Rapid, non-genomic actions of Retinoic Acid on Phosphatidyl-Inositol-3-Kinase signaling pathway mediated by the Retinoic Acid Receptor

Short title: **RA-induced activation of PI3K**

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Disclosure Statement: The authors have nothing to disclose.

ABSTRACT

Retinoic Acid (RA) treatment of SH-SY5Y neuroblastoma cells results in activation of phosphatidyl-inositol-3-kinase (PI3K) signaling pathway, and this activation is required for RA-induced differentiation. Here we show that RA activates PI3K and ERK1/2 MAP Kinase signaling pathways through a rapid, non-genomic mechanism that does not require new gene transcription or newly synthesized proteins. Activation of PI3K by RA appears to involve the classical nuclear receptor RAR, on the basis of the pharmacological profile of the activation, loss and gain of function experiments with MEF-RAR($\alpha\beta\gamma$)$^{L-/L-}$ null cells, and the physical association between liganded RAR and PI3K kinase activity. The association of RAR with the two subunits of PI3K was differentially regulated by the ligand. Immunoprecipitation experiments performed in SH-SY5Y cells showed stable association between RARα and p85, the regulatory subunit of PI3K, independently of the presence of RA. In contrast, ligand administration increased the association of p110, the catalytic subunit of PI3K, to this complex. The intracellular localization of RAR resulted to be relevant for PI3K activation. A chimerical RAR receptor fusing c-*Src* myristylation domain to the N-terminal of RARα (*Myr*-RARα) was targeted to plasma membrane. Transfection of *Myr*-RARα to MEF-RAR($\alpha\beta\gamma$)$^{L-/L-}$ null cells and COS-7 cells results in strong activation of PI3K signaling pathway, although both in the absence as well in the presence of RA. Our results support a mechanism in which ligand binding to RAR would play a major role in the assembly and intracellular location of a signaling complex involving RAR and the subunits of PI3K.

Keywords: **RAR, nuclear hormone receptors, RA, Retinoic Acid, neuroblastoma, PI3K, non-genomic actions**

INTRODUCTION

Retinoic Acid (RA), the biologically active form of Vitamin A, plays a major role as physiological regulator of important biological processes, as the early embryonic development, the development of certain organs and systems, etc. (1). RA is an important signaling molecule for the development of the nervous system, especially by controlling proliferation and differentiation of neural cells (2, 3). The actions of RA and its derivatives are mediated by two types of receptors, RARs and RXRs, belonging to the Nuclear Hormone Receptor superfamily, which encompasses receptors for steroid and thyroid hormones, vitamins A and D, and many other signaling molecules (4). Nuclear hormone receptors have been traditionally considered as ligand-regulated transcription factors, acting through binding to specific DNA elements on the promoters of target genes and ultimately leading to changes in the expression levels of those genes. However, novel non-genomic mechanisms of signal transduction through nuclear hormone receptors have been described. These rapid, non-genomic actions do not rely on gene transcription or protein synthesis, but involve ligand-induced modulation of signal transduction pathways (5-8).

Administration of RA to neuroblastoma cells *in vitro* leads to proliferative arrest and neuronal differentiation (9-11). These results prompted the introduction of RA and its derivatives into therapeutic protocols for neuroblastoma patients, with success especially in the treatment of minimal residual disease (12, 13). In a previous paper we have described that RA treatment of SH-SY5Y neuroblastoma cells results in activation of phosphatidyl-inositol-3-kinase (PI3K) signaling pathway, and this activation is required for RA-induced differentiation (14). Although the mechanisms by which RA regulates gene transcription are well understood, very little is known about the molecular mechanisms underlying the activation of PI3K/Akt signaling pathway by RA. We report here that activation of PI3K by RA occurs through a non-genomic action of the *classical* nuclear RA receptor RAR. In contrast to what has been reported for steroid receptors, where interaction of the receptor with the regulatory subunit of PI3K (p85) was under the control of ligand, (15-17) we show that RAR forms a stable complex with p85-PI3K. Activation of PI3K by ligand-bound RAR is mediated by a novel mechanism in which the binding of RAR by its cognate ligand results in the formation of a signaling complex by recruiting the catalytic subunit of PI3K. In addition, the experiments shown here suggest a role for RA in regulating the intracellular location of that complex including RAR and the two subunits of PI3K.

RESULTS

Rapid, non-genomic activation of PI3K and ERK1/2 MAP kinase pathways by RA.

Administration of RA to SH-SY5Y neuroblastoma cells results in activation of PI3K signaling pathway (14). When this phenomenon was analyzed in its right timeframe, we observed that RA rapidly activated PI3K signaling pathway, and specific phosphorylation of Akt kinase in Ser_{473} could be detected within 5 min of RA treatment (Fig. 1A). Rapid activation of PI3K signaling pathway by RA argued for an atypical, non-genomic activation event and against a classical transcriptional action of RA. In fact, RA treatment could induce phosphorylation of Akt kinase in the absence of newly synthesized proteins in Cycloheximide-treated cells as well in the absence of new transcription in Actinomycin D-treated cells (Fig. 1B). Activation of signaling pathways by RA is not limited to PI3K/Akt, but also the ERK1/2 MAP Kinase pathway was rapidly activated in RA-treated neuroblastoma cells, as detected by western blot with phosphorylation-specific ERK1/2 antibodies (Fig. 1C).

Rapid activation of signaling pathways by RA appeared not restricted to neuroblastoma cells. Mouse embryo fibroblast NIH-3T3 cells expressed considerable amounts of RARγ (Fig. 2A), and responded to RA treatment by activating PI3K (Fig. 2B) and ERK1/2 (Fig. 2D) signaling pathways (see also (18)). Activation of PI3K signaling pathway in response to RA treatment occurred both in serum-deprived NIH-3T3 cells (Fig. 2B), as well in cells growing in the presence of 10% newborn calf serum (Fig. 2C). Although in serum-fed cells Akt phosphorylation basal

levels were higher than in serum-starved cells, RA treatment resulted in increased Akt phosphorylation (Fig 2C). As was the case for neuroblastoma cells, activation of PI3K by RA in NIH-3T3 cells was not prevented by Cycloheximide or Actinomycin D treatments. As expected, RA-induced phosphorylation of Akt was impaired by treatment with the specific PI3K inhibitor LY294002 (Fig. 2C).

Involvement of nuclear receptor RAR in RA-induced rapid non-genomic activation of PI3K.

The first type of evidence supporting the involvement of the nuclear RA receptor RAR in RA-induced non-genomic actions came out from the pharmacological features of the activation of PI3K by RA in neuroblastoma cells. First, the concentrations of RA required for activation of PI3K signaling pathway (as low as 10 nM, see Fig. 3A) are compatible with the observed Kd of RARs for RA in the nanomolar range. (19) Second, all the different RAR ligands tested up to now, including RAR-specific synthetic retinoids, have been able to activate PI3K similarly to all trans-RA. As shown in Figure 3B, both natural and synthetic RAR-selective agonists (*all-trans*-RA, *13-cis*-RA, TTNPB, Am580, 4-HPR, and ALRT1550) as well as pan-RAR agonists (*9-cis*-RA and LG 100567) induced phosphorylation of Akt in Ser_{473} (Fig. 3B). Next, we tested whether the activation of PI3K signaling pathway by RA or a RAR-selective agonist like TTNPB could be prevented by the pan-RAR antagonist AGN193109 (20). Surprisingly, treatment of cells with AGN193109 (5 μM) could not block the activation of PI3K by RA or TTNPB (0.5 μM). Moreover, AGN193109 itself act as an agonist and activated Akt phosphorylation (Fig. 3C). However, in the same concentrations AGN193109 prevented RA-induced transcriptional activation of *RARB*, one of the prominent RA–regulated genes in neuroblastoma cells, as shown by Northern Blot hybridization (Fig. 3D). Therefore, AGN193109 showed a dissociated antagonist/agonist profile, having a potent transcriptional antagonistic activity, but acting as an agonist in respect to PI3K activation.

The second type of evidence supporting the involvement of nuclear RA receptor RAR in the activation of PI3K was obtained by loss and gain of function experiments. Cultured mouse embryo fibroblasts from wild-type animals (MEF-wt cells) responded to RA treatment by activating PI3K/Akt pathway (Fig. 4A). However, activation of PI3K/Akt pathway by RA was abolished in a mouse embryo fibroblast cell line devoid of all the tree RAR isoforms, named MEF(RARαβγ)$^{L-/L-}$ (21) (Fig. 4B). Re-introduction of a functional RARα gene through a retroviral expression vector resulted in restoration of RA-induced PI3K/Akt activation (Fig. 4C).

A third type of evidence supporting the involvement of RAR in RA-induced activation of PI3K/Akt signaling pathway was obtained by assaying PI3K activity on immunoprecipitated fractions of control and RA-treated SH-SY5Y cells (Figure 5). Anti-RARα immunoprecipitated fractions were assayed together with anti-PI3K-p110 immunoprecipitates as positive control and control IgG immunoprecipitates as negative control. PI3K activity could be detected associated to RARα immunocomplexes only in RA-treated cells, whereas no activity above background levels was detected in RARα-immunoprecipitated fractions from untreated cells.

All these experiments together strongly support a role for the classical RA nuclear receptor RAR in rapid, non-genomic activation of PI3K signaling pathway by RA.

