

ORIGINAL ARTICLE

 β -arrestin 2 modulates the activity of nuclear receptor RAR β 2 through activation of ERK2 kinase

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The activity of retinoid receptors activity can be regulated by various extracellular stimuli. In an effort to understand the molecular basis for this phenomenon, the role of β -arrestins was investigated. β -Arrestins constitute a class of proteins involved in the internalization of agonist-activated receptors. They have also been linked to MAPK activation suggesting a direct involvement in signaling cascades. Here, we report that β -arrestin 2 stimulates the transcriptional activation of the retinoid RAR and RXR receptors. Of all the retinoid receptors, the RAR β 2 subtype showed the strongest sensitivity to β -arrestin 2 action. Interestingly, this event requires the presence of the MAP kinase ERK2, but not that of JNK or P38. Site-directed mutagenesis showed that Ser 22 and Leu 217 are critical residues of the RAR β 2 receptor through which β -arrestin 2 effects are mediated. More importantly, we demonstrate that the induction of PC12 growth inhibition by Nerve Growth Factor is indeed dependent upon RAR β 2 transcriptional activation in a β -arrestin 2- and ERK2-dependent manner.

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Introduction

β -Arrestins constitute a class of proteins that facilitate clathrin-mediated endocytosis of G-protein-coupled receptors (GPCRs). Evidence suggests that other receptor types can also be targeted for internalization, namely receptor tyrosine kinases (RTKs) (Lin *et al.*, 1998). The physical interaction of β -arrestins with the targeted receptor is agonist-dependent and results in the inhibition of further signal transduction. Recently, aside from their role in receptor desensitization, β -arrestins have been involved *per se* in the activation of signaling pathways. Notably, β -arrestin 2 has been associated to initiating MAPK activation, in particular that of JNK3

(McDonald *et al.*, 2000) and ERK2 (Luttrell *et al.*, 2001) kinases.

Retinoid acid (RAR α , NR1B1; RAR β , NR1B2; RAR γ , NR1B3) and retinoid X (RXR α , NR2B1; RXR β , NR2B2; RXR γ , NR3B3) receptors belong to the steroid family of nuclear receptors, a class of ligand-activated transcription factors whose activities are regulated in multiple ways, primarily through the binding of agonists. In the absence of ligand, retinoid receptors are bound to corepressors. Retinoids, such as all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9-*cis* RA), act as ligands for the RAR and RXR receptors, respectively. Ligand binding triggers a conformational change in the ligand-binding domain of the receptors. Corepressors are then displaced allowing coactivators to bind, resulting in the transcriptional activation of the receptors.

While transcriptional activation of retinoid receptors in response to ligand binding has been extensively studied, less prominent ways of activation have also been reported. For instance, various external stimuli modulate retinoid receptors. Stress signals such as ultraviolet irradiation impair ligand-induced transcription of target genes by RAR and RXR (Wang *et al.*, 1999). Forskolin, an activator of protein kinase A, enhances RAR α activity (Rochette-Egly *et al.*, 1995). Moreover, low redox levels achieved through hypoxic culture conditions increase RAR-dependent transactivation (Demary *et al.*, 2001). Targeting for ubiquitin-mediated proteolytic degradation defines another level of regulation (Kyriakis, 2000). A common feature of these regulatory functions is the phosphorylation of specific amino-acid residues of the retinoid receptors. A number of kinases such as protein kinase A (Rochette-Egly *et al.*, 1995; Harish *et al.*, 2000), protein kinase C (Delmotte *et al.*, 1999) and MAP kinases (Adam-Stitah *et al.*, 1999; Lee *et al.*, 2000) have been shown to phosphorylate various residues on the retinoid receptors in a specific manner.

As external stimuli are known to modulate retinoid receptors activity, we tested the hypothesis that β -arrestins, in response to extracellular signals, could affect directly or indirectly the transcriptional properties of the RAR and RXR receptors. Here we present evidence that β -arrestin 2 strongly enhances the activity of RAR β 2, and that of the other retinoid receptors to a smaller extent. This effect is dependent upon the recruitment of the ERK2 kinase and its direct

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interaction with RAR β 2. We also identified specific residues in the RAR β 2 receptor that define ERK2-dependent phosphorylation and docking sites. Finally, we uncovered physiological situations in which such regulatory pathways are evident.

Results

We investigated whether β -arrestins could affect the activity of the different retinoid receptors subtypes. To assess this hypothesis, the functional assay R-SATTM (Receptor Selection and Amplification Technology) assay was used, that allows to monitor proliferation of gene targets in a ligand-dependent fashion. Such assays have been developed for a number of receptor families, ranging from GPCRs (Brauner-Osborne and Brann, 1996) to RTKs (Burstein *et al.*, 1998; F.Piu unpublished) and cytokine receptors (Piu *et al.*, 2002). NIH-3T3 fibroblasts were transiently transfected with the individual RAR and RXR receptor subtype, in the presence or absence of β -arrestins. Cells were then incubated for 5 days with 1 μ M AM-580, a pan-retinoid agonist ligand that activates both RAR and RXR receptor subunits. Ligand-dependent proliferation was monitored by cotransfection of a constitutively expressed β -galactosidase reporter gene. All RAR and RXR receptors displayed ligand-dependent activation as quantified by the measurement of absorbance at 405 nm (Figure 1a). Responses were ligand and receptor specific as no activity was detected in nontransfected cells incubated with AM-580, or in RAR- and RXR-transfected cells in the absence of AM-580 (data not shown). When β -arrestin 1 was expressed, no potentiation of nuclear receptors activity could be observed. In contrast, the presence of β -arrestin 2 led to a significant increase in RAR and RXR receptors activation. Interestingly, the effect of β -arrestin 2 was similar for all RAR/RXR receptors with the marked exception of RAR β 2: while the increase was in the range of 1.3–1.4 fold for all nuclear receptors, it was consistently above twofold for RAR β 2 (Figure 1b). The enhancement of retinoid receptors activity by β -arrestin 2 was selective and dependent upon the presence of the RAR, RXR receptors and their ligands, and not the result of a general effect on cellular proliferation as transfection of β -arrestin 2 alone or in the presence of RAR β 2 did not increase the assay baseline (Figure 1d and data not shown).

In addition to its role as targeting receptors for endocytosis via clathrin-coated vesicles, β -arrestin 2 can also act as an adapter protein to promote activation of MAPK signaling. To distinguish between these two functions, a β -arrestin 2 mutant lacking a functional clathrin-binding domain was generated (β Arr2 AAEA mutant) in which the critical hydrophobic residues LIEF of the clathrin domain (aa 373–376) were mutated to alanine residues. This mutant is significantly impaired in its ability to bind clathrin-coated vesicles (<10%) and is unable to promote internalization of agonist-activated GPCRs (Krupnick *et al.*, 1997). Interestingly, the β Arr2

AAEA mutant was still able to potentiate RAR and RXR nuclear receptors activity in a manner similar to that of wild-type β -arrestin 2 (Figure 1c). Thus, the ability of β -arrestin 2 to bind clathrin-coated vesicles is not critical to its stimulation of nuclear receptor activity. Most likely, it is β -arrestin 2 function as an adapter protein for MAP kinases or other signaling molecules that is responsible for the observed increase in RAR and RXR receptor subtypes activity.

