Down-regulation of the Phosphatidylinositol 3-Kinase/Akt Pathway Is Involved in Retinoic Acid-induced Phosphorylation, Degradation, and Transcriptional Activity of Retinoic Acid Receptor γ2*

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Nuclear retinoic acid (RA) receptors (RARs) are phosphorylated at conserved serine residues located in their N-terminal domain. Phosphorylation of RARγ; at these residues is increased in response to RA subsequently to the activation of p38MAPK. We show here that this RA-induced phosphorylation of RARγ; resulted from the down-regulation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. By overexpressing Akt and by using agents that activated or inhibited the PI3K/Akt pathway, we also demonstrated that the RA-induced down-regulation of the PI3K/Akt pathway targeted not only the phosphorylation of RARγ; but also the turnover and transcriptional activity of the receptor. Altogether these data indicate that the PI3K/Akt pathway plays an important role in retinoid acid signaling.

The effects of retinoid acid (RA)1 are mediated by two families of nuclear receptors, the retinoic acid receptors (RARs), -β, and -γ and the retinoid X receptors (RXRs, -β, and -γ), which are ligand-dependent transcriptional regulators functioning as RAR/RXR heterodimers both in vivo and in vitro (1–3). A ligand-independent activation domain called AF-1, which is present in the N-terminal A/B region of RARs, contains serine residues (see Fig. 2A) that are constitutively (i.e. in the absence of ligand) phosphorylated by the Cdk7 subunit of the general transcription factor TFIIH (4, 5). We recently demonstrated that phosphorylation of RARγ; at these residues is markedly increased in response to RA through activation of p38MAPK.2 This RA-induced phosphorylation is important for both RARγ;-mediated transcription of RA target genes and degradation of the receptor by the ubiquitin-proteasome pathway. The aim of the present study was to investigate how p38MAPK is activated in response to RA.

Activation of p38MAPK has been traditionally associated with stress responses through a cascade of phosphorylation reactions involving upstream kinases (MAPKKK, MAPKK, and MAPK) (Refs. 6–9 and references therein). However, it has been recently reported that p38MAPK activity could be regulated through cross-talks with the PI3K/Akt pathway (10–13). We show here that the RA-induced activation of p38MAPK and therefore the subsequent increase in RARγ; phosphorylation resulted from the inhibition of the PI3K/Akt pathway. This down-regulation of the PI3K/Akt pathway was crucial for RA-induced degradation and transactivation activity of RARγ; indicating that it is a key step in retinoid signaling.

EXPERIMENTAL PROCEDURES

Reagents, Plasmids, and Chemicals—The pSG5-based expression vectors for mouse (m) RARγ;, mRARγ;S66A/S68A, and the DR5-tk-CAT reporter gene were described previously (5, 14). All-trans-retinoic acid, LY294002, and wortmannin were from Sigma-Aldrich. STI571 was a gift from Dr. Barbara Willi (Novartis Pharma AG). The vectors for dominant active and negative Akt containing a Myc tag were purchased from Upstate Biotechnology Inc. The cDNA for p38MAPK was provided by P. Cohen (Dundee, UK) and cloned into the pSG5 expression vector.

Rabbit polyclonal antibodies against RARγ; (RPγ;F) have been described previously (15). Tyr(P) 4G10 monoclonal antibodies were from Zymed Laboratories Inc. Rabbit polyclonal antibodies against p38MAPK and Akt and their active phosphorylated forms, P-p38MAPK (Thr-180/Tyr-182) and P-Akt (Ser-473), were from Cell Signaling Technology, Inc. Rabbit polyclonal antibodies against c-Abl (K-12), c-Abl antibodies conjugated to agarose beads, and goat polyclonal antibodies against actin (C-11) were from Santa Cruz Biotechnology Inc. Anti-Myc tag antibodies were from Upstate Biotechnology Inc.

Polyclonal antibodies specific to RARγ; phosphorylated at Ser-66 or Ser-68 were prepared by immunizing rabbits with synthetic phosphopeptides followed by column chromatography with SulfoLink gel columns (Pierce) coupled to the corresponding immunizing phosphorylated peptide. After elution, the antibodies reacting with unphosphorylated RARγ; were depleted by chromatography on a column coupled to the unphosphorylated peptide.

