P38MAPK-dependent phosphorylation and degradation of SRC-3/AIB1 and RAR α -mediated transcription

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Nuclear retinoic acid (RA) receptors (RARs) activate gene expression through dynamic interactions with coregulators in coordination with the ligand and phosphorylation processes. Here we show that during RA-dependent activation of the RAR α isotype, the p160 coactivator pCIP/ACTR/AIB-1/RAC-3/TRAM-1/SRC-3 is phosphorylated by p38MAPK. SRC-3 phosphorylation has been correlated to an initial facilitation of RAR α -target genes activation, via the control of the dynamics of the interactions of the coactivator with RAR α . Then, phosphorylation inhibits transcription via promoting the degradation of SRC-3. In line with this, inhibition of p38MAPK markedly enhances RAR α -mediated transcription and RA-dependent induction of cell differentiation. SRC-3 phosphorylation and degradation occur only within the context of RAR α complexes, suggesting that the RAR isotype defines a phosphorylation code through dictating the accessibility of the coactivator to p38MAPK. We propose a model in which RAR α transcriptional activity is regulated by SRC-3 through coordinated events that are fine-tuned by RA and p38MAPK.

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Introduction

Retinoic acid (RA) influences the proliferation, differentiation and apoptosis of a variety of cell types through modifications in the expression of subsets of RA-target genes. The RA response is mediated by two classes of nuclear receptors, the RA receptors (RARs) (α , β and γ) and the RXRs (α , β and γ), which function as ligand-dependent heterodimeric RAR/

RXR transcription activators (Laudet and Gronemeyer, 2001; Lefebvre *et al.*, 2005).

The basic mechanism of RARs transcriptional activity relies on their ability to recruit coactivators after RA-induced conformational changes of the ligand-binding domain (LBD) (Chambon, 1996). Among the large number of coactivators that have been identified, the steroid receptor coactivator SRC/p160 family (SRC-1/NCoA1; SRC-2/TIF2/GRIP-1; SRC-3/pCIP/RAC3/ACTR/AIB-1/TRAM-1) and p300/CBP stand out. Their physiological importance in RAR activity has been substantiated through gene deletion studies in mice (Xu and Li, 2003). P160 coactivators interact with RARs through a central conserved domain (NID) (Figure 1B) with three LXXLL motifs (Chen, 2000; McKenna and O'Malley, 2002). They are endowed with intrinsic acetyltransferase activity and contain two conserved C-terminal transcriptional activation domains, AD1 and AD2 (Figure 1B), involved in the recruitment of proteins with histone acetyltransferase (p300/CBP and p/CAF) and methyltransferase (CARM1 and PRMT1) activities. Functionally, recruitment of these proteins by SRCs is critical for RAR-directed chromatin remodeling and decondensation, and favors the generation of a transcriptionally permissive environment at the promoter. All these coregulators cycle on and off the promoter of target genes in a dynamic fashion and a sequential order (Metivier *et al.*, 2003; Dennis and O'Malley, 2005; Rochette-Egly, 2005).

A concept that has developed over the last years is that RARs (Rochette-Egly, 2003; Bastien and Rochette-Egly, 2004) and SRC coactivators (Wu *et al.*, 2005) are subjected to rapid modifications such as phosphorylations. RARs are phosphorylated in their N-terminal domain at one serine residue (S77 in RAR α 1 and S68 in RAR γ 2) by the cyclin H-dependent kinase cdk7 associated to the general transcription factor TFIIH (Figure 1A). In the particular case of the RAR γ isotype, an additional nearby residue (S66 in RAR γ 2) is phosphorylated by p38MAPK. Concerning SRCs, phosphorylation occurs in response to different stimuli and involves a wide range of kinases including MAPKs. It was proposed that phosphorylation regulates transcription through controlling the interactions of RARs with coactivators and/or other coregulators (Bour *et al.*, 2005b; Rochette-Egly, 2005; Yi *et al.*, 2005). Finally, it emerged recently that RARs and coactivators are targets for the ubiquitin–proteasome pathway (Rochette-Egly, 2005). However, there is no direct evidence demonstrating that any specific phosphorylation and/or degradation of coactivators occurs in response to RA, thereby regulating RAR-mediated transcription.

Consequently, we investigated whether coactivators are targets for kinases and/or the proteasome in response to RA. We focused on SRC-3 which contributes significantly to RAR α -dependent transcriptional activation (Brown *et al.*, 2003). We show that, in response to RA, SRC-3 is phosphorylated by p38MAPK and then degraded by the proteasome

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Figure 1 Schematic representation of (A) RAR α 1 and RAR γ 2 proteins with the phosphorylation sites and (B) SRC-3 with the known functional domains and the p38MAPK phosphorylation sites. The truncated mutants are also shown.

pathway. Phosphorylation of SRC-3 has a biphasic effect on RAR α -mediated transcription with facilitation followed by restriction. These results define a new critical role of SRC-3 in RAR α -mediated transcription through its phosphorylation by p38MAPK.

Results

RA induces proteasomal degradation of SRC-3

As SRC-3 is a target for the proteasome (Yan *et al*, 2003; Shao *et al*, 2004), we investigated whether SRC-3 could be degraded through this pathway in response to RA. COS-1 cells were cotransfected with a full-length tagged-SRC-3 (B10-SRC-3) vector, RAR α and a DR5 reporter gene. At 16 h, a significant decrease in the amount of SRC-3 was observed, and at 48 h no SRC-3 could be seen (Figure 2A, lanes 6 and 8). This decrease was prevented by the proteasome inhibitor MG132 (Figure 2B). As, under the same experimental conditions, SRC-3 transcripts were not affected (data not shown), these data indicate that the RA-induced decrease in SRC-3 results from a degradation process involving the proteasome. The degradation of SRC-3 occurred later than that of RAR α (Bastien and Rochette-Egly, 2004), which was detected as early as 2–4 h following RA addition (Figure 2A, lane 2). In contrast to what was observed for SRC-3, RAR α levels were not further affected up to 48 h and thus still significant.