Ligand differentially regulates the association between RAR and PI3K subunits

The kinase assays performed on immunoprecipitates shown in Figure 5 suggest the idea of a physical association between RAR and PI3K that could be regulated by ligand binding to RAR, and prompted us to analyze the interactions of the PI3K subunits with RAR in SH-SY5Y neuroblastoma cells. Surprisingly, immunoprecipitation experiments followed by Western blot detection demonstrated the existence of a stable physical interaction between RARα and p85 regulatory subunit of PI3K in SH-SY5Y neuroblastoma cells. Antibodies against RARα immunoprecipitated p85-PI3K from SH-SY5Y cell extract, but not an irrelevant antibody used as control (Fig. 6A). Conversely, a p85-PI3K

antiserum immunoprecipitated RARα (Fig. 6B). However, association of p85 and RARα was observed independently of the presence of RA (Fig. 6A and 6B). This association could be detected in the cell nucleus, as demonstrated by immunoprecipitation experiments carried out in nuclear extracts (Fig. 6C). In contrast, RA-induced association of p110-PI3K to RARα was observed in immunoprecipitates from RARα antibodies (Fig. 6A). Immunoprecipitation experiments with an antibody against p110-PI3K showed association between p110, p85 and RARα, and ligand binding increased notably complex formation between p110 and p85 and RARα (Fig. 6D). Taken together, the results of the immunoprecipitation experiments support the idea of a stable complex between RARα and p85, the regulatory subunit of PI3K. Ligand binding to RAR would facilitate the formation of a signaling complex, by increasing the association of the p110 catalytic subunit of PI3K.

We wanted to know whether a direct protein-protein interaction determines the association between RAR and p85-PI3K. This was tested *in vitro* by GST pull-down assay, using recombinant GST-RARα fusion protein expressed in *E. coli* and extracts from cells overexpressing p85-PI3K tagged with the HA epitope. As shown in Figure 7A, binding of p85-PI3K to GST-RARα could not be detected, independently of the presence of RA. As positive control experiment, the assay readily detected the interaction of GST-RARα and RXRα, which are known to form heterodimers *in vitro* (22). Consistently with that, immunoprecipitation experiments performed after co-transfection of expression plasmids for RARα and p85α-HA into COS-7 cells did not resulted in the formation of a binary complex (Fig. 7B). Although most of p85-PI3K was in the precipitated fraction, RARα was recovered quantitatively from the supernatant of the immunoprecipitation. Taken together, the results pointed out that the interaction between RAR and p85-PI3K is not direct. Therefore, other yet unknown proteins mediating or stabilizing RAR-p85 interactions may be involved in complex formation, which may became limiting in overexpression experiments.

Influence of RAR intracellular localization on RA-induced PI3K activation.

A paradox arises from the fact that PI3K activity takes place at the plasma membrane, whereas RAR, as other members of the nuclear receptor superfamily, is located mainly in the nucleus. Therefore, intracellular localization of RAR might be relevant for RA-induced activation of PI3K. To address this point, we have examined whether ectopic localization of RAR at plasma membrane would affect the activation of PI3K by RA. For this purpose we have constructed a chimerical receptor fusing c-*Src* myristylation domain (23) to the N-terminus of RARα (*Myr*-RARα). We have overexpressed this chimerical *Myr*-RARα and wild-type RARα in NIH-3T3 cells through retroviral vectors, and examined their intracellular localization in conventional cell fractionation experiments. The results showed that most of the *Myr*-RARα receptor could be located in the nuclear fraction, as was the case for wild-type RARα. However, considerable amounts of *Myr*-RARα could be detected in the microsomal fraction and in purified plasma membrane, whereas wild-type RARα was not detected in membrane fractions (Fig. 8A). In detergent-partition experiments *Myr*-RARα receptor could be detected in the detergent-resistant fraction, whereas wild type RARα could not (data not shown). As compared to those of wild type RARα, the levels of *Myr*-RARα receptor were strongly increased in detergent-resistant membrane microdomain fractions (lipid rafts), co-migrating with caveolin 1 in sucrose gradients (Fig. 8B). Therefore, these experiments demonstrated that *Myr*-RARα receptor shows increased presence in plasma membrane fractions, as compared to wild-type RARα.

The ability of *Myr*-RARα receptor to activate PI3K signaling pathway was tested in retrovirally transfected MEF(RARαβγ)$^{L-/L-}$ cells. As compared to wild-type RARα, *Myr*-RARα was a potent activator of PI3K in the absence of RA, and surprisingly the addition of RA did not further increased PI3K activity (Fig. 8C). Similar results were obtained in transient transfection experiments in COS-7 cells (Fig. 8D). These results showed that the presence of the receptor at the plasma membrane results in increased activation of

PI3K, although in those conditions binding of the ligand appears to be dispensable.

The above results are compatible with a model in which the binding of the ligand may play a role in the localization of RAR to the plasma membrane, allowing there interactions with components of the signal transduction machinery leading to PI3K activation. To test that hypothesis we have examined the intracellular location of RAR. Attempts to show the presence of RAR in plasma membrane from SH-SY5Y or NIH-3T3 cells by immunofluorescent confocal microscopy with a variety of commercially available anti-RAR antibodies failed (data not shown). A Green Fluorescent Protein-tagged RARα version (GFP-RARα) was generated, and its expression vector was transiently or stably transfected to COS-7 cells. When analyzed by fluorescent confocal microscopy, the cells showed strong nuclear staining with few cells showing plasma membrane localization of GFP-RARα fusion protein. However, a consistent plasma membrane pattern could not be detected, and the addition of RA did not affected GFP-RARα intracellular distribution (data not shown). As alternative approach, we have analyzed the distribution of endogenous RARα in SH-SY5Y neuroblastoma cells by means of conventional biochemical cell fractionation experiments. RARα could not be detected in microsomal or purified plasma membrane fractions by western blot immunodetection (data not shown). Similar fractionation experiments were performed in NIH-3T3 cells. As expected, most of the RARγ receptor was detected in the nuclear fraction. Administration of RA to NIH-3T3 cells resulted in a strong increase in the levels of RARγ into the membrane fractions (microsomal and purified plasma membranes) (Fig. 9A). Similarly, RARα-p85 complexes could be detected in the microsomal fraction of SH-SY5Y neuroblastoma cells, in experiments involving a combination of cell fractionation and immunoprecipitation. A RA-induced increase in the levels of those complexes in membrane fractions could be observed (Fig. 9B). Both experiments suggest the idea of a ligand-induced redistribution of the receptor to plasma membrane.

DISCUSSION

Although the classical transcriptional effects of RA have been extensively studied ((4, 24), for recent reviews), little information is available about the non-classical, rapid non-genomic effects of RA on signal transduction pathways and their physiological implications. A considerable amount of information on non-genomic actions of steroid hormones has accumulated during the last few years ((6-8, 25), for recent reviews), but only a few examples of non-genomic actions of RA have been reported (14, 18, 26-34).

We have obtained insight into the mechanistic of activation of PI3K by RA, and the results reported here support a novel mechanism, in which a stable complex including RAR, the regulatory subunit p85-PI3K and other unknown proteins occurs in the absence of RA. Ligand binding to RAR facilitates the association of p110, the catalytic subunit of PI3K, to that complex, and therefore promoting PI3K activity. In addition, we have shown experiments suggesting a role for RA in the regulation of the intracellular location of that signaling complex that could be relevant for PI3K activation, and recruitment of the signaling complex to plasma membrane resulted in increased PI3K activity. We believe that this novel mechanism could be extended to other members of the Nuclear Receptor superfamily, especially for those belonging to the retinoic/thyroid hormone /vitamin D subfamily of receptors.

The involvement of the classical nuclear hormone receptors in the non-genomic actions of steroid hormones and other ligands has been controversial over the past decade. However considerable evidence has accumulated supporting that nuclear hormone receptors are also responsible of the rapid non-genomic actions of steroid hormones, especially for ER (35-39). The participation of nuclear RA receptor RAR in RA-mediated non genomic actions is supported by the pharmacological profile of PI3K activation, by loss and gain function experiments performed in MEF(RARαβγ)$^{L-/L-}$ cells, and by the physical association between liganded RARα and PI3K activity, as demonstrated in immunoprecipitation/PI3K assays. Although the failure of the RAR antagonist AGN193109 in preventing the activation of PI3K by RA or TTNPB could argue against the participation of RAR, AGN193109 itself is able to activate

PI3K, suggesting a dissociated transcriptional antagonist/non-genomic agonist profile, as has been reported for other compounds like the specific ER modulators estren and raloxifen, (40, 41), or synthetic vitamin D_3 analogs (42).

PI3K is an important signaling node on the control of a variety of cellular responses, including survival, growth and differentiation ((43) for review). The mechanism depicted here involves RA-induced formation of a signaling complex, probably through stimulation of the association of p85 and p110 subunits of PI3K. The impact of the regulatory subunit p85 on PI3K activity has been controversial, and evidences supporting both positive and negative effect of p85 on PI3K activity exist in the literature (44, 45). Our results suggest that heterodimer formation between p85 and p110 result in increased PI3K activity. In addition, recruitment of p110 subunit to plasma membrane in the proximity of its substrate could affect its activation, as previously reported (46). A mechanism involving Cellular Retinol-Binding Protein I (CRBP-I) in the regulation of PI3K subunit heterodimerization has been reported recently in breast cancer cells, where RA signaling would have negative effects on PI3K subunit association and PI3K activity. However, the different context between adoptive long-term effects (47) and the rapid effects analyzed here could account for the controversy.

