A coordinated phosphorylation cascade initiated by p38MAPK/MSK1 directs RAR α to target promoters

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The nuclear retinoic acid (RA) receptor alpha (RAR α) is a transcriptional transregulator that controls the expression of specific gene subsets through binding at response elements and dynamic interactions with coregulators, which are coordinated by the ligand. Here, we highlighted a novel paradigm in which the transcription of RAR α target genes is controlled by phosphorylation cascades initiated by the rapid RA activation of the p38MAPK/MSK1 pathway. We demonstrate that MSK1 phosphorylates RAR α at S369 located in the ligand-binding domain, allowing the binding of TFIID and thereby phosphorylation of the N-terminal domain at S77 by cdk7/cyclin H. MSK1 also phosphorylates histone H3 at S10. Finally, the phosphorylation cascade initiated by MSK1 controls the recruitment of RAR α /TFIID complexes to response elements and subsequently RAR α target gene activation. Cancer cells characterized by a deregulated p38MAPK/MSK1 pathway, do not respond to RA, outlining the essential contribution of the RA-triggered phosphorylation cascade in RA signalling.

The EMBO Journal (2009) 28, 34–47. doi:10.1038/emboj.2008.256; Published online 11 December 2008

Subject Categories: chromatin & transcription

Keywords: MSK1; nuclear receptor; phosphorylation; retinoic acid; transcription

Introduction

Nuclear retinoic acid (RA) receptors (RARs) consist of three subtypes, α (NR1B1), β (NR1B2) and γ (NR1B3) (Laudet and Gronemeyer, 2001; Germain *et al.*, 2006a, b), which function as ligand-dependent transcriptional regulators heterodimerized with retinoid X receptors (RXRs). The basic mechanism for transcriptional regulation by RARs relies on binding to specific

response elements (RAREs) located in the promoters of target genes and on ligand-induced structural rearrangements in the ligand-binding domain (LBD) that direct the association/dissociation of coregulator complexes with different enzymatic activities, including histone acetyltransferases/deacetylases, histone methyltransferases/demethylases, kinases/phosphatases, ubiquitin ligases/deubiquitinases or DNA-dependent ATPases (Bastien and Rochette-Egly, 2004; Lefebvre *et al.*, 2005; Zhao *et al.*, 2008). The exchanges between coregulatory complexes and RARs are dynamic and coordinated (Rochette-Egly, 2005; Rosenfeld *et al.*, 2006). In fine, they cooperate to alter the chromatin structure surrounding the promoter of target genes, paving the way for the recruitment of the transcription machinery, including RNA polymerase II and the general transcription factors (Dilworth and Chambon, 2001).

A concept that developed over the last several years is that RARs are subject to phosphorylations (Rochette-Egly, 2003), which have an important and ever-growing function in the transcription of RA target genes (Taneja *et al.*, 1997; Keriell *et al.*, 2002; Bour *et al.*, 2006, 2007). However, the process appeared to be complex and nothing is known about the upstream kinases that govern RAR phosphorylation in response to RA and how RAR phosphorylation impacts the transcription of target genes. Because p38MAPK is activated in response to RA (Alsayed *et al.*, 2001; Gianni *et al.*, 2002, 2006), we explored whether there is a connection among this kinase signalling pathway, RAR α phosphorylation and the induction of RAR α target genes.

Results

RA activates p38MAPK leading to MSK1 activation

In mouse embryonic fibroblasts (MEFs), p38MAPK α was activated rapidly and transiently following RA treatment. This activation started within minutes, peaked at 15 min and was reversed by 30–60 min (Figure 1A). p38MAPK was similarly activated by a synthetic RAR α agonist but not by RAR γ and RAR β agonists nor by RAR α antagonists (Figure 1A). p38MAPK activation was not observed in MEF knockout for the three RARs, MEF (RAR α , β , γ)^{−/−}, but was restored upon reexpression of RAR α WT (Figure 1B), indicating that activation of p38MAPK by RA requires RAR α . p38MAPK was also rapidly activated in human breast cancer cell lines, such as MCF7 cells (Figure 1C). In contrast, though present, p38MAPK was not activated in SKBR3 cells (Figure 1C), which overexpress the ERBB2/HER2 oncogene with a downstream hyperactivity of the PI3K/Akt pathway (Liao and Hung, 2003; She *et al.*, 2008).

Downstream of p38MAPK, there is MSK1 (Deak *et al.*, 1998), which contributes to the regulation of the expression of several genes (Chow and Davis, 2006; Vicent *et al.*, 2006). After RA treatment of MCF7 cells and MEFs, there was an increase in the activated, phosphorylated form of MSK1 that

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Received: 10 August 2008; accepted: 11 November 2008; published online: 11 December 2008

Figure 1 *In vivo*, RA activates the p38MAPK/MSK1 pathway. (A) Analysis of p38MAPK α activation (phospho p38MAPK ELISA) in MEF WT treated with RA (10^{-7} M), synthetic retinoids specific for RAR α (BMS 753, 10^{-7} M), RAR γ (BMS 961, 10^{-7} M), RAR β (BMS 493, 10^{-7} M) or a RAR α antagonist (BMS 614, 10^{-6} M). The results are an average $\pm$ s.d. of three experiments. (B) Same experiments with MEFs WT, RAR (α, β, γ)-/- or reexpressing RAR α WT in a RAR (α, β, γ)-null background. Western blots show the expression of RAR α in the different cell lines (right panel). (C) p38MAPK activation in MCF7 and SKBR3 cells treated with RA (10^{-7} M). Western blots show the expression levels of p38MAPK (D) Immunoblotting analysis of active/phosphorylated p38MAPK and MSK1 in extracts from RA-treated MCF7 cells applied onto phospho-protein affinity columns. (E) Comparison of MSK1 activation/phosphorylation in RA-treated MEFs, MCF7 and SKBR3 cells by immunoblotting. (F) Knockdown of p38MAPK α with siRNAs abrogates the activation of MSK1 in RA-treated MEFs.