SRC-3 contains three LXXLL motifs located in the NID that mediate direct binding to the LBD of RARs. In line with this, SRC-3 interacted with RAR α in response to RA, in a co-immunoprecipitation procedure (Klein *et al*, 2000), including a synthetic double-stranded oligonucleotide containing a DR5 retinoic acid response element (RARE) (Figure 2C, lanes 5 and 6). No interaction could be detected with a mutated RARE unable to bind the heterodimers (Figure 2C, lane 7). The SRC-3-AAA mutant that contains mutated LXXLL motifs (Zheng *et al*, 2005) was unable to bind RAR α (Figure 2C, lanes 3 and 4) and could not be degraded (Figure 2D),

indicating that a direct interaction with RAR α is necessary for RA-dependent degradation of SRC-3. In line with this, SRC-3 degradation occurred in the presence of overexpressed RAR α and was more efficient upon cotransfection of its heterodimeric partner, RXR α (Figure 2E, lane 4). It was less evident in the absence of a RARE (data not shown), suggesting that to be degraded, SRC-3 has to interact with RAR α /RXR α heterodimers bound to a RARE.

To ensure that the degradation process occurs with the endogenous protein as well, SRC-3 degradation was analyzed in HeLa, HL60 and NB4 cells. In the three cell lines, SRC-3 was degraded in response to RA and this process was reversed by MG132 (Supplementary Figure S1), indicating that RA-induced SRC-3 degradation is a general phenomenon. Finally, we determined whether other p160 coactivators such as SRC-1/NCoA1 or SRC-2/TIF2/GRIP-1, which are also proteasome targets, could be degraded in response to RA. None of these coactivators was significantly degraded when cotransfected with RAR α in COS-1 cells (Figure 2F). The same observation was made with other coactivators such as p300/CBP and SUG1 (data not shown).

Phosphorylation by p38MAPK signals RA-induced SRC-3 degradation

Increasing data indicate that degradation by the ubiquitin-proteasome pathway is signalled by phosphorylation processes (Bastien and Rochette-Egly, 2004). As SRC-3 can be phosphorylated at several residues by MAPKs (Figure 1B and Wu *et al*, 2004; Zheng *et al*, 2005), in response to multiple signals, we investigated whether RA can induce SRC-3 phosphorylation in transfected COS-1 cells labeled with [32 P] orthophosphate. As shown in Figure 3A, RA induced the phosphorylation of SRC-3. This phosphorylation was abrogated upon cotransfection of a vector encoding a dominant negative mutant of p38MAPK (dnp38MAPK) (Figure 3B, lane 4) and by p38MAPK inhibitors (SB 203580 or PD169316, data not shown), but was not affected by PD98059, which inhibits

Figure 2 RA induces the degradation of SRC-3 by the proteasome. (A) COS-1 cells were cotransfected with the full-length B10-SRC-3 vector (0.2 μ g) along with RAR α (0.1 μ g) and the DR5-tk-CAT reporter gene (1 μ g), and treated with RA (1 μ M). Whole-cell extracts (WCEs) were analyzed by immunoblotting with B10, RAR α and β -actin antibodies. (B) MG132 reverses the degradation of SRC-3 induced by RA at 16 h in COS-1 cells transfected as in (A). (C) Extracts from COS-1 cells cotransfected with Flag-tagged SRC3 (WT or AAA), RAR α and RXR α vectors were incubated with a DR5 RARE, RA-treated and immunoprecipitated with RAR α antibodies. Bound RAR α and SRC-3 were analyzed by immunoblotting. Lanes 1 and 2 correspond to 1% of the amount of immunoprecipitated extracts. (D) Unlike SRC-3 WT, SRC-3AAA overexpressed in COS-1 cells as in (A), and is not degraded in response to RA. (E) Same as in (A) except that RXR α was cotransfected and RA was added for 16 h. (F) COS-1 cells were cotransfected with increasing concentrations of the SRC-1, TIF2/SRC-2 or B10-SRC-3 vectors along with RAR α and the DR5-tk-CAT reporter gene and RA-treated for 16 h. WCEs were immunoblotted with SRC-1, TIF2, B10 or β -actin antibodies.

the activation of Erks (data not shown). These data suggest that RA induces the phosphorylation of SRC-3 and that this effect involves p38MAPK. In line with this, p38MAPK was activated in transfected COS-1 cells (Figure 3C) as well as in several cell lines (Supplementary Figure S2) (Alsayed *et al*, 2001; Gianni *et al*, 2002a). This activation was abrogated by dnp38MAPK (Figure 3D) and p38MAPK inhibitors (data not shown).

To further demonstrate that SRC-3 is a target for RA-activated p38MAPK, *in vitro* phosphorylation experiments were performed using the GST-SRC-3 (aa 481–1417) fusion protein (Figure 1B). GST-SRC-3 was phosphorylated by active phospho-p38MAPK isolated from RA-treated COS-1 cells (Figure 3E). It was also phosphorylated by recombinant p38MAPK (Figure 3F, lane 2) and to a lesser extent by p42/p44MAPK (Erks) (Figure 3F, lane 3). In contrast, the NID of SRC-3 (aa 611–831) (Figure 1B) was not phosphorylated (data not shown), consistent with the absence of phosphorylation sites (Wu *et al*, 2004). Collectively, these results converge towards the conclusion that RA induces the phosphorylation of SRC-3 via the activation of p38MAPK.

Interestingly, inhibition of p38MAPK, but not of Erks, also inhibited the SRC-3 degradation (Figure 3G, lanes 4 and 6), suggesting that phosphorylation by p38MAPK signals its degradation. O'Malley *et al* demonstrated that SRC-3 is

phosphorylated by p38MAPK at four residues, that is, S505, S543, S860 and S867 (Wu *et al*, 2004; Zheng *et al*, 2005). To support the importance of p38MAPK-mediated phosphorylation in the RA-induced degradation of SRC-3, we analyzed the degradation of the phosphorylation-defective mutants. Mutation of S505, S543 and S867 to alanine did not affect the ability of SRC-3 to be degraded (Figure 3H, lanes 4, 6, 8 and 12). As for SRC-3 WT, the degradation of these mutants was reversed by the dnp38MAPK vector (data not shown). However, mutation of S860 to alanine abolished the degradation of SRC-3 (Figure 3H, lane 10), indicating that S860 is involved, at least in part, in the RA-p38MAPK-mediated phosphorylation/degradation of SRC-3.