The presence of steroid hormone receptors at the plasma membrane and in caveolae and its importance for the cellular effects of steroid hormones are increasingly accepted. However how the receptors are tethered to the membrane is still unclear, although the few reports addressing this point suggest that membrane localization could result from receptor interaction with specific membrane proteins and/or lipid modifications (for review see (25)). Because RAR, as other members of the nuclear receptor superfamily, is located mainly in the nucleus, an intriguing paradox arises from the fact that rapid non genomic actions of nuclear receptors appear to be initiated at the plasma membrane. Any model wanting to explain the mechanism of PI3K activation by RAR needs to address this point. The importance of the localization of the receptor at the plasma membrane became evident because ectopic expression of RARα to plasma membrane by the introduction of a myristylation domain resulted in increased PI3K activation. Increased non-genomic signaling was also observed by targeting ER to the plasma membrane, but full activation required the addition of hormone (48). Conversely, chimerical or mutant ER with reduced plasma membrane localization show reduced non-genomic activity (40, 49, 50) Some experiments shown here suggest that ligand binding may control the localization of the receptor, promoting the presence of ligand-bound RAR at the plasma membrane. However, we have failed to obtain formal demonstration for this hypothesis by confocal microscopy. Probably only a minor part of the RAR pool is engaged in non-genomic actions (less than 5%, as estimated from immunoprecipitation experiments). This could be close to the threshold level for conventional detection methodologies. As suggested here for RAR, a rapid ligand-induced association of Vitamin D_3 receptor to plasma membrane has been also reported (51, 52).

The mechanism proposed here shows striking differences with the prevalent model proposed to explain the non genomic actions of steroid receptors as the ER (for a review (25)). In that case a pool of *resident* plasma membrane ER molecules interact there with the components of the signal transduction machinery in a way that those interactions are under the control of the ligand, as described for the interactions between ER, GR or AR and p85, the regulatory subunit of PI3K (15-17, 53). The different dynamics of the two subfamilies of nuclear hormone receptors and their peculiarities may have imposed different regulatory solutions to ultimately obtain the ligand-dependent regulation of PI3K activation. The unliganded receptors of the retinoic/thyroid subfamily are mainly nuclear and they don't form inactive complexes with hsp90 and other proteins. A stable interaction of p85-PI3K with the receptor, shown here for RAR, was recently reported for thyroid hormone receptor (54, 55). If the presence of the complex including the receptor and p85-PI3K at the plasma membrane may increase upon ligand binding, as indicated by the experiments shown here, this may facilitate the recruitment of the catalytic subunit p110-PI3K to the complex and could allow interactions with other components of the signal transduction machinery. On the contrary, unliganded steroid receptors form inactive

complex with hsp90 and other proteins that keep the receptor in an inactive form. It appears conceivable that these interactions with hsp90 and/or other of the proteins within the unliganded steroid receptor complex could preclude an interaction with p85-PI3K, which only takes place once the complex is dissociated upon ligand binding.

MATERIAL AND METHODS

Cell Culture and treatments.

SH-SY5Y human neuroblastoma cells, COS-7 cells, MEF-wt, MEF(RARαβγ)$^{L-/L-}$ cells and BOSC23 cells were cultured in DMEM medium with 10% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. NIH-3T3 cells were cultured in DMEM medium with 10% newborn calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Cell cultures were kept in a humidified incubator at 37ºC with 5% CO_2. The medium was replaced every 3 days and the cells were split before they reached confluence. Cycloheximide (CHX) Actinomycin D (AMD), Puromycin, LY294002, *all-trans*-retinoic acid (RA), *9-cis*-retinoic acid (9-c-RA), *13-cis*-retinoic acid (13-c-RA), TTNPB, Am580, and N-(4-Hydroxyphenyl)-retinamide (4-HPR, fenretinide) were purchased from Sigma. AGN193109 (UVI2109) (20), ALRT1550 (UVI2103) (56), and LG100567 (UVI2104) (57) were synthesized and their spectroscopic and analytical data matched those reported in the literature. The different compounds were dissolved in ethanol or DMSO and added to the culture medium at the indicated concentrations.

RNA analysis

Northern Blot analysis of total RNA from SH-SY5Y cells with a [^{32}P]-labeled probe was performed as previously described (14). *RARB* probe consisted in a 1.4 kb DNA fragment containing the human RARβ$_2$ cDNA (58).

Western Blot and Immunoprecipitation

Whole cell extracts were obtained by lysis of the cells in RIPA buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1 mM EGTA) containing 0.5% Nonidet P-40, protease (1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin) and phosphatase inhibitors (1 mM sodium ortovanadate, 1 mM NaF). After 10 min incubation on ice, the lysate was cleared by centrifugation (16,100 g, 10 min, 4ºC), and protein concentration determined. Western blot analysis of proteins from whole cell extracts was performed as described (14). Antibodies against, RARα, RARγ, RXRα, Caveolin 1, ERK2, Akt 1/2, and the phosphorylated (Tyr$_{204}$) form of ERK1/2 were purchased from Santa Cruz Biotechnology. Antibodies against phosphorylated Akt (Ser$_{473}$), RARα and Lamin A were obtained from Cell Signal Technologies. Antibodies against PI3K-p85 and –p110 were from Upstate. HA.11 monoclonal antibody was purchased from Covance. Monoclonal antibody against p110-PI3K was a gift of Dr. A. C. Carrera (CNB, CSIC-UAM, Madrid). Horseradish-peroxidase-conjugated secondary antibodies were obtained from GE Healthcare, Jackson ImmunoResearch and Santa Cruz Biotechnology. Chemiluminiscent signals were developed with ECL or ECL Plus (GE Healthcare).

For immunoprecipitation cells were treated with RA (1 µM, 10 min) or vehicle and cell extracts prepared in 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet P-40, and protease (1 mM PMSF, 40 µg/ml aprotinin and 40 µg/ml leupeptin) and phosphatase inhibitors (1 mM sodium ortovanadate, 1 mM NaF). Alternatively, nuclear extract or microsomal fractions were prepared as described below in *Subcellular Fractionation*. Approximately 150 µg protein from control or RA-treated cell extracts were incubated with 5 µg of antibodies against p85-PI3K, RARα, or p110-PI3K as indicated. As control, parallel reactions were set with an unrelated antibody. Immunocomplexes were precipitated using anti-rabbit IgG beads (eBioscience), following the manufacturer's instructions, and suspended in sample buffer containing freshly added 50 mM DTT. After Western blot as above, the filters were sequentially developed with the antibodies indicated in the figure, using Horseradish-peroxidase-conjugated Rabbit TrueBlot (eBioscience) as secondary antibodies. Chemiluminiscent signals were developed with ECL (GE Healthcare).

GST pull-down assay

GST-RARα (59) was expressed in *E. coli* BL21 cells and purified by using standard techniques. For the pull-down assays, 20 ng of

GST-RARα or GST protein immobilized in glutathione-agarose beads were added to cell lysates (160 μg protein) from COS-7 cells expressing p85-PI3K-HA or RXRα in 500 μl of 50 mM Tris pH 7.5, 150 mM Nacl, 10% Glycerol, 1% Nonidet P-40, 1 mM PMSF, 40 μg/ml aprotinin, 40 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM NaF and 1 mM sodium ortovanadate. Extract from non-transfected COS-7 cells was used as negative control. Where indicated, 1 μM RA was included in the incubation mixture. After incubation (18 h, 4ºC), the beads were washed three times with the same buffer, and the bound proteins detected by Western blot.

PI3K Assay

Extracts from control or RA-treated (1μM RA, 10 min) SH-SY5Y cells, containing 150 μg protein were incubated with 5 μg of RARα antibodies, p110-PI3K antibodies or an unrelated control IgG, and the immunocomplexes recovered with protein A/G Plus-Agarose (Santa Cruz Biotecnology). The complexes were washed 3 times with 10 mM Hepes, pH 7.5, 0.1 mM EGTA. Kinase reactions were set by mixing 20 μl immunoprecipitates, 20 μl of 0.5 mg/ml L-α-phosphatidyl-inositol (Sigma) in 10 mM HEPES pH 7.4, 0.1 mM EGTA and 10 μl of 50 mM HEPES pH 7.4, 25 mM $MgCl_2$ containing 10 μCi of [γ-^{32}P]-ATP (NEN, spec. act 3000 Ci/mmol). Kinase assays were performed as described (14). Quantification of the radioactivity on the spots was carried out with Phosphor capture screens in a Fujifilm FLA5000 laser scanner.

Transient and retrovirus-mediated transfection

Myr-RARα expression vector was generated by in-frame fusion of RARα (2-462) to the 21 residues of the chick c-*Src* myristylation domain present in pCEFL-*Myr* expression vector (a gift of Dr. P. Crespo, IIB-Univ. de Cantabria). Semi-confluent COS-7 cells were transfected by the Calcium Phosphate method with expression vectors for RARα and *Myr*-RARα. After 8-14 h of exposure to the precipitates, the medium was replenished. RA treatments took place after 24 h, and cells were lysed and cell extracts processed for Western Blotting. Retroviral particles for the expression of RARα and *Myr*-RARα and its derivatives were prepared by transfecting retroviral vector expression vectors based on pBABE (60) into BOSC23 packaging cells (61) by the Calcium Phosphate method. After 2 days of incubation, the supernatants containing retrovirus were used to infect semi-confluent MEF(RARαβγ)$^{L-/L-}$ or NIH-3T3 cells, and pools of transfected cells were selected with Puromycin (2.5 μg/ml). For immunoprecipitation, semi-confluent COS-7 cells were co-transfected with expression vectors for hRARα and p85-PI3K-HA (62) by the Calcium Phosphate method. After 8-14 h of exposure to the precipitates, the medium was replenished, and after further 24 h the cells were lysed and cell extracts processed for immunoprecipitation.