paralleled the increase in phosphorylated p38MAPK (Figure 1D and E). Activation of MSK1 was inhibited upon knockdown of p38MAPK α with specific short interfering RNA (siRNA) (Figure 1F) or by SB203580, a selective inhibitor of p38MAPK α (data not shown), indicating that activation of MSK1 occurs downstream of p38MAPK α in RA signalling (Cuenda and Rousseau, 2007). Accordingly, MSK1 was not activated in SKBR3 cells, which are defective in RA induction of p38MAPK (Figure 1E). Other signalling pathways that have been shown to participate in hormone signalling such as Erks and the cAMP/PKA pathways were not activated after RA treatment (data not shown).

RA-activated MSK1 phosphorylates RAR α at S369, *in vivo*

Then we wondered whether the rapid and transient RA activation of the p38MAPK/MSK1 pathway was associated with a change in the phosphorylation status of RAR α . The RAR α protein can be phosphorylated at two serine residues, S77 and S369 located in the N- and C-terminal domains, respectively (Rochette-Egly, 2003) (Figure 2A). S77 is phosphorylated by the CAK subcomplex of the general transcription factor TFIIF either *in vitro* (Figure 2B) or *in vivo* (Rochette-Egly *et al*, 1997; Keriell *et al*, 2002). However the kinase that phosphorylates *in vivo* S369, in response to RA, has not yet been identified. S369 belongs to an Arg-Lys-rich

motif, which corresponds to a consensus phosphorylation site for several kinases including MSK1 (Figure 2A). In *in vitro* phosphorylation experiments, active recombinant MSK1 phosphorylated purified bacterially expressed RAR α at S369 (and not at S77), as assessed by immunoblotting with antibodies recognizing specifically RAR α phosphorylated at these residues. (Figure 2B).

We also analysed the *in vivo* phosphorylation of endogenous RAR α in MCF7 cells, after phosphoprotein affinity purification. The amount of phosphorylated RAR α markedly increased 5–30 min after RA treatment and decreased at 60 min (Figure 2C), in parallel to the activation of p38MAPK/MSK1 (see Figure 1D). This increase in RAR α phosphorylation concerned S369 (Figure 2D). It was inhibited upon knockdown of MSK1 (Figure 2E, left panel) or p38MAPK α , the upstream kinase (Figure 2E, right panel) or by SB203580, a selective inhibitor of p38MAPK (Supplementary Figure 1A). Finally, it did not occur in SKBR3 cells, which are defective in MSK1 activation (Figure 2D), highlighting the importance of the initial RA activation of MSK1 in RAR α phosphorylation at S369.

In vivo, S369 phosphorylation directs RAR α interaction with TFIIF and thereby S77 phosphorylation

RAR α was also rapidly phosphorylated at S77 after RA addition (Figure 2D). Unexpectedly, though S77 was not a

Figure 2 RA-activated MSK1 phosphorylates RAR α at S369. (A) Schematic representation of the RAR α 1 protein with the functional domains and phosphorylation sites. (B) *In vitro* phosphorylation of purified bacterially expressed RAR α with highly purified CAK and active recombinant MSK1 was analysed by immunoblotting with antibodies recognizing RAR α phosphorylated at S369 or S77. (C) Kinetics of RAR α phosphorylation in RA-treated MCF7 cells after phosphoprotein affinity purification. (D) Immunoblots showing, after phosphoprotein affinity purification, that RA induces RAR α phosphorylation at S369 and S77 in MCF7 cells but not in SKBR3 cells. (E) In MCF7 cells, knockdown of MSK1 or p38MAPK α abrogates the phosphorylation of RAR α induced by RA at 5 min.

target for MSK1, phosphorylation of this residue was inhibited by siRNAs against MSK1 or p38MAPK (Figure 2E). Therefore, we wondered whether phosphorylation of S77 might be controlled by that of S369.

MEF expressing RAR α WT, S77A or S369A, as the sole RA-dependent transcriptional activator in a (RAR α , β , γ)^{-/-} background were generated and compared for RAR α phosphorylation at 5-min intervals following RA addition. In each rescue cell line, MEF (RAR α WT), MEF (RAR α S369A) and MEF (RAR α S77A), the RAR α protein was expressed at similar amounts (Figure 3A) and the p38MAPK/MSK1 pathway was similarly activated in response to RA (Supplementary Figure 1C). In RA-treated MEF (RAR α WT), the amount of RAR α phosphorylated at S369 and S77 also increased rapidly at 5–15 min (Figure 3B, lanes 1–4), and was abrogated upon MSK1 knockdown (data not shown). In MEF (RAR α S369A)

(Figure 3B, lanes 5–8) and MEF (RAR α S77A) (Figure 3B, lanes 9–12), no signal was obtained by immunoblotting with the antibodies recognizing specifically RAR α phosphorylated at S369 and S77, respectively, validating their specificity. In MEF (RAR α S77A), the ability of RAR α to be phosphorylated at S369 was not affected. In contrast, in MEF (RAR α S369A), RAR α was not phosphorylated at S77. This indicates that phosphorylation of S369 controls that of S77 and not vice versa.

