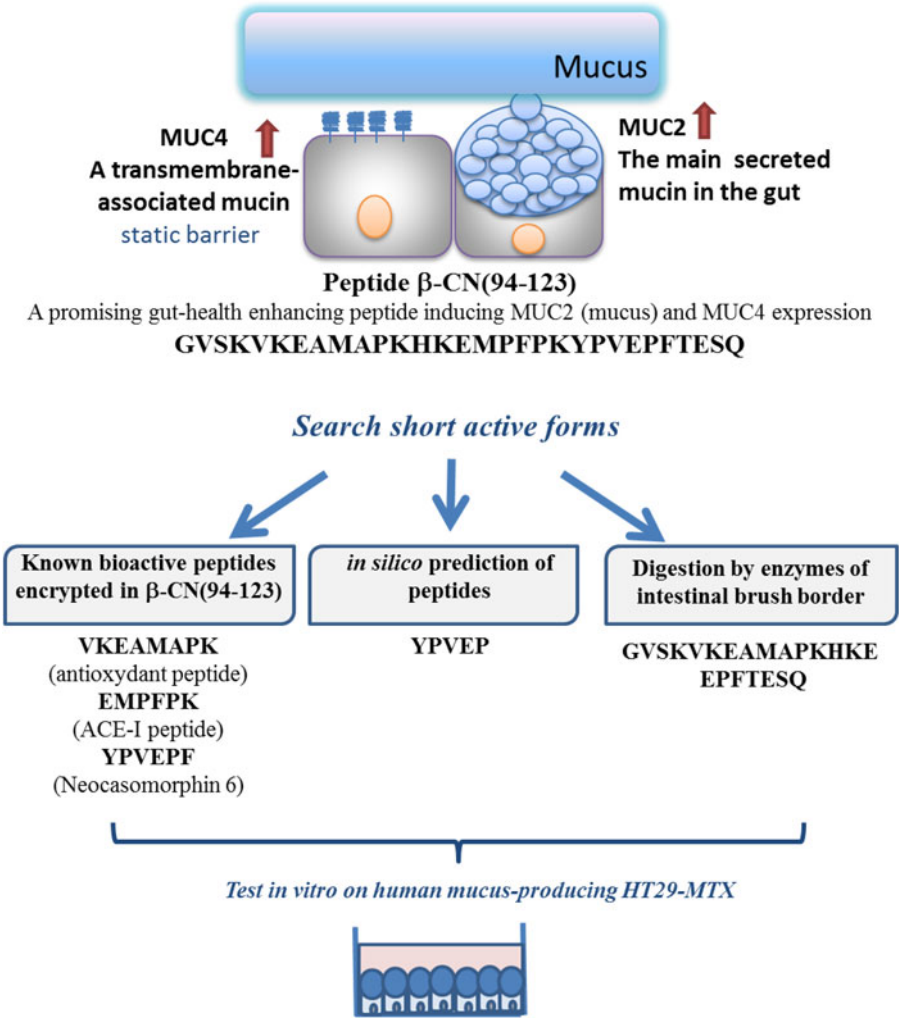

Fig. 1. Strategies used to search the minimal sequence responsible for the biological activity of peptide β-CN(94-123).

as previously described (Plaisancie et al. 2013). Calculations were performed according to the $2^{-\Delta\Delta Ct}$ method with correction for efficiency and the final value was adjusted so that controls had a mean relative mRNA level of 1 (Livak & Schmittgen, 2001; Pfaffl et al. 2002).

Western blot analysis. Proteins (30 µg) were separated using Tris acetate 3–8% and then used for western blot analysis as described previously (Plaisancie et al. 2013).

Dot blot of MUC2 in apical media. Sixty microlitres vigorously mixed apical medium of each well were spotted onto nitrocellulose membrane (45 µM) using a Bio-Dot apparatus (Bio-Rad Laboratories, Hercules, CA). After rinse with distilled water, the membrane was dried and blocked during 2 h and then incubated 3 h at room temperature with a monoclonal anti-MUC2 antibody (Abcam, Paris, France; 1/2000). Blots were developed with a commercial kit (WesternBreeze Chemiluminescent, Invitrogen, France). The optical density of spots was visualised and pixelised with the 'Image System' (ImageMAster VDS-CL, Amersham Bio-sciences) and densitometrically analysed with Quantity one image analysis software (Biorad Laboratory, Hercules, CA, USA).

In silico prediction of bioactive sequences

PattinProt software (http://npsa-pbil.ibcp.fr/) was used to identify putative consensus sequences for opioid peptides. The obtained pattern Y-P-x(2,3)-P was searched against the sequence of the β-CN(94-123) peptide using the ScanProsite search engine located on http://prosite.expasy.org/scanprosite/.

