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Cholecystokinin 1 receptor modulates the MEKK1-induced c-Jun trans-activation: structural requirements of the receptor

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1 In cells overexpressing active MEKK1 to enhance c-Jun trans-activation, expression of rat cholecystokinin 1 receptor increased the activity of c-Jun while in the same experimental conditions overexpression of mouse cholecystokinin 1 receptor repressed it.

2 This differential trans-activation is specific, since it was not observed for either the other overexpressed kinases (MEK, PKA) or for other transcription factors (ATF2, ELK-1, CREB). This differential behaviour was also detected in a human colon adenocarcinoma cell-line naturally producing high levels of endogenous MEKK1.

3 This differential behaviour between the two receptors on the MEKK1-induced c-Jun trans-activation was independent of the activation state of JNK, of the phosphorylation level of c-Jun and of its ability to bind its specific DNA responsive elements.

4 Two amino acids (Val43 and Phe50 in the mouse cholecystokinin 1 receptor, replaced by Leu43 and Ileu50 in the rat cholecystokinin 1 receptor) localized in the first transmembrane domain were found to play a crucial role in this differential behaviour.

5 MEKK1 probably activates a transcriptional partner of c-Jun whose activity is maintained or increased in the presence of the rat cholecystokinin 1 receptor but repressed in the presence of the mouse cholecystokinin 1 receptor.
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Abbreviations: AP-1, activating protein 1; CBP, CREB-binding protein; CCK, cholecystokinin; CCK-R, cholecystokinin receptor; CCK-8s, sulfated CCK-8 (H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2); CREB, cAMP-responsive element-binding protein; MAP/ERK kinase kinase 1, MEKK1, mitogen extracellular regulated kinase kinase 1; MEK, mitogen extracellular regulated kinase 1; PKA, protein kinase A

Introduction

Cholecystokinin (CCK) was originally described in the gastrointestinal tract and later in the mammalian central nervous system thus classifying CCK as both a peptide hormone and a neurotransmitter. Biological effects of cholecystokinin are initiated upon binding to membrane receptors which belong to the class I family of the G protein-coupled receptors (GPCR) (Bockaert & Pin, 2000). Cholecystokinin receptors (CCK-R) have been biologically and pharmacologically classified into two main types, the type 1 CCK receptor (CCK_1-R) and the type 2 CCK receptor (CCK_2-R), on the basis of their affinity for the two endogenous peptide agonists CCK and gastrin, as well as their affinity for selective antagonists (Wank, 1995; Noble *et al*., 1999). The CCK_1-R is mainly found in the peripheral system, and to a lesser extent in localized areas of the central nervous system, whereas the CCK_2-R is mainly found throughout the central nervous system but is also located in the stomach where it is involved in stimulation of acid secretion. The CCK_1-R regulates several biological functions related to nutrient homeostasis such as physiological stimulation of gall bladder contraction, secretion of pancreatic enzymes, gastric emptying and enteric transit regulations, and induction of satiety. Owing to these wide spectrum of biological activities on various organs and cell types, the CCK_1-R has been quickly considered as an important therapeutic target. While others have investigated the hormone-binding domain to develop highly selective and potent drugs (Kennedy *et al*., 1997; Gigoux *et al*., 1998; 1999; Pellegrini & Mierke 1999; Ding & Miller, 2001; Escrieut *et al*., 2002), we decided to investigate the differential behaviour between the mouse and the rat CCK_1-R which was previously described (Galas *et al*., 1988; Matozaki *et al*., 1989; Stark *et al*., 1989; Bianchi *et al*., 1994). More recently, we have shown that even in the absence of ligand, the rat CCK_1-R has an intrinsic potency significantly higher than that of the mouse CCK_1-R in inducing c-fos gene expression and AP-1 responsive genes (Poosti *et al*., 2000). In agreement with these results it was suggested that both intrinsic parameters, like primary structure of the CCK_1-R, and extrinsic parameters, like differential coupling to G proteins, or structure and organization of the lipidic environment of the cell membrane, could explain this differential behaviour

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(Ghanekar *et al*., 1997; Logsdon, 1999; Ji *et al*., 2000; Poosti *et al*., 2000).

Owing to the crucial role of c-Jun in the formation of AP-1 complexes and of its important involvement in biological responses such as cell proliferation, tumorigenesis, apoptosis and embryonic development, we addressed the question of the role of c-Jun trans-activation in the presence of rat or mouse CCK_1-R, and studied the c-Jun N-terminal kinase (JNK) pathway using a reporter gene strategy, as well as Western immunoblotting and pull-down experiments.

c-Jun is known to be phosphorylated by JNK at two N-terminal sites: Ser-63 and Ser-73 (Barr & Bogoyevitch, 2001; Vogt, 2001). The immediate upstream activators of JNK are MAP kinase kinases (MKKs), known as c-Jun N-terminal kinase kinases (JNKKs) (Deacon & Blank, 1997; Holland *et al*., 1997), that are phosphorylated by MAP kinase kinase kinases (MKKKs) such as MEKK1 (MAP/ERK kinase kinase 1) (Hagemann & Blank, 2001).