It has been reported that β -arrestin 2 associates with a number of signaling molecules that link to MAPK signaling cascades. For instance, β -arrestin 2 can physically interact with Raf-1 (DeFea *et al.*, 2000b), c-Src (DeFea *et al.*, 2000a) and the MAP kinases JNK (McDonald *et al.*, 2000) and ERK (Luttrell *et al.*, 2001). We sought to investigate which signaling pathway(s) triggered by β -arrestin 2 would lead to RAR β 2 activation. Cells were thus transfected with RAR β 2 and β -arrestin 2, along with constructs encoding dominant negative forms of various signaling proteins. The dominant negative properties and selectivities of these mutants have been documented elsewhere (Burstein *et al.*, 1998; Piu *et al.*, 2002). While some mutants affected the baseline activity of the assay, they did not significantly influence the agonist-dependent activity of the RAR β 2 in response to AM-580 (Figure 2a, see no drug data). Expression of the dominant negative mutants Raf-1(–) and Ras(–) significantly inhibited the effect of β -arrestin 2 on RAR β 2 activity, while no effect was seen with the dominant negative Rac(–) mutant (Figure 2a). Raf and Ras proteins have been extensively linked to MAP kinase signaling (Avruch *et al.*, 2001). We then investigated which MAPK enzyme(s) could mimic β -arrestin 2 potentiation of RAR β 2 activity. Three representatives of the mammalian MAPK cascades were used: the stress-activated protein kinase JNK1, the extracellular signal-regulated kinase ERK2 and the p38 protein kinase (Figure 2b). Neither JNK1 nor p38 could significantly affect RAR β 2 ligand-dependent activity. On the other hand, ERK2 strongly stimulated RAR β 2 activity to an extent comparable to the response observed in the presence of β -arrestin 2. Thus, only the ERK2 kinase, but not JNK1 nor p38, is able to mimic β -arrestin 2 action in potentiating RAR β 2 agonist-dependent activity. This result is compatible with the observation that Raf-1 and Ras dominant negative mutants inhibit the effect of β -arrestin 2, and that expression of Ras can mimic β -arrestin 2 action (Figure 2b). Indeed, Raf-1 and Ras are known activators of the ERK pathway, acting upstream as MAP kinase kinase kinases (MAPKKKs) (Kolch, 2000).

Further, we tested selective protein kinase inhibitors to investigate the contribution of the different MAP kinases in β -arrestin 2-mediated effects. 5-Iodotubercidin and U0126 are standard potent competitive inhibitors of ERK2 (Favata *et al.*, 1998; Fox *et al.*, 1998). SB-203580 and JNK inhibitor II are potent inhibitors of p38 (Cuenda *et al.*, 1995) and JNKs (Bennett *et al.*, 2001), respectively. All compounds display at least a 50–100-fold selectivity for the particular MAP kinase

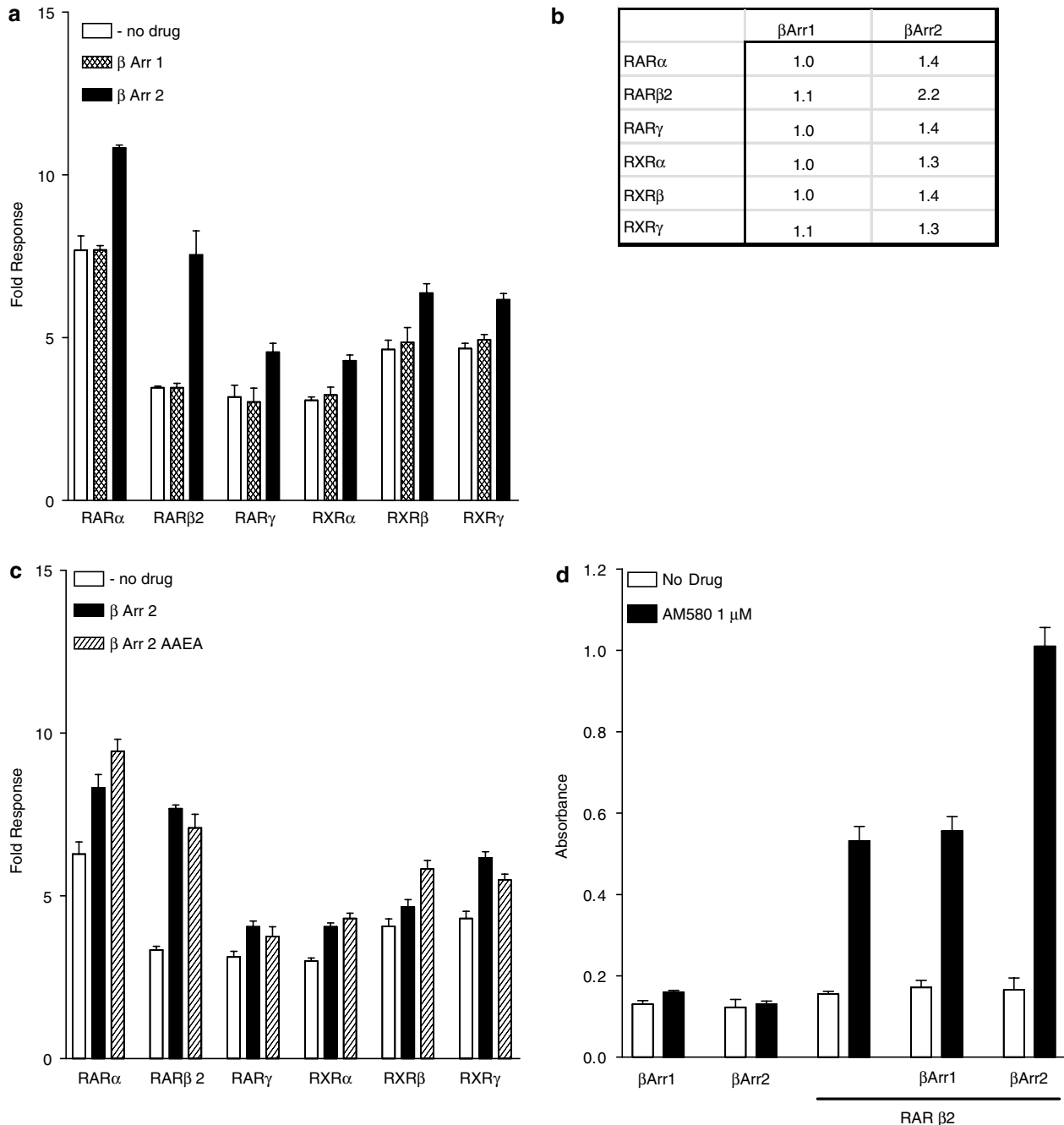


Figure 1 β -Arrestin 2 potentiates the activity of retinoid receptors independently of its ability to promote endocytosis. Cells were transfected with each of the RAR and RXR receptor subunits either alone or in the presence of β -arrestin 1, β -arrestin 2 (a), or the β -arrestin 2 AAEA mutant (c). After overnight transfection, cells were incubated in the presence of the pan-retinoid agonist AM-580 (1 μ M). Nuclear receptor activity was determined by monitoring proliferation using R-SATTM, quantifying the degree of β -galactosidase activity after 5 days. (b) Fold inductions for β -arrestins were normalized for each receptor. The value 1.0 was defined as the activity of the retinoid receptor in the absence of β -arrestins. (d) Cells were transfected with RAR β 2, β -arrestin 1, β -arrestin 2, alone or in combination. Upon transfection, cells were incubated in the presence or absence of 1 μ M AM-580. Activity was determined by measuring absorbance at 405 nm. Experiments were performed in duplicate and data represent the mean \pm s.d. of three independent experiments.

they inhibit, and IC₅₀ against the targeted MAP kinase are in the 20–500 nM range. To rule out any potential cellular toxic effects of the kinases inhibitors at high doses in our system, cells were transfected with the oncogene *c-myc*. In R-SATTM, *c-myc* leads to constitutive

activation of the system. Cells were then exposed to 10 μ M of each kinase inhibitor. None of the kinase inhibitors showed cytotoxic effects at doses 10 times superior to the doses used to test their kinase inhibitory functions (Figure 2d). NIH-3T3 fibroblasts were then

transfected with RAR β 2 and β -arrestin 2, and the effects on the kinase inhibitors were investigated. RAR β 2 activity was not affected by the treatment with SB-203580 nor JNK inhibitor II, indicating that JNK and p38 do not contribute significantly to the transcriptional activation of this nuclear receptor (Figure 2c). Furthermore, β -arrestin 2 stimulation of RAR β 2 activity was not impaired by the JNK or p38 kinase inhibitors. In contrast, both ERK2 inhibitors, 5-iodotubercidin and U0126, partially inhibited RAR β 2 activity in the absence of β -arrestin 2, and completely eliminated the stimulatory action of β -arrestin 2 on RAR β 2. To rule out any nonspecific effects, titration of the ERK2 kinase inhibitors was performed (Figures 2e and f). Dose-dependent effects were seen with IC₅₀ values of 400–600 nM for 5-iodotubercidin and 30–50 nM for U0126, respectively (Fox *et al.*, 1998). Thus, RAR β 2 activity is increased by β -arrestin 2 through activation of an ERK2-dependent pathway.