S369 is located in the LBD, in loop 9–10 in close proximity to the binding domain of cyclin H (Loop 8–9 and the beginning of H9) (Bour *et al*, 2005a), which forms with cdk7 and MAT1 the CAK subcomplex of TFIIF that phosphorylates RAR α at S77 (Rochette-Egly *et al*, 1997; Keriell *et al*, 2002). In an earlier study (Gaillard *et al*, 2006), we demonstrated that, *in vitro*, prior phosphorylation of RAR α at S369 enhanced the ability of the cdk7/cyclin H complex to

Figure 3 RAR α phosphorylation at S369 induces interaction with and phosphorylation by CAK within TFIH. (A) Immunoblots showing the reexpression of RAR α WT, S369A and S77A in MEF (RAR α , β , γ)^{-/-}. (B) RAR α phosphorylation in extracts from MEF (RAR α WT), (RAR α S369A) and (RAR α S77A) after phosphoprotein affinity purification. (C) *In vitro* phosphorylation by MSK1 at S369 increases the ability of bacterially expressed RAR α to be subsequently phosphorylated at S77 by CAK. (D) ChIP western experiments performed with MEF (RAR α WT), MEF (RAR α S77A) and MEF (RAR α S369), treated with RA for the indicated times. The complexes immunoprecipitated with RAR α antibodies were analysed by immunoblotting as indicated.

phosphorylate S77. This observation has been confirmed herein upon prior phosphorylation of RAR α by MSK1 (Figure 3C). Therefore, we hypothesized that, *in vivo*, the RA-induced phosphorylation of S369 would be the signal allowing the interaction of RAR α with TFIH and subsequently phosphorylation of S77 by cdk7.

With that aim, RAR α phosphorylation and interaction with TFIH were analysed in ChIP western experiments performed with the MEF (RAR α WT), MEF (RAR α S369A) or MEF (RAR α S77A) rescue lines, at 5-min intervals following RA

addition (Figure 3D). In the absence of RA, immunoprecipitated RAR α WT was not phosphorylated and did not retain TFIH (lane 1). As soon as 5 min following RA addition, RAR α WT became phosphorylated at S369. Then, at 10 min, RAR α interacted with TFIH and became phosphorylated at S77. RAR α phosphorylation and interaction with TFIH were maintained up to 50 min and then decreased at 60 min. RAR α S77A retained the ability to be phosphorylated at S369 and to recruit TFIH. In contrast, RAR α S369A was unable to interact with TFIH and therefore was not phosphorylated at

Figure 4 MSK1 is required for the RA-induced activation of the *Cyp26A1* gene. (A) The RA-induced expression of the *Cyp26A1* gene, which is abolished in MEF (RAR α, β, γ) $^{-/-}$, is rescued upon reexpression of RAR α WT, but not of RAR α S369A or S77A, as monitored by qRT-PCR. (B) The *Cyp26A1* gene is activated in MCF7 but not in SKBR3 cells in response to RA, in qRT-PCR experiments. (C, D) Knockdown of MSK1 or p38MAPK α inhibits the RA-induced expression of *Cyp26A1* in MEFs and MCF7 cells.

S77. Similar results were obtained in immunoprecipitation experiments performed with cyclin H antibodies (Supplementary Figure 1D). Collectively, these results indicate that, *in vivo*, phosphorylation of S369 promotes TFIIF binding and thereby S77 phosphorylation, confirming our earlier *in vitro* studies (Gaillard *et al*, 2006).

The integrity of the p38MAPK/MSK1 pathway and of the RAR α phosphorylation sites is required for the RA induction of RAR α target genes

Next, we asked whether activation of MSK1 is significant for the activation of RAR α target genes. In MEFs and MCF7 cells, RA treatment enhances transcription of the *Cyp26A1* gene, which is the paradigm of the RA target genes, as assessed by quantitative RT (qRT)-PCR (Figure 4A and B). *Cyp26A1* markedly decreased upon knockdown of MSK1 or p38MAPK α (Figure 4C and D) or in the presence of SB203580 (Supplementary Figure 1B). *Cyp26A1* was not activated in SKBR3 cells, which are refractory to p38MAPK/MSK1 activation (Figure 4B), highlighting the significance of MSK1 activation for RA induction of RAR α target genes.

The RA-induced activation of CYP26A1 was also abrogated in MEFs in which all RARs have been deleted, but was re-established upon reintroduction of RAR α WT (Figure 4A). However, the RA response was not restored upon reintroduction of the RAR α S369A and RAR α S77A mutants (Figure 4A), indicating that phosphorylation of S77 and S369 is also required for RAR α -mediated transcription.

Upon RA treatment, RAR α is recruited with TFIIF to the promoter of the *Cyp26A1* gene

Next, we used chromatin immunoprecipitation (ChIP) experiments to gain mechanistic insight into the role of MSK1 activation and/or RAR α phosphorylation in the activation of *Cyp26A1* expression. The *CYP26A1* gene promoter contains two functional DR5 RAREs, a proximal one (R1) and a more distal one (R2) (Figure 5A), which work synergistically *in vivo*, to provide a maximal response to RA (Loudig *et al*,

2005; Pozzi *et al*, 2006). We assessed the RAR α occupancy of these promoter regions in ChIP experiments performed at 5-min intervals, up to 1 h, after RA addition to MEF (RAR α WT). The specificity of our experimental conditions was checked in the absence of antibodies and with the promoter of the control 36B4 gene, which does not contain any RARE (Supplementary Figure 2A).

In the absence of RA, RAR α was hardly detected at the distal R2 region of *Cyp26A1* (Figure 5B, lane 1). However, some of the R1 elements were already occupied by RAR α (Figure 5C, lane 1). Then at 30 min after RA addition, there was an enrichment of RAR α bound at both R1 and R2, which peaked at 40–55 min, decreased by 60 min (Figure 5D and E) and came back close to the initial values at 2 h (Supplementary Figure 2B). No additional variations were detected between 2 and 16 h (Supplementary Figure 2B).

It is interesting to note that the corepressor SMRT was bound to R1 in the absence of RA, in line with the RAR α occupancy of some of these elements, and dissociated rapidly after RA addition (Figure 5E). However, SMRT could not be detected at R2 consistent with the absence of RAR α (Figure 5D).