When looking at the c-Jun trans-activation in cells over-expressing the active form of MEKK1, a kinase belonging to the JNK pathway, we noticed a difference in the c-Jun trans-activation depending on the presence of the rat or mouse CCK_1-R. Indeed, while the presence of the rat CCK_1-R resulted in a high level of c-Jun trans-activation upon MEKK1 induction, the presence of the mouse CCK_1-R repressed it.

In addition, we demonstrated that this differential behaviour between rat and mouse CCK_1-R was specific to the c-Jun transcription factor and to MEKK1, however, it was independent of the activation state of JNK, of the phosphorylation level of c-Jun and of its ability to bind to its specific DNA responsive elements. Furthermore, additional experiments using mutated forms of these receptors suggested a major role of two amino acids localized in the first trans-membrane domain.

Methods

Cell culture

Human cervical carcinoma and human colon adenocarcinoma cell lines (HeLa, DLD-1) were routinely cultured at 37°C, in a humidified 5% CO_2 atmosphere in DMEM (Dulbecco's modified Eagle's medium) containing phenol red and supplemented with FCS (foetal calf serum – 10% (v/v)), glutamine (2.5 mM), and penicillin–streptomycin (150 U–150 μg).

Plasmids and transient transfection

pFR-Luc, pFA2-c-Jun, pFA2-ATF2, pFA2-CREB, pFA2-ELK-1, pFC-MEK, pFC-MEKK1 and pFC-PKA plasmids were purchased from Stratagene (Ozyme, France). The reporter plasmid pFR-Luc placed the coding sequence of the firefly luciferase (Luc) under the control of five tandem repeats of the Gal_4 binding element (Xing *et al*., 1998; Hocevar *et al*., 1999). Plasmids pFA2-c-Jun, pFA2-ATF2, pFA2-CREB, pFA2-ELK-1 led to expression of transcription factors in which the Gal_4 DNA-binding domain was fused to the trans-activation domain of c-Jun, ATF2, CREB, and ELK-1, respectively. The plasmids encoding the CCK_1-Rs of rat, mouse and of mouse mutants have been described previously (Poosti *et al*., 2000). Other mutants were constructed by the two-step PCR procedure using purified oligonucleotides synthesized by Genome Express (Meylan, France). pFC-MEK, pFC-MEKK1 and pFC-PKA plasmids encode active forms of MEK (mitogen extracellular regulated kinase), MEKK1, and PKA (protein kinase A), respectively. The plasmid $p(TRE)_3$-tk-Luc has been previously described (Astruc *et al*., 1995).

Transient transfection experiments were performed as follows: cells were harvested with trypsin/EDTA, and washed with the washing buffer (KCl 120 mM, $CaCl_2$ 0.15 mM, $MgCl_2$ 5 mM, K_2HPO_4/KH_2PO_4 10 mM pH 7.6, EGTA 2 mM pH 7.6). Cells were then suspended in 500 μl of electroporation buffer (10 million cells, washing buffer supplemented with ATP 2 μM, gluthathione 5 μM), incubated with the plasmid for 10 min and then electroporated using an Easyject Optima apparatus (Equibio, Kent, U.K.) at 270 V and 1350 μF for HeLa cells, and 250 V and 1500 μF for DLD-1 cells. A typical electroporation was performed by mixing 5 μg of reporter plasmid (pFR-Luc or $p(TRE)_3$-tk-Luc), 3 μg of vector encoding either rat, mouse, or mutated mouse CCK_1-R, 3 μg of vector encoding either natural c-Jun or one of the chimaeric transcription factors Gal_4-c-Jun, Gal_4-ATF2, Gal_4-CREB, or Gal_4-ELK-1, with or without 10 ng of either pFC-MEK, pFC-MEKK1 or pFC-PKA plasmids. After the pulse, cells were immediately re-suspended in complete medium without phenol red (500 μl) and transferred to 1.5 ml tubes for a 10 min incubation. Cells were then transferred into a 12-well plate and grown for 24 h in DMEM without phenol red supplemented with 10% FCS 10%. The medium was then replaced with DMEM without phenol red, supplemented with FCS 0.3% for an additional period of 24 h. Transfected cells were then stimulated with CCK-8s (30 nM) for 8 h and lysed using 100 μl of a buffer containing 125 mM Tris, 10 mM CDTA, DTT 10 mM DTT, 50% glycerol, 5% Triton X-100, pH 7.6. Detection of luciferase activity was performed using a Wallac 1450 Microbeta Jet (Perkin-Elmer, France). Measurements were performed for 2 s upon injection of luciferase detection buffer (KH_2PO_4/K_2HPO_4 15 mM, $MgCl_2$ 1.05 mM, $MgSO_4$, 2.7 mM, EDTA, 0.2 mM, ATP, 530 μM, coenzyme A 270 μM, luciferin 500 μM, pH 7.4; 100 μl) to the cell lysate (100 μl). Results are expressed as luminescence arbitrary units corrected for the background signal. Each value represents the mean $\pm$ s.d. of at least three separate experiments performed in triplicate.