Inhibition of SRC-3 phosphorylation by p38MAPK increases the interaction of SRC-3 with RAR α

Physical interaction with nuclear receptors being at the basis of SRC-3 functions, we asked whether phosphorylation by p38MAPK affects RA-induced interaction of SRC-3 with RAR α . Extracts from transfected COS-1 cells were subjected to RAR α immunoprecipitation and analysis of SRC-3 binding. SRC-3 interacted with RAR α in response to RA (Figure 4A, lane 3). This interaction was more evident when the co-immunoprecipitation procedure included a synthetic double-stranded oligonucleotide containing a DR5 RARE (Figure 4B,

Figure 3 Phosphorylation by p38MAPK signals SRC-3 degradation. (A) COS-1 cells cotransfected as in Figure 2A were RA-treated and labelled with [32 P] orthophosphate. SRC-3 phosphorylation was analyzed by autoradiography and immunoblotting after immunoprecipitation of the extracts with B10 antibodies. (B) Same as in (A) with and without dnp38MAPK (0.2 μ g). (C) Kinetics of p38MAPK activation as assessed by *in vitro* phosphorylation of equal amounts of recombinant ATF2 by phospho-p38MAPK immunoprecipitated from extracts of RA-treated COS-1 cells. Phosphorylated ATF-2, immunoprecipitated P-p38 MAPK and p38MAPK in the extracts before immunoprecipitation were revealed by immunoblotting. (D) Same as in (C) in the absence and presence of dnp38MAPK. (E) *In vitro* phosphorylation of immobilized GST or GST-SRC-3 (aa 481–1417) proteins with P-p38MAPK immunoprecipitated from COS-1 cells treated or not with RA for 16 h was analyzed by autoradiography and immunoblotting. (F) Same as in (E), with recombinant p38MAPK or p42/p44MAPK. (G) Effect of dnp38MAPK overexpression and PD98059 (5 μ M) on the degradation of SRC-3 in COS-1 cells treated with RA for 16 h. Protein loading and dnp38MAPK expression were checked by reprobing the membrane with β -actin and p38MAPK antibodies. (H) COS-1 cells were transfected with the vectors expressing FLAG-SRC-3 WT or the phosphorylation-deficient Flag-SRC-3 mutants (S505A, S547A, S860A and S867A) and RA-treated for 16 h. SRC-3 degradation was analyzed by immunoblotting with Flag antibodies.

lane 4 and Figure 4C, lane 3) and was not affected by addition of a synthetic peptide corresponding to the LXXLL motif, LXD3 within the NID (Figure 4B, lane 5). However, peptides corresponding to the LXD2 and LXD1 motifs had a dramatic inhibitory effect (Figure 4B, lanes 6 and 7 and Figure 4C, lanes 4–6) in line with the requirement of these motifs for the interaction of SRC-3 with RARs (Klein *et al*, 2000).

Overexpression of the dnp38MAPK vector increased slightly the amount of SRC-3 interacting with RAR α , in the presence of RA (Figure 4A, lane 5 and Figure 4C, lane 7). Under these experimental conditions, only the highest concentrations of peptides LXD1 and LXD2 were effective in inhibiting the interaction between SRC-3 and RAR α

(Figure 4C, lanes 8–10 and data not shown). This suggests that inhibition of the overall SRC-3 phosphorylation strengthens the interaction of SRC-3 with RAR α . Note however that, in the presence of RA, the SRC-3(S860A) mutant interacted with RAR α as efficiently as the WT coactivator (Supplementary Figure S3).

Finally, we investigated whether, in transfected COS-1 cells, p38MAPK also modulates the interaction of another p160 coactivator, SRC-1/N-CoA1, with RAR α . RA induced the recruitment of SRC-1 to RAR α /RXR α /RARE ternary complexes (Figure 4D, lane 3) through the LXD2 and LXD3 motifs, but not the LXD1 motif (Figure 4D, lanes 4–6). However, overexpression of dnp38MAPK did not affect this

Figure 4 Inhibition of p38MAPK enhances the interaction of SRC-3 with RAR α . (A) COS-1 cells cotransfected with the B10-SRC-3, RAR α , RXR α and dnp38MAPK vectors were RA-treated for 2 h. WCEs were immunoprecipitated with RAR α antibodies and bound RAR α and SRC-3 proteins were analyzed by immunoblotting. The expression of dnp38MAPK was tested by immunoblotting of the extracts before immunoprecipitation. Lane 1 corresponds to 5% of the amount of immunoprecipitated extracts. (B) Extracts from COS-1 cells cotransfected as in (A) were incubated with a DR5 RARE in the absence or presence of the peptides corresponding to the LXD1, LXD2 and LXD3 motifs (40 μ M), RA-treated and immunoprecipitated. In lane 3, a mutated DR5 (mDR5) element was used. (C) Same as in (B) with WCEs from cells cotransfected or not with the dnp38MAPK vector and with increasing concentrations (40, 120 and 240 μ M) of the LXD2 peptide. (D) COS-1 cells cotransfected with the SRC-1 vector along with RAR α , RXR α and dnp38MAPK were processed as in (C), in the absence or presence of the peptides specific for each SRC-1 LXXLL domain (40 μ M).

interaction or the ability of the LXD2 and LXD3 peptides to decrease the recruitment of SRC-1 (Figure 4D, lanes 7–10).

Altogether, these results indicate that the efficiency of the interaction between SRC-3 and RAR α is increased upon inhibition of the phosphorylation of the coactivator by p38MAPK. However, this is not true for SRC-1.