As RAR α binds TFIIF after RA addition (see Figure 3D), we also examined the recruitment of TFIIF in ChIP experiments performed with antibodies against its cyclin H, cdk7 or XPB subunits. In the absence of RA, R2 was not occupied by the TFIIF subunits (Figure 5B, lane 4). In contrast, some of the proximal R1 regions, which encompass the DR5 R1, the TATA box and the transcription start site, were already occupied (Figure 5C, lane 4). In response to RA, the levels of R1 and R2 bound to TFIIF increased markedly and reached a peak at 40–50 min (Figure 5F and G), which was concomitant with that of RAR α .

The same results were obtained with MCF7 cells, RAR α and the XPB, cdk7 and cyclin H subunits of TFIIF being recruited to both the R1 and R2 RAREs of the *Cyp26A1* promoter (Figure 6B–G and data not shown) with a peak at 40–55 min following RA addition.

Figure 5 In response to RA, RAR α is recruited with TFIID to the *Cyp26A1* gene promoter. **(A)** Schematic representation of the promoter regions of the *Cyp26A1* gene, with the primer pairs used for qPCR amplification. **(B, C)** Chromatin immunoprecipitation (ChIP) experiments determining the basal (in the absence of RA) occupancy of some R1 regions (and not R2) by RAR α and the XPB subunit of TFIID, in the different rescue MEF lines. Values (% of the inputs) are the mean $\pm$ s.d. of triplicates performed on three separate chromatin preparations. Data were analysed using Microsoft Excel 2002 and pairwise compared by using unpaired Student's *t*-test. *Depicts a statistically significant difference in RAR α or TFIID recruitment versus the signals obtained in the absence of antibody ($P < 0.05$). **(D, E)** Kinetic ChIP experiments performed with RA-treated MEF (RAR α WT) and determining the recruitment of RAR α , RNA PolII and SMRT to the R1 and R2 promoter regions. Values correspond to a representative experiment among five. **(F, G)** Kinetic ChIP experiments determining the recruitment of the cdk7, cyclin H and XPB subunits of TFIID to R1 and R2. Values correspond to a representative experiment among five. **(H, I)** ChIP and reChIP experiments performed with the indicated antibodies, showing that, at 45 min following RA addition, DNA-bound RAR α is associated with TFIID and phosphorylated at S77. Lanes 1 and 4 correspond to ChIP and reChIP, respectively, performed with a control antibody. Results are an average of two experiments that agreed within 20%.

Then to investigate whether TFIIH and RAR α are present in the same complex on the promoter, MEF (RAR α WT) was subjected to sequential ChIP (reChIP) experiments, first with anti-RAR α and then with anti-TFIIH antibodies. Note that in the absence of RA, the R1 region, which was already occupied to some extent by TFIIH and RAR α , was not enriched, indicating that, at this stage, TFIIH is not associated with

RAR α (Figure 5I, lane 5). However, at 45 min following RA treatment, both the R1 and R2 regions were specifically enriched (Figure 5H and I, lanes 5), demonstrating that TFIIH and RAR α form a complex on DNA in a RA-dependent manner.

Finally, ChIP experiments were performed with antibodies recognizing specifically RAR α phosphorylated at S77, the

Figure 6 Activation of the p38MAPK/MSK1 pathway is required for the recruitment of RAR α and TFIIH to the *Cyp26A1* gene promoter. (A) Schematic representation of the *Cyp26A1* gene promoter. (B, C) Same as in Figure 5B and C with ChIPs determining the basal (in the absence of RA) occupancy of some R1 elements (but not R2) by RAR α and the XPB subunit of TFIIH, in MCF7 cells. (D–G) Kinetic ChIP experiments showing that RAR α and the XPB subunit of TFIIH are recruited to R2 and R1 in RA-treated MCF7 but not in SKBR3 cells. Values correspond to a representative experiment among three. (H, I) Knockdown of p38MAPK α in MCF7 cells abrogates the recruitment of RAR α and TFIIH to R1 and R2 observed 50 min after RA addition.

cdk7 site. At 45 min following RA addition, RAR α recruited to the R1 and R2 regions of the CYP26A1 promoter was phosphorylated at S77 (Figure 5H and I, lanes 6). Altogether, these results indicate that RAR α bound at the promoter is associated with TFIIF and phosphorylated at S77. Note that RNA PolII was also recruited to R1 and R2 in parallel to RAR α and TFIIF (Figure 5D and E).

The phosphorylation cascade initiated at S369 and ending at S77 controls the recruitment of both RAR α and TFIIF to the Cyp26A1 promoter

To investigate whether activation of the p38MAPK/MSK1 pathway is required for promoter recruitment of RAR α , ChIP experiments were performed with MEF (RAR α WT) and MCF7 cells transfected with siRNAs targeting specifically MSK1 or p38MAPK α . In both cases, the recruitment of RAR α at R1 and R2 was abrogated (Figures 6H and I, and 7B and C). The recruitment of TFIIF was also abrogated (Figures 6H and I, and 7D and E). Similar results were obtained upon treatment of the cells with the MSK1 inhibitor H89 or the p38MAPK inhibitor SB203580 (Figure 7F and G). Finally, in SKBR3 cells, which are defective in p38MAPK/MSK1 activation compared with MCF7 cells, RAR α and TFIIF recruitment to R2 and R1 remained low and unchanged, irrespective of the RA treatment duration (Figure 6D–G). Collectively, these results indicate that the activation of the p38MAPK/MSK1 kinase cascade is required for the recruitment of the RAR α –TFIIF complexes to the Cyp26A1 promoter.