Digestion of β-CN(94-123) by enzymes of intestinal brush border membrane vesicles (BBMV)

Preparation of intestinal BBMV. BBMV from the ileum of a freshly killed pig were prepared as described

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(Boutrou et al. 2008). Purification and enrichment of the BBMV were checked by determination of the marker enzymes alkaline phosphatase (EC 3.1.3.1) and dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5). Samples were diluted 1 : 100 in 0·1 M sodium carbonate buffer pH 9·4 and mixed to an equal volume of *para*nitrophenyl phosphate. The absorbance at 405 nm was measured each min during 10 min to determine the activity. To measure the activity of DPP IV, samples were diluted 1 : 80 in 0·02 M Tris-HCl buffer pH 7·5. Fifty microlitres were incubated with 50 µl 0·66 mM Phe-Pro β-naphtylamide at 37 °C. The reaction was stopped by adding 50 µl of a mixture containing 1 mg Fast Garnet/ml, 10% (v:v) Triton X-100 and 1 M sodium acetate pH 4·0 after 0, 5, 10, 15 and 20 min and the absorbance at 550 nm was measured. Protein concentration was determined by using the Bradford reagent (Sigma, St.-Quentin Fallavier, France) with bovine serum albumin as standard. The specific alkaline phosphatase and DPP IV activities were 19·6 and 17·0 fold enriched, respectively in the final BBMV fraction.

Digestion of β-CN(94-123) by BBMV enzymes. Digestion of the peptide β-CN(94-123) by BBMV enzymes was performed at 37 °C in 35 mM Hepes-Tris buffer, 0·15 M KCl pH 7·0. Digestion was started by mixing equal volume of substrate solution (5 g/l) and BBMV preparation diluted 1 : 10 (v:v) in Hepes-Tris buffer. At selected times 0·3 ml samples were withdrawn and the reaction was stopped by removing the BBMV (centrifugation at 2000 ***g*** for 1 min). The supernatants were stored at − 20 °C until analysis. A blank sample was obtained by replacing the substrate with buffer. A control was obtained by replacing BBMV preparation with buffer.

Identification of peptides by nano LC-ESI/MS-MS. All mass spectra were performed using a hybrid quadrupole time of flight (Q/TOF) mass spectrometer (MS) QStar XL (MDS Sciex, Toronto, Canada). The instrument was calibrated with a multi-point calibration using fragment ions that resulted from the collision-induced decomposition of a peptide from β-casein, β-CN(193-209) (NeoMPS S.A., Strasbourg, France). After 1 : 1000 dilution in 0·1% trifluoroacetic acid (TFA; Pierce, Touzart et Matignon, Vitry-sur-Seine, France), the peptide fraction (10 µl) was trapped onto a micro-pre-column cartridge C_{18} PepMap 100 (300 µM i.d. × 5 mm, Dionex) before separation of peptides onto a column C_{18} PepMap 100 (75 µM i.d. × 150 mm, Dionex). The separation started with 10% solvent B for 10 min and a linear gradient from 10 to 50% of solvent B for 40 min was performed at a flow rate of 300 nl/min. Solvent A contained 2% acetonitrile, 0·08% formic acid and 0·01% TFA in LC grade water; and solvent B contained 95% acetonitrile, 0·08% formic acid and 0·01% TFA in LC grade water. The online separated peptides were analysed by ESI Q-TOF in positive ion mode. An optimised voltage of 2·8 kV was applied to the nanoelectrospray ion source (Proxeon Biosystems A/S, Odense, Denmark). MS and MS/MS data were acquired in continuum mode. Data-direct analysis was employed to perform MS/MS analysis on 1^+ to 4^+ charged precursor ions. Precursor selection was based upon ion intensity, charge state and if the precursors had been previously selected for fragmentation they were excluded for the rest of the analysis. Spectra were collected in the selected mass range 400–1500 *m/z* for MS spectra and 60–2000 *m/z* for MS/MS. The mass spectrometer was operated in data-dependant mode automatically switching between MS and MS/MS acquisition using Analyst QS 1.1 software (Applied Biosystems, Framingham, MA) when the intensity of the ions was above ten cps. To identify peptides, all data (MS and MS/MS) were submitted to MASCOT software (v. 2.2). The search was performed against a homemade database dealing with major milk proteins which represents a portion of the Swissprot database (http://www.uniprot.org); consequently the endogenous proteins were not identified in the present study. No specific enzyme cleavage was used and the peptide mass tolerance was set to 0·2 Da for MS and 0·15 Da for MS/MS. For each peptide identified, a minimum MASCOT score corresponding to a *P*-value $<0{\cdot}05$ was considered as a prerequisite for peptide validation.

Statistical analysis

In vitro data were compared using repeated-measures ANOVA, followed by the Mann–Whitney *U*-test when appropriate or Mann–Whitney test alone for single comparisons. All the data are expressed as mean ± SEM. Differences with $P<0{\cdot}05$ were considered significant. Statistical analyses were performed with XLSTAT, Version 2009.4.06 (Addinsoft, Paris, France).

Results

In vitro effects of bioactive peptides encrypted in β-CN (94-123)

Bioactive peptides previously identified in the literature and encrypted within the amino-acids sequence of β-CN (94-123) are: VKEAMAPK (β-CN f98-105, an antioxidant peptide), EMPFPK (β-CN f108-113, an ACE-inhibitory peptide) and YPVEPF (β-CN f114-119, an opioid peptide named neocasomorphin-6). We studied their impact on the secreted mucin MUC2 and on the transmembrane-associated mucin MUC4 which are known to be stimulated by β-CN(94-123). Expression of the major mucin produced by HT29-MTX cells, the mucin MUC5AC, was also considered.