Subcellular fractionation

Membrane fractions were obtained according to (52) with some modifications. SH-SY5Y neuroblastoma cells, wild-type or retrovirally transfected NIH-3T3 cells cells overexpressing RARα or Myr-RARα were washed with PBS and harvested. Cells were pelleted by centrifugation and resuspended in TEDK buffer (10 mM Tris-HCl, pH 7.4; 0.3 M KCl, 1 mM EDTA, 1mM DTT) containing protease (1 mM PMSF, 40 μg/ml aprotinin, 40 μg/ml leupeptin) and phosphatase inhibitors (1 mM sodium ortovanadate, 1 mM NaF). Cell suspension was homogenized in a glass-teflon homogenizer and the lysate was centrifuged first at 200 g for 10 min to eliminate cell debris, and later to 16,100 g for 20 min to pellet the nuclei and mitochondria. The cleared lysate was centrifuged at 100,000 g for 45 min. in a Beckmann SW60 rotor. The pellet containing the microsomal fraction was resuspended in 1.5 ml of TEDK containing 15% sucrose and laid on top of a 30-45% sucrose step gradient. Gradients were run at 76,000 g for 3 h and the interface between 15-30% sucrose, containing purified plasma membranes, was picked up, diluted in TEDK and the membranes re-pelleted at 100,000 g for 1 h. The purified plasma membrane was resuspended in TEDK buffer containing inhibitors.

Nuclear fraction was obtained resuspending cells in 60 mM KCl, 15 mM NaCl, 20 mM Tris-HCl pH 8, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 0.5 mM EGTA. Cell suspension was mixed with one volume of the same buffer containing 0.5% Nonidet P-

40 and incubated for 5 min on ice. Nuclei were pelleted by centrifugation at 1,500 g for 10 min at 4ºC, and washed with the same buffer without detergent. The Nuclei were re-pelleted and lysated with RIPA buffer containing 0.5% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS.

Detergent-resistant membrane microdomains (lipid rafts) from NIH-3T3 cells overexpressing RARα and *Myr*-RARα were fractionated in sucrose step-gradients as previously described (63, 64), with modifications. About 15 $x10^6$ cells were suspended in 500 µl of ice-cold lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25% TritonX-100) plus inhibitors (1 mM PMSF, 40 µg/ml aprotinin, 40 µg/ml leupeptin, 1 mM sodium ortovanadate, 1 mM NaF) and solubilized for 30 min at 4 °C. Sucrose concentration of the lysates was adjusted to 41% before they were overlaid with 2.7 ml of 35% sucrose and 0.8 ml of 16% sucrose prepared in 10 mM Tris-HCl, pH 7.4. Sucrose gradients were ultracentrifuged (40,000 rpm in a SW60 rotor, 18 h, 4 °C), and 10 0.4 ml fractions were collected from each gradient (from the top to the bottom, fractions 1–10; fraction 10 included the pellet), precipitated with 6.5% trichloroacetic acid in the presence of 0.05% sodium deoxycholate, washed with 80% cold acetone, dissolved, and boiled in 2x Laemmli sample buffer. Fractions were analyzed by SDS-PAGE followed by Western blotting.

Acknowledgements

The authors are indebted to J. M. Escamilla and A. Llopis for excellent technical assistance, to Dr. R. Alvarez y Dr. M. J. Vega for preparing ALRT1550 and LG100567, to G. Sarrió and M. Tatay for help with plasmid cloning, to Dr. H. Gronemeyer for the gift of the MEF(RARαβγ)$^{L-/L-}$ cells, to Dr. A. Aranda for providing the GST-RARα construct and to V. Andrés, A. Díez-Juan, A. C. Carrera, P. Crespo, T. Espert, C. Gallego, T. Iglesias, B. Jiménez, A. Muñoz, F. Palau, V. Rubio and P. Sanz for suggestions, protocols, gifts of materials and use of equipment. S. M. was the recipient of a pre-doctoral fellowship (FPI) from the Ministry of Science and Technology and a contract from Programa de contratación de Técnicos, Ministry of Education and Science. This work was supported by grants of the Spanish former Ministry of Science and Technology and Ministry of Education and Science (SAF2003-00311 and SAF2006-00647 to D. B.; SAF2004-07131 to A. R. de L.), European Commission (QLK3-2002-02029 "Anticancer Retinoids" to A. R. de L.), Generalitat Valenciana (GRUPOS 03/15 and ACOMP 06/212 to D. B.) and FEDER.

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LEGEND TO FIGURES

Figure 1. Rapid, non-genomic activation of PI3K/Akt and ERK1/-2 signaling pathways by RA treatment in SH-SY5Y neuroblastoma cells

A. Rapid activation of PI3K signaling pathway by RA. Cells were treated with RA (1 μM) for the times indicated in the figure and total cell extracts were prepared. The phosphorylation state of Akt was analyzed by Western Blot with specific antibodies against Akt phosphorylated in Ser_{473} (AKT-P). The filter was reprobed with antibodies against total Akt (AKT). Each lane contains 25 μg of total protein.

B. Rapid activation of PI3K by RA did not required new gene transcription or newly synthesized proteins. Neuroblastoma cells were pre-treated for 30 min with the protein synthesis inhibitor Cycloheximide (CHX, 10 μg/ml) or the transcription inhibitor Actinomycin D (AMD, 1 μg/ml). Afterwards they were treated with RA (1μM) or vehicle for 5 min, and the phosphorylation state of Akt was analyzed by Western Blot as described in the legend of Fig. 1A.

C. Rapid activation of ERK1/2 signaling pathway by RA. Cells were treated with RA (1μM) for the times shown in the figure and total cell extracts were prepared. The phosphorylation state of ERK1/2 MAP kinases was analyzed by Western Blot with specific antibodies against the phosphorylated (Tyr_{204}) form of ERK 1/2 (ERK-P). The filter was reprobed with antibodies against ERK2 (ERK2). Each lane contains 25 μg of total protein.

Figure 2. Activation of PI3K/Akt and ERK1/2 signaling pathways by RA in NIH-3T3 cells

A. RARγ is expressed in NIH-3T3 cells. RARγ (approximately 50 KDa, arrow) was detected in total cell extract by Western Blot with specific antibodies. The positions of the corresponding marker bands and their molecular weights (in KDa) are shown on the left side of the blot. Gel lane contained 25 μg total proteins.

B. Rapid activation of PI3K by RA in NIH-3T3 cells. Cells were serum-starved for 14 h and then treated with RA (1μM) for the times indicated. The phosphorylation state of Akt was analyzed by Western Blot as described in the legend of Fig. 1A.

C. Rapid activation of PI3K by RA did not required new gene transcription or newly synthesized proteins. NIH-3T3 cells were pre-treated for 30 min with the transcription inhibitor Actinomycin D (AMD, 1 μg/ml), the protein synthesis inhibitor Cycloheximide (CHX, 10 μg/ml), or the PI3K specific inhibitor LY294002 (LY, 10 μM). Afterwards they were treated with RA (1μM) or vehicle for 10 min, and the phosphorylation state of Akt was analyzed by Western Blot as described in the legend of Fig. 1A.

D. Rapid activation of ERK1/2 signaling pathway by RA in NIH-3T3 cells. Cells were serum-starved for 14 h and then treated with RA (1μM) for the times indicated. Total cell extracts were prepared, and the phosphorylation state of ERK1/2 MAP kinases was analyzed by Western Blot as described in the legend of Fig. 1C.

Figure 3. Pharmacological profile of the activation of the PI3K/Akt signaling pathway by RA and its derivatives in neuroblastoma cells

A. Neuroblastoma cells were treated during 10 min with different RA concentrations as indicated in the figure, and the phosphorylation state of Akt was analyzed by Western Blot as described in the legend of Fig. 1A.

B. Activation of PI3K by different RAR agonists. Neuroblastoma cells were treated during 10 min with *all-trans*-RA (RA), *9-cis*-RA (9c-RA), *13-cis*-RA (13c-RA), TTNPB, Am580, N-(4-Hydroxyphenyl)-retinamide (fenretinide, 4-HPR), ALRT1550 (ALRT), and LG100567 (LG) at 1 μM. Afterwards, the phosphorylation state of Akt was analyzed by Western Blot as described in the legend of Fig. 1A.

C. Effect of the RAR transcriptional antagonist AGN193109 on the activation of PI3K by RAR agonists. Neuroblastoma cells were treated during 10 min with 0.5 μM RA (RA), 0.5 μM TTNPB (TTNPB), 0.5 μM RA and 5 μM AGN193109 (RA+AGN), 0.5 μM TTNPB and 5 μM AGN193109

(TTNPB+AGN), and 5 μM AGN193109 alone (AGN). Afterwards, the phosphorylation state of Akt was analyzed by Western Blot as described in the legend of Fig. 1A.
D. As a control, the effect of the RAR antagonist AGN193109 on the transcriptional induction of *RARB*, encoding the RARβ receptor, in neuroblastoma cells was tested. Cells were treated with 0.5 μM RA (RA), or 0.5 μM RA and 5 μM AGN193109 (RA+AGN) during 24 h. Afterwards, total RNA was prepared and the expression of *RARB* transcript (approximately 3 Kb) was analyzed by Northern Blot with a [^{32}P]-labeled *RARB* probe. Each lane contained 15 μg of total RNA. The 28S ribosomal RNA band in the blot stained with methylene blue is shown as internal loading control (rRNA).