Antibodies, Western-immunoblotting and pull-down experiments

Polyclonal anti-phospho-SAPK/JNK (Thr183/Thr185, rabbit), anti-MEKK1 (rabbit), anti-phospho-c-Jun (Ser63, rabbit), and anti-rabbit secondary antibodies were from New England Biolabs (Beverly, MA). Cells were treated as described above, except that they were placed in a 100-mm culture dish, following electroporation, which was performed in the absence of pFR-Luc and pFA2-c-Jun. For Western-immunoblotting experiments, cells were stimulated with CCK-8s (30 nM) for 15 min, then extensively washed with cold PBS and further lysed using 200 μl of the following buffer: 195 mM DTT, 12.5 mM Tris buffer pH 6.8, 0.1 M SDS, 2.2 M glycerol. Cell lysates were then boiled for 10 min and stored at −80°C. Lysates for pull-down experiments were prepared in a similar manner and the detection was performed according to

the manufacturer's instructions (SAPK/JNK assay kit, New England Biolabs, Ozyme, France).

For both Western-immunoblotting and pull-down experiments, cell lysates (20 μg protein) were resolved on 10% SDS–PAGE and transferred to a nitrocellulose membrane (ECL, Amersham, France) using a Bio-Rad Mini Protein III apparatus. Specific proteins were detected with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies using the Phototope-HRP (Phototope-horseradish peroxidase) Western-blot detection system. The detection was performed using a Biomax ML scientific imaging film (Eastman Kodak Co.) exposed for less than 1 min.

DNA-binding experiments

HeLa cells were cotransfected as described above with plasmids encoding native c-Jun, active MEKK1, and either mouse or rat CCK_1-R. Cells were then distributed in 150 mm Petri dishes and cultured as described for the immunoblotting experiments. Cells were stimulated for 3–4 h with CCK-8s (30 nM) and their cellular content extracted using the 'mercury transfector extraction kit' (Clontech/Ozyme-France). Cellular extracts (30 μg protein) were then tested for their ability to bind to TRE (12-*O*-tetradecanoylphorbol-13-acetate responsive element) sequences according to an ELISA protocol provided by the same manufacturer.

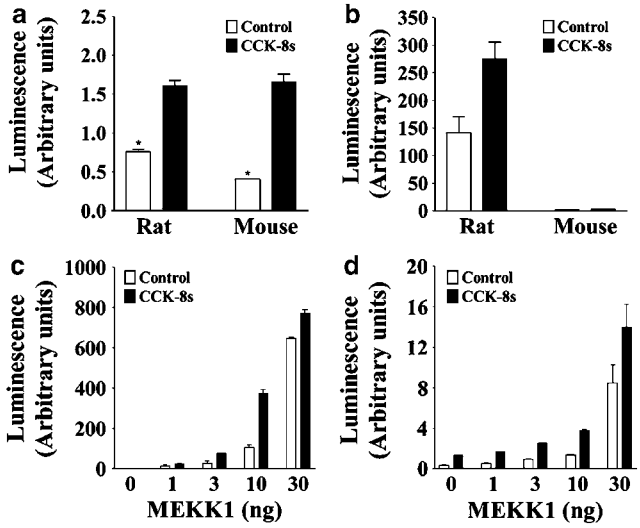

Figure 1 Effect of active MEKK1 on Gal_4-c-Jun trans-activation. HeLa cells were transiently cotransfected with the reporter plasmid pFR-Luc (5 μg), pFA2-c-Jun (3 μg) and vectors encoding either rat or mouse CCK_1-R (3 μg), without (a) or with (b) pFC-MEKK1 (10 ng). Transfected cells were incubated either in the presence (black histogram) or in the absence (white histogram) of CCK-8s (30 nM) for 8 h and then lysed for luciferase measurement. The effect of increasing amounts of pFC-MEKK1 on Gal_4-c-Jun trans-activation was studied according to the coexpressed CCK_1-R (c: rat CCK_1-R, d: mouse CCK_1-R).

Results

Differential effects of Gal4-c-Jun trans-activation, in the presence of rat or mouse CCK_1-R

To investigate the role of rat and mouse CCK_1-R on c-Jun trans-activation, HeLa cells were transiently cotransfected with vectors encoding the Gal_4-c-Jun construct (pFA2-c-Jun), its appropriate reporter plasmid (pFR-Luc) and vectors encoding either rat or mouse CCK_1-R. In the presence of rat and mouse CCK_1-R, CCK-8s induced a low luciferase activity (Figure 1a). All experiments performed in the absence of CCK-8s indicated that rat CCK_1-R was significantly ($^*P<0.001$, $n=50$) more potent than mouse CCK_1-R in increasing Gal_4-c-Jun trans-activation despite the low luminescence values (Figure 1a). This differential behaviour was strongly accentuated when active MEKK1, a kinase known to predominantly activate the JNK cascade *in vivo*, was coexpressed (Hagemann & Blank, 2001). Accordingly, cells expressing rat CCK_1-R were characterized by a high level of Gal_4-c-Jun trans-activation, while cells expressing mouse CCK_1-R had a poor Gal_4-c-Jun trans-activation (Figure 1b), this differential behaviour between these two CCK_1-Rs was significant both in the absence and in the presence of CCK-8s ($P<0.001$). For both receptors, we confirmed these observations by transfection of increasing amounts of pFC-MEKK1 (0–30 ng) and determined that with up to 10 ng, the activity of Gal_4-c-Jun was significantly increased upon binding of CCK-8s ($P<0.001$) (Figure 1c and d). When 30 ng of plasmid encoding the active MEKK1 was transfected, the effect of the ligand was less significant ($P<0.01$, when the rat CCK_1-R was coexpressed (Figure 1c) and not significant when the mouse CCK_1-R was coexpressed (Figure 1d)). Additional binding experiments revealed that this differential behaviour was not due to a difference in receptor expression levels (data not shown).