Addition of VKEAMAPK to the incubation medium (0·01–100 µmol/l, 4 h) did not modulate the mRNA level of *MUC2, MUC4* and *MUC5AC* (Fig. 2a). As shown in Fig. 2, EMPFPK and YPVEPF dose-dependently increased the expression of the transmembrane-associated mucin *MUC4* with a maximal response achieved with 100 µmol/l EMPFPK (142 ± 8% of control, $P<0{\cdot}05$) or YPVEPF (152 ± 22%

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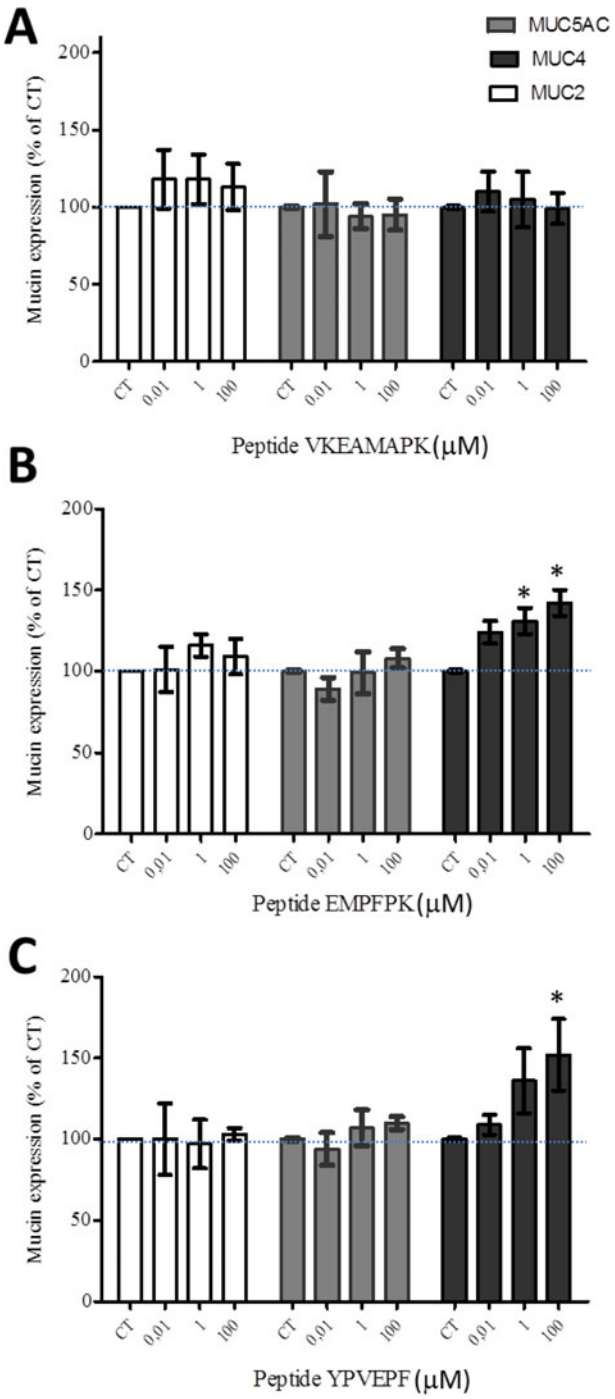

Fig. 2. Effect of known bioactive peptides encrypted in β-CN (94-123) on *MUC2, MUC5AC* and *MUC4* expression in HT29-MTX cells (4 h exposure). After overnight serum starvation, the cells were incubated without (control, CT) or with known bioactive peptides à 37 °C for 4 h. Total RNA was isolated and mucin mRNA levels were analysed by quantitative RT-PCR. *Cyclophilin A* mRNA, used as internal control, were unaffected by treatment. A/ Effect of VKEAMAPK (an antioxidant peptide); B/ Effect of EMPFPK (an ACE-inhibitor peptide) and C/ Effect of YPVEPF (neocasomorphin-6, an opioid peptide). Each bar represents the mean per cent increase *vs.* controls ± SEM of 4 experiments performed in triplicate. *$P<0{\cdot}05$ *vs.* controls.

Table 1. A pattern was determined from sequences of endogenous opioid peptides and searched against the sequence of the β-CN (94-123) peptide

Entry	Pattern	Scan against β-CN(94-123)
Bovin casein β-CN(60-66) YPFPGPI	Y-P-x(2,3)-P	YPVEP (114-118)
Human casein β-CN(51-57) YPFVEPI		YPVEP (114-118)
Bovin casein β-CN(114-119) YPVEPF		YPVEP (114-118)

of control, $P<0{\cdot}05$). In contrast, they did not alter the expression of *MUC2* and *MUC5AC*. They were also without effect on mucus secretion *(*data not shown*)*. The effect of EMPFPK on *MUC4* was not reproduced by Captopril (D-3-mercapto-2-methyl-propionyl-L-proline) (0·01–100 µmol/l), a strong and specific ACE-Inhibitor (data not shown).

In vitro effects of in silico predicted peptides

As several milk peptides with effects on intestinal goblet cells act as opioid agonists (Zoghbi et al. 2006), we have compared the sequence of β-CN(94-123) with that of endogenous opioid. A consensus sequence was determined, and its search in the peptide β-CN(94-123) revealed the sequence YPVEP (Table 1). The peptide was chemically synthesised and tested on HT29-MTX cells (0·01, 1 and 100 µmol/l) for 4 h. YPVEP did not induce an increase in *MUC2, MUC4* and *MUC5AC* mRNA levels (data not shown).