Figure 4. RA-induced PI3K activation was abolished in RAR null MEF cells, but was restored by re-introduction of RAR expression.
A. RA treatment rapidly activates PI3K in wild-type MEF cells. Wild-type MEF cells were serum-starved for 14 h, treated with 1 μM RA for the times indicated on the figure and total cell extracts were prepared. The phosphorylation state of Akt was analyzed by Western Blot as described in the legend of Fig. 1A.
B. RA-induced PI3K activation is abolished in MEF(RARαβγ)$^{L-/L-}$ cells. MEF(RARαβγ)$^{L-/L-}$ cells were serum-starved for 14 h, treated with 1 μM RA for the times indicated on the figure and total cell extracts were prepared. As control for the integrity of the PI3K signaling pathway, cells were serum-stimulated with 10% FBS for 10 min. (lane labeled SE). The phosphorylation state of Akt was analyzed by Western Blot as described in the legend of Fig. 1A.
C. Retrovirus-mediated re-expression of RARα restored RA-induced PI3K activation. MEF(RARαβγ)$^{L-/L-}$ cells and a derivative cell line expressing RARα through retroviral transfection, MEF(RARαβγ)$^{L-/L-}$+RARα, were serum-starved for 14 h, treated with 1 μM RA for the times indicated on the figure and total cell extracts were prepared. The phosphorylation state of Akt was analyzed by Western Blot as described in the legend of Fig. 1A.The filter was reprobed additionally with antibodies against RARα.

Figure 5. PI3K activity was physically associated to ligand-bound RARα.
PI3K activity was assayed *in vitro* on immunoprecipitated fractions of control (-) and RA-treated (1 μM, 10 min.) (+) SH-SY5Y neuroblastoma cells. Immunoprecipitation reactions were set with anti-RARα and anti-p110-PI3K antibodies, and parallel control reactions with a non-related antibody (n. r.) were set as controls. PI3K activity was assayed on the immunoprecipitates in the presence of Phosphatidyl-inositol and [γ-^{32}P]-ATP as described in the Material and Methods section. The caption shows a detail of the autoradiogram of the thin layer chromatography plate showing the generation of Phosphatidyl-inositol-3-phosphate (PIP) *in vitro*. Activity data (in arbitrary units) were obtained by quantification of the radioactivity in the spots with Phosphor capture screens in a Fujifilm FLA5000 laser scanner.

Figure 6. Interactions between RARα and PI3K subunits.
A. Total cell extracts were prepared from neuroblastoma cells treated with 1μM RA or vehicle for 10 min. About 150 μg protein from control or RA-treated cell extracts were incubated with 5 μg of RARα antibody. As control, parallel reactions were set with an unrelated antibody. Immunocomplexes were precipitated using anti-rabbit IgG beads (eBioscience), following the manufacturer's instructions, and suspended in sample buffer containing freshly added 50 mM DTT. After Western blot the filter was sequentially developed with anti-p85-PI3K and anti-RARα, using Horseradish-peroxidase-conjugated Rabbit TrueBlot (eBioscience) as secondary antibodies. The filter was reprobed additionally with an anti-p110-PI3K monoclonal antibody. Note the anomalous migration of RARα (approx. 50 KDa) in the immunoprecipitate lanes, that is due to co-migration with IgG heavy chain (approx. 50 KDa), which in its reduced form was not detected by the TrueBlot secondary antibodies.
B. Total cell extracts were prepared from neuroblastoma cells treated with 1μM RA or vehicle for 10 min. About 150 μg protein from control or RA-treated cell extracts were incubated with 5 μg of

p85-PI3K antibody. As control, parallel reactions were set with an unrelated antibody. Immunocomplexes were precipitated as described in the legend of Fig. 6A. After Western blot, the filters were sequentially developed with anti-RARα and anti-p85-PI3K, using Horseradish-peroxidase-conjugated Rabbit TrueBlot (eBioscience) as secondary antibodies. Note the anomalous migration of RARα (approx. 50 KDa) in the immunoprecipitate lanes, that is due to co-migration with IgG heavy chain (approx. 50 KDa), which in its reduced form was not detected by the TrueBlot secondary antibodies.

C. Nuclear extract was prepared from untreated neuroblastoma cells, and 150 μg protein from nuclear extract were incubated with 5 μg of p85-PI3K antibody. As control, a parallel reaction was set with an unrelated antibody. Immunocomplexes were precipitated and Western Blot was carried out as described in the legend of Fig. 6A. Note the anomalous migration of RARα (approx. 50 KDa) in the immunoprecipitate lanes, that is due to co-migration with IgG heavy chain (approx. 50 KDa), which in its reduced form was not detected by the TrueBlot secondary antibodies

D. RA administration increases association of p110-PI3K to RARα-p85-PI3K complex in neuroblastoma cells. Total cell extracts were prepared from neuroblastoma cells treated with 1μM RA or vehicle for 10 min. About 150 μg protein from control or RA-treated cell extracts were incubated with 5 μg of p110-PI3K antibody. As control, parallel reactions were set with an unrelated antibody. Immunocomplexes were precipitated as described in the legend of Fig. 6A. After Western blot, the filters were sequentially developed with anti-RARα and anti-p85-PI3K using Horseradish-peroxidase-conjugated Rabbit TrueBlot (eBioscience) as secondary antibodies. Note the anomalous migration of RARα (approx. 50 KDa) in the immunoprecipitate lanes, that is due to co-migration with IgG heavy chain (approx. 50 KDa), which in its reduced form was not detected by the TrueBlot secondary antibodies.

Figure 7. The interaction between RAR and p85-PI3K does not appear to be direct.

A. RARα and p85-PI3K do not interact *in vitro* in GST pull-down assays. Recombinant GST-RARα and GST protein were expressed in *E. coli* and bound to glutathione-agarose beads. GST-RARα or GST beads were incubated with cell lysates of COS-7 cells expressing p85-PI3K-HA (HA-p85) or human RXRα (RXRα). Extract from non transfected cells is used as negative control (C). Where indicated 1 μM RA (+) or vehicle (-) was added to the pull-down reaction. Bound proteins were detected by Western Blot, and the filter sequentially developed with anti-HA and anti-RXRα antibodies. For comparison, the amount of extracts loaded in the lanes labeled input corresponds to 25% of the amount loaded from the pull-down assays.

B. RARα and p85-PI3K did not form a complex when co-expressed in COS-7 cells. COS-7 cells were co-transfected with RARα and p85-HA expression plasmids. Total cell extract (150 μg protein) was incubated with 5 μg of anti-p85-PI3K antibody. As control a parallel immunoprecipitation was set with an unrelated IgG (n. r.). Immunocomplexes were precipitated as described in the legend of Fig. 6A. The supernatant of the p85 immunoprecipitation (snat) was also included in the gel After Western blot, the filters were sequentially developed with anti-RARα and anti-p85-PI3K, using Horseradish-peroxidase-conjugated Rabbit TrueBlot (eBioscience) as secondary antibodies. Note the anomalous migration of RARα (approx. 50 KDa) in the immunoprecipitate lanes, that is due to co-migration with IgG heavy chain (approx. 50 KDa), which in its reduced form was not detected by the TrueBlot secondary antibodies.

Figure 8. Ectopic expression of RARα in the plasma membrane results in increased activation of the PI3K/Akt signaling pathway.

A. *Myr*-RARα is targeted to plasma membrane. Whole cell extract, nuclear fraction, microsomal fraction, and plasma membrane fraction purified from sucrose gradients were obtained from retrovirally transfected NIH-3T3 cells overexpressing wild type RARα or *Myr*-RARα chimerical receptor. RARα and *Myr*-RARα were detected by Western Blot with antibodies against RARα. Equal amounts of protein from RARα- and *Myr*-RARα-expressing cells were loaded.

B. *Myr*-RARα localizes in detergent-resistant membrane micro domains (*lipid rafts*). TritonX-100 extracts from NIH-3T3 cells overexpressing RARα and *Myr*-RARα were fractionated in parallel

sucrose step-gradients as described in the Material and Methods section. Fractions were collected from each gradient (10 0.4 ml fractions from the top to the bottom, fractions 1–10; fraction 10 included the pellet). The presence of RARα in total cell extract (T) and the different fractions (1-10) was analyzed by Western Blotting with specific antibodies. The filter was reproved with antibodies against Caveolin-1 (cav1) to mark the low-density *lipid rafts*-containing fractions.

