RAPID, NON-GENOMIC ACTIONS OF RETINOIC ACID ON PHOSPHATIDYL-INOSITOL-3-KINASE SIGNALLING PATHWAY MEDIATED BY THE RETINOIC ACID RECEPTOR

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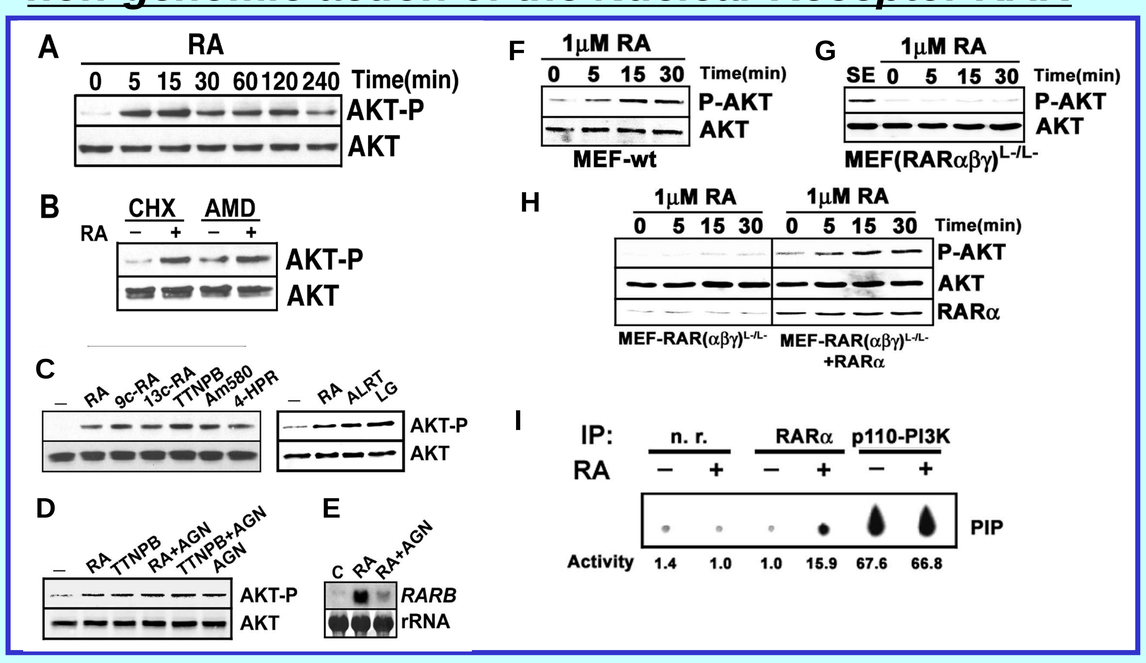
Retinoic Acid (RA) treatment of SH-SY5Y neuroblastoma cells results in activation of phosphatidyl-inositol-3-kinase (PI3K) signalling pathway, and this activation is required for RA-induced differentiation. RA activates PI3K and ERK1/2 MAP Kinase signalling pathways through a rapid, non-genomic mechanism 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. Ligand binding differentially regulated the association of RAR with the two subunits of PI3K. 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 signalling pathway, although both in the absence as well in the presence of RA. Our results suggest a mechanism in which ligand binding to RAR would play a major role in the assembly and intracellular location of a signalling complex involving RAR and the subunits of PI3K.