In vitro effects of β-CN(94-108) and β-CN(117-123) identified throughout β-CN(94-123) digestion by enzymes of intestinal BBMV

Digestion by enzymes of intestinal BBMV. The peptide β-CN(94-123) was digested through the action of enzymes from the brush border membrane. The digestion products were identified using HPLC techniques coupled to mass spectrometry. Peptides were not detected in the digested sample after 3 h. The peptide β-CN(94-108) was identified from 0·25 to 3 h digestion. The peptide β-CN(117-123) was later released; it also appeared stable because identified from 1·5 to 3 h digestion. To investigate a direct effect of these peptides on the induction of *MUC2* or *MUC4* expression, we exposed HT29-MTX cells to each of them.

In vitro effects of β-CN(94-108) and β-CN(117-123) peptides – Effects on intestinal HT29-MTX cells. Surprisingly, the peptide β-CN(117-123) induced a strong decrease in *MUC2* expression after 4 h of stimulation. The first significant decrease in transcripts was observed with a concentration of 0·01 µmol/l (41 ± 6% of CT, $P<0{\cdot}05$) (Fig. 3a). Following this result, an intermediate concentration

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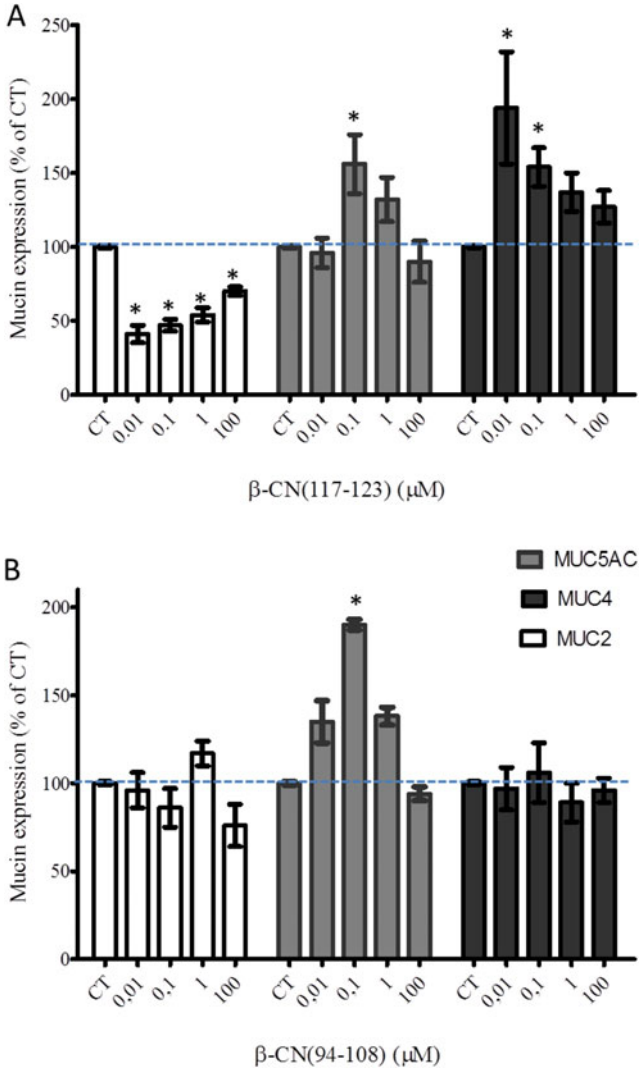

Fig. 3. Effect of the peptides β-CN(94-108) and β-CN(117-123) on *MUC2, MUC5AC* and *MUC4* expression in HT29-MTX cells (4 h exposure). After overnight serum starvation, the cells were incubated without (control, CT) or with the peptides β-CN(94-108) and β-CN(117-123) à 37 °C for 4 h. Total RNA was isolated and mucin mRNA levels were analysed by quantitative RT-PCR. *Cyclophilin A* mRNA, used as internal control, were unaffected by treatment. Each bar represents the mean per cent increase *vs.* controls ± SEM of 4 experiments performed in triplicate. A/ Effect of peptide β-CN(117-123) B/ Effect of peptide β-CN(94-108).