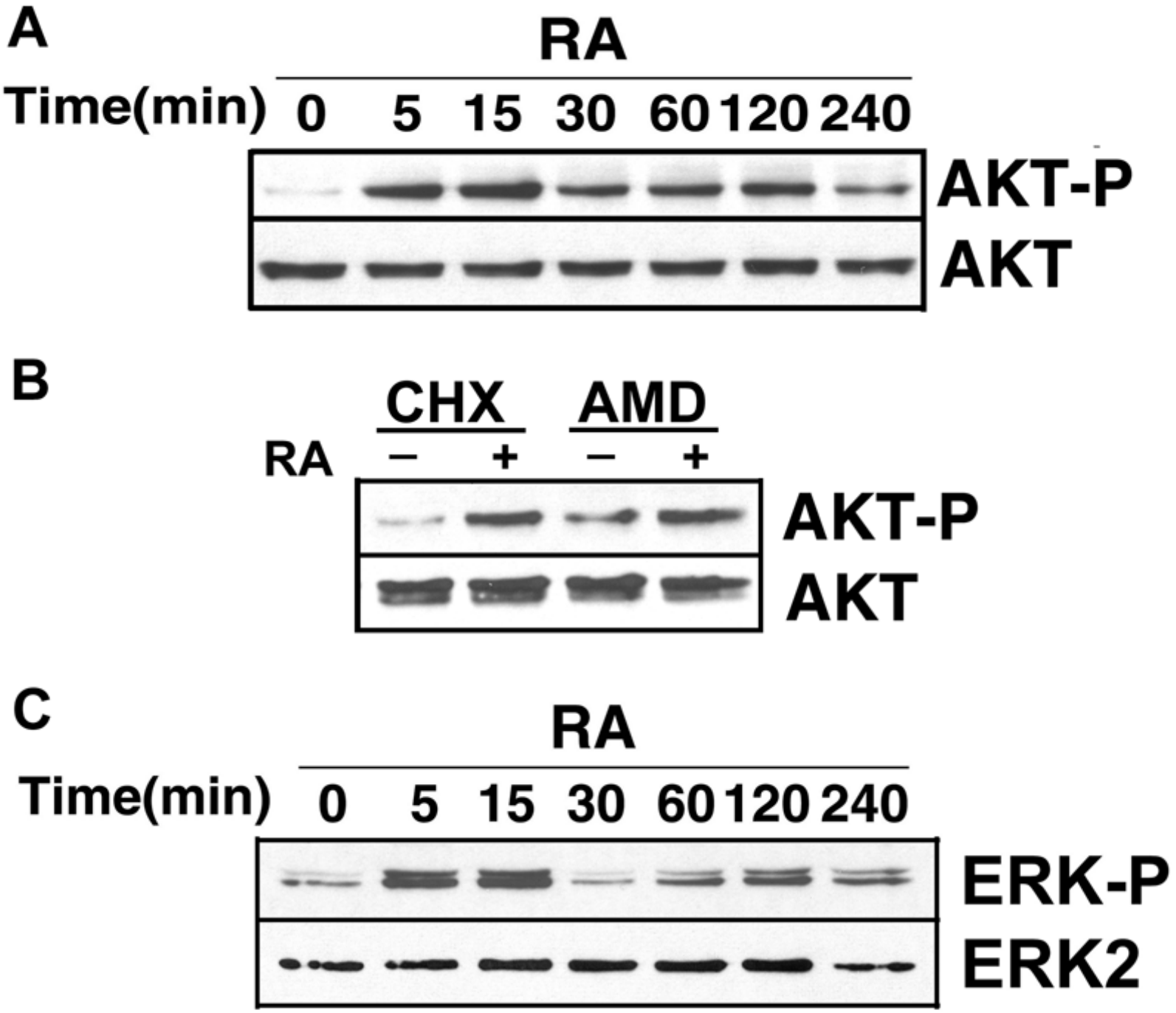
C. Expression of *Myr*-RARα increases the activity of PI3K/Akt signaling pathway. Parental MEF(RARαβγ)$^{L-/L-}$ cells and its derivatives expressing RARα and *Myr*-RARα through retroviral vectors were serum-starved for 14 h, treated with vehicle (-) or RA (1 μM, 10 min)(+) as indicated on the figure and total cell extracts were prepared. The phosphorylation state of Akt was analyzed by Western Blot as described in the legend of Fig. 1A.The filter was reprobed additionally with antibodies against RARα.

D. Transfection of *Myr*-RARα increases the activity of PI3K/Akt signaling pathway. COS-7 cells were transfected with expression vectors encoding RARα, *Myr*-RARα or empty vector (mock) by the calcium phosphate protocol. Medium was replenished after transfection and 24 h later the cells were treated with vehicle or 1 μM RA for 10 min. Total cell extracts were prepared and the phosphorylation state of Akt was analyzed by Western Blot as described in the legend of Fig. 1A. The filter was reprobed additionally with antibodies against RARα.

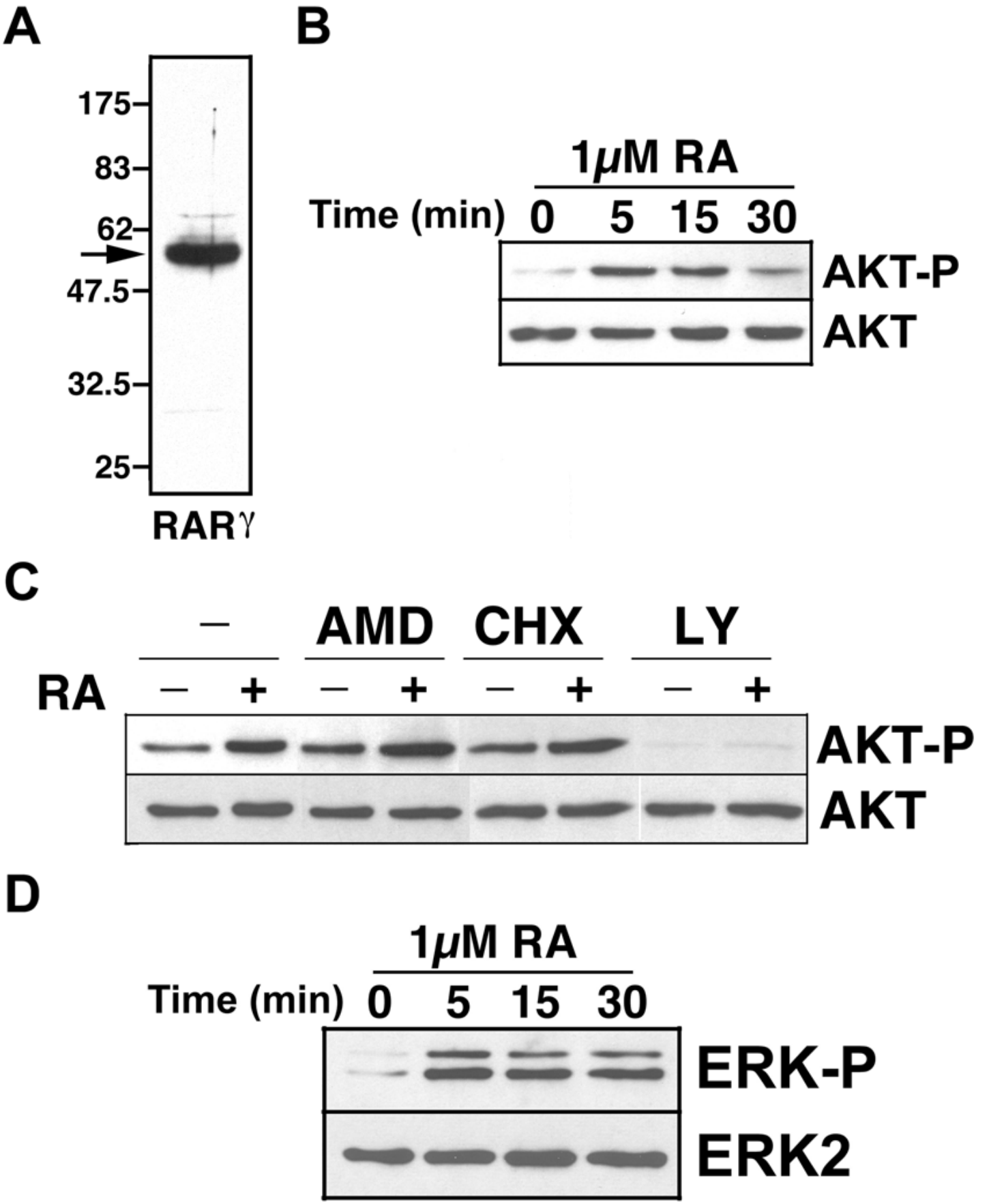
Figure 9. Ligand-induced localization of RAR to Plasma Membrane.

A. Liganded RARγ is targeted to plasma membrane in RA-treated NIH-3T3 cells. Cells were treated with vehicle (-) or 1 μM RA (+) for 10 min. Whole cell extract, nuclear fraction, microsomal fraction, and plasma membrane fraction purified from sucrose gradients were obtained as described in the Material and Methods section and analyzed by Western Blotting. Equal amounts of protein from untreated and RA-treated cells were loaded. The filters were probed sequentially with antibodies against RARγ, Caveolin-1 (Cav1) and Lamin A (LamA). (n.d., not done).