Kinase and transcription factor specificity

To determine whether this differential behaviour was specific to the MEKK1 pathway and the Gal_4-c-Jun factor, we expressed active MEK or PKA, instead of MEKK1, and also tested three other chimaeric transcription factors (Gal_4-ATF2, Gal_4-ELK-1 and Gal_4-CREB). In experiments carried out with active MEK or PKA, no significant increase in Gal_4-c-Jun trans-activation was observed, regardless of whether mouse or rat CCK_1-R was coexpressed, thus suggesting a specific role of MEKK1 (Figure 2). In experiments carried out with each of the three other chimaeric transcription factors, the expression of active MEKK1 led to an increase of luciferase activity for both basal and ligand-induced levels, independently of receptor (rat or mouse) and coexpressed chimaeric transcription factors (Gal_4-ELK-1, Gal_4-ATF2 and Gal_4-CREB, Figure 3: compare 3b vs 3a, 3d vs 3c, 3f vs 3e). Although the expression of active MEKK1 led to a higher increase in luciferase activity when Gal_4-ATF2 was expressed (Figure 3b) as compared with Gal_4-ELK-1 (Figure 3d) or Gal_4-CREB (Figure 3f) no noticeable difference was observed between rat and mouse CCK_1-R, suggesting that Gal_4-c-Jun is a specific target that is activated or repressed according to the coexpressed CCK_1-R (Figure 3).

Dominant-negative effect of the mouse CCK_1-R

To determine which receptor behaved as the dominant one, we cotransfected increasing amounts of plasmid encoding mouse CCK_1-R (30 ng–3 μg) with a fixed amount of plasmid encoding

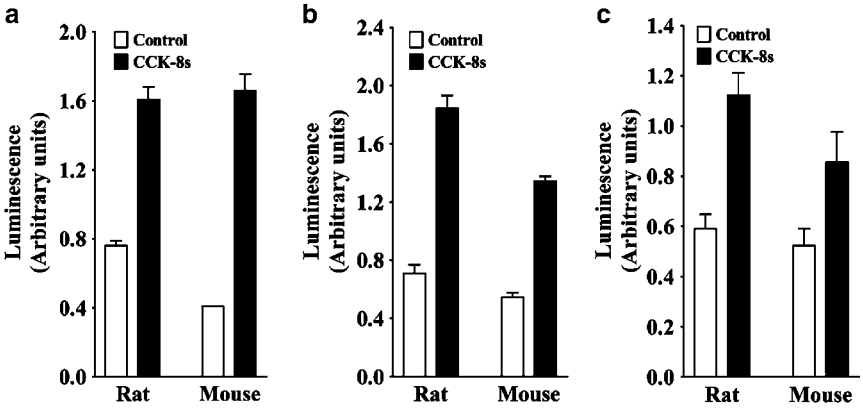

Figure 2 Effect of active MEK and active PKA on Gal_4-c-Jun trans-activation. HeLa cells were treated as described in the legend of Figure 1 except that they were cotransfected in the absence (a) or in the presence of either pFC-MEK (10 ng (b)) or pFC-PKA (10 ng (c)) instead of pFC-MEKK1.