ERK2 phosphorylates serine and threonine residues through an S/TP motif present on substrate proteins. The tight interaction between the protein kinase and its substrate is dependent upon a short sequence on the substrate known as a docking site (Holland and Cooper, 1999). ERK2 docking sites are usually of the type +_nX_{1–5}LXL where + is a basic residue, X is any amino-acid and L is leucine (Sharrocks *et al.*, 2000). A rapid analysis of the RAR β 2 protein sequence suggested five potential serine and two threonine residues in a configuration compatible with ERK2 phosphorylation sites (Figure 3). A potential docking site for ERK2 was also evident (aa 209–217). While being within the RAR β 2 ligand-binding domain, the docking site is located between the α helices H2 and H3, away from the core-binding pocket (Figure 3). Individual mutants in which each potential phosphorylation site was inactivated through site-directed mutagenesis were generated. Similarly, the critical leucine residues of the potential docking site were mutated. All RAR β 2 Ser/Thr mutants displayed ligand-dependent activation at least as strong as wild-type RAR β 2, with the exception of the mutant S432A (Figure 4a). Interestingly, two RAR β 2 mutants T417A and S444A displayed slightly increased activity when compared to wild type. Most likely, these sites are the target of inhibitory signals. Of the two docking site mutants, only RAR β 2 L217A showed a significant decrease (45%) in its activity when compared to wild-type. Next, we analysed which of the RAR β 2 mutants would retain their responsiveness to β -arrestin 2 (Figure 4b). All mutants, except for S22A and L217A, retained the ability to be potentiated by β -arrestin 2. Thus, β -arrestin 2 promotes RAR β 2 activity through phosphorylation of Ser 22, and also requires the integrity of Leu 217 in the docking site. We then compared the responsiveness of these two RAR β 2 mutants (S22A and L217A) to the wild-type receptor in their capacity to respond to ERK2 stimulation (Figure 4c). RAR β 2 activity was stimulated by ERK2 kinase to an extent similar to β -arrestin 2, whereas the two RAR β 2 mutants S22A and L217A were completely unresponsive to either β -arrestin 2 or

ERK2. The p38 kinase did not potentiate the activity of the wild type nor that of the RAR β 2 mutants S22A and L217A. Thus, our results provide strong evidence that ERK2 stimulates RAR β 2 activity.

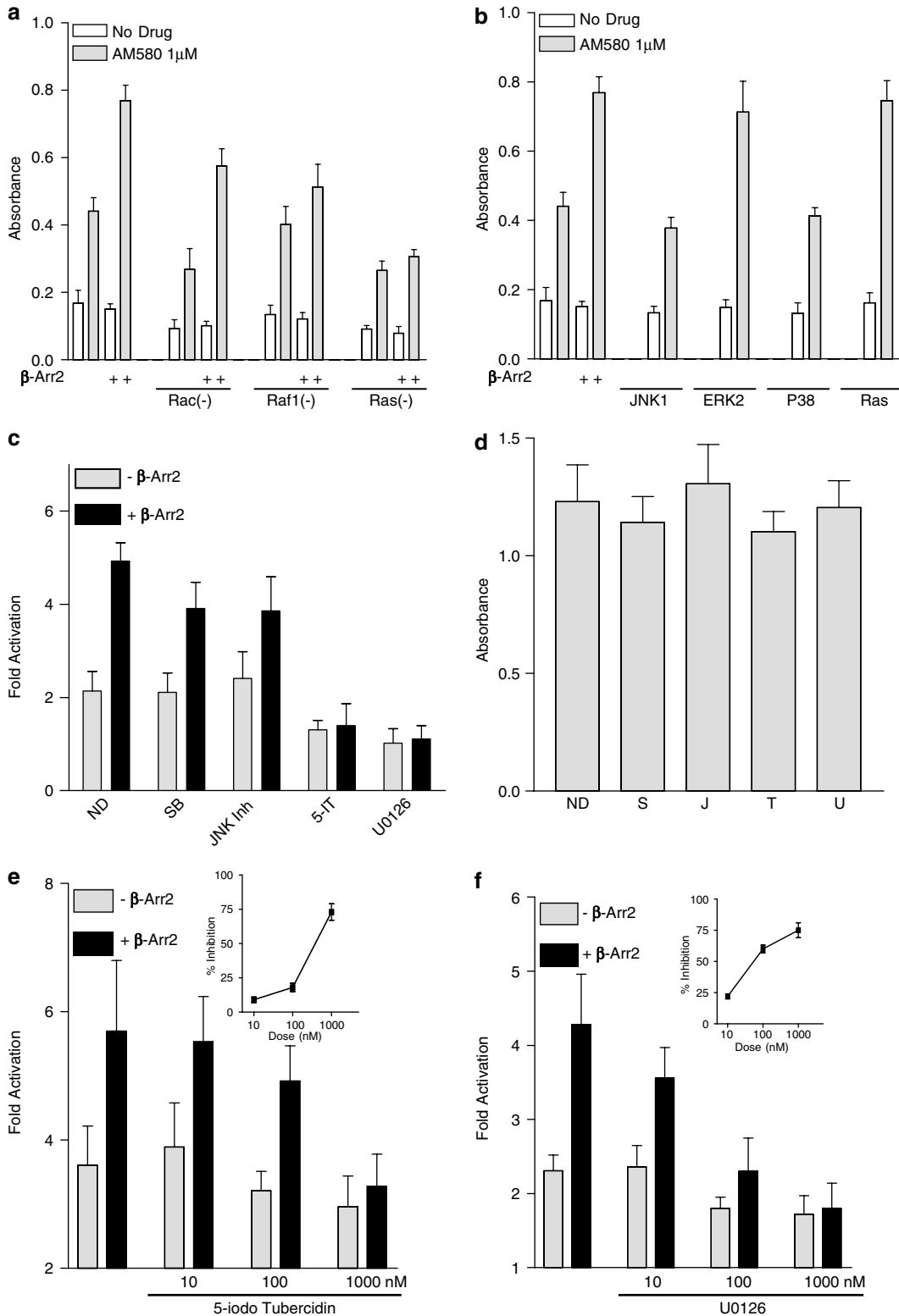
The nature of the interaction between RAR β 2 and ERK2 was further investigated. First, the ability of β -arrestin 2 to phosphorylate Ser 22 of RAR β 2 was assessed using immunoprecipitation techniques (Figure 5a). RAR β 2 is naturally phosphorylated, predominantly at Ser 22 since the S22A mutant is significantly less phosphorylated than its wild-type counterpart. Similarly, the native phosphorylation levels of the mutant L217A are reduced, suggesting that MAP kinases contribute notably to the phosphorylation state of RAR β 2. Expression of β -arrestin 2 leads to a sixfold increase in the phosphorylation levels of RAR β 2, but not of the mutants S22A and L217A. These results suggest that Ser 22 is a major phosphorylation site of RAR β 2 and the target of β -arrestin 2 action, which relies upon the functional MAPK docking site located in the RAR β 2 ligand-binding domain. Second, we sought to investigate whether RAR β 2, β -arrestin 2 and MAP kinases could physically interact. Immunoprecipitation studies of transfected cells with RAR β 2 and MAP kinases indicate that RAR β 2 co-precipitates with ERK2, but not JNK1 nor P38 (Figure 5b). Additionally, similar studies where ERK2, RAR β 2 and β -arrestin 2 were cotransfected indicated a strong physical interaction between ERK2 and β -arrestin 2, but not between β -arrestin 2 and RAR β 2 (Figure 5c). To investigate if ERK2 can directly phosphorylate RAR β 2, protein extracts of cells transfected with RAR β 2 wild type or S22A mutant were incubated *in vitro* with ERK2 and then immuno-precipitated (Figure 5d). Indeed, increasing amounts of ERK2 led to a significant increase in the serine phosphorylation status of RAR β 2 wild type, but not that of the mutant S22A. Finally, the effects of PD 98059, a selective MEK1 inhibitor, on the phosphorylation status of RAR β 2 were assessed. Cells were cotransfected with RAR β 2 wild type or mutant S22A in the presence of β -arrestin 2 then immunoprecipitated following treatment with PD 98059 (Figure 5e). In presence of β -arrestin 2, the serine phosphorylation levels of wild-type RAR β 2 were robust. Treatment with the MEK1 inhibitor PD 98059, upstream of ERK2 in the MAP kinase signaling pathway, decreased significantly serine phosphorylation of wild-type RAR β 2. In contrast, the serine phosphorylation levels of RAR β 2 S22A in the presence of β -arrestin 2 were low and not affected by treatment with PD 98059.