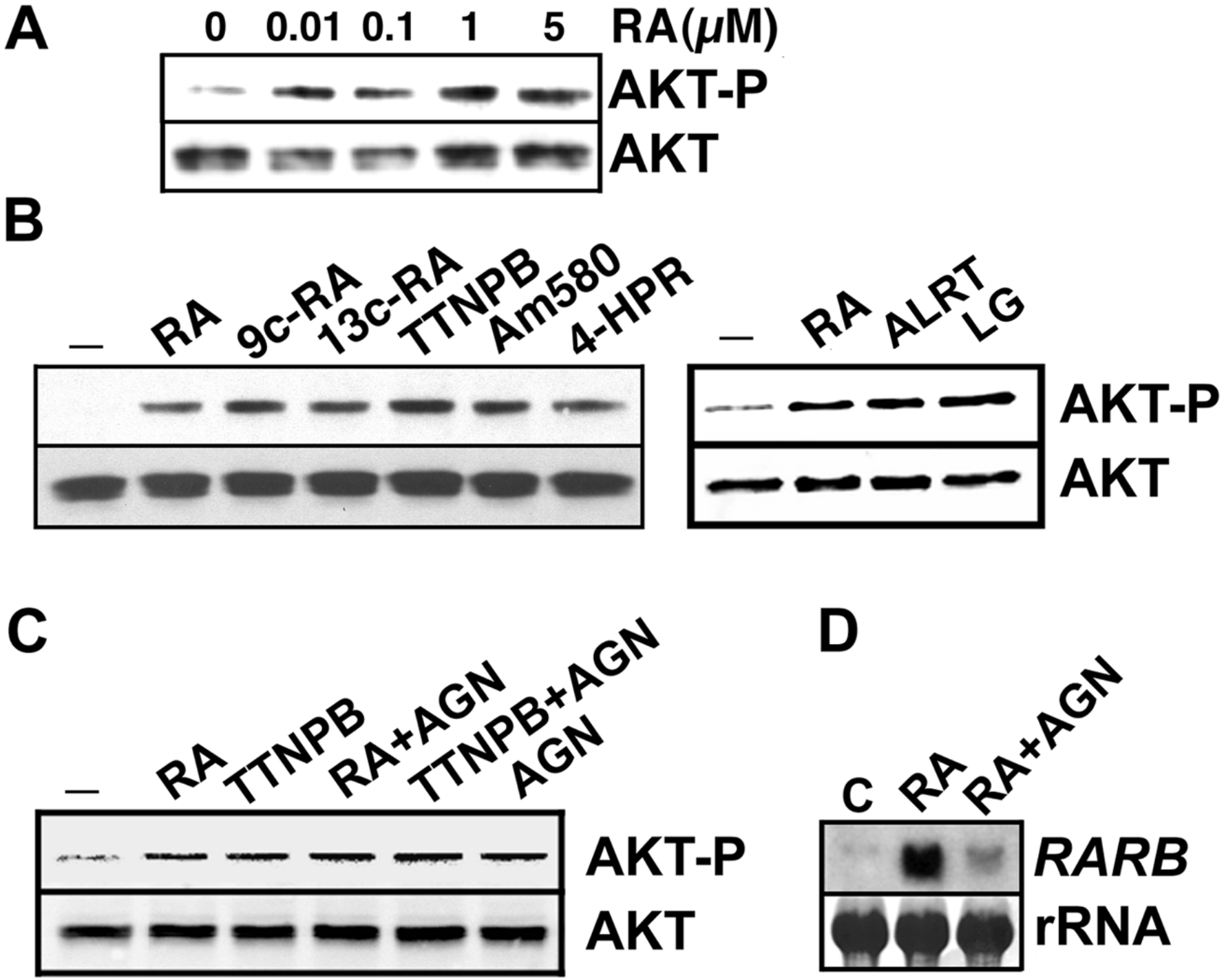
B. Detection of RARα-p85 complexes in membrane fractions from SH-SY5Y cells. Microsomal fractions were prepared from neuroblastoma cells treated for 10 min with 1μM RA (+) or vehicle (-). About 150 μg protein from microsomal fractions from control or RA-treated cells were incubated with 5 μg of p85-PI3K antibodies as indicated. Immunocomplexes were precipitated as described in the legend of Fig. 6A. After Western blot, the filters were sequentially developed with anti-RARα and anti-p85-PI3K, using Horseradish-peroxidase-conjugated Rabbit TrueBlot (eBioscience) as secondary antibodies. Note the anomalous migration of RARα (approx. 50 KDa) in the immunoprecipitate lanes, that is due to co-migration with IgG heavy chain (approx. 50 KDa), which in its reduced form was not detected by the TrueBlot secondary antibodies. Total cell extract from SH-SY5Y cells was included as reference (T):



Masia S. et al. Fig. 1ABC



Masia S. et al. Fig. 2ABCD



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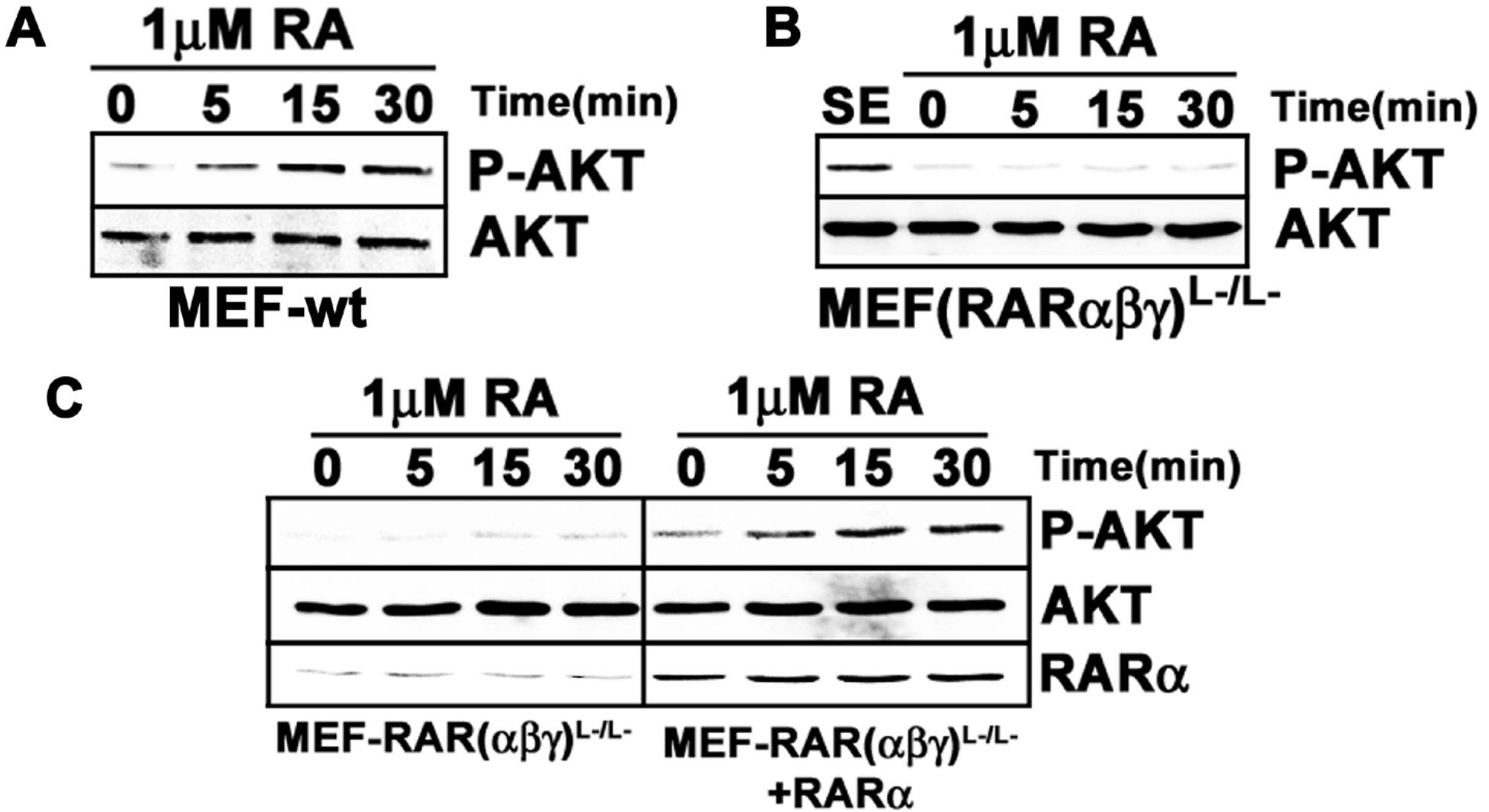