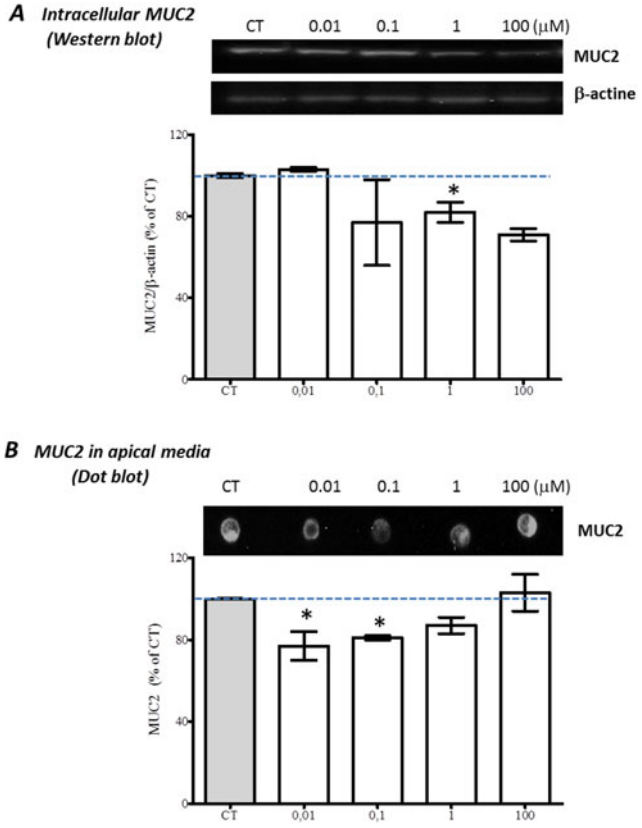

Fig. 4. Effect of the peptide β-CN(117-123) on the secreted mucin MUC2 in HT29-MTX cells (4 h exposure). After overnight serum starvation, the cells were incubated without (control, CT) or with the peptide β-CN(117-123) à 37 °C for 4 h. (A) Western blot analysis of MUC2 in lysates of HT29-MTX cells treated with β-CN(117-123). The expression of MUC2 was normalised to the reference protein level (β-actin) in each sample. Each bar represents the mean per cent increase *vs.* controls ± SEM of 4 experiments performed in triplicate. Picture: Representative western blot picture of MUC2 and β-actin in control and treated cells (peptide β-CN(117-123), 0·01–100 μmol/l). Images are representative of 4 separated experiments performed in triplicate. (B) Dot blot analysis of MUC2 in the cell culture medium ($P<0·05$). Each bar represents the mean per cent increase *vs.* controls ± SEM of 4 experiments performed in triplicate. *$P<0·05$ *vs.* controls. Picture: Representative dot blot picture of MUC2 in control and treated cells (peptide β-CN(117-123), 0·01–100 μmol/l). Images are representative of 4 separated experiments performed in triplicate.

(0·1 μmol) was tested. Similar inhibition of *MUC2* expression was observed again. Analyses of western blot and of dot blot revealed that this decreased MUC2 mRNA level was reflected either by a reduced intracellular MUC2 protein level in HT29-MTX cells (Fig. 4a) or by a lower secretion of MUC2 in cell culture medium ($P<0·05$) (Fig. 4b). For example, upon stimulation with the lowest dose of peptide β-CN(117-123), HT29-MTX cells have maintained an intracellular content of MUC2 identical to that of control cells but to the detriment of MUC2 secretion. The peptide β-CN(117-123) also induced a significant increase in the expression of *MUC5AC* (156 ± 20% of CT at 0·1 μM, $P<0·05$) and of *MUC4* (194 ± 38% of CT, $P<0·05$ and 152 ± 13% of CT, $P<0·05$ at 0·01 and 0·1 μM, respectively) (Fig. 3a). This effect of peptide β-CN(117-123) on the expression of *MUC5AC* and *MUC4* was not observed when tested at higher concentrations (1 and 100 μM). One possible explanation for these results may be that the interaction of β-CN (117-123) with a putative receptor would reach saturation.

Incubation of HT29-MTX cells in presence of the peptide β-CN(94-108) increased *MUC5AC* expression (190·0 ± 3·0% of CT; $P<0·05$ when tested at the concentration of 0·1 μM)

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Table 2. Effects of the tested peptides on HT29-MTX cells

Peptides	Start	Stop	Sequence	Observed effect
Peptide β-CN(94-123)	94	123	GVSKVKEAMAPKHKEMPFPKYPVEPFTESQ	↑ **MUC2**, MUC4 No effect on MUC5AC (Plaisancie et al. 2013)
Identified in the literature	98	105	VKEAMAPK	No effect
	108	113	EMPFPK	↑ MUC4
	114	119	YPVEPF	↑ MUC4
In silico predicted	114	118	YPVEP	No effect
From BBMV digestion	94	108	GVSKVKEAMAPKHKE	↑ MUC5AC
	117	123	EPFTESQ	↑ MUC5AC, MUC4 ↓ MUC2

but did not modify the expression of *MUC2* and *MUC4* (Fig. 3b). Western blot and dot blot analysis also showed that β-CN(94-108) did not modulate MUC2 secretion or intracellular MUC2 production (data not shown).

The effects of all the tested peptides on HT29-MTX cells are summarised on Table 2.

Effects on an airway epithelial cell line. Although the mucus is an important factor of airway protection, an excessive production contributes to obstruction in several lung diseases (asthma, chronic obstructive pulmonary diseases, cystic fibrosis . . .). In this context, a peptide inhibiting the production of secreted mucins could be a help in aerosol therapies. To examine this hypothesis, we used confluent NCI-H292 cells, a human pulmonary mucoepidermoid cell line, which is frequently used for studying production of airway mucins (Kai et al. 1996; Rose et al. 2000). We observed that peptide β-CN(117-123) significantly decreased *MUC2* gene expression and MUC2 protein production after 4 h incubation (Fig. 5). In this airway epithelial cell line, β-CN(117-123) also induced a diminution in *MUC5AC* mRNA level at 0·01 and 0·1 μM ($P<0·05$). Western blotting could not be achieved with MUC5AC because the amount of protein produced was too low. The decreased expression of *MUC2* and *MUC5AC* was maintained after 24 h exposure with peptide β-CN(117-123) (Fig. 6). In this experimental condition, the maximal effect was observed when β-CN(117-123) was tested at 1 μM.

Discussion

Recently, we demonstrated that a novel bioactive peptide derived from bovine β-casein, the peptide β-CN(94-123), was active on mucus cells, a population of the intestinal epithelium implicated in gut defence. The present study was dedicated to the search of minimal sequences responsible for its biological activity. We focused our attention on bioactive peptides described in the literature and encrypted in β-CN(94-123), *in silico* predicted and the ones which resist *in vitro* intestinal digestion. The results are discussed according to their activity on expression and production of mucins.