P38MAPK controls RAR α -mediated transcription via SRC-3 phosphorylation

The relevance of SRC-3 phosphorylation by p38MAPK for RAR α -mediated transcription was investigated using transient transfection assays. In COS-1 cells cotransfected with RAR α and a CAT reporter gene controlled by a DR5 RARE (DR5-tk-CAT), CAT activity was increased in response to RA, with a peak at 16–24 h and a downmodulation at 48 h (Figure 5A).

Upon overexpression of dnp38MAPK, a slight decrease in the RA-induced CAT activity was observed at 8–10 h (Figure 5B, lanes 2 and 5 and Figure 5C, lane 1) followed by a marked increase at 16–24 h (Figure 5B, lane 8 and Figure 5C, lane 2). No more effect could be seen at 48 h (Figure 5B, lane 11). No significant variations were observed upon overexpression of WT p38MAPK (Figure 5B, lanes 3, 6, 9 and 12). Such effects were not the consequence of variations in the phosphorylation of RAR α at Ser77 (Figure 5D), which is the only potential phosphorylation site for MAPKs.

This is in line with our previous reports demonstrating that, *in vivo*, this residue is phosphorylated by cdk7 (Rochette-Egly, 2005). They were not the consequence either of variations in the decrease in RAR α levels that occurs in response to RA (Figure 5E). Finally, transcription mediated by RAR α with S77 mutated to alanine (RAR α S77A), which is deficient compared to that mediated by WT RAR α (Figure 5C), was enhanced as efficiently as that of the WT receptor upon overexpression of dnp38MAPK (Figure 5C, compare lanes 2 and 4).

From these data, we aimed at determining whether p38MAPK modulates RAR α -mediated transcription through SRC-3 phosphorylation. We analyzed the ability of overexpressed SRC-3 to coactivate RAR α . RAR α -mediated transcription was not affected at 10 h (Supplementary Figure S4), in line with the absence of any degradation of the coactivator at that time (see Figure 2A). However, at 16–24 h, overexpression of SRC-3 enhanced transcription as efficiently as dnp38MAPK (Figure 6A, lanes 8–10 and 2–4), suggesting that limiting amounts of SRC-3 are present in COS-1 cells and that overexpression of the coactivator (Figure 6B) compensates for RA-induced degradation. The SRC-3(S860A) mutant which is neither phosphorylated nor degraded in response to RA (Figure 6D) was more efficient than the WT coactivator to enhance RAR α -mediated transcription (Figure 6C, compare lanes 2 and 3). The other SRC-3 mutants

Figure 5 p38MAPK controls RAR α -mediated transcription through SRC-3 phosphorylation. (A) COS-1 cells transfected with the DR5-tk-CAT reporter gene along with RAR α were RA-treated for the indicated times and analyzed for CAT activity. The results, which were normalized to the CAT activity in the absence of RA, are the mean $\pm$ s.d. of at least four experiments. (B) Effect of dnp38MAPK, and p38MAPK on RAR α -mediated activation of the DR5-tk-CAT reporter gene. (C) RAR α S77A is less efficient than RAR α WT in inducing CAT activity but its activity is similarly enhanced at 16 h by dnp38MAPK. The results are the average of two experiments that agreed within 15%. (D) Overexpression of dnp38MAPK does not affect the phosphorylation of RAR α as determined by immunoblotting with antibodies recognizing specifically RAR α , RAR α phosphorylated at S77 and p38MAPK. (E) Overexpression of dnp38MAPK in COS-1 cells does not affect the degradation of RAR α induced by RA.

(S505A, S543A and S867A) behaved as the WT coactivators (data not shown) in line with their ability to be degraded. Overexpression of the other coactivators, SRC-1 and SRC-2, did not alter RAR α -mediated transcription, suggesting an unexpected degree of coactivator specificity (Figure 6A, lanes 7–10). Note that RAR α levels, although decreased in response to RA, were not affected by SRC-3 overexpression (Figure 6B and D). Collectively, these results indicate that inhibition of the p38MAPK-mediated phosphorylation/degradation of SRC-3 contributes to increase RAR α -mediated transcription.

RNA interference was used to further demonstrate that SRC-3 is the target of p38MAPK. We analyzed whether knockdown of SRC-3 by siRNAs affects RAR α transcriptional activity and the potentiating effect of dnp38MAPK observed at 16 h. Transfection of COS-1 cells with SRC-3 siRNA reduced the levels of overexpressed SRC-3 (Figure 7A, lanes 5 and 6) but not of SRC-1 (Figure 7B, lanes 3 and 4). It did not change the expression and the degradation of RAR α (Figure 7A and B). SiSRC-3 prevented the enhancing effect of overexpressed SRC-3 on RAR α -mediated transcription (Figure 7C, lanes 2 and 3). Interestingly, it also abrogated the enhancing effect of dnp38MAPK (Figure 7C, lanes 8 and 9). In contrast, selective siRNA targeting of SRC-1 or SRC-2 (Figures 7A and B and

data not shown) was irrelevant both in terms of the enhancing effects of overexpressed SRC-3 (Figure 7C, lane 4) and dnp38MAPK (Figure 7C, lane 10).

Similar results were obtained with endogenous SRC-3 in HeLa cells. Indeed, selective knockdown of SRC-3 (Figure 7D, inset) abrogated RA-induced CAT activity (Figure 7D, lane 2 and Figure 7E, lanes 1–3) and the enhancing effects of both overexpressed SRC-3 (Figure 7D, lanes 3 and 4) and dnp38MAPK (Figure 7E, lanes 4 and 5).

Collectively, our results indicate that RA-induced phosphorylation of SRC-3 by p38MAPK is an event that restricts RAR α -mediated transcription. Such an event can be prevented by inhibition of p38MAPK or compensated by overexpression of SRC-3.