Then we investigated whether phosphorylation of RAR α is also required by performing ChIP experiments with MEF (RAR α S369A) and MEF (RAR α S77A). As described above with MEF (RAR α WT), the two RAR α mutants and TFIIF were hardly detected at the distal R2 region of Cyp26A1 (Figure 5B, lanes 2, 3, 5 and 6), whereas some of the R1 elements were already occupied (Figure 5C, lanes 2, 3, 5 and 6). However, after RA addition, neither the RAR α mutants nor TFIIF was recruited to R2 and R1 (Figure 7H–K). RNA PolII was not recruited either (data not shown). We have demonstrated above that RAR α S369A cannot be phosphorylated at S77 due to its inability to interact with TFIIF, whereas RAR α S77A has retained the ability to be phosphorylated at S369 and to interact with TFIIF (see Figure 3D). Thus, one can conclude that the last step of the phosphorylation cascade, that is, S77 phosphorylation is required for the RA-induced promoter recruitment of RAR α –TFIIF complexes.

Phosphorylated RAR α controls the formation of a bridge between the R1 and R2 regions of the Cyp26A1 promoter

According to several recent studies, DNA looping may juxtapose promoter DNA with enhancer elements that lie far upstream (Resendes and Rosmarin, 2006). Given that, in response to RA, RAR α is recruited to both the R1 and R2 regions of the Cyp26A1 promoter, in association with TFIIF and RNA PolII, we raised the hypothesis that RAR α would bridge R1 and R2 to form an enhanceosome. To demonstrate the existence of such a gene looping, we performed ChIP loop assays (Simonis *et al*, 2007), which combine chromosome conformation capture (3C) with ChIP (Figure 8A).

In MEF (RAR α WT) RA-treated for 50 min, PCR amplification of non-digested immunoprecipitated material with pri-

Figure 7 MSK1 activation and RAR α phosphorylation are required for the recruitment of RAR α and TFIIF to the Cyp26A1 gene promoter. (A) Schematic representation of the Cyp26A1 gene promoter. (B–E) Kinetic ChIP experiments showing that the recruitment of RAR α or TFIIF to R1 and R2 is abrogated upon MSK1 knockdown in MEF (RAR α WT). (F, G) Inhibition of p38MAPK or MSK1 with SB203580 and H89, respectively, in MEF (RAR α WT), also abrogates the recruitment of RAR α and TFIIF to R1 and R2. (H–K) Kinetic ChIP experiments determining the recruitment of RAR α and the XPB subunit of TFIIF at R1 and R2 in MEF (RAR α WT), (RAR α S369A) and (RAR α S77A).

mers corresponding to R1 and R2 (Figure 8A, panel 2) produced a 1930-bp fragment (Figure 8B, upper panel, lane 2). Upon digestion with PvuII, which cuts twice between R1 and R2 (Figure 8A, panel 4), a new 1300-bp band was generated (Figure 8B, upper panel, lanes 8–15), which

represents loss of a 630-bp fragment and religation of the R1 and R2 regions that remained in close proximity due to bridging complexes. Religation was confirmed by PCR amplification with primers on both sides of the junction that generated a 360-bp fragment instead of a 1000 bp one (Figure 8B, lower panel). The 1300- and 360-bp fragments were detected at 30 min up to 60–75 min following RA addition (Figure 8B and C), when RAR α was recruited to both R1 and R2 in ChIP experiments (see Figure 5). Similar results were obtained when immunoprecipitation was performed with TFIIH or RNA PolII antibodies (Figure 8C, lanes 1–9). Collectively, these results indicate that the R1 and R2 regions

become juxtaposed in response to RA, through the recruitment of complexes involving RAR α as well as TFIIH and RNA PolII.

In contrast, in MEF (RAR α S77A) (Figure 8B, lanes 16–28 and Figure 8C, lanes 10–18), as well as in MEF (RAR α S369A) (data not shown), only a transient and delayed bridge was detected when ChIP loop experiments were performed with RAR α or TFIIH antibodies, indicating that efficient looping relies on the promoter recruitment of the phosphorylated form of RAR α . Note that no bridging occurred when immunoprecipitation was performed with RNA PolII antibodies.

Figure 8 ChIP loop assay showing that RAR α bridges the R1 and R2 regions of the *Cyp26A1* promoter. **(A)** Schematic representation of the *Cyp26A1* gene promoter with R1 and R2, the *PvuII* restriction sites and the primers used to amplify the whole promoter (primers 1 and 2) or the junction (primers 3 and 4). **(B)** ChIP loop assay performed with MEF (RAR α WT) and MEF (RAR α S77A) showing that RAR α phosphorylated at S77 controls the formation of a bridge between R1 and R2. Chromatin from control and RA-treated cells was immunoprecipitated with RAR α antibodies without or with prior *PvuII* digestion–ligation of DNA, as indicated. Then qPCR was performed with primer pairs 1 and 2 (upper panels) or 3 and 4 (lower panels). **(C)** Same as in (B) with RAR α , TFIIH and RNA PolII antibodies.

Activated MSK1 is also recruited to the *Cyp26A1* promoter and phosphorylates histones H3 at S10

Next, as MSK1 has been shown to have an essential function in preparing promoter chromatin for gene activation, we investigated whether MSK1 is recruited to the *Cyp26A1* promoter after RA addition and whether it phosphorylates histones H3 at S10 (H3S10p), a hallmark of gene transcription activation (Vicent *et al*, 2006). ChIP assays performed with antibodies specific for phosphorylated MSK1 showed that, after RA addition to MEF (RAR α WT), the activated kinase is recruited to the R1 and R2 regions (Figure 9B and C) with a peak at 45 min. H3 was phosphorylated at S10 in the same time slot as assessed in ChIPs with antibodies against H3S10p (Figure 9B and C). Histones H4 were also acetylated at K5, K8 and K12 (Figure 9B and C). Note that in the context of the R1 region, an additional peak of H4 acetylation was observed at 10–15 min, probably reflecting the recruitment of histone acetylases by RAR α already bound to some of these regions. Knockdown of MSK1 reduced significantly the accumulation of active MSK1 and H3S10 phosphorylation on both R1 and R2 (Figure 9D and E), confirming that the RA-induced phosphorylation of H3 is executed by MSK1.