Taken altogether, these studies provide strong and direct evidence that (i) β -arrestin 2 physically interacts with ERK2, (ii) ERK2 directly phosphorylates and interacts with RAR β 2 and (iii) this event is mediated through phosphorylation of Ser22 of RAR β 2, and depends upon the integrity of the docking site.

To investigate the contribution of endogenous β -arrestin 2 to the activity of RAR β 2, a number of experiments using silencing RNAs (SiRNAs) were designed. Following transfection of commercially available selective β -arrestin 2 SiRNAs, protein expression of

β -arrestin 2 was decreased by at least 75% (see Figures 6a, 7c and d). Cells were transfected with high amounts of RAR β 2 in order to maximize the constitutive activity of RAR β 2 (Figure 6a). Increasing doses of the murine selective β -arrestin 2 SiRNAs led to a significant

decrease in β -arrestin 2 protein levels and a concomitant dose-dependent decrease in RAR β 2 activity, indicating that β -arrestin 2 plays a direct role in the basal activity of RAR β 2. Ligand-dependent activation of RAR β 2 by AM580 was not affected by depletion of



endogenous β -arrestin 2 RNAs (Figure 6b). As expected, the murine β -arrestin 2 SiRNAs did not affect the fold activation response seen in the presence of transfected human β -arrestin 1 and β -arrestin 2 (Figure 6b, fold responses). However, the overall apparent response was significantly diminished, indicating a major contribution of β -arrestin 2 to the basal activity of RAR β 2.

Nerve Growth Factor (NGF) promotes the growth arrest and subsequent differentiation of the pheochromocytoma cell line PC12. This cellular model is well

studied and involves activation of the MAP kinase pathway (Rakhit *et al.*, 2001) and of the nuclear receptor RAR β 2 (Cosgaya and Aranda, 2001). In particular, NGF-dependent activation of p42/p44 MAPK relies on β -arrestin and associated clathrin-mediated endocytic signaling. Moreover, Cosgaya and colleagues demonstrated the existence of a Ras-dependent NGF pathway leading to promoter activation of RAR β 2. Based on our evidence that β -arrestin 2 stimulates ERK2 activity, which in turn stimulates RAR β 2 transcriptional activation, we investigated the relevance of our findings in the physiological model of NGF-induced PC12 growth arrest. Cells were transiently transfected with RAR β 2 and β -arrestin 2 alone or in combination. The effect on DNA synthesis was then evaluated in the absence or presence of NGF. As expected, treatment of control PC12 cells with NGF resulted in a strong decrease in DNA synthesis as measured by thymidine incorporation (Figure 7a). In the absence of NGF, expression of RAR β 2 alone slightly decreased DNA synthesis while β -arrestin 2 strongly inhibited thymidine incorporation. These effects were potentiated in the presence of NGF. Interestingly, when both β -arrestin 2 and RAR β 2 were cotransfected, the extent of the inhibition of DNA synthesis was similar to the one seen with untransfected PC12 treated with NGF, suggesting that NGF activates RAR β 2 in a β -arrestin 2-dependent manner. To confirm the ability of β -arrestin 2 to stimulate the transcriptional activity of RAR β 2, reporter gene assays in PC12 cells were performed (Figure 7b). β -Arrestin 2 stimulated RAR β 2 transcriptional activation to the same extent as NGF or retinoic acid (RA). No such activation by β -arrestin 2 (nor NGF) was seen with the phosphorylation-defective mutant RAR β 2 S22A. Furthermore, inhibition of ERK2 kinase activity by U0126 was sufficient to impair RAR β 2 transcriptional activation. To further evaluate the contribution of β -arrestin 2 in NGF-mediated growth arrest of PC12, experiments with SiRNAs were devised. Increased amounts of cotransfected β -arrestin 2 SiRNAs led to an almost complete loss of endogenous β -arrestin 2 protein levels (Figure 7c and d). Depletion of endogenous β -arrestin 2 in PC12 significantly impairs NGF-mediated inhibition of DNA synthesis (Figure 7c) and was the consequence of decreased transcriptional

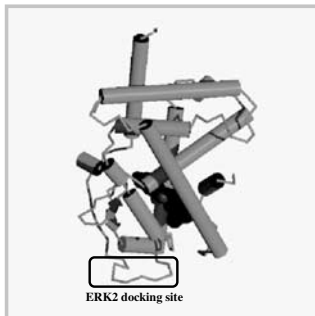
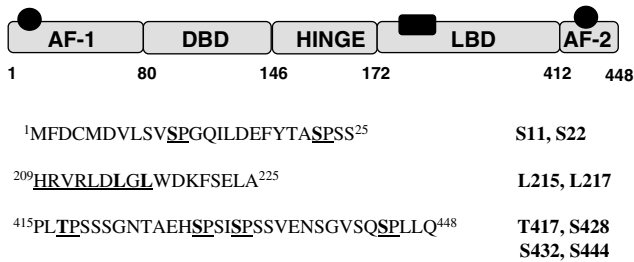


Figure 3 Potential ERK2 phosphorylation and docking sites in RAR β 2. An analysis for the presence of ERK2 phosphorylation sites was performed using the motif S/TP as a consensus for proline-directed Ser/Thr phosphorylation sites. A number of these are apparent throughout the transcriptional activation domains AF-1 and AF-2 of the retinoid RAR β 2 receptor. A similar search was devised to locate potential docking site(s) using the consensus $+_nX_{1-5}LXL$, where $+$ is defined as any basic amino acid, X can be any residue and L is a leucine. Only one potential ERK2 docking site (aa 209–217) was identified, contained in a noncritical region of the ligand-binding domain (LBD). The relative position of the ERK2 docking site in the LBD three-dimensional structure is indicated. It is located between helices H2 and H3, 'below' the core ligand binding pocket and away from any physical interaction with the ligand.

Figure 2 Activation of RAR β 2 by β -arrestin 2 is dependent upon Ras/ERK2. (a) Increasing amounts of the Rac (–), Raf1 (–) and Ras (–) dominant negative mutants were transfected along with expression vectors for RAR β 2 and β -arrestin 2. Cells were then exposed to 1 μ M AM-580 for 5 days, after which the activity was assessed using R-SATTM. Results represent mean \pm s.d. of a representative experiment were performed in triplicate. (b) Fibroblasts were transiently transfected with RAR β 2 alone or along with JNK1, ERK2 or p38 expression vectors. Ras and β -arrestin 2 were used as positive controls. Upon transfection, cells were incubated in the presence of 1 μ M AM-580 for 5 days. RAR β 2 activation was measured using R-SATTM. Data represent the mean \pm s.d. of two independent experiments that was performed in duplicate. (c) Cells were transfected with RAR β 2 encoding vectors with or without β -arrestin 2. Upon transfection cells were incubated for 5 days with 1 μ M AM-580. Activity was evaluated using R-SATTM. Cells were simultaneously treated with a specific P38 kinase inhibitor SB-203580 (SB), a JNK inhibitor JNK Inhibitor II (JNK Inh) or two different ERK2 kinase inhibitors 5-iodo Tubercidin (5-IT) and U0126. A unique dose of 1 μ M was used. (d) To rule cellular toxic effects, cells were transfected with the oncogene *c-myc* and then exposed to 10 μ M of the following kinase inhibitors: SB-203580 (S), JNK inhibitor II (J), 5-iodo tubercidin (T), U0126 (U). Dose–response analyses of 5-iodotubercidin (e) and U0126 (f) were also performed. Activity is presented as fold activation. The percentage inhibition of β -arrestin 2-dependent RAR β 2 activation by 5-iodotubercidin or U0126 is indicated for all three doses tested (insets). Data represent the mean \pm s.d. of two independent experiments performed in duplicate. Activity was determined using R-SATTM, measuring absorbance at 405 nm. ND: no drug.