P38MAPK controls maturation of RA-sensitive cells through SRC-3

Since inhibition of p38MAPK increases RAR α -mediated transcription, we asked whether the phenomenon has an impact on RA-induced cellular differentiation. Therefore, we analyzed the consequences of p38MAPK inhibition on RA-dependent differentiation of the NB4 and HL60 leukemia cells. In these cells, RA activates p38MAPK (Supplementary

Figure 6 Overexpressed SRC-3 compensates for RA-induced SRC-3 degradation. (A) Increasing amounts of dnp38MAPK, p38MAPK, SRC-3, SRC-1 and SRC-2 vectors were transfected in COS-1 cells along with the DR5-tk-CAT reporter gene and RAR α . CAT activity was determined 16 h after RA treatment. The results are the mean $\pm$ s.d. of three experiments. (B) Extracts corresponding to lanes 1 and 8–10 in (A) were analyzed for expression and degradation of SRC-3 and RAR α by immunoblotting. (C) COS-1 cells were transfected with the SRC-3 WT or SRC-3(S860A) vectors along with DR5-tk-CAT reporter gene and RAR α . CAT activity was analyzed as in (A). (D) Extracts from lanes 2 and 3 in (C) were analyzed for expression and degradation of RAR α and SRC-3 by immunoblotting.

Figure S2) and subsequently the degradation of SRC-3 (Figure 8A).

It is well known that RA induces the granulocytic differentiation of NB4 cells as assessed by morphological and biochemical criteria. RA increases the proportion of cells with lobated nuclei, cytoplasmic granules and/or elevated cytoplasm/nucleus volume ratio as well as acquisition of NADPH oxidase activity measured by nitrobluetetrazolium reduction (NBT-R) (Parrella *et al*, 2004). The p38MAPK inhibitor, PD169316, which does not exhibit any differentiating activity on its own, markedly enhanced the effect of RA as assessed by the measurement of NBT-R activity (Figure 8B, lane 2) and the number of NBT-R-positive cells (data not shown). Similar results were evident with SB203580 (Figure 8B, lane 3) another p38MAPK inhibitor, while SP600125 (Figure 8B, lane 4) and PD 98059 (data not shown) which are inhibitors of JNK and Erks, respectively, were devoid of any significant effect. The enhancing effect of PD169316 extended to the surface differentiation markers, CD11b and CD11c (Figure 8C). In contrast, the number of CD33-positive cells, which was not affected by RA, was unaffected by the p38MAPK inhibitor (Figure 8C). P38MAPK inhibition enhanced RA-induced expression of other important mediators of granulocytic maturation like cEBP β and STAT1 (Parrella *et al*, 2004), both at the mRNA (data not shown) and protein levels (Figure 8D). It also exerted a significant enhancing effect on STAT1 tyrosine phosphorylation (data not shown) and potentiated the expression of RA-target genes such as RAR α 2 as assessed by real time RT-PCR (Supplementary Figure S5). Altogether, these results indicate that specific inhibition of p38MAPK increase NB4 maturation.

Inhibition of p38MAPK also resulted in the amplification of the RA-induced maturation of HL60 cells (Figure 8B and C). Although the magnitude of the RA effects was lower than in NB4 cells, the potentiating effect of the p38MAPK inhibitor was similar, if not superior. For instance, STAT1 expression which was not affected by RA alone was strongly induced when combined to PD169316 (Figure 8D). Altogether, these data indicate that inhibition of p38MAPK results in the amplification of the differentiating action of RA.

To further demonstrate that inhibition of SRC-3 phosphorylation is at the basis of the effects of p38MAPK inhibition, we analyzed the consequences of siRNA-mediated knockdown of SRC-3. SRC-3 siRNA electroporation reduced temporary SRC-3 levels in NB4 cells (Figure 8E, lane 3). Knockdown of SRC-3 decreased the RA-induced expression of cEBP β (Figure 8F, lane 6) and the increase in NBT reducing activity (Figure 8G), underscoring the importance of SRC-3 in the differentiating activity of RA. Interestingly, knockdown of SRC-3 also reduced the potentiating effect of the p38MAPK inhibitor on RA-induced maturation of NB4 cells, both in terms of cEBP β expression (Figure 8F, lane 8) and NBT reducing activity (Figure 8G). Collectively, these results corroborate that p38MAPK controls the maturation of RA-sensitive cells via targeting SRC-3.

SRC-3 is phosphorylated by RA-activated p38MAPK, only within RAR α complexes

To investigate the RAR isotype specificity of SRC-3 phosphorylation and degradation in RA signaling, we investigated whether similar effects are observed in the case of RAR γ ,

Figure 7 siRNA-induced knockdown of SRC-3 abrogates the enhancing effect of dnp38MAPK on RAR α -mediated transcription. (A) COS-1 cells transfected with control, SRC-1 or SRC-3 siRNAs (50 nM) were subsequently transfected with the SRC-3 and RAR α vectors and the DR5-tk-CAT reporter gene and RA-treated for 16 h. Knockdown of overexpressed SRC-3 was analyzed by immunoblotting as well as RAR α expression and degradation. Protein loading was controlled by immunoblotting of β -actin. (B) Same as in (A) with overexpressed SRC-1. (C) siRNA against SRC-3 and not siRNA against SRC-1 abrogate the enhancing effect of overexpressed SRC-3 and dnp38MAPK on RAR α -mediated activation of a DR5-tk-CAT reporter gene. (D) In HeLa cells, siRNA against SRC-3 also reduce the levels of endogenous and overexpressed SRC-3 and decrease the activation of a DR5-tk-CAT reporter gene. (E) In HeLa cells, siSRC-3 and not siSRC-1 abrogate the enhancing effect of dnp38MAPK on CAT activity. The results are an average of two experiments that agreed within 15%.