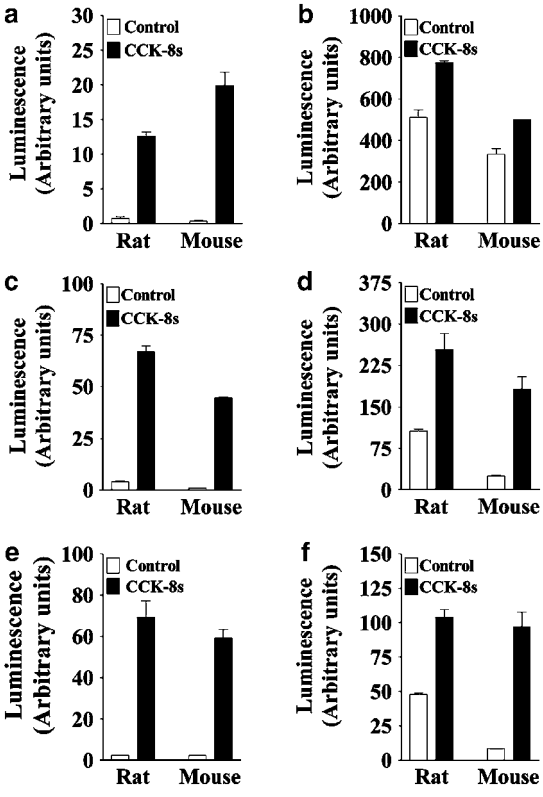

Figure 3 Effect of active MEKK1 on the trans-activation of Gal_4-ATF-2, Gal_4-ELK-1, Gal_4-CREB. HeLa cells were treated as described in the legend of Figure 1 except that they were cotransfected with either pFA2-ATF2 (a, b), pFA2-ELK-1 (c, d) or pFA2-CREB (e, f) instead of pFA2-c-Jun in the absence (a, c, e), or in the presence (b, d, f) of pFC-MEKK1.

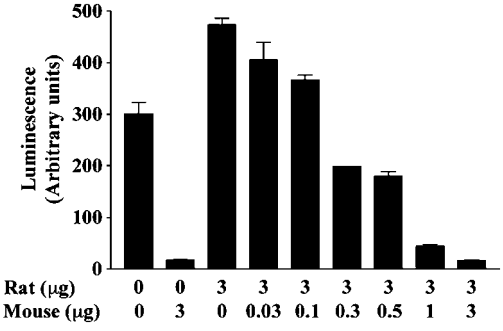

Figure 4 Effect of rat and mouse CCK_1-R coexpression on Gal_4-c-Jun trans-activation. HeLa cells were transiently cotransfected with plasmids encoding active MEKK1 (10 ng), Gal_4-c-Jun (3 μg), rat CCK_1-R (3 μg), and increasing amounts of plasmid encoding mouse CCK_1-R (30 ng–3 μg). Cells were then treated as described in the legend of Figure 1.

rat CCK_1-R (3 μg) (Figure 4). Interestingly, the inhibition profile of Gal_4-c-Jun trans-activation appeared to be 'dose-dependent' with a significant ($P<0.001$) effect at 1% of mouse CCK_1-R encoding plasmid over rat CCK_1-R encoding plasmid. The fact that Gal_4-c-Jun trans-activation was completely abolished when identical amounts of each plasmid were transfected (last histogram) suggests that mouse CCK_1-R behaves as a strong negative dominant (not significantly different from the control level obtained when the mouse CCK_1-R is coexpressed).

No difference in DNA-binding of c-Jun

We next determined whether this differential behaviour was observed for the natural c-Jun protein. For this purpose, HeLa cells were cotransfected with vectors encoding either rat or mouse CCK_1-R, active MEKK1, and $p(TRE)_3$-tk-Luc, a reporter plasmid specifically designed to study the activation of AP-1, a complex known to be largely activated by the c-Jun protein (Vogt, 2001). To prevent the involvement of protein partners other than c-Jun itself at the TRE sequences, we also overexpressed c-Jun. While rat or mouse CCK_1-R gave similar results in the absence of overexpressed c-Jun, a significant inhibition of AP-1 trans-activation was only observed for cells coexpressing c-Jun and mouse CCK_1-R ($P<0.001$) (Figure 5a). When the binding of c-Jun to its TRE sequences was examined (ELISA, Figure 5b), we noticed that the level obtained for the control experiment (MEKK1 alone, first histogram) was identical to that obtained in the presence of mouse CCK_1-R (not significant) but increased in the presence of rat CCK_1-R ($P<0.001$). As control, no or a poor binding of c-Jun at TRE sequences was observed when competitor or

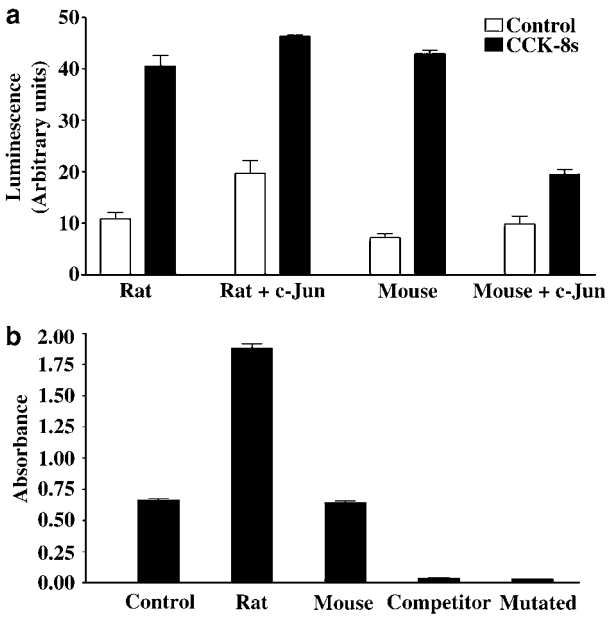

Figure 5 Effect of active MEKK1 on both trans-activation and DNA-binding properties of c-Jun. (a) HeLa cells were transiently cotransfected with the $p(TRE)_3$-tk-Luc reporter plasmid (5 μg), pFC-MEKK1 (10 ng), vectors encoding either rat or mouse CCK_1-R (3 μg) with or without a vector encoding the natural c-Jun protein (3 μg). Cells were then treated as described in the legend of Figure 1. (b) HeLa cells were transfected as above in the presence of plasmid encoding the native c-Jun protein. Cells were incubated with CCK-8s (30 nM) for 4 h and lysed. Cellular extracts were then tested for their ability to bind to immobilized TRE sequences (TGACTCA) using an ELISA method. c-Jun specificity was confirmed by co-incubation of cellular extracts obtained from cells expressing no CCK_1-R (Control), with an excess of TRE competitor (Competitor) or to immobilized mutated TRE sequence (TGACTTG, Mutated).