Our study revealed that peptide β-CN(117-123) inhibited the expression as well as the production of MUC2 in intestinal cells. After 4 h stimulation, this decreased MUC2 expression generates either a reduction of the intracellular MUC2 protein level or a lesser secretion of MUC2 in culture medium. It is likely that a longer stimulation with this peptide could lead to total depletion of MUC2 and could therefore have a deleterious effect on intestinal defences. This peptide has been chosen because it is resistant to proteases throughout the intestinal tract, a prerequisite for milk-derived peptides to exert biological activities in the intestine. However, it is important to note that the probability of release of β-CN(117-123) *in vivo* seems low. Indeed, although intestinal BBMV procedure is a very interesting model to study enzymatic hydrolyses of amino-acid sequences, the *in vivo* digestion is a much more complex process including degradation by acid and by pepsin in the stomach, an endopeptidase with broad specificity, and then by pancreatic proteases before the action of the brush border membrane enzymes. So that both peptides β-CN(117-123) and β-CN(94-108) may not be produced, or not be produced in sufficient quantities, in the intestinal lumen. In agreement with this hypothesis, we recently investigated peptides released in the jejunum of human volunteers fed with casein (Boutrou et al. 2013) and we demonstrated that the β-CN(94-108) and β-CN(117-123) peptides were not detected. It is also to be noted that we did not observe such an inhibitory effect on intestinal Muc2 after oral administration of β-CN(94-123) in rats (Plaisancie et al. 2013).

We established that β-CN(94-108) and β-CN(117-123) induced expression of the secreted mucin *MUC5AC*, whereas β-CN(94-123) was without effect on this one (Plaisancie et al. 2013). As in physiological conditions, MUC5AC is mainly produced in the airways and in the stomach, this effect cannot strengthen or restore intestinal homoeostasis. As discussed above, the peptide β-CN(117-123) decreased expression and production of MUC2, but it also induced expression of MUC5AC. While many studies have focused on the mechanisms controlling the expression of MUC2 alone or of MUC5 alone, experiments on factors that regulate the expression of these mucins in opposite direction are rare. Some studies have only observed

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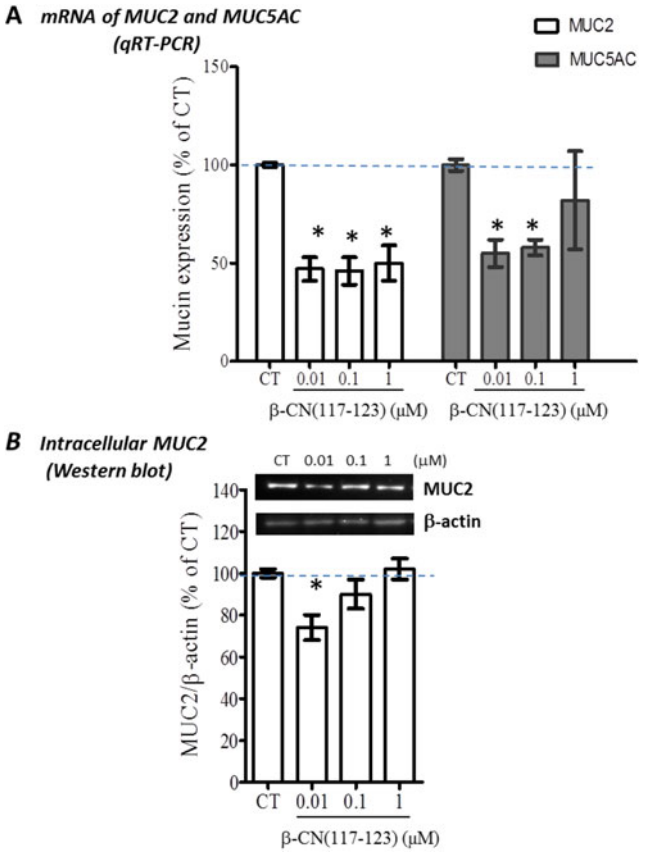

Fig. 5. Effect of peptide β-CN(117–123) on the secreted mucin MUC2 and MUC5AC in NCI-H292 cells (4 h exposure) (A) *MUC2* and *MUC5AC* expression: Total RNA was isolated and mucin mRNA levels were analysed by quantitative RT-PCR. Each bar represents the mean per cent increase *vs.* controls ± SEM of 4 experiments performed in triplicate. (B) Western blot analysis of MUC2 in lysates of NCI-H292 cells treated with β-CN(117-123). The expression of MUC2 was normalised to the reference protein level (β-actin) in each sample. Each bar represents the mean per cent increase *vs.* controls ± SEM of 4 experiments performed in triplicate. **P* < 0·05 *vs.* controls. Picture: Representative Western blot picture of MUC2 and β-actin in control and treated cells (peptide β-CN (117-123), 0·01–1 μmol/l). Images are representative of 4 separated experiments performed in triplicate.