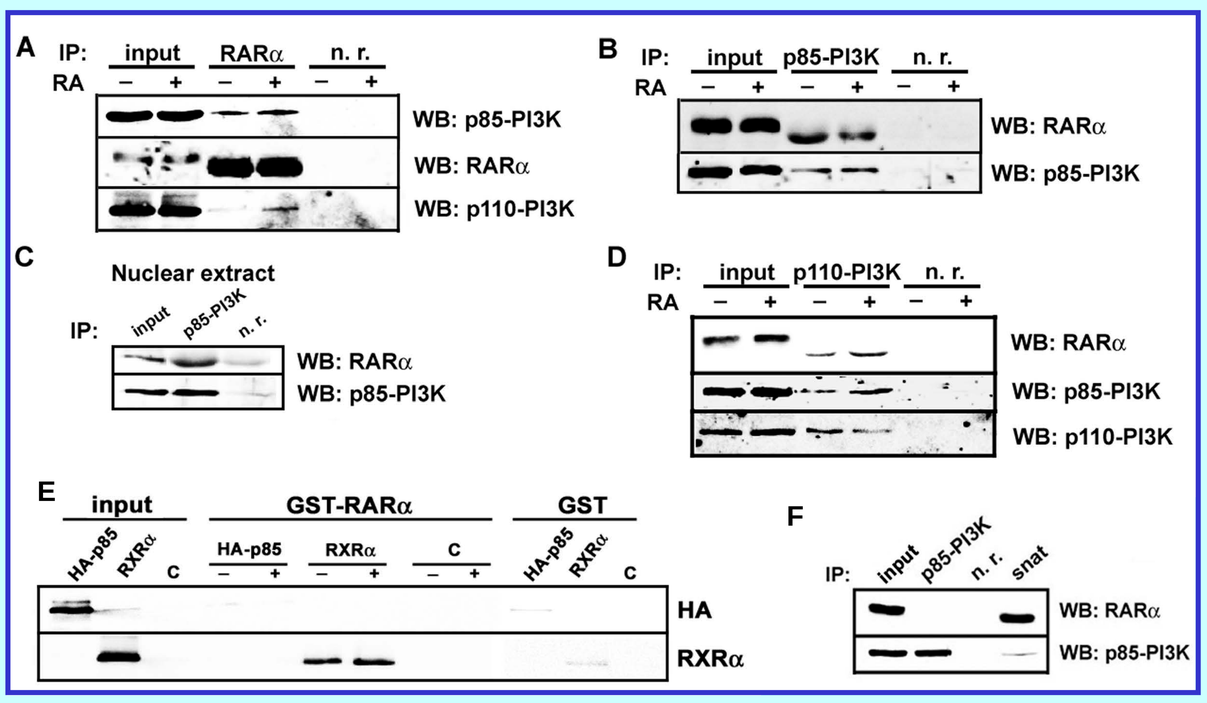
Next we have investigated the consequences of the activation of signalling pathways by RA in neuroblastoma cells. Downstream targets of the PI3K/Akt signalling pathway, like mTOR or p70S6K, are activated upon RA addition. In addition we show that RA addition rapidly increased phosphorylation of several Akt kinase target proteins in the nucleus, identified by an antibody recognizing the phosphorylated Akt motif. We have analyzed possible nuclear protein targets for RA-induced phosphorylation, with the aim of finding functional links between non-genomic actions and classical transcriptional actions mediated by RAR. We have found that RA treatment rapidly results in phosphorylation of chromatin proteins (core histone H3), as well as proteins involved in transcriptional activation (transcription factor CREB). In a second approach, nuclear phospho-proteins from control and RA-treated neuroblastoma cells were purified by affinity chromatography and analysed by 2D-electrophoresis. The differentially expressed spots were identified by Mass Spectrometry. We have found that RA treatment induces phosphorylation of chromatin proteins (HMGB1 and Histone H1.5) and proteins involved in the processing and transport of mRNA (PABP, hnRNP-K, hnRNP-C1/C2, Nucleophosmin).

1. *Rapid Activation of PI3K/AKT by RA through a non-genomic action of the Nuclear Receptor RAR*