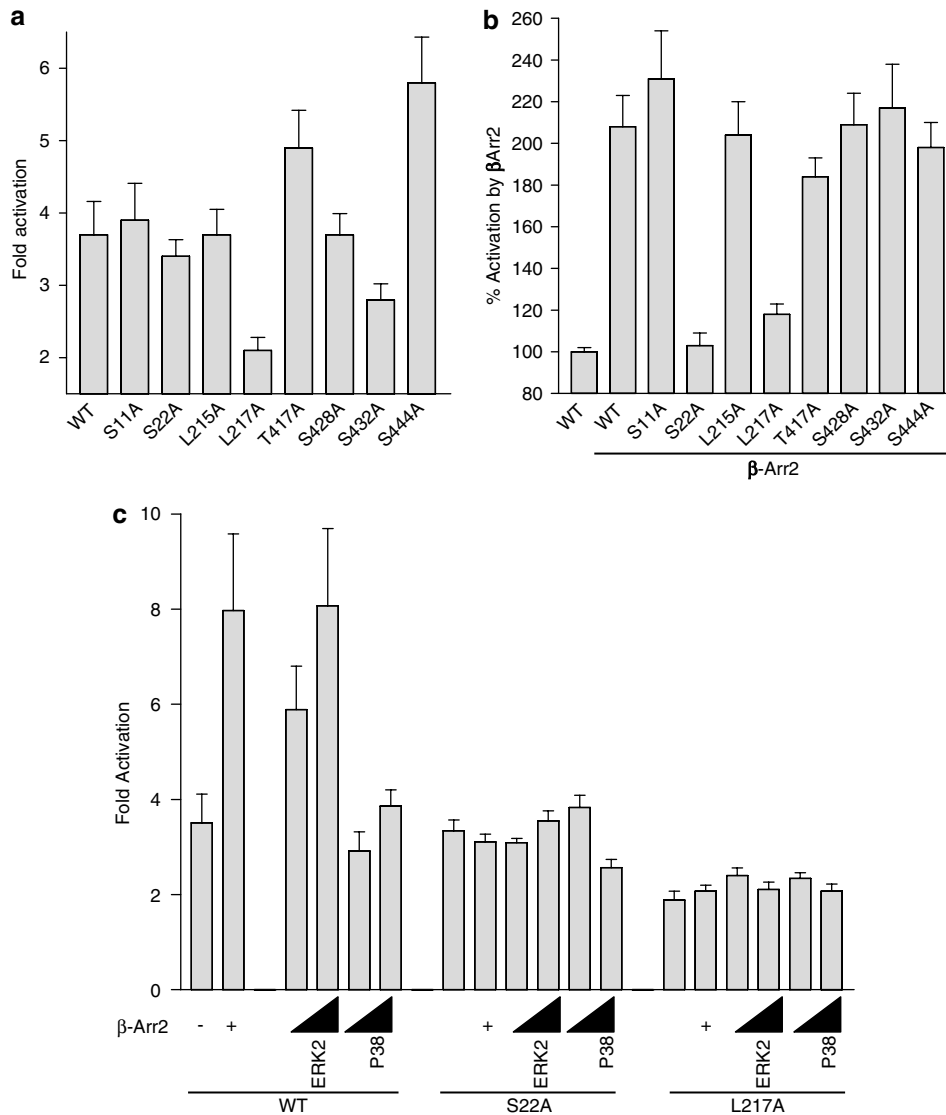


Figure 4 Mutation of S22A and L217A renders RAR β 2 insensitive to β -arrestin 2. Cells were transfected with the different RAR β 2 mutants either in the absence (a) or presence (b) of β -arrestin 2. Transfected cells were incubated for 5 days with 1 μ M AM-580. Activity was assessed by monitoring proliferation using R-SATTM. In (b), activity was normalized to wild-type RAR β 2 in the absence of β -arrestin 2. Data represent mean \pm s.d. of two independent experiments were performed in duplicate. (c) Cells were transfected with RAR β 2 wild type (WT) or the mutants S22A and L217A. Expression vectors for β -arrestin 2, ERK2 or P38 were transfected along. Increasing doses of ERK2 and P38 were used. Cells were thereafter incubated for 5 days with 1 μ M AM-580. Fold activation indicate the activity as measured by R-SATTM. Data are the mean \pm s.d. of two independent experiments performed in triplicate.

activity of RAR β 2 (Figure 7d). Taken together, these results indicate that the physiological effect of NGF on growth arrest and differentiation of PC12 cells is, in part, mediated by direct activation of RAR β 2 transcriptional activity through a β -arrestin 2- and ERK2-dependent pathway.

Discussion

Our results shed new light on the regulation of retinoid receptors, especially RAR β 2, by extracellular stimuli and provide a mechanistic basis for this phenomenon.

We demonstrated that RAR β 2 transcriptional activity is enhanced by β -arrestin 2, a scaffold protein involved in receptor internalization. This event involves the recruitment of the mitogen-activated protein kinase ERK2. Moreover, we demonstrated that the RAR β 2 amino-acid residues Ser 22 and Leu 217 are critical elements to the interaction for ERK2 with the retinoid receptor. Finally, we provided evidence of a physical interaction between ERK2 and RAR β 2, in the context of β -arrestin 2 recruitment.

We uncovered that the activity of retinoid receptor subtypes is significantly enhanced by the constitutive expression of β -arrestin 2 but not that of β -arrestin 1.

Arrestins act as scaffold proteins that couple GPCR activation to MAPK signaling pathways. Moreover, β -arrestins can modulate signaling pathways other than MAP kinases by recruiting, for example, Src family kinase members (Barlic *et al.*, 2000). While structurally similar, β -arrestins differ widely in their specificity towards receptors and signaling pathways. Thus, it is reasonable to assume that the ability of β -arrestin 2 but not β -arrestin 1 to modulate retinoid receptors activity depends on structural features not shared by both proteins. Indeed, a motif responsible for the strong JNK3 signaling was identified in the C-terminus of

β -arrestin 2 (Miller *et al.*, 2001). This motif is not present in β -arrestin 1 and as such β -arrestin 1 is a very weak activator of JNK3 signaling. This further suggests that nuclear receptors can be controlled differently by a number of extracellular signals based upon the preferences of the stimuli to activate various β -arrestin and kinase pathways.

Interestingly, we were able to dissociate the ability of β -arrestin 2 to stimulate retinoid receptors from its role in endocytosis of agonist-bound GPCRs and other cell surface receptors. A β -arrestin 2 mutant with a non-functional clathrin-binding domain (β -Arr2 AAEA) enhanced retinoid receptor transcriptional activity as strongly as the wild-type protein. While indicating that these two functions can be structurally separated, it is likely that, in a physiological situation, β -arrestin 2 activity is increased in response to agonist-dependent endocytosis of surface receptors, leading to downstream activation of retinoid receptors functions. Our results are thus in agreement with published reports that indicate the activity of nuclear receptors, and retinoid receptors in particular, can be significantly affected by extracellular stimuli (Tahayato *et al.*, 1993; Vo *et al.*, 1998; Ishaq *et al.*, 2000).