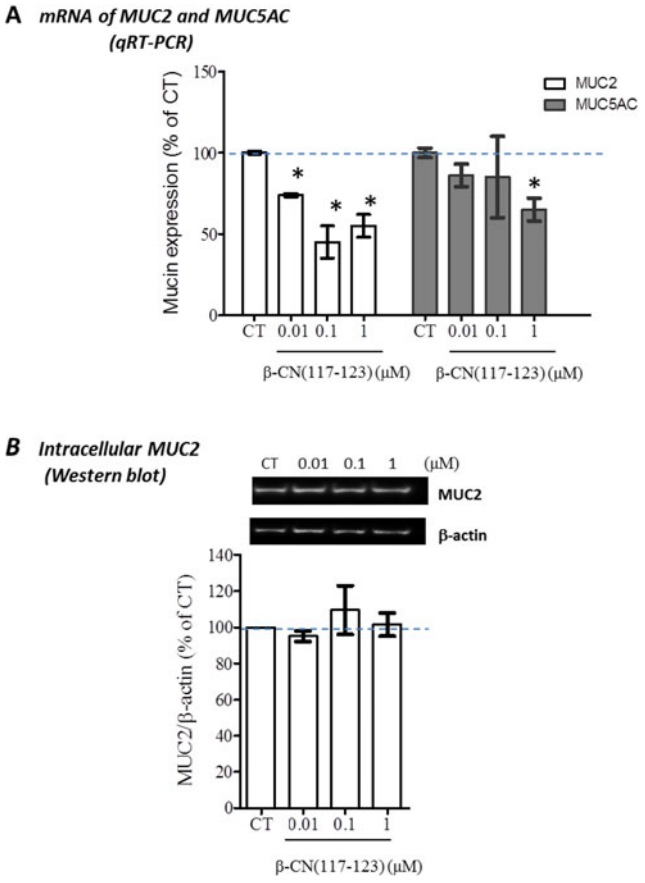

Fig. 6. Effect of peptide β-CN(117-123) on the secreted mucin MUC2 and MUC5AC in NCI-H292 cells (24 h exposure) (A) *MUC2* and *MUC5AC* expression: Total RNA was isolated and mucin mRNA levels were analysed by quantitative RT-PCR. Each bar represents the mean per cent increase *vs.* controls ± SEM of 4 experiments performed in triplicate. (B) Western blot analysis of MUC2 in lysates of NCI-H292: The expression of MUC2 was normalised to the reference protein level (β-actin) in each sample. Each bar represents the mean per cent increase *vs.* controls ± SEM of 4 experiments performed in triplicate. **P* < 0·05 *vs.* controls. Picture: Representative Western blot picture of MUC2 and β-actin in control and treated cells (peptide β-CN(117-123), 0·01–1 μmol/l). Images are representative of 4 separated experiments performed in triplicate.

a temporal correlation between de novo expression of *MUC5AC* and a decreased expression of *MUC2* in colonic preneoplastic lesions (Sylvester et al. 2001; Zoghbi et al. 2007). If MUC2 and MUC5AC may have a simultaneous expression in various cell lines, thus indicating common regulatory mechanisms (Han et al. 2000; Gaudier et al. 2004; Gosalia et al. 2013), their cell- and tissue-specific distribution also implies the involvement of other pathways. For example, Gum et al. (1999) revealed the existence of cell-specific elements responsible for *MUC2* expression in intestinal goblet cells. Therefore, we could hypothesise that peptides may act on such cell-specific factors of regulation, at least for a mucin.

It is interesting to note that β-CN(117-123) decreased the expression of *MUC2* and *MUC5AC* in NCI-H292 cells, a human airway epithelial cell line. Such a peptide could therefore be interesting to mitigate symptoms of some lung diseases. Indeed, overexpression of the gel-forming mucin MUC5AC is a characteristic of inflammatory pulmonary diseases including asthma, chronic bronchitis, and cystic fibrosis. Mucus overproduction can then block the conducting airways, impair the gas exchange and therefore, contribute to the pathogenesis of respiratory diseases. Furthermore, although the production of MUC2 has not been demonstrated yet in normal airway mucus, there is evidence that expression of this secreted mucin is up

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regulated in cystic fibrosis but also following exposition with *Pseudomonas aeruginosa* (Li et al. 1997; Voynow et al. 2006). Thus, adding synthetic peptide β-CN(117-123) in aerosol therapy could present advantages in the treatment of several lung diseases. Several experiments will be required to verify this hypothesis.

We demonstrated that among the seven peptides tested, 3 of them (EMPFPK, YPVEPF and EPFTESQ) induce expression of the transmembrane-associated mucin MUC4. One of these peptides, and in particular the peptide EPFTESQ, could be at the origin of the effect of peptide β-CN(94-123) on intestinal expression of MUC4. Indeed, we previously showed that the effect of β-CN(94-123) on the expression of MUC4 by HT29-MTX cells was the most prominent at a concentration of 0·01 µmol/l and that this effect was reversed when β-CN(94-123) was tested at 100 µmol/l. Our results showed that the peptide EPFTESQ has a similar effect on MUC4, which is not the case of peptides EMPFPK and YPVEPF. Note that the sequence β-CN f108-113, i.e., the casokinin EMPFPK, is known to act by blocking the Angiotensin Converting Enzyme (ACE), a dipeptidyl peptidase also known as kininase II (EC 3.4.15.1). In the present study, we observed that EMPFPK significantly increased expression of the transmembrane-associated mucin *MUC4* when tested at 100 µM. This effect was not reproduced by captopril (D-3-mercapto-2-methyl-propionyl-L-proline), a strong and specific ACE inhibitor often used in the treatment of hypertension, thus suggesting that EMPFPK acts on goblet-like cells through mechanisms independent of ACE inhibition pathway. Like EMPFPK, the sequence YPVEPF, also named neocasomorphin-6 (Teschemacher, 2003), induced a significant rise in *MUC4* expression in HT29-MTX cells and was without effect on the expression of *MUC2* and *MUC5AC*. In keeping with these results, Martínez-Maqueda et al. (2012) demonstrated that neocasomorphin-6 did not provoke mucin secretion by HT29-MTX cells. It is worth noting that YPVEP was without effect on HT29-MTX cells, thus suggesting that the C-terminal phenylalanine in the sequence YPVEPF plays a critical role in the action of neocasomorphin-6 on *MUC4* expression.