another RAR isotype. RA induced the interaction of RAR γ with SRC-3 through the LXD1 and LXD2 motifs in DNA co-immunoprecipitation (Figure 9A, lanes 3–6) as described above in the case of RAR α . However, dnp38MAPK did not affect this interaction nor the ability of the LXD1 and LXD2 peptides to decrease the interaction of SRC-3 with RAR γ (Figure 9A, lanes 7–10). Thus, the interaction of SRC-3 with RAR γ does not appear to be modulated by a p38MAPK-mediated phosphorylation process. Accordingly, SRC-3 was not phosphorylated (Figure 9B) nor degraded in response to RA (Figure 9C, lane 2). In fact, p38MAPK induced the

phosphorylation (Figure 9D, lane 2) and the degradation (Figure 9C, lane 2) of RAR γ itself (Gianni *et al*, 2002a), and both processes were blocked upon inhibition of p38MAPK (Figure 9C and D, lane 4). In addition, inhibition of p38MAPK rather decreased RAR γ -mediated transcription (Figure 9E, lanes 4–7), consistent with the known requirement of RAR γ phosphorylation by p38MAPK for maximal activity (Gianni *et al*, 2002a). In line with this, the MEK1 inhibitor PD98059 and WT p38MAPK had no effect and increased the RA response, respectively (Figure 9E, lanes 2 and 3). Collectively, these results suggest that p38MAPK-mediated phos-

Figure 8 p38MAPK controls the RA-induced maturation of NB4 and HL60 cells through SRC-3. NB4 and HL60 cells, seeded at an initial concentration of 150 000 cells/ml, were treated for 3 days with vehicle, RA (0.01 μ M for NB4 cells and 1 μ M for HL60 cells), PD 169316 (1 μ M), SB203580 (5 μ M), SP600125 (5 μ M) either alone or in combination. In all experiments, the percentage of viable cells was >90%. (A) Immunoblotting analysis of RA-induced SRC-3 degradation. (B) NBT-R activity was measured in cell homogenates. Each value represents the mean $\pm$ s.d. of three independent culture flasks from two independent experiments. (C) FACS analysis of the CD11b, CD11c and CD33 markers. The results correspond to the mean associated fluorescence (MAF) for each marker. (D) Western blot analysis of STAT1(p91), β -actin and cEBP β (the 36–38 kDa LAP form) at 5 and 72 h. (E) Knockdown of SRC-3 in NB4 cells electroporated with siSRC-3 (80 and 160 nM) was demonstrated by immunoblotting with siGAPDH (100 nM) as a negative control. Protein loading was controlled by immunoblotting of β -actin. Knockdown of SRC-3 by siRNA decreased the effects of RA and PD 169316 on the expression of cEBP β (F) and NBT-R activity (G).

phorylation and degradation of SRC-3 is RAR α specific. They also indicate that SRC-3 is not always a target of p38MAPK and that the RAR complex determines the accessibility of the coactivator to the kinase.

To further substantiate the specificity of our results, we investigated the effects of dnp38MAPK on the function of SRC-3 in another signaling pathway. Indeed, SRC-3 is phosphorylated in response to TNF α and functions as a coactivator

Figure 9 When recruited to RAR γ , SRC-3 is not phosphorylated nor degraded in response to RA. (A) COS-1 cells were cotransfected with the B10-SRC-3, RXR α , RAR γ and dnp38MAPK vectors and WCEs were incubated with a DR5 RARE, in the absence or presence of the peptides specific for each SRC-3 LXXLL domain (40 μ M) and RA-treated. After RAR γ immunoprecipitation, bound proteins were analyzed by immunoblotting. Lane 1 corresponds to 5% of the amount of immunoprecipitated extracts. (B) SRC-3 phosphorylation was analyzed in transfected COS-1 cells treated with RA either alone or in combination with PD169316 (1 μ M) and labeled with [³²P] orthophosphate as in Figure 3A. (C) SRC-3 and RAR γ degradation was analyzed by immunoblotting. (D) RAR γ phosphorylation was analyzed by immunoblotting with antibodies recognizing RAR γ and its phosphorylated form. (E) CAT activity in COS-1 cells cotransfected with the RAR γ vector (WT or mutated) and a DR5-tk-CAT reporter, in the absence or presence of WT or dnp38MAPK and treated with RA alone or combined to PD98059 (5 μ M) or PD169316 (1 μ M). The results are the mean $\pm$ s.d. of at least three experiments. (F–G) HeLa cells were cotransfected with the SRC-3 and p65 vectors along with an NF- κ B-luciferase reporter gene and dnp38MAPK, and treated with TNF α (100 nM) for 16 h. Extracts were immunoprecipitated with p65 antibodies and analyzed for bound SRC-3 and p65 proteins by immunoblotting (F) or for luciferase activity (G). The results are the mean $\pm$ s.d. of three experiments.

for other transcription activators like NF- κ B (Wu *et al*, 2004). In HeLa cells engineered to express an NF- κ B-reporter gene along with the p65 subunit of NF κ B and B10-SRC-3, SRC-3 co-immunoprecipitated with p65 in response to TNF α (Figure 9F, lane 3). However, this interaction was not modulated by dnp38MAPK (Figure 9F, lane 4). Significantly, the activation of the NF- κ B-luciferase reporter gene observed in response to TNF α was also left unaffected (Figure 9G, lane 3), indicating that it does not involve phosphorylation by p38MAPK and corroborating that distinct physiological signaling pathways involve differential phosphorylation processes (Wu *et al*, 2004).

Discussion

SRC-3 associated to RAR α is phosphorylated by p38MAPK and degraded by the proteasome

Although it is generally accepted that regulation of gene transcription is the result of alterations in the amounts and/or activities of DNA-binding activators (Dennis and O'Malley, 2005; Rochette-Egly, 2005), there is increasing evidence that regulation of the p160 coactivators also plays an important role. Coactivators are recruited to cognate gene promoters by nuclear receptors through direct contacts involving their NID

and transmit the activation signal through their AD1 and AD2 domains, which serve as platforms to recruit other factors that contribute to transcriptional activation. Here, we identified a new mechanism underlying the activation of the nuclear receptor RAR α , which involves the coactivator SRC-3 and p38MAPK. We show that in response to RA, SRC-3 is phosphorylated by p38MAPK and subsequently degraded by the proteasome. Recent studies reported that SRC-3 is phosphorylated by p38MAPK at several serine residues in response to steroid hormones, all of them being required for the activity of the coactivator (Wu *et al*, 2004). However, phosphorylation of only one of the p38MAPK phosphorylatable residues (S860) appears to be required for the RA-induced degradation of SRC-3, corroborating the hypothesis that the specificity of the SRC-3 response to cellular signalings is defined by the activation of a specific phosphorylation code (Wu *et al*, 2005). Although SRC-1 and SRC-2 can also be phosphorylated in response to several stimuli and regulated by the ubiquitin–proteasome pathway (Yan *et al*, 2003; Wu *et al*, 2005), they were not degraded upon RA activation of RAR α , suggesting a relative target-specific coactivator effect of RA.