Interestingly, the recruitment of MSK1 was concomitant with that of RAR α . Coimmunoprecipitation experiments were performed to investigate whether MSK1 was associated with RAR α (Figure 9F). MSK1 interacted rapidly (within minutes) with RAR α in response to RA (lane 5). However, at 50 min following RA treatment, there was no more interaction, suggesting that chromatin-bound MSK1 was not associated with RAR α (lane 7). Collectively, these results suggest an additional role for MSK1 in the activation of RAR α target promoters through histone phosphorylation.

Activated MSK1 and RAR α phosphorylation also control the RA induction of other target gene promoters such as the *RAR β 2* gene

In MEFs, other well-known RA target genes such as the *RAR β 2* gene are also inhibited upon knockdown of MSK1 or knockout of the three RARs. The RA induction of *RAR β 2* was reestablished upon reexpression of RAR α WT, but not of RAR α S77A or RAR α S369A (Supplementary Figure 3A). The *RAR β 2* gene promoter contains only one DR5 RARE at position –60 (de The *et al*, 1990) (Supplementary Figure 3B) and ChIP experiments (Supplementary Figure 3C–J) gave results

Figure 9 MSK1 is recruited to the *Cyp26A1* promoter and phosphorylates histones. (A) Schematic representation of the *Cyp26A1* gene promoter. (B, C) Kinetic ChIP experiments performed with MEF (RAR α WT) showing that after RA addition, P-MSK1 is recruited to R1 and R2 and that H3S10 become phosphorylated and H4K5; 8; 12 acetylated. (D, E) Knockdown of MSK1 with siRNAs abrogates H3S10 phosphorylation. (F) Coimmunoprecipitation experiments performed with MEF (RAR α WT) and showing that when recruited to the *Cyp26A1* promoter, MSK1 is no more associated with RAR α .

that were very similar to those obtained for the proximal R1 region of the Cyp26A1 promoter. Indeed, prior to RA treatment, RAR α , TFIIH (panel C, lanes 1 and 4) and the corepressor SMRT (panel D) already occupied some promoters. After RA addition, the corepressor SMRT dissociated rapidly but the levels of DR5 region bound to RAR α , TFIIH and RNA PolII increased markedly with a peak at 45–55 min (panels D and E). After RA treatment, DNA-recruited RAR α was associated with TFIIH and was phosphorylated at S77 (panel F). The recruitment of RAR α and TFIIH was abrogated upon mutation of the RAR α phosphorylation sites (S369 and S77) (panels G and H), or upon knockdown of MSK1 (panel J). Finally, phosphorylated MSK1 was also recruited to the promoter, which became phosphorylated at H3S10 and acetylated at H4K5; 8; 12 (panel I). These results confirm that MSK1 is the upstream signal promoting phosphorylation cascades targeting RAR α and subsequently the recruitment of RAR α –TFIIH complexes to promoters.

Discussion

In this study, we demonstrated that the activation of the p38MAPK pathway by RA (Alsayed *et al*, 2001; Gianni *et al*, 2006) is required for the induction of RAR α target genes. We outlined a new paradigm in which, *in vivo*, RA induces a phosphorylation cascade, starting with the rapid activation of p38MAPK, leading to MSK1 activation, phosphorylation of RAR α and finally to promoter recruitment of RAR α , TFIIH and the transcription machinery (Figure 10). To our knowledge, this is the first report of a coordinated phosphorylation cascade induced by RA and coupled to promoter activation. It was unexpected and shed light on a new level of complexity in the fine-tuning of RAR α -mediated transcription.

The mechanism of activation of p38MAPK/MSK1 is out of scope of this study, but involves very likely a non-genomic activation event (Masia *et al*, 2007), similar to that described for steroid receptors (Castoria *et al*, 2001; Vicent *et al*, 2006) and requiring RAR α . One of the targets of MSK1 is RAR α , which becomes rapidly phosphorylated at S369 located in the LBD, and we found that phosphorylation of this residue is critical for the binding of TFIIH and the subsequent phosphorylation of the NTD at S77 by cdk7. Given that S369 is located in close proximity of the cyclin H-binding domain (Bour *et al*, 2005a), we propose that, *in vivo*, phosphorylation of this residue by MSK1 propagates a signal to the cyclin H-binding surface, allowing the recruitment of CAK within TFIIH, in line with our previous *in vitro* results (Gaillard *et al*, 2006). Ultimately, the consequence of the last step of this cascade, that is, S77 phosphorylation, is to anchor RAR α –TFIIH complexes to target promoters. To gain insight into such a model, additional investigations are required to determine how S77 phosphorylation affects the binding of RAR α to its response elements. Experiments are in progress to address whether S77 phosphorylation modifies the dynamic properties of the nearby DBD (Pufall *et al*, 2005) and/or induces the dissociation of inhibitory proteins.

Interestingly, in the context of a promoter with two RAREs, a proximal and a more distal one, as exemplified with the Cyp26A1 promoter, phosphorylated RAR α bridges both RAREs, in association with TFIIH and RNA PolII. Whether and how other chromatin remodelling and/or transcription

Figure 10 Recapitulation of the phosphorylation cascade induced by RA. In response to RA, p38MAPK and the downstream protein kinase MSK1 are activated. MSK1 interacts with RAR α and phosphorylates its LBD at S369, subsequently allowing the docking of cyclin H within TFIIH and the formation of a RAR α –TFIIH complex. Then the cdk7 subunit of TFIIH phosphorylates the NTD of RAR α at S77. Finally, RAR α phosphorylated and associated with TFIIH is recruited to response elements located in the promoter of target genes. MSK1 is also recruited and phosphorylates H3S10.

factors are also involved to form an enhanceosome (Resendes and Rosmarin, 2006) awaits further studies.