The results presented herein demonstrate in several ways that activation of MAPK signaling, and especially of the Ser/Thr protein kinase ERK2, is crucial in β -arrestin 2-mediated transcriptional activation of the retinoid receptor RAR β 2. Indeed, we showed that proteins activating ERK2 signaling affect RAR β 2 activity in a similar fashion as β -arrestin 2. Moreover, proteins that do not activate or mediate ERK2 signaling (such as Rac, p38 or JNK) had no effect on RAR β 2 activity. The use of specific protein kinase inhibitors further confirmed the critical requirement of ERK2 kinase in β -arrestin 2-dependent activation of RAR β 2. Various immunoprecipitation studies indicated that RAR β 2 co-precipitates with ERK2, and also that β -arrestin 2 co-precipitates with ERK2, demonstrating

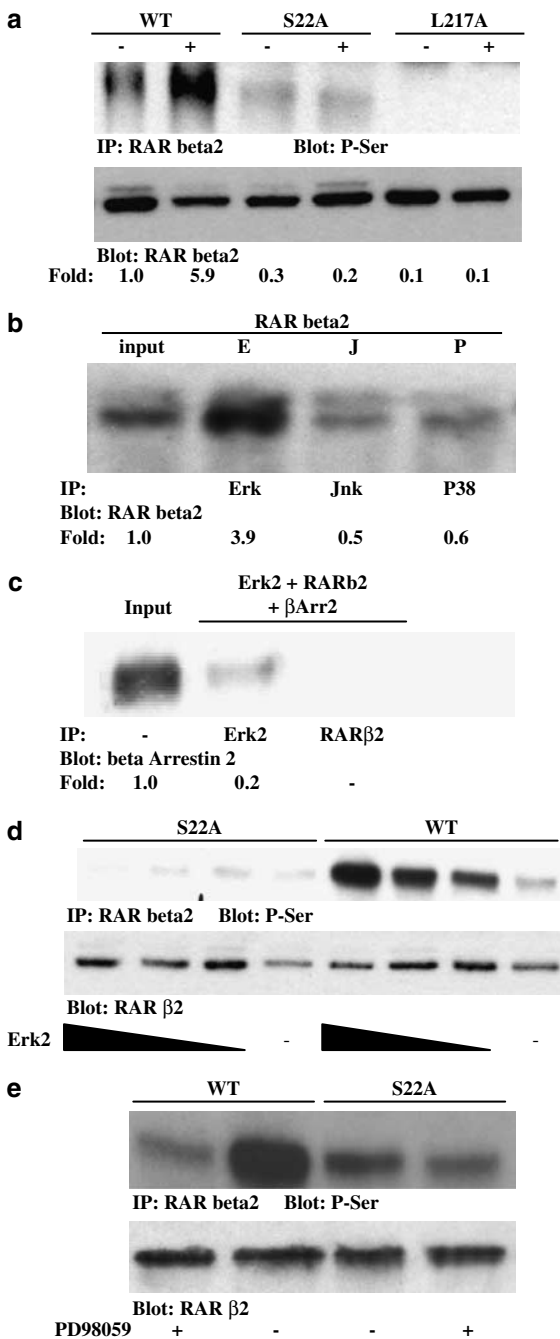


Figure 5 ERK2 phosphorylates serine 22 and physically interacts with RAR β 2. (a) Cells were transfected with RAR β 2 wild-type (WT) or the mutants S22A and L217A with (+) or without (-) ERK2. Protein extracts were harvested and immunoprecipitated (IP) with a specific RAR β 2 antibody. The various immune complexes were then analysed by Western blotting with a phosphoserine antibody. (b) Protein extracts of transfected cells with ERK2, JNK1 or P38 along with RAR β 2-encoding vectors were harvested. Samples were immunoprecipitated with specific antibodies against ERK2, JNK1 or P38. Immunocomplexes were then treated by Western blot using a selective RAR β 2 antibody. Input represents 10% of the whole sample. (c) Cells were transfected with RAR β 2, β -arrestin 2 and ERK2. Protein extracts were immunoprecipitated with specific antibodies against either ERK2 or RAR β 2 and then analysed by Western blotting using a β -arrestin 2 antibody. The input lane constitutes 50% of the sample. (d) Protein extracts of cells transfected with RAR β 2 wild type or mutant S22A were incubated *in vitro* with increasing amounts of ERK2 protein. Samples were then immunoprecipitated with a specific RAR β 2 antibody and blotted with a phospho-serine antibody. (e) Cells were transfected with RAR β 2 and β -arrestin 2 and then treated with PD 98059 (1 μ M). Protein extracts were harvested, immunoprecipitated with an RAR β 2 antibody and then analysed by Western blotting using a selective phospho-serine antibody.

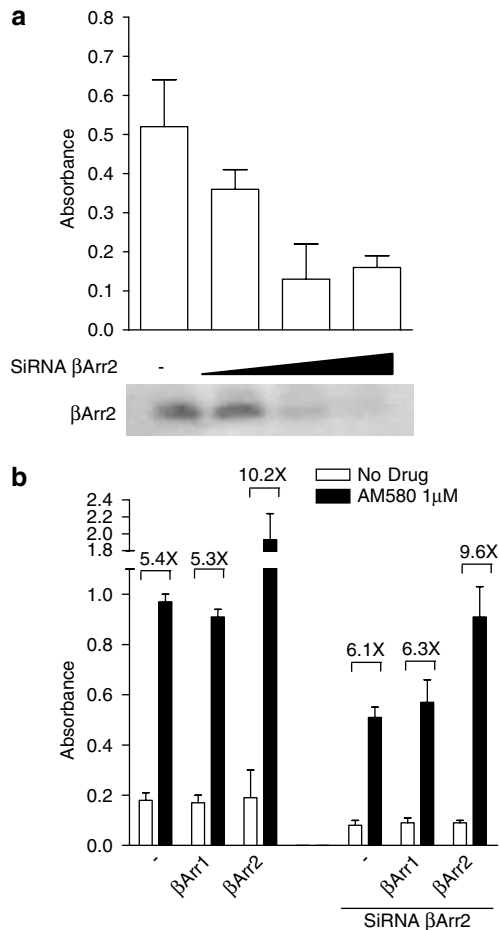


Figure 6 Endogenous β -arrestin 2 mediates RAR β 2 constitutive activity. (a) Cells were transfected with high amounts of RAR β 2 (30 ng/well) and increasing amounts of murine β -arrestin 2 siRNAs (30, 60 and 120 pmol). Upon transfection, cells were incubated for 5 days. RAR β 2 activation was measured using R-SATTM. Data represent the mean \pm s.d. of three independent experiments performed in duplicate. (b) Cells were transfected with regular amounts of RAR β 2 in the presence or absence of β -arrestin 1 and β -arrestin 2, as well as with or without murine β -arrestin 2 siRNAs (60 pmol). Cells were further incubated for 5 days with AM580 (1 μ M). R-SATTM data represent the mean \pm s.d. of three independent experiments carried out in duplicate. The fold activation (AM580/No Drug) is indicated for each condition set.

physical interaction between these three components. More importantly, we also showed that ERK2 directly phosphorylates RAR β 2 *in vitro*.

A number of potential ERK2 phosphorylation sites were identified in RAR β 2. All Ser/Thr residues seem confined to either the AF-1 or AF-2 domains. Both regions are involved in transcriptional activation in a ligand-independent and dependent manner, respectively. Moreover, the presence of a potential MAPK docking site was identified in the ligand-binding domain, spanning aa 209–217. Disruption of Leu 217, one of the two leucine residues critical to the integrity of the docking site, impaired RAR β 2 ligand-dependent transcriptional activity quite significantly. It could be that this residue is important to the conformation of the ligand-binding pocket and its interaction with the

ligand. However, when compared to the crystal structure of other retinoid receptors bound to their ligands (Klaholz *et al.*, 1998; Bourguet *et al.*, 2000; Egea *et al.*, 2000), Leu 217 and the docking site containing it are located between helices H2 and H3 of the LBD. The ligand-binding pocket is formed of 12 α helices but none of the residues defining the MAPK docking site have been reported to involve interaction with the ligand (Klaholz *et al.*, 2000). Moreover, the substitution from leucine with alanine is a relatively minor one from a steric perspective and as such should not affect the overall three-dimensional state of the ligand pocket. Moreover, the position of Leu 217 appears clearly outside of the core ligand-binding pocket (Figure 4), as defined by various crystal structure studies (Renaud *et al.*, 1995; Wurtz *et al.*, 1996; Ostrowski *et al.*, 1998). Thus, the decrease seen in the overall RAR β 2 activity in the L217A mutant stems most likely from the loss of a functional docking site rather than a lack of binding of the agonist. Interestingly, this docking site appears to be conserved among the RAR subtypes and among different species as well. However, we could not identify a similar motif in the RXR receptors even though it has been reported that RXR receptors activity can be modulated by MAP kinases (Adam-Stitah *et al.*, 1999; Lee *et al.*, 2000).