SRC-3 phosphorylation has a biphasic effect on RAR α -mediated transcription, with facilitation followed by restriction

Our finding that SRC-3 is phosphorylated by p38MAPK and then degraded in response to RA, led us to determine the functional role of these processes. The present study indicates that RA-dependent phosphorylation of SRC-3 has a biphasic action on RAR α activity, with a positive influence followed by a restrictive one. We provide evidence that in an early phase, inhibition of p38MAPK decreases RAR α -mediated transcription. Unexpectedly, our data correlated this transcriptional effect to an increased interaction of SRC-3 with RAR α . Normally, an increased interaction of coactivators with nuclear receptors contributes positively to transcriptional activation. However, the new arising concept is that a continuous exchange of nuclear receptors and coactivators is required for the formation of the complexes at the promoter and therefore for transcription to proceed (Metivier *et al*, 2003; Dennis and O'Malley, 2005). Moreover, there is increasing evidence that phosphorylation leads to conformational changes that alter interaction surfaces (Foulds *et al*, 2004). In line with these new ideas, we propose that, at the beginning, phosphorylation by p38MAPK regulates positively transcription through controlling the dynamics of the interactions of SRC-3 with RAR α , thereby preventing the interactions from stalling (Dennis and O'Malley, 2005; Rochette-Egly, 2005). Whether phosphorylation also facilitates the interaction of SRC-3 with other coregulators (Yi *et al*, 2005) will require further investigations.

In contrast, later on, inhibition of p38MAPK increases RAR α -mediated transcription. Using phosphorylation-deficient mutants and siRNAs, this effect has been correlated to the abrogation of SRC-3 phosphorylation at S860 and thereby of SRC-3 degradation. We excluded the possibility that it reflects variations in RAR α phosphorylation and turn over. As SRC-3 degradation occurs rather late (16 h), relative to activation of p38MAPK (within 1 h), it is tempting to speculate that phosphorylation of S860 occurs later than that of the other p38MAPK sites. Such a delay might reflect the requirement of a specific conformational context for selective

phosphorylation of S860 and/or subsequent recruitment of the ubiquitin–proteasome system (Shao *et al*, 2004; Verma *et al*, 2004). Finally, these data led us to suggest that p38MAPK-mediated degradation of SRC-3 contributes to transcriptional inhibition through disrupting the fine-tuned exchanges between RAR α and the various components of the transcription machinery. Thus, SRC-3 must be removed in order for transcription to cease.

Given that p38MAPK can phosphorylate four serine residues on SRC-3, we propose a model whereby its biphasic effect on RAR α -mediated transcription results from a series of successive and coordinated phosphorylation events targeting different residues of the coactivator. During the first hours of transcription, combinations of phosphorylations may induce conformational changes facilitating interactions and exchanges and thereby transcriptional activation. Subsequently, phosphorylation of other residues including S860 may promote the degradation of SRC-3 thereby restricting transcription. In conclusion, phosphorylation of SRC-3 works as a clock, regulating the transcription of RAR α -target genes with an initial facilitation followed by a restriction due to the degradation of the coactivator.

Regulation of SRC-3 by p38MAPK has far reaching functional implications. Indeed, inhibition of the kinase also potentiates RA-induced maturation of leukemia cells. Since RA is used in the treatment of acute promyelocytic leukemia (APL) and several other malignancies (Clarke *et al*, 2004), the combined use of pharmacological inhibitors of p38MAPK may have clinical potential.

SRC-3 was originally identified on the basis of its frequent amplification in several malignancies (Lonard and O'Malley, 2005; Wu *et al*, 2005). Moreover, several kinases including MAPKs are frequently overexpressed or aberrantly active in a large number of tumors (Blume-Jensen and Hunter, 2001). Whether such cancers are associated with aberrant phosphorylation and degradation of SRC-3 in response to RA remains to be determined. Nevertheless, given the unique function of SRC-3 phosphorylation by p38MAPK in regulating RAR α activity, the present study raises the possibility that p38MAPK inhibitors could be exploited at the clinical level to improve retinoid therapy and/or reverse retinoid resistance.

RA-induced phosphorylation of SRC-3 by p38MAPK is RAR isotype specific

SRC-3 phosphorylation has been shown to regulate its transcriptional coactivation in response to different cellular signals (Wu *et al*, 2004). However, the current study demonstrates that SRC-3 is phosphorylated in response to RA only in the context of RAR α complexes, suggesting a relative and unexpected degree of RAR isotype specificity in the phosphorylation process. Indeed, when recruited to another RAR isotype (RAR γ), neither is SRC-3 phosphorylated by p38MAPK nor is it degraded by the proteasome. In this particular context, it is RAR γ and not SRC-3, which is phosphorylated and degraded. It must be noted that RAR γ contains two vicinal serine residues (Figure 1A), one of them being phosphorylated by p38MAPK in response to RA. In a separate study, we evidenced the critical role of RAR γ phosphorylation by p38MAPK for both the activity and the degradation of the receptor (Gianni *et al*, 2002a).

The reason(s) for p38MAPK selection of the RAR or the coactivator partner in different isotype complexes is not yet

known. Nevertheless, it is tempting to speculate that RAR α - or RAR γ -interacting protein complexes are endowed with different and specific conformations, which dictate docking of p38MAPK and selection of the target. We can suggest that p38MAPK generates, in cooperation with RA, a phosphorylation code defined by the target, allowing at least, in part, accurate and specific regulation of transcription. Nevertheless, it is possible that, in addition to RARs and SRC-3, other corepressors and/or coregulators (Jonas and Privalsky, 2004) are phosphorylated by p38MAPK and contribute, in a combinatorial fashion, to the modulation of the processes described here.