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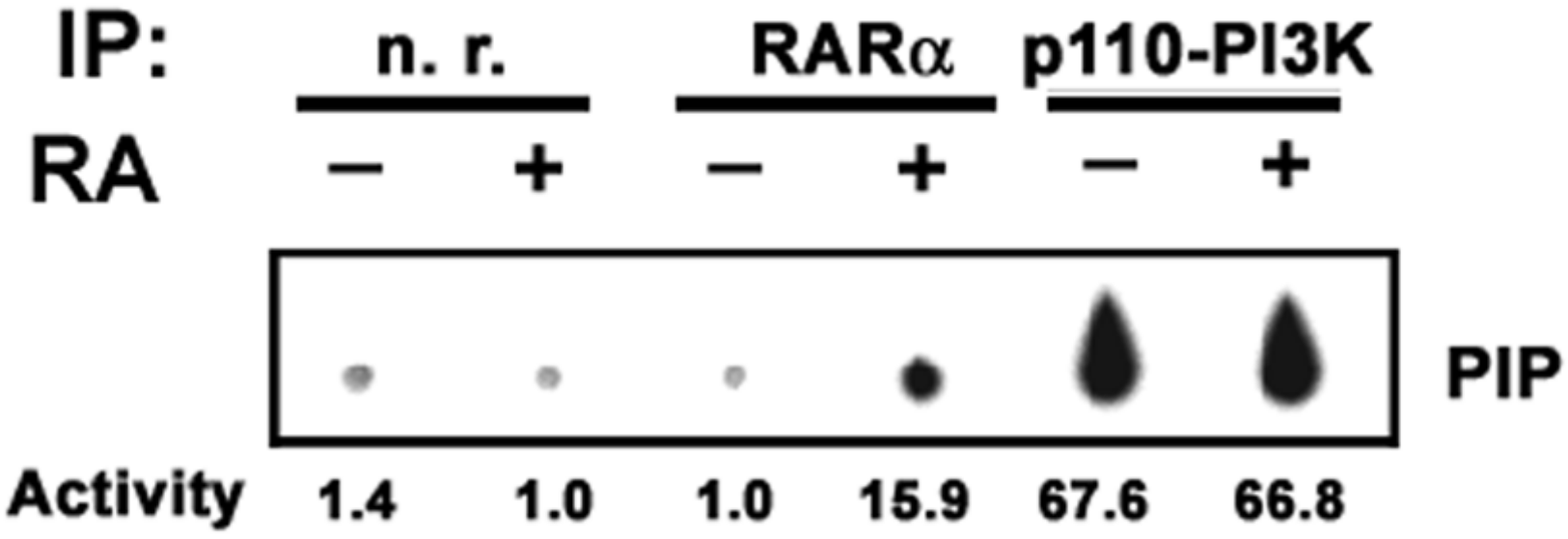
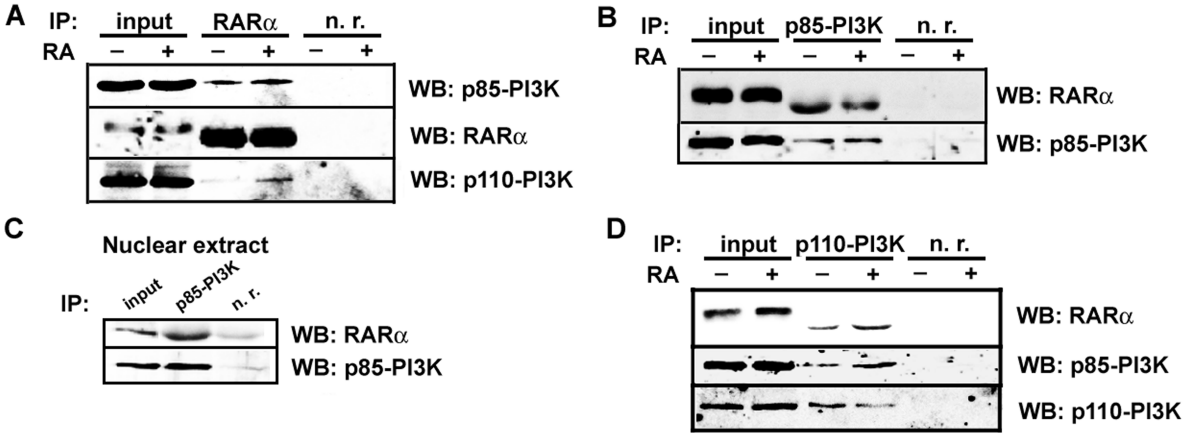
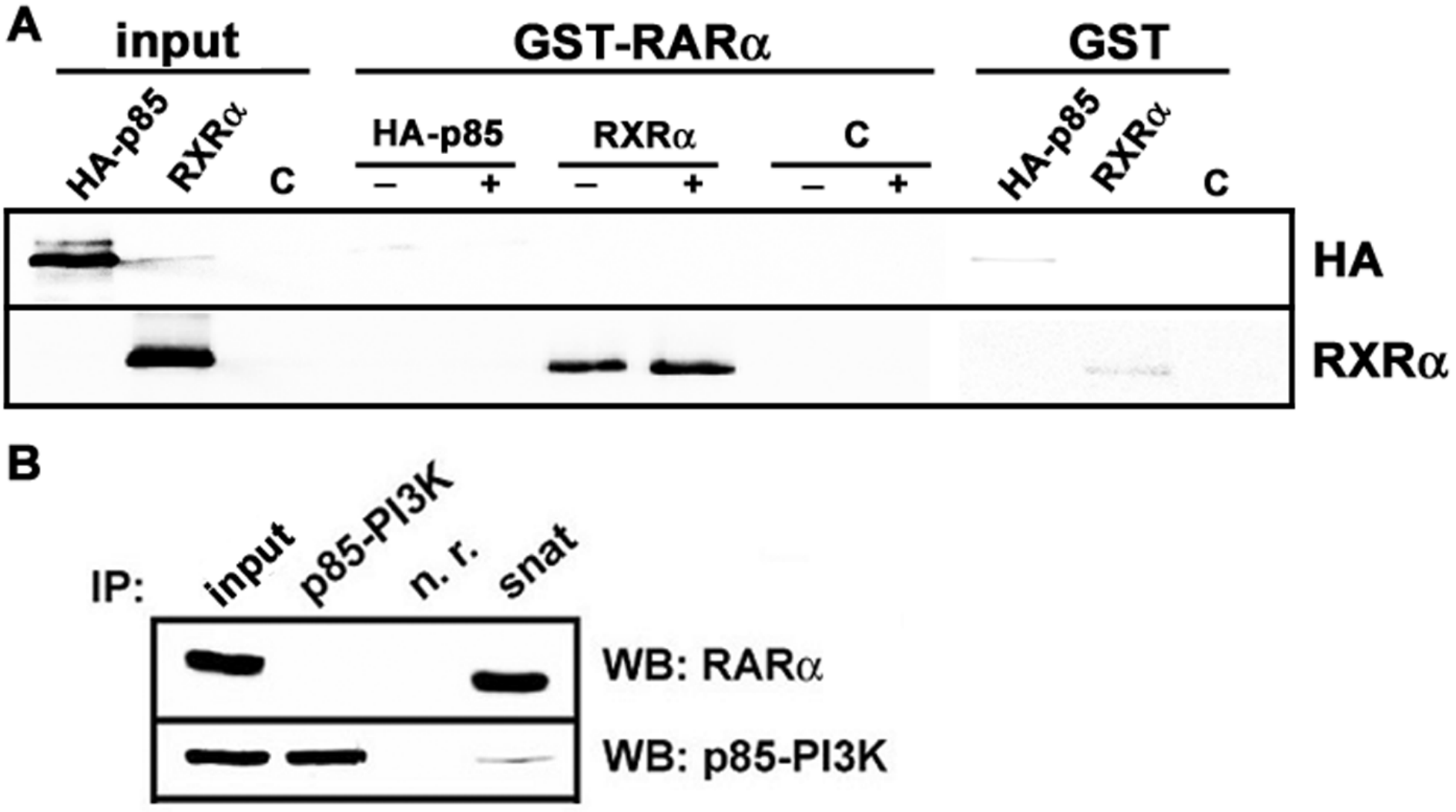


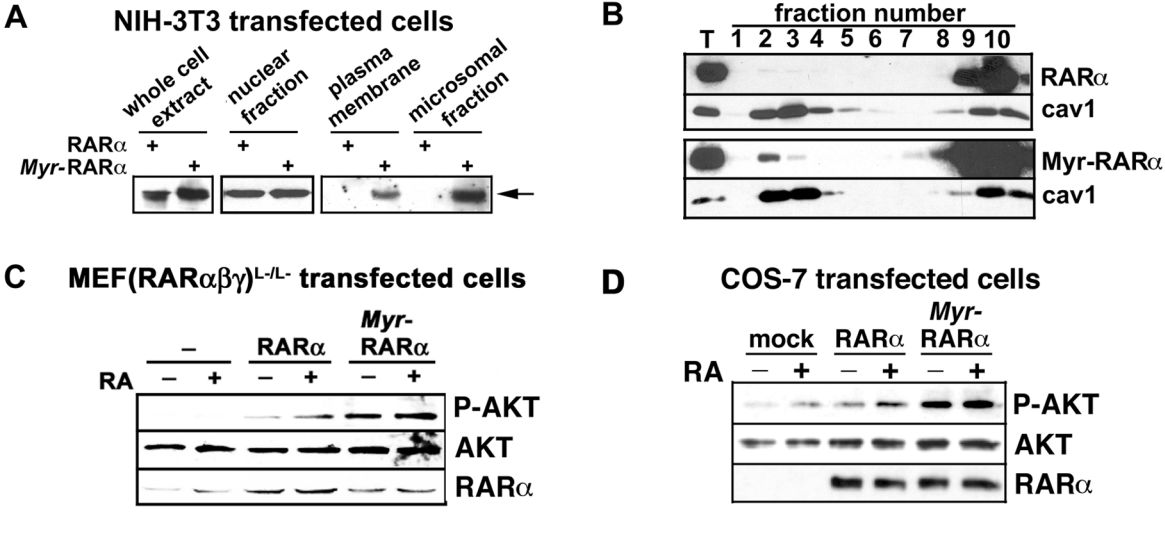
Masia S. et al. Fig. 5



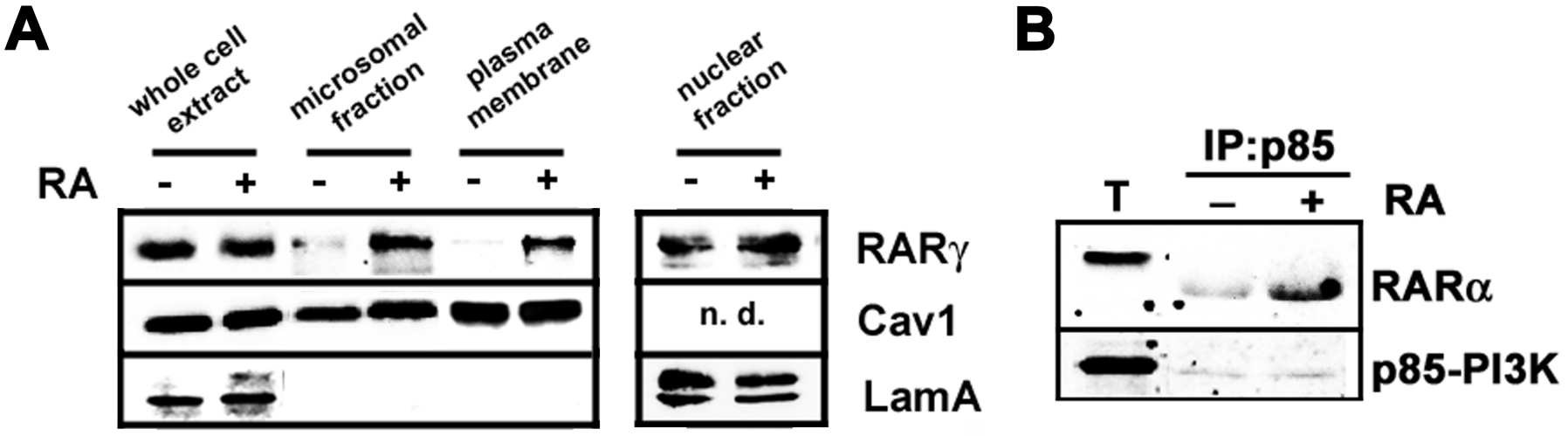
Masia S. et al. Fig 6ABCD



Masia S. et al. Fig. 7AB



Masia S. et al., Fig 8ABCD



Masia S. et al. Fig. 9AB