The potentiating action of β -arrestin 2 on RAR β 2 activity, through the ERK2 kinase, is associated with the presence of Ser 22. For instance, we demonstrated that the mutant S22A is phosphorylation defective with regard to β -arrestin 2 treatment and that ERK2 specifically phosphorylates RAR β 2 at the Ser 22 amino acid. This residue does not appear to be particularly conserved among the RAR or RXR receptors. Interestingly though, similar to RAR β 2, a number of potential phosphorylation sites (based upon the proline-directed S/TP motif) are present in the AF-1 domain of all RAR and RXR subtypes. The physiological importance of Ser 22 in the RAR β 2 receptor is also evident from its strong conservation throughout evolution. Indeed, a rapid search of the GenBank database revealed that Ser 22 is conserved among chicken, hamster, rat, mouse and human species.

Our findings also emphasize that the ligand-independent transcriptional activity of RAR β 2 is modulated by the expression levels of β -arrestin 2. Depletion of endogenous β -arrestin 2 by transfection of selective siRNAs is associated with a significant decrease in RAR β 2 constitutive activity. This underlies the fact that RAR β 2 constitutive activity is largely a consequence of β -arrestin 2 activation and, by extension, highly dependent upon extracellular stimuli. The ligand-independent activity constitutes a significant percentage of the overall activity potential of RAR β 2. Since β -arrestins are broadly distributed, it follows that the modulation of RAR β 2 constitutive activity by extracellular stimuli is probably a very common occurrence.

The physiological relevance of our findings is especially evident in the context of NGF-induced growth arrest and subsequent differentiation of PC12 cells. Previous reports have emphasized the ability of the

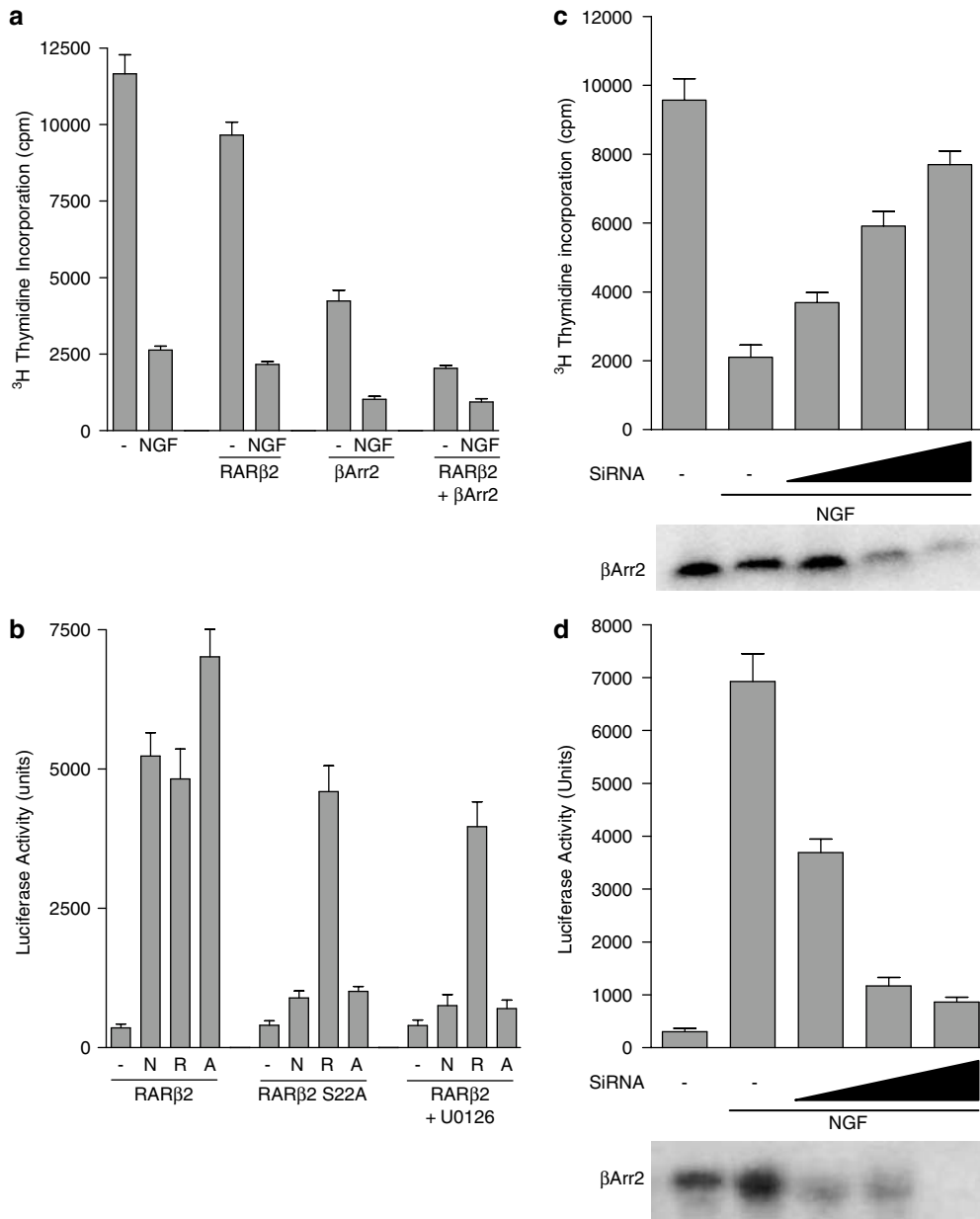


Figure 7 β -Arrestin 2 stimulates RAR β 2 transcriptional activity in NGF-induced growth inhibition of PC12. (a, c) PC12 cells were transfected with RAR β 2 and (a) β -arrestin 2 alone or in combination or (c) with increasing amounts of β -arrestin 2 SiRNAs (30, 60 and 120 pmol). Radiolabeled thymidine accumulation was measured for 24 h, a marker of DNA synthesis, in the absence or presence of NGF (50 ng/ml). Results are expressed in cpm of [^3H]thymidine incorporation ($n=3$). (b, d) PC12 cells were transfected with (a) expression vectors for RAR β 2 wild type and S22A, β -arrestin 2, or with (d) increasing amounts of β -arrestin 2 SiRNAs (30, 60 and 120 pmol) in the absence or presence of NGF (50 ng/ml) or RA (1 μM). A 3X-DR1-Luc was used as the reporter gene for transcriptional activation of RAR β 2. ERK2 inhibitor U0126 was used at 1 μM . Data are expressed in arbitrary luciferase units (Avg. \pm s.d., $n=3$). N = NGF, RA = retinoic acid, A = β -arrestin 2.

receptor tyrosine kinase TrkA, the cognate receptor for NGF, to activate ‘GPCR-like’ pathways leading to β -arrestin recruitment and subsequent MAPK activation (Rakhit *et al.*, 2001). This Ras–Raf-dependent signaling pathway leads to promoter activation of RAR β 2 as well as synthesis of retinoic acid, the cognate ligand for RAR β 2 (Corcoran and Maden, 1999; Cosgaya and Aranda, 2001). These events are dependent upon cooperation between NGF and RA. Our results fit

well within this differentiation model, but add another layer of complexity. Indeed, we established that, in addition, NGF can directly stimulate retinoid receptors transcriptional activity (RAR β 2) through a β -arrestin 2/ERK2 pathway ultimately leading to PC12 cells differentiation. This event is essential to NGF-mediated differentiation as depletion of endogenous β -arrestin 2 through selective SiRNAs completely impaired DNA synthesis inhibition of PC12 cells by NGF. It follows

that a parallel and intricate system of regulation is in place: NGF triggers β -arrestin 2 activation, which on the one hand, through classical internalization of agonist-activated GPCRs, leads to increased RAR β 2 expression and synthesis of its cognate ligand retinoic acid, and on the other hand, through direct stimulation of MAP kinase, leads to transcriptional activation of RAR β 2.