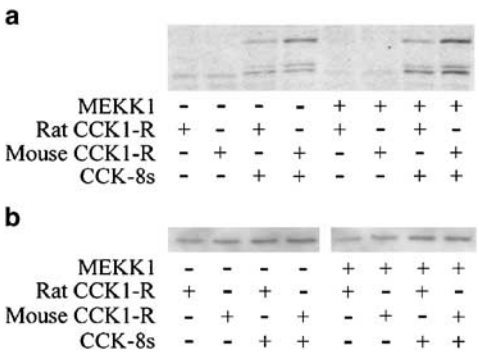

Figure 6 Immunodetection of phospho-JNK and of phospho-c-Jun: effect of active MEKK1. HeLa cells were cotransfected with vectors encoding either rat or mouse CCK_1-R, in the presence (+) or in the absence (−) of vector encoding active MEKK1. Cells were then incubated with (+) or without (−) CCK-8s (30 nM, 15 min), and then lysed for immunodetection of phospho-JNK (a, Western immunoblotting) or of phospho-c-Jun to detect JNK activity (b, pull down).

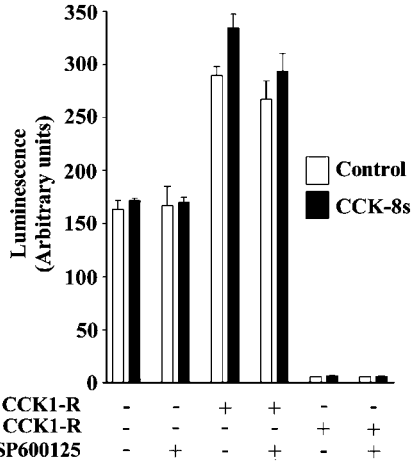

Figure 7 Effect of SP600125 on the MEKK-1-induced Gal_4-c-Jun trans-activation. HeLa cells were cotransfected as in Figure 1b, and incubated with (+) or without (−) a specific JNK inhibitor (SP600125, 5 μM) for 12 h. Cells were further incubated with (black histogram) or without (white histogram) CCK-8s (30 nM) for 8 h and lysed for luciferase detection.

mutated TRE sequences were used. This suggests that the inhibition of AP-1 transcriptional activity was not the result of an inhibition of the binding of c-Jun to its specific TRE sequences.

No differential activation of the SAPK/JNK pathway

To explore the role of JNK in the differential behaviour of mouse and rat CCK_1-R, we measured its phosphorylation state using Western blots and monitored its kinase activity using pull-down assays. In the absence of CCK-8s, the phosphorylation state of the JNK isoforms was relatively low, with CCK_1-R expressed from both species. Upon CCK-8s stimulation, we observed a moderate and equivalent JNK activation for cells expressing rat or mouse CCK_1-R, both with and without active MEKK1, regardless of the expressed CCK_1-R (Figure 6a). Using pull-down experiments, we tested the ability of endogenous JNK to phosphorylate a GST-c-Jun fusion protein (Glutathione S-transferase-c-Jun fusion protein). We showed that rat and mouse CCK_1-Rs were indistinguishable regarding the phosphorylation of the exogenous fusion protein, whether active MEKK1 was expressed or not regardless of the presence of CCK-8s (Figure 6b). We further confirmed that the differential behaviour was independent of the phosphorylation state of JNK since SP600125, a specific cell permeable inhibitor of JNK (Bennett *et al*., 2001) had no significant effect on the differential trans-activation of Gal_4-c-Jun (Figure 7).

The differential behaviour between rat and mouse CCK_1-R on Gal_4-c-Jun trans-activation was observed in a cell line, which naturally expresses a high level of MEKK1

To determine whether our experimental model might reflect physiological conditions, we selected the human colorectal adenocarcinoma cell line (DLD-1) expected to endogenously express high levels of MEKK1 (Licato *et al*., 1997; Licato & Brenner, 1998; Hirano *et al*., 2002), and performed transfection experiments as described for our HeLa model except that this was performed in the absence of any overexpression of active MEKK1. As expected, Gal_4-c-Jun trans-activation was high and similar for both control and rat CCK_1-R (no significant difference), while the expression of mouse CCK_1-R