Cell Lines, Transfections, and CAT Assays—F9 cells were cultured and treated with 10−7 M RA as described previously (16). COS-1 cells were grown and transiently transfected in six-well plates using the

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FIG. 1. Down-regulation of PI3K/Akt is involved in RA-induced activation of p38MAPK. A, COS-1 cells were cotransfected with the RARγ expression vector, in the absence or presence of either the dn or the da Akt expression vector, and treated with vehicle or RA. WCEs were immunoprecipitated with immobilized p38MAPK antibodies and immunoblotted with antibodies against either c-Abl or phosphotyrosine. WCEs were also immunoprecipitated with immobilized c-Abl antibodies and immunoblotted with antibodies against Akt or its phosphorylated form (P-Akt). C, RARγ-transfected COS-1 cells and F9 cells were treated with RA or STI571 either alone or in combination as indicated. WCEs were immunoprecipitated with immobilized p38MAPK antibodies and immunoblotted with antibodies against Akt or its phosphorylated form (P-Akt). D, transfected COS-1 cells and F9 cells were grown without or with addition of wortmannin (100 nm) or LY294002 (10 μM) for 16 or 2 h, respectively, before harvesting. WCEs were immunoblotted with antibodies against Akt or its phosphorylated form.

RESULTS

RA-induced Activation of p38MAPK Results from the Down-regulation of the PI3K/Akt Pathway—In RARγ-transfected COS-1 cells, p38MAPK phosphorylation was induced after 24 h of RA treatment as assessed by WB analysis with specific antibodies recognizing the active phosphorylated form of the kinase (Fig. 1A, P-p38, compare lanes 1 and 2). To investigate whether this increase in p38MAPK activity involves Akt, we activated or inhibited the PI3K/Akt pathway. First, a constitutively active (da) form of Akt was coexpressed with RARγ in COS-1 cells. This markedly decreased the RA-induced activation of p38MAPK (Fig. 1A, lanes 3–6). On the other hand, overexpression of a dominant negative (dn) form of Akt enhanced p38MAPK phosphorylation (Fig. 1A, lanes 7–10). These results were corroborated by using STI571 (18), an inhibitor of the non-receptor tyrosine kinase c-Abl that down-regulates the PI3K/Akt pathway (19, 20). As expected, STI571 (10 μM) decreased the level of c-Abl tyrosine phosphorylation (Fig. 1B) while it increased the amount of active phosphorylated Akt (Fig. 1B, P-Akt) as assessed by WB analysis with antibodies specific for the phosphorylated form of Akt. STI571 also suppressed the RA-induced increase in phosphorylated p38MAPK (Fig. 1C, compare lanes 2 and 4). The effects of PI3K inhibitors (LY299002 and wortmannin) on p38MAPK phosphorylation were also evaluated. LY299002 (10 μM) and wortmannin (100 nm) decreased the amount of constitutively phosphorylated and activated Akt (Fig. 1D, P-Akt) and increased the activation of p38MAPK induced by RA (Fig. 1E, compare lanes 2 and 3).

RA also activated p38MAPK in mouse embryocarcinoma cells (F9 cells)2 (Fig. 1C, lane 2). Moreover, as in transfected COS-1 cells, STI571 and LY294002 abrogated (Fig. 1C, compare lanes 2 and 4) and increased (Fig. 1E, compare lanes 2 and 3), respectively, the activation of p38MAPK induced by RA in these cells. Altogether these results indicate that the RA-induced activation of p38MAPK involves the inhibition of the PI3K/Akt pathway.
Additionally, the increase in RAR and lanes 2 enhanced upon overexpression of p38MAPK (Fig. 2A). Altogether these results further support the conclusion that the amount of RAR phosphorylated at serine 66 (P-RAR) increased in response to RA.2 These results were confirmed by WB analysis using antibodies recognizing specifically RAR (Fig. 2B, lane 2), indicating that RA increases the phosphorylation of both residues. No increase was observed in COS-1 cells expressing a RAR mutant in which the two serine residues are mutated into alanine (RAR/S66A/S68A) (Fig. 2B, lane 4).