Interestingly, our experimental data indicate that, depending on the cellular context, transcriptional activation of RAR β 2 by β -arrestin 2 can lead to different end points: proliferation in NIH-3T3, growth arrest in PC12 cells. Such differential effects of retinoid receptors have been previously described (for review see Ahuja *et al.*, 2003; Lefebvre *et al.*, 2005). Several explanations can be proposed to explain these opposite outputs. For instance, in PC12 cells, sustained activation of ERK2 kinase is required in order to trigger NGF-mediated growth arrest. Thus, differences in kinetics and intensities of activation can result in different physiological outcomes. Also RARs are known to heterodimerize with RXRs for functional activation and as such the RXR partner could influence the nature of the target genes recognized by this transcriptional complex. Further, transcriptional activation of nuclear receptors is dependent upon the recruitment of a variety of coactivators and corepressors, which can display tissue selectivities. Finally, β -arrestin 2 integrates receptor-mediated extracellular stimuli responses, which vary depending on the cell type. Taken together, these various elements contribute to defining the ultimate nature of the cellular output.

Our findings provide for a more general basis of modulation of retinoid receptors activity, and other nuclear receptors, by extracellular stimuli. In particular, it appears that β -arrestins and their ability to link surface receptors such as GPCRs and RTKs to MAPK signaling are critical effectors of the transcriptional activation of nuclear receptors. For instance, induction of cellular stress by ultraviolet irradiation inhibits ligand-induced transcription of RXR-RAR target genes (Wang *et al.*, 1999). Peptide growth factors block the biological effects of retinoids by inhibiting the activity of the RAR and RXR receptors (Miller *et al.*, 1993; Andreatta-Van Leyen *et al.*, 1994). Interestingly, other studies involved insulin like growth factors (IGFs) and their interaction with retinoid signaling. Receptors for IGFs have been shown to display alternative signaling properties through the recruitment of and interaction with effector molecules of the GPCR pathways (Dalle *et al.*, 2001). Moreover, the potentiation of nuclear receptors activity by a number of GPCRs and tyrosine kinase receptors (RTKs) has been reported. For example, transactivation of the glucocorticoid receptor is increased by the β -adrenergic receptor (Schmidt *et al.*, 2001) as well as by the IGF-1 and FGF receptor tyrosine kinases (Koutsilieris *et al.*, 1997; Schmidt *et al.*, 2001). Our findings establish such a physical link by demonstrating that β -arrestin 2 enhances retinoid receptors ligand-independent transcriptional activity through recruitment of the ERK2 kinase in response to extracellular stimuli.

Materials and methods

Constructs

Retinoid receptor subunits were cloned by PCR using cDNAs from various human tissues. The sequences of primers used were: RAR α 5' CTTCTGACTGTGGCCGCTTG, RAR α 3' GCCTCTGTCCAAGGAGTCGTG, RAR β 5' GCACTTTGCGACATTTCAG, RAR β 3' ATCCTGGAACCTGAAGGTAC, RAR γ 5' GAAGACCTCGCCCGCCCACTG, RAR γ 3' CTCATTGGAAGGGGTGGGG, RXR α 5' GGCCGGCATGAGTTAGTCG, RXR α 3' GAGAAGAACAGCTGGCGTCCAG, RXR β 5' GGGAAATCCCTGGGACCTCTTCG, RXR β 3' GCTCCTCCAGTGTGGAGAA, RXR γ 5' GAGAGGAACATGAACTGACG, RXR γ 3' GCATCACATTTTGGGGACAG. PCR fragments were cloned into PCR2.1 TOPO (Invitrogen) and then subcloned into mammalian expression vectors. All constructs were sequence verified. Single point mutants of the RAR β 2 receptor (S11A, S22A, L215A, L217A, T417A, S428A, S432A, S444A) were generated using the Quick Change site-directed mutagenesis procedure according to the manufacturer's instructions (Stratagene). Similarly, the β -arrestin 2 mutant AAEA was created. Other constructs have been described (Piu *et al.*, 2002).

Materials

AM-580 and RA were purchased from Calbiochem. The kinase inhibitors 5-iodotubercidin, JNK inhibitor II, U0126, SB-203580 and PD98509 were from Calbiochem and Biomol. NGF was purchased from Peprotech. All ligands were stored in DMSO as 10 mM stock solutions. siRNAs targeting β -arrestin 2 were purchased from Dharmacon. Antibodies were purchased from Santa Cruz.

R-SATTM assays

R-SATTM (Receptor Selection and Amplification Technology) is a proprietary cell-based functional assay that allows one to monitor receptor-dependent proliferative responses and has been described elsewhere (Piu *et al.*, 2002). The technology has been validated for a number of receptors ranging from GPCRs (Brauner-Osborne and Brann, 1996), RTKs (Burstein *et al.*, 1998) and cytokine receptors (Piu *et al.*, 2002). Its principle resides in the genetic selection and amplification of the nuclear receptors in a ligand-dependent manner. This process is achieved by partial cellular transformation through the overcoming of contact inhibition and the loss of growth factor dependency. Monitoring is achieved by transfecting the cells with a β -galactosidase reporter gene vector whose expression is under a constitutively active promoter. Briefly, NIH-3T3 fibroblasts were plated overnight in 96-well plates in DMEM 10% calf serum (Gibco BRL) and grown to 60–70% confluency prior to transfection. Transient transfections were performed using Superfect or Polyfect (Qiagen) according to the manufacturer's instructions. Typically, a transfection mix would consist of the receptor and the β -galactosidase expression vectors. At 16 h post-transfection, cells were incubated with different doses of ligand in DMEM containing 2% CytoSF3 (Kemp Technologies) to generate a dose response curve. After 5 days, plates were developed by adding onto the washed cells a solution containing the β -galactosidase substrate *o*-nitrophenyl-*d*-galactopyranoside (ONPG; in phosphate-buffered saline with 5% Nonidet P-40 detergent) as described (Piu *et al.*, 2002). Plates were read using a microplate reader at 405 nm. Data from R-SATTM assays were fit to the equation $r = A + B(x/(x + c))$, where A is the minimum response, B the maximum response minus minimum response, c is EC₅₀, r is response and x is the concentration of ligand. Curves were

generated using the curve fitting softwares Excel Fit and GraphPad Prism (San Diego, CA, USA).

Immunoprecipitation and Western blot assays

Serum-starved NIH-3T3 cells were scraped off plates following treatment, spun down, and resuspended in lysis buffer (25 mM HEPES, 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5% Triton and a protease inhibitor cocktail). Cell extracts (50 μ g) were precleared with serum, incubated with protein A/G sepharose and a selective antibody for 2 h, and then washed extensively. Immune complexes were then separated on denaturing polyacrylamide gels by SDS-PAGE and the proteins blotted onto Immobilon-P membranes. Western blotting was performed as described (Piu *et al.*, 2002).

DNA synthesis assays

Six-well plates were seeded at 50 000 PC12 cells/plate. The next day, cells were transfected using Polyfect (Qiagen) according to the manufacturer's protocol. At 16 h following transfection,

cells were incubated with [³H]thymidine (0.5 μ Ci/ml) for 24 h in the presence or absence of NGF (50 ng/ml). Cells were then harvested, TCA precipitated and DNA incorporation measured.

Transient expression assays

Briefly, 50 000 PC12 cells were plated per 60 mm plate. The next day, cells were transfected using Polyfect (Qiagen) according to the manufacturer's recommendations. At 16 h post-transfection, cells were incubated in the presence of the appropriate ligands for 24 h. Cells were then assayed for luciferase activity as previously described (Piu *et al.*, 2002).

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