An unexpected finding in this study is that RAR α –TFIIH complexes started to be recruited to target promoters only at 30 min after RA addition, whereas RAR α phosphorylation and interaction with TFIIH occurred as soon as 5 min. Such a discrepancy suggests that TFIIH interacts with RAR α out of the promoters that are not accessible yet, and that other RA-dependent events such as chromatin modifications and remodelling are required. Which histone modifications are induced by RA during the first 30 min is out of the scope of the present study, but histones H4 become acetylated (Figure 9). Whatever be the case, several events have to be initiated and coordinated according to the ‘histone code’ so that they serve as a ‘transcriptional time clock’ to make the response elements available for RAR α recruitment.

Note however that some elements such as the R1 element of the Cyp26A1 promoter or the DR5 of the RAR β 2 promoter can be already occupied by RAR α and TFIIH in the absence of RA, indicating that, in some cells, they would be accessible. However, RAR α was not associated with TFIIH, suggesting

that this occupancy would rely on a different mechanism from that observed in response to RA.

Also unexpected, is that the recruitment of RAR α -TFIIH complexes was transient and occurred in a narrow time slot (from 30 to 60–75 min after RA addition). This might be the basis of the absence of variations in the RAR α occupancy of the RAR β 2 promoter observed by others (Pavri *et al*, 2005; Flajollet *et al*, 2006). The RA-induced phosphorylation cascade targeting RAR α was also transient, suggesting the existence of phosphatases that would antagonize the functions of chromatin-tethered kinases (Chow and Davis, 2006), MSK1 and cdk7 within TFIIH.

The other novelty of this study is that MSK1 is recruited to RAR α target promoters in the active phosphorylated form, leading to increased phosphorylation of S10 on H3. A similar mechanism was recently reported for the regulation of other nuclear receptor target genes (Vicent *et al*, 2006). Though activated rapidly, MSK1 was recruited to target promoters only at 30 min after RA addition, concomitantly with RAR α . However, chromatin-bound MSK1 was not associated with RAR α . How MSK1 is recruited to RA target promoters will require further investigations but again, RA-dependent chromatin modifications might be required for MSK1 recruitment in agreement with a 'transcriptional time clock'. Then the question is how MSK1-mediated phosphorylation of H3 contributes to RAR α target gene induction. One possibility is that phosphorylated H3S10 is a chromatin mark, which in cooperation with other histone modifications, accounts for the dissociation of repressive complexes and/or the recruitment of chromatin-remodelling complexes (Vicent *et al*, 2006). In conclusion, the present study highlighted the importance of MSK1 in the RA response. Whether the closely related MSK2 is also activated through the same pathway is not excluded, but will require further investigations.

Finally, the present study demonstrated that in ERBB2-positive human breast cancer SKBR3 cells, the p38MAPK/MSK1 pathway is deregulated, compared with the control MCF7 cells. Consequently, the phosphorylation cascade and RAR α -mediated transcription are significantly blunted. Whether the deregulation of the p38MAPK/MSK1 pathway reflects the overexpression of the upstream ERBB2/HER2 oncogene (Tari *et al*, 2002; Neri *et al*, 2003) awaits further studies. Nevertheless, it raises the issue of a contribution of the p38MAPK/MSK1 pathway (Liao and Hung, 2003) and of RAR α phosphorylation in the RA resistance of ERB2/HER2-positive tumours. In this context, it is interesting to note that MEF (RAR α S369A) and MEF (RAR α S77A) grow faster than the RAR α WT counterpart (our unpublished observations). Therefore, specific modulation of the p38MAPK/MSK1 pathway and of RAR α phosphorylation might be a promising therapeutic strategy in the treatment of RA-resistant cancers. Given that the S369 phosphorylation site is shared by MSK1 and PKA, our data are also particularly attractive in terms of the synergy between RA and cAMP in the treatment of many leukaemia cells (Vitoux *et al*, 2007).

Materials and methods

Materials

RA was from Sigma Aldrich Corporation (USA). The synthetic retinoid agonists and antagonists were as described (Taneja *et al*, 1997). Rabbit polyclonal antibodies against RAR α , RP α (F), and

mouse monoclonal antibodies recognizing RAR α phosphorylated at S77 or S369 were described earlier (Bour *et al*, 2005a; Gaillard *et al*, 2006). Monoclonal antibodies against RNA PolII phosphorylated at S5 in the carboxyl terminal repeat domain, cyclin H and acetylated histones (H4K5; 12 and H4K5; 8; 12) were produced by the IGBMC facility. Antibodies against MSK1 and β -actin (C11) were purchased from Santa Cruz Biotechnology as well as polyclonal antibodies against RAR α (C-20), SMRT (H-300) and the XPB (S-19) and cdk7 (C-19) subunits of TFIIH used in the ChIP experiments. Antibodies against P-MSK1, P-p38MAPK and p38MAPK were from Cell Signaling. Those against H3S10p were from Upstate Biotechnology (Millipore). SB203580 was from Promega and H89 from VWR International SAS.