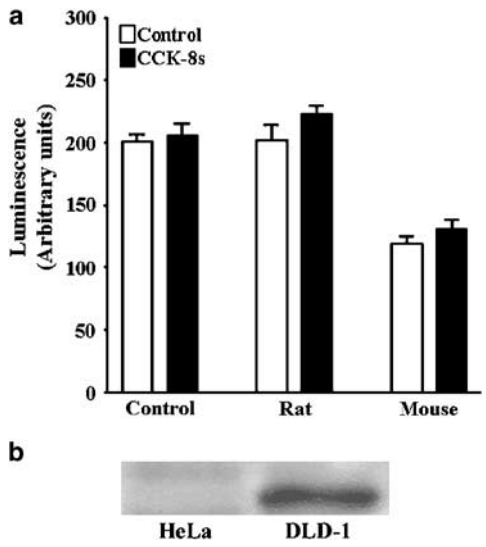

Figure 8 Gal_4-c-Jun trans-activation in DLD-1 cells and determination of their MEKK1 content. (a) DLD-1 cells were cotransfected with the pFR-Luc reporter plasmid and vectors encoding Gal_4-c-Jun and either rat or mouse CCK_1-R, in the absence of the plasmid encoding active MEKK1. Cells were then treated as described in the legend of Figure 1. (b) Immunoblotting of endogenous MEKK1 from untransfected HeLa and DLD-1 cells.

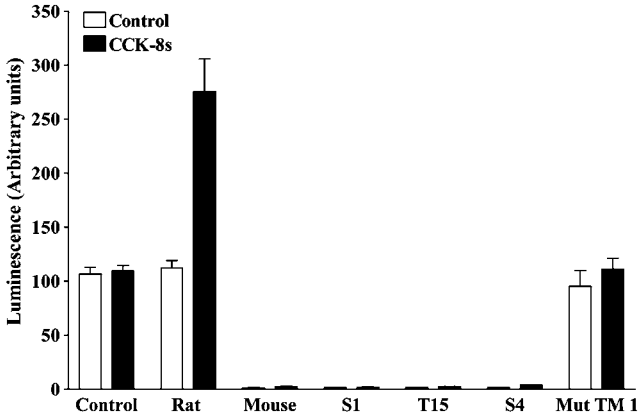

Figure 9 MEKK1-induced Gal_4-c-Jun trans-activation: effect of various mutations in the mouse CCK_1-R. HeLa cells were cotransfected with the pFR-Luc reporter plasmid, and vectors encoding Gal_4-c-Jun (pFA2-c-Jun), active MEKK1 (pFC-MEKK1) and either rat CCK_1-R, mouse CCK_1-R or mutated mouse CCK_1-Rs ('S1', 'T15', 'S4' and 'Mut TM1'). 'S1' was obtained by substitution of the third intracellular loop of the mouse CCK_1-R for one of the rat CCK_1-R. 'T15' was obtained by substitution of amino-acids 294–308 of the mouse CCK_1-R for those of the rat CCK_1-R. 'S4' was generated by deletion of the polyGly domain of the third intracellular loop of the mouse CCK_1-R. 'Mut TM1' was obtained by substitution of two amino acids localized in the first transmembrane domain of the mouse CCK_1-R for the corresponding amino acids of the rat CCK_1-R.

significantly reduced it ($P<0.001$) regardless of whether CCK-8s was added or not (Figure 8a). We further checked the endogenous presence of MEKK1 by immunoblotting experiments and clearly observed that DLD-1 cells naturally expressed higher levels of MEKK1 than HeLa cells (Figure 8b).

Differences in the primary structure of rat and mouse CCK_1-R contribute to explaining their differential behaviour on Gal4-c-Jun trans-activation induced by active MEKK1

Owing to the high sequence homology between rat and mouse CCK_1-Rs, we were interested in identifying the residues in the primary structure of these receptors involved in their differential behaviour. By using mouse CCK_1-R mutants called S1, T15, S4, in which the third intracellular loop was totally or partially replaced by that of the rat CCK_1-R (Poosti *et al*., 2000), we clearly observed that this region was not directly involved in the differential behaviour of these receptors since their expression did not significantly modify the effect of the mouse CCK_1-R (Figure 9). We then tested a mutant of the mouse CCK_1-R called 'Mut TM1', in which the two amino acids Valine 43 and Phenylalanine 50 localized in the first transmembrane domain of the mouse CCK_1-R were substituted for Leucine 43 and Isoleucine 50, residues of the rat CCK_1-R. Interestingly, an increase in the MEKK1-induced Gal_4-c-Jun trans-activation was observed. This was shown both in the presence or absence of CCK-8s, suggesting the key role of these two amino-acid residues in the differential behaviour of the two rat and mouse CCK_1-R. In fact, the expression of 'Mut TM1' led to a partial reversion since we do not observe any significant effect of CCK-8s to 'Mut TM1'.

Discussion

We have previously reported a differential behaviour between rat and mouse CCK_1-R on the basis of their ability to modulate the expression of AP-1 responsive genes, the rat CCK_1-R being more efficient to activate these genes than the mouse CCK_1-R (Poosti *et al*., 2000). Since the AP-1 complex is mainly composed of c-Jun homo-dimers, or of c-Jun hetero-dimers involving members of the c-Jun and c-Fos families, we specifically investigated the role of rat and mouse CCK_1-Rs on c-Jun trans-activation.