The high reactivity of MUC4 is surprising and could be related to a specific protective function in newborns. Indeed, MUC4 is present in breast milk, suggesting again a major role in neonate defences. Actually, several studies demonstrated that the membrane-associated mucins MUC1 and MUC4 present in milk strongly block the attachment of pathogens to host cell membranes (Ruvoen-Clouet et al. 2006; Habte et al. 2007, 2008; Liu et al. 2012).

In conclusion, we demonstrated that different peptides encrypted in the sequence of β-CN(94-123) interact with intestinal goblet cells (Fig. 7). Three of them, β-CN(108-113), β-CN(114-119) and β-CN(117-123) induced *MUC4* expression and could be partly responsible for the activity of β-CN(94-123) on this transmembrane-associated mucin. Two others, β-CN(94-108) and β-CN(117-123), modulated the expression of the secreted mucin *MUC5AC* while β-CN

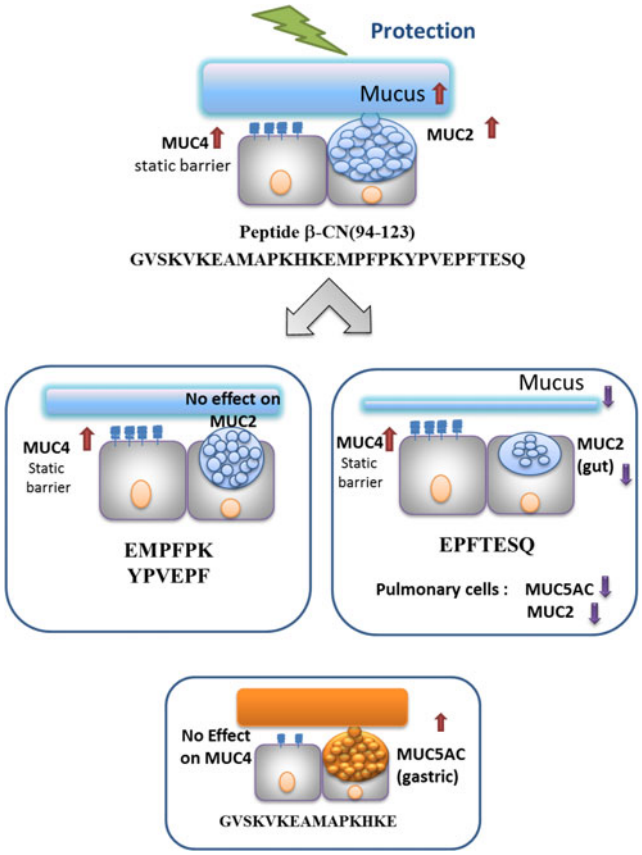

Fig. 7. Summary diagram. Only the sequence (94-123) of bovine β-casein stimulates the production of MUC2, a crucial factor of intestinal protection. The peptide β-CN(94-123), derived from bovine β-casein and identified in yoghurt, represents a novel molecule with potential health applications. It enhances the expression of the secreted mucin *MUC2* and of the transmembrane-associated mucin *MUC4*. The mucin MUC2 is secreted by goblet cells and forms a viscous gel (mucus layer) on the intestinal epithelial surface. Mucus lubricates the intestinal surface, limits passage of luminal molecules into the mucosa, functions as a defensive barrier against enzymes, acid and enteric pathogens. The membrane-associated mucin MUC4 is believed to play a role in the protection of cell surfaces. Tested on human intestinal HT29-MTX cells, the peptides EMPFPK and YPVEPF increased the expression of *MUC4* and could thus enhance static protection. None of them modulated production of the secreted mucin MUC2, a major player in the intestinal protection. The peptide EPFTESQ also induced expression of *MUC4*, but it greatly reduced *MUC2* expression. This surprising effect was observed on intestinal cells (HT29-MTX) and also on cells of the airways (NCI-H292). In the latter case, the peptide EPFTESQ also decreased the expression of *MUC5AC* that is highly expressed in the lungs. This peptide could thus be of interest in the treatment of lung diseases characterised by an overproduction of mucus. Note that the peptide GVSKVKEAMAPKHKE has increased expression of the secreted mucin MUC5AC but this is produced in the stomach and will therefore not strengthen or restore intestinal homoeostasis.

(117-123) also decreased that of *MUC2*. However, none of them reproduced the stimulatory effect of β-CN(94-123) on the prominent intestinal mucin MUC2, thus suggesting

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that the 30 amino-acids of β-CN(94-123) are necessary for reinforcing intestinal protection. In contrast, the peptide β-CN(117-123) inhibited the expression as well as the production of MUC2 in intestinal cells. This peptide could therefore have a deleterious effect on intestinal defences.

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