The RA-induced increase in phosphorylated RAR detected with the phospho-RAR antibodies was impaired upon incubation of the transfected cells with SB203580 (10 μM), a highly specific inhibitor of p38MAPK (21) (Fig. 2C, lane 3), while the MEK1 inhibitor PD98059 (5 μM) had no effect (Fig. 2C, lane 4). Additionally, the increase in RAR phosphorylation was enhanced upon overexpression of p38MAPK (Fig. 2D, compare lanes 2 and 4) and appeared earlier (at 10 h instead of 24 h). Altogether these results further support the conclusion that the RA-induced increase in RAR phosphorylation results from the activation of p38MAPK.2

We then investigated whether, as expected from the above results, modulating the activity of the PI3K/Akt pathway would affect RA-induced RAR phosphorylation. Overexpression of the da form of Akt inhibited the increase in RAR phosphorylation (Fig. 3A, lanes 3 and 4), whereas a dn form of Akt enhanced RAR phosphorylation (Fig. 3A, lanes 5 and 6). Addition of STI571 also blocked the RA-induced increase in RAR phosphorylation (Fig. 3B, compare lanes 2 and 4), while in contrast, it was enhanced by the PI3K inhibitors LY294002 and wortmannin (Fig. 3C, compare lanes 2 and 4). Similar results were obtained with F9 cells (data not shown). Collectively these results indicate that the RA-induced RAR phosphorylation results from the activation of p38MAPK through inhibition of the PI3K/Akt pathway.

**Fig. 3.** Inhibition of the PI3K/Akt pathway is involved in RA-induced RAR phosphorylation. A, COS-1 cells were cotransfected with the mRAR2 expression vector, in the absence or presence of either the dn or the da Akt expression vector, and treated with vehicle or RA. WCEs were immunoblotted with P-RAR, RPγ(F), or actin antibodies. Akt overexpression was checked by immunoblotting with antibodies recognizing the Myc tag. B, RAR2-transfected COS-1 cells were treated with RA or STI571 either alone or in combination as indicated. WCEs containing equal amounts of RAR2 were immunoblotted with P-RAR, RPγ(F) and RPγ antibodies. C, RAR2-transfected COS-1 cells were treated with vehicle or RA, and LY294002 (10 μM) was added 2 h before harvesting as indicated. WCEs were immunoblotted with P-RAR, RPγ(F), or actin antibodies.
tk-CAT), the expression of da Akt (Fig. 4B) decreased the RA-induced increase in CAT activity. Addition of STI571 had similar effects (Fig. 4D). In contrast, the PI3K inhibitors LY294002 and wortmannin enhanced CAT activity (Fig. 4F). Collectively these results indicate that the down-regulation of the PI3K/Akt pathway is involved in both RA-induced degradation and transcriptional activity of RARγ2.

DISCUSSION

We previously found that the RA-induced increase in RARγ2 phosphorylation is mediated through activation of p38MAPK. Here we report that this activation implicates the down-regulation of the PI3K/Akt pathway. Indeed, blocking PI3K with wortmannin or LY294002 amplified the observed RA-induced increase in p38MAPK activity and RARγ2 phosphorylation. Reciprocally, stimulation of the PI3K/Akt pathway upon STI571 treatment or overexpression of da Akt down-regulated these processes.

Our present results are in agreement with recent reports demonstrating that Akt negatively regulates p38MAPK and that disruption of the PI3K/Akt pathway prevents these effects, resulting in the activation of the p38MAPK (13). How RA inhibits the PI3K/Akt pathway was recently elucidated in mouse embryonic cells (F9 cells) by Bastien et al. who have shown that RA acts at two levels, phosphorylation of the phosphatase PTEN and inhibition of PI3K through its p85α subunit, both of them leading to Akt inhibition.

Interestingly our present study has demonstrated that the RA-induced down-regulation of the PI3K/Akt pathway targets not only the phosphorylation of RARγ2 but also its transcriptional activity and its degradation by the proteasome. Thus, RARγ2 phosphorylation, RARγ2 turnover, and RARγ2-mediated transcription of RA target genes are interrelated events resulting from the RA-induced down-regulation of the PI3K/Akt pathway, which therefore plays an important role in RA signaling.

It is interesting to note that Akt is a mediator of cell growth and survival, while RA has pronounced antiproliferative potential that is usually linked to its capacity to induce differentiation. In keeping with this activity, RA is used in the treatment of several cancers (23, 24). As a number of tumoral processes have been correlated with constitutively high Akt activity (11, 25, 26) and therefore to aberrant downstream kinase activities, one can speculate that inhibition of this pathway would improve the efficiency of RA therapy. In that respect it should be noted that STI571, which not only inhibits c-Abl tyrosine kinase but also other receptor tyrosine kinases that are often amplified in carcinoma (27) and lead to increased activation of the PI3K/Akt pathway, is currently used in cancer therapy (28–31). Moreover it synergizes with retinoids in terms of cytodifferentiation and growth inhibition (32) and is capable of partially reversing the RA resistance of some acute promyelo-
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