We report here that coexpression of either rat or mouse CCK_1-Rs with active MEKK1, a kinase well known to predominantly activate the JNK cascade *in vivo* (Hagemann & Blank, 2001) and having an important role in cell proliferation, survival and apoptosis in various cells (Okutomi *et al*., 2003), resulted in a differential trans-activation of Gal_4-c-Jun. Indeed, we clearly observed that cells expressing the rat CCK_1-R were characterized by a high level of Gal_4-c-Jun trans-activation compared to cells expressing the mouse CCK_1-R. We showed the crucial role of Valine 43 and Phenylalanine 50, two amino-acid residues located in the first transmembrane domain of the mouse CCK_1-R in this Gal_4-c-Jun trans-activation. We first thought that rat CCK_1-R was constitutively able to activate c-Jun. However, we subsequently observed that (i) strong inductions of Gal_4-c-Jun trans-activation were measured in cells expressing active MEKK1 alone, (ii) inhibition of AP-1 responsive genes was obtained only for cells co-expressing mouse CCK_1-R, active MEKK1 and native c-Jun and (iii) mouse CCK_1-R behaved as a negative dominant to the rat CCK_1-R. Taken together, these results suggest that the differential behaviour observed between these two receptors is much more related to a repressive mechanism mediated by the mouse CCK_1-R rather

than to an activation mechanism mediated by the rat CCK_1-R. Although we determined that this differential trans-activation was MEKK1 as well as Gal_4-c-Jun specific, we postulated whether it would reflect more the physiological conditions. Therefore, we first confirmed that the differential behaviour also occurred for the native c-Jun protein and determined that it was neither related to the activation state of JNK, its phosphorylation level, nor to its ability to bind its specific responsive elements. To determine the physiological relevance of our observations, we performed similar experiments in DLD-1 cells, a system expressing endogenous MEKK1 at levels comparable to those relevant to physiological disorders involved in some cancers. This human colorectal adenocarcinoma cell line was selected on account that (i) several data suggested a constitutive activity of the SAPK/JNK pathway in various human cancers such as pancreatic and colon cancers, tissues that usually express CCK_1-Rs (Licato *et al*., 1997; Licato and Brenner, 1998); (ii) DLD-1 cells were described to express a mutant of Ras (K-Ras) able to strongly activate the JNK pathway once stably transfected in NIH3T3 cells (Guerrero *et al*., 2000). In such cells, we were able to reproduce the same differential behaviour between the two CCK_1-Rs previously observed in HeLa cells. Moreover, in DLD-1 cells we observed that addition of CCK-8s was unable to increase Gal4-c-Jun trans-activation whatever the rat or mouse CCK_1-R that was expressed. This result is similar to that obtained in HeLa cells when 30 ng of plasmid encoding active form of MEKK1 was transfected (compare Figure 1c and d with Figure 8a); this result is in agreement with previous studies suggesting that MEKK1 is highly activated in DLD1 cells (Licato *et al*., 1997; Licato & Brenner, 1998; Hirano *et al*., 2002).

On the basis of these results, we hypothesized that the CCK_1-Rs-induced differential trans-activation of c-Jun might involve an interacting partner of c-Jun. Indeed, a plethora of such proteins have been described to interact with c-Jun or with the AP-1 complex (Chinenov & Kerppola, 2001). Among them, proteins involved in the formation of transcription regulatory multi-protein complexes, binding at specific promoter and enhancer regions (for example coactivators and chromatin remodelling factors), offer various interesting candidates such as members of the CBP (CREB-binding protein)/p300 family and various subunits of the COP9 signalosome. For example, MEKK1 was described to enhance p300-mediated transcription without requiring the JNK pathway, but rather through the phosphorylation of p300, either by MEKK1 itself or by other not yet identified kinases (See *et al*., 2001; Vo & Godmann, 2001). These findings support our results attributing a major role to MEKK1 in a pathway independent of the JNK activation.

Conclusions

We have shown in this piece of work that G-protein coupled receptors, that is, mouse and rat CCK_1-Rs, were able to modify intracellular events (c-Jun trans-activation) in cells overexpressing active MEKK1, independently of their activation by their natural ligand CCK-8s. The rat CCK_1-R increased c-Jun activity, while the mouse CCK_1-R repressed it. Two amino acids (Leu43, Ile50 in rat, Val43, Phe50 in mouse) of the first putative transmembrane domain of rat and mouse CCK_1-Rs were found crucial for the differential trans-activation of c-Jun. This localization in a region usually not involved in the intracellular signalization argues in favour of a model in which the conformational structure of a receptor would be responsible for a particular signalling pathway. We therefore assume that the conformational structure of each CCK_1-R would have opposite contributions on c-Jun trans-activation when MEKK1 is activated. Finally we suggest that a specific partner of c-Jun would be differentially activated by MEKK1 depending on the CCK_1-R that is coexpressed.

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