In conclusion, the present study has uncovered a novel level of transcriptional regulation of RA-responsive genes by p38MAPK. Although differentially integrated by the different RAR isotypes and the coactivator SRC-3, p38MAPK signaling appears to be a common regulatory pathway mediating the right response of the right gene at the right time.

Materials and methods

Plasmids and reagents

The pSG5-based expression vectors for mouse (m) RXR α 1, human (h) RAR α 1, hRAR α S77A, mRAR γ 2, mRAR γ 2S66/68A, SRC-1, TIF2/SRC-2 and the DR5-tk-CAT reporter gene were previously described (Gianni *et al*, 2002a; Bour *et al*, 2005a). The cDNA for SRC-3 was provided by Don Chen. The vector for B10-tagged SRC-3 was constructed by inserting amino acids 151–165 of the B region of hER into a pTL2 vector containing HA-Flag-tagged full-length SRC-3 and provided by T Lerouge. The expression vectors for full-length Flag-tagged SRC-3 either WT or mutated at the three LXXLL motifs (SRC-3AAA) and at the phosphorylation sites (S505A, S543A, S860A and S867A) were provided by O'Malley (Wu *et al*, 2004; Zheng *et al*, 2005).

The vector for p38MAPK was described previously (Gianni *et al*, 2002b) and that for dominant negative p38MAPK (KRSPA Flag p38 K-M) was a gift from R Krug and UR Rapp (Wurzburg, Germany). The (kB)₄-IL2-Luc reporter gene was provided by A Sica (Milano, Italy). The procaryotic vectors pGEX2T-SRC-3 (481–1427) and pGEX2T-SRC-3 (611–831) were constructed and provided by C Erb. MG132, SB203580, PD169316, SP600125 and PD98059 were from Calbiochem Inc. (Merck Biosciences).

Antibodies

Antibodies against GST, RAR α , RAR γ and RXR α were previously described (Gianni *et al*, 2002a; Bour *et al*, 2005b) as well as the antibodies specific to RAR α phosphorylated at S77 and to RAR γ phosphorylated at S66 (Gianni *et al*, 2002b; Keriell *et al*, 2002). Antibodies against the epitope B of the N-terminal A/B domain of the estrogen receptor (MAb B10) and TIF2/SRC-2 were as described (Ali *et al*, 1993; Gianni *et al*, 2002a). The antibodies against the p65 subunit of NF- κ B, p38MAPK and its phosphorylated form, P-p38MAPK (Thr180/Tyr182), and the phosphorylated form of ATF-2 were from Cell Signaling Technology, Inc. (USA). The antibodies against β -actin (C-11), SRC-1, cEBP β and STAT1 were from Santa Cruz Biotechnology Inc. (USA). Antibodies against SRC-3 and the phosphorylated form of STAT-1 were from Upstate Biotechnology Inc. (USA) and those against FLAG tag from Sigma.

Cells, transfections, immunoprecipitations, immunoblotting and differentiation assays

COS-1 and HeLa cells were transiently transfected in six-well plates using the DMR1E-C and Lipofectamine 2000 reagents, respectively,

according to the manufacturer's protocol (Invitrogen-Life Technologies). CAT and luciferase activities were determined according to standard procedures (Bour *et al*, 2005a). Whole-cell extracts (WCEs) were prepared and subjected to immunoprecipitation and immunoblotting as previously described (Bour *et al*, 2005b).

NB4 and HL60 cells were cultured and analyzed for NBT-R-positive cells, NBT-R activity and surface expression of the myeloid markers (FACS analysis) as described (Parrella *et al*, 2004).

Short interfering (si) RNA

The smartpool short interfering RNA (siRNA) against human SRC-3 (NM_006534) and SRC-1 (NM_003743) were purchased from Dharmacon (Lafayette CO) as well as scrambled siRNA. SiRNAs were transfected into COS-1 or HeLa cells according to the manufacturer's protocol. In the case of cotransfection of siRNA and plasmid DNA experiments, cells were transfected with siRNA first. Then 6 h later, they were cotransfected with siRNA and DNA plasmid and processed as for plasmid DNA transfection alone. Predesigned siRNAs against SRC-3 and GAPDH (Ambion, Inc., USA) were transfected into NB4 cells as described in Kamashev *et al* (2004).

DNA co-immunoprecipitation experiments

WCEs from transfected COS-1 cells were incubated with annealed double-strand oligonucleotides (DR5 RARE, 5' tcgagggtagggttcaccga aagttcactcg 3'; mutant RARE: 5' tcgagggtagggttcaccgaagttcactcg 3') in the absence or presence of RA as in Klein *et al* (2000) and immunoprecipitated with monoclonal RAR antibodies. When indicated, synthetic peptides corresponding to the LXXLL motifs (LXD1, LXD2 and LXD3) of SRC-3 or SRC-1 (Klein *et al*, 2000) were added before the addition of RA.

In vivo and in vitro phosphorylation

In vivo phosphorylation experiments were performed with transfected COS-1 cells labelled with [³²P] orthophosphate as previously described (Rochette-Egly *et al*, 1995). For *in vitro* phosphorylation, GST-SRC-3 fusion proteins expressed in *Escherichia coli* and immobilized on Glutathione-Sepharose beads were incubated with 20 μ Ci ³²P γ -ATP and purified recombinant p44 or p38 MAPKs (20 ng, Upstate Biotechnology Incorporated, Lake Placid, NY, USA), or P-p38MAPK immunopurified with Phospho-p38MAPK antibodies. Phosphorylated proteins were resolved by SDS-PAGE and analyzed by autoradiography and immunoblotting. Phosphorylation of ATF-2 by p38MAPK was performed as in Gianni *et al* (2002a).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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