Cell lines and immunoblotting

MEFs with all three RARs deleted, MEF (RAR α , β , γ)^{-/-}, were as described (Chapellier *et al*, 2002; Altucci *et al*, 2005). RAR α WT, S369A or S77A cDNAs subcloned from the pSG5 constructs (Gaillard *et al*, 2006) into pMSCV neo plasmid were reintroduced into these cells by retroviral transfer followed by G418 selection. MCF7 and SKBR3 human breast cancer cells were cultured under standard conditions. When 80–90% confluent, cells were treated with RA (10⁻⁷ M) after 24 h in a medium containing 1% dextran-charcoal-treated fetal calf serum. Extracts were prepared and immunoblotted as described (Bour *et al*, 2005b). Immunoprecipitations were performed in 20 mM Tris-HCl pH 7.8 containing 100–500 mM KCl, 10% glycerol and 0.1 mM EDTA and bound proteins were analysed by immunoblotting (Bour *et al*, 2005b).

RNA isolation and qRT-PCR

Total RNAs were isolated and subjected to qRT-PCR as described (Bour *et al*, 2005b). Primer sequences for mouse and human 36B4 and for human Cyp26A1 were described earlier (Bour *et al*, 2005b; Gaillard *et al*, 2006). Those for mouse Cyp26A1 are as follows: 5'-GGGCTTACTTTGCAAGAGCA-3' and 5'-GAAGGCCTCTCCAAA TGCA-3'.

ChIPs, reChIPs and ChIP western experiments

Subconfluent cells were treated with RA (10⁻⁷ M) and ChIP experiments were performed according to the protocol described by Upstate Biotechnology. Control ChIP was performed without antibody. In reChIP experiments, complexes were eluted by incubation for 30 min at 37°C in 250 μ l 95 mM NaHCO₃ containing 1% SDS, diluted 20 times with reChIP buffer (1% Triton X-100, 50 mM Tris-HCl, (pH 7.5), 2 mM EDTA and 150 mM NaCl) and subjected again to the ChIP procedure. Immunoprecipitated DNA was amplified by qPCR using primer pairs designed using the Primer3 software (Rozen and Skaletsky, 2000): Mouse Cyp26A1: R2, 5'-AAACAGGAGCAGGCTGAAC-3' and 5'-CGCTGCCACTGTCA TATCT-3'; R1, 5'-GGTAACTCGGAGCTCTGCAC-3' and 5'-CCAGGT TACTGCCACGTTA-3'; human Cyp26A1: R1, 5'-GCGGAACAAACG GTTAAAGA-3' and 5'-GCAGTACAGTCCCAGAGCTT-3'; R2, 5'-GA GTTCACTCGATGTCACG-3' and 5'-ATCGCGTGGAGGTAATCT-3'. Primer amplification specificity and efficiency were verified on DNA serial dilutions. Occupancy of the promoters was calculated by normalizing the PCR signals from the IP samples to the signals obtained from the input DNA.

For ChIP western experiments, the precipitated chromatin complexes were proceeded as described (Das *et al*, 2004) and bound proteins were revealed by immunoblotting.

ChIP loop assay

Cells crosslinked with formaldehyde were digested with *Pvu*II (New England Biolabs) (600 U, overnight, 37°C), religated (4 h at 16°C using T4 DNA ligase) as described in Resendes and Rosmarin (2006) and immunoprecipitated with RAR α , TFIIH (XPB subunit) or RNA PolII antibodies. Controls were performed without antibody. Ligation frequencies of restriction fragments were analysed by qPCR with the following primer pairs. Primers 1 and 2 (R1 and R2), 5'-AGGCTCACGATCGATCGGC-3' and 5'-ACGCAGTACAGGCCCA GAG-3'; primers 3 and 4 (junction), 5'-TATTGCAAGGGTTACCG GAC-3' and 5'-GTAGTAATACGCCCAAGCCG-3'.

In vitro RAR α phosphorylation and detection of in vivo phosphorylated RAR α and p38MAPK

In vitro phosphorylation of purified recombinant RAR α expressed in *Escherichia coli* by the purified CAK complex (Bour *et al*, 2005a) or

recombinant active MSK1 (Upstate Biotechnology) was performed as described earlier (Keriel *et al*, 2002) followed by immunoblotting with antibodies recognizing RAR α or RAR α phosphorylated at S77 or S369.

For detection of *in vivo* phosphorylated RAR α , cell extracts were prepared and applied to phosphoprotein affinity purification columns (PhosphoProtein Purification System; Qiagen SA) according to the manufacturer-supplied instructions. After washing, column eluates containing protein peaks were concentrated and analysed by immunoblotting. Phosphorylated p38^{MAPK} was detected by using a Phospho p38^{MAPK} (Thr (P)-180/Tyr (P)-182) ELISA kit (Biosource Invitrogen Corporation).

siRNA

The smartpool siRNA against human MSK1 (M-004665-01), mouse MSK1 (L-040751-00), mouse p38MAPK α (M-040125-00) or human p38MAPK α (L-003512-00) were purchased from Dharmacon (Lafayette, CO) as well as the control non-targeting siRNA pool (D-001206-13). siRNAs (50 nM) were transfected by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Then cells were vehicle- or RA-treated 48 h post-transfection.

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We are grateful to JL Plassat for help in qRT-PCR. We also thank members of the cell culture facilities. Special thanks to P Chambon and N Ghyselinck for RAR (α , β , γ) null MEFs. We warmly thank F Coin, N Le-May and D Motta, from JM Egly team for helpful suggestions and comments on the paper. Many thanks also to I Davidson, L Tora, G Bour, S Lalevee and V Duong. This study was supported by funds from CNRS, INSERM, the Agence Nationale pour la Recherche (no. ANR-05-BLAN-0390-02) and the Institut National du Cancer (INCa-PL06-095). The teams of CR-E and HdeT were supported in partnership by the Association pour la Recherche sur le Cancer (ARC A05/2/3139) and INCa (PL07-96099). NB was supported by the Ligue Nationale Contre le Cancer and the ARC. CF was supported by the Ministère de la Recherche et de l'Enseignement Supérieur. VD was supported by the ARC.

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