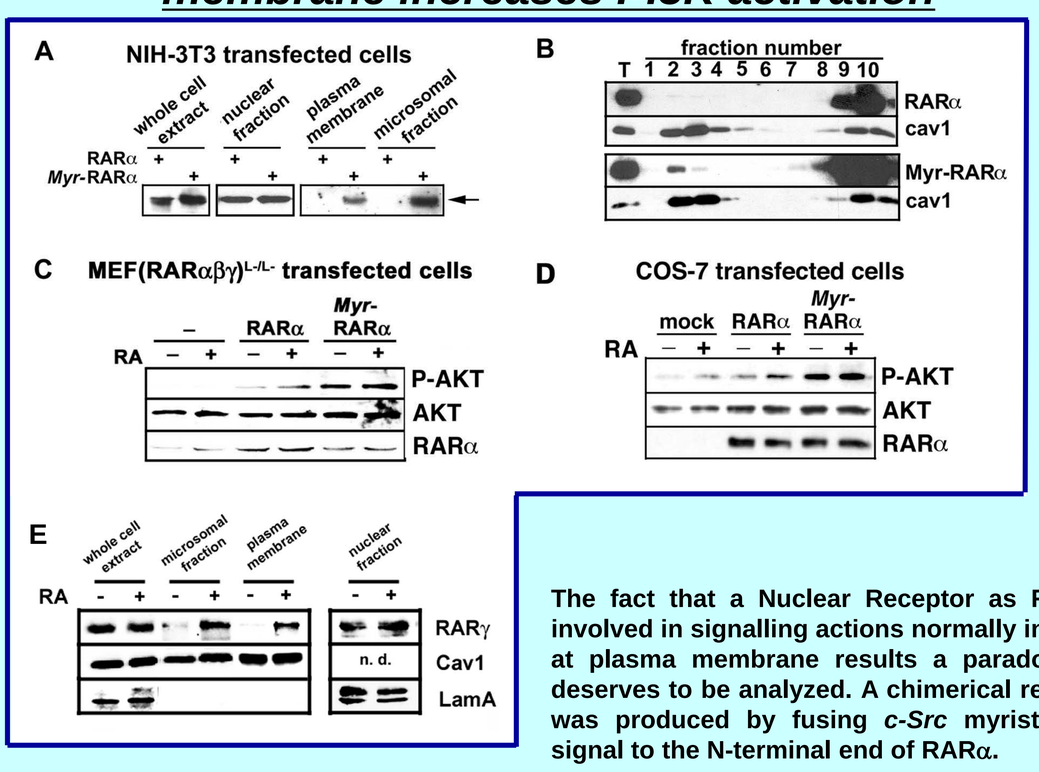
Activation of PI3K/Akt signalling by RA in SH-SY5Y neuroblastoma cells is rapid (within 5 min. of treatment) (Fig. 1A), and did not required newly synthesized proteins or newly transcribed genes (Fig. 1B). This suggests that activation of PI3K/Akt by RA occurs through an atypical non-genomic mechanism. Involvement of the Nuclear Receptor RAR in the activation of PI3K/Akt is supported by three types of evidences: 1. The pharmacological specificity of the activation, because all RAR agonists tested are good activators of PI3K/Akt (Fig. 1C). Surprisingly, RA transcriptional antagonist AGN193109 did not prevented activation of PI3K by RA or TTNPB (Fig. 1D), and acts as an agonist activating PI3K/Akt. However, AGN193109 avoided RA-induced transcriptional activation of *RARB* (Fig.1E), suggesting a mixed agonist/antagonist profile for AGN193109. 2. Loss and gain of function experiments with MEF-RAR($\alpha\beta\gamma$)$^{L-/L-}$ cells. RAR null cells have lost the ability of activating PI3K/Akt signalling (Fig. 1G), as compared with MEF-wt cells (Fig. 1F). Activation of PI3K/Akt by RA could be restored by expressing a functional RARα (Fig. 1H). 3. PI3K activity is associated to liganded RAR in SH-SY5Y cells, as shown in PI3K assays performed on RARα immunoprecipitates (Fig. 1 I).

2. *Association between RARα and PI3K subunits*



The experiment shown in Fig. 1I argues for a physical interaction between RAR and the subunits of PI3K. A stable association between RARα and the regulatory subunit of PI3K, p85, which was not affected by the administration of RA, could be detected in SH-SY5Y neuroblastoma cells by immunoprecipitation (Fig. 2A and 2B). That complex could be immunoprecipitated from nuclear extracts (Fig. 2C). However, association of the catalytic subunit p110-PI3K to that complex depends on the administration of RA (Fig. 2A and Fig. 2D), suggesting that activation of RARα by its cognate ligand results in the formation of a signalling complex including RARα, and the regulatory and catalytic subunits of PI3K. The interaction between RARα and p85-PI3K does not appear to be direct, since a protein-protein interaction could not be detected *in vitro* by GST-pulldown experiments (Fig. 2E). In the same conditions, the experiment readily detected the interaction between RARα and its dimerisation partner RXRα. Consistently with that, binary complexes including RARα and p85-PI3K could not be detected when co-expressed in COS-7 cells (Fig. 2F). The experiment suggests that other yet unknown proteins mediating or stabilizing the interactions between RAR and p85-PI3K might be part of the signalling complex.

3. *Ectopic expression of RARα to the plasma membrane increases PI3K activation*



The fact that a Nuclear Receptor as RAR is involved in signalling actions normally initiated at plasma membrane results a paradox and deserves to be analyzed. A chimerical receptor was produced by fusing *c-Src* myristylation signal to the N-terminal end of RARα.

This *Myr*-RARα is mainly located in the nucleus, but could be detected in the plasma membrane at significative levels (Fig. 3A). Moreover, *Myr*-RARα was present in the *lipid rafts* fractions, plasma membrane microdomains involved in signalling (Fig. 3B). Transfection of *Myr*-RARα chimerical receptor to MEF-RAR($\alpha\beta\gamma$)$^{L-/L-}$ or COS-7 cells resulted in strong activation of PI3K/Akt, that nevertheless could be detected in the absence of RA (Fig. 3C and 3D). The experiment underscores a novel role for RAR at the membrane, and suggests a requirement of the ligand for the location of RAR at the plasma membrane, that could be overcome by ectopically targeting of the receptor to the membrane. 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). Experiments with a fluorescent GFP-RARα fusion protein did not show a consistent membrane distribution pattern (data not shown). In conventional biochemical cell fractionation experiments performed in SH-SY5Y cells 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), suggesting the idea of a ligand-induced translocation of the receptor to plasma membrane (Fig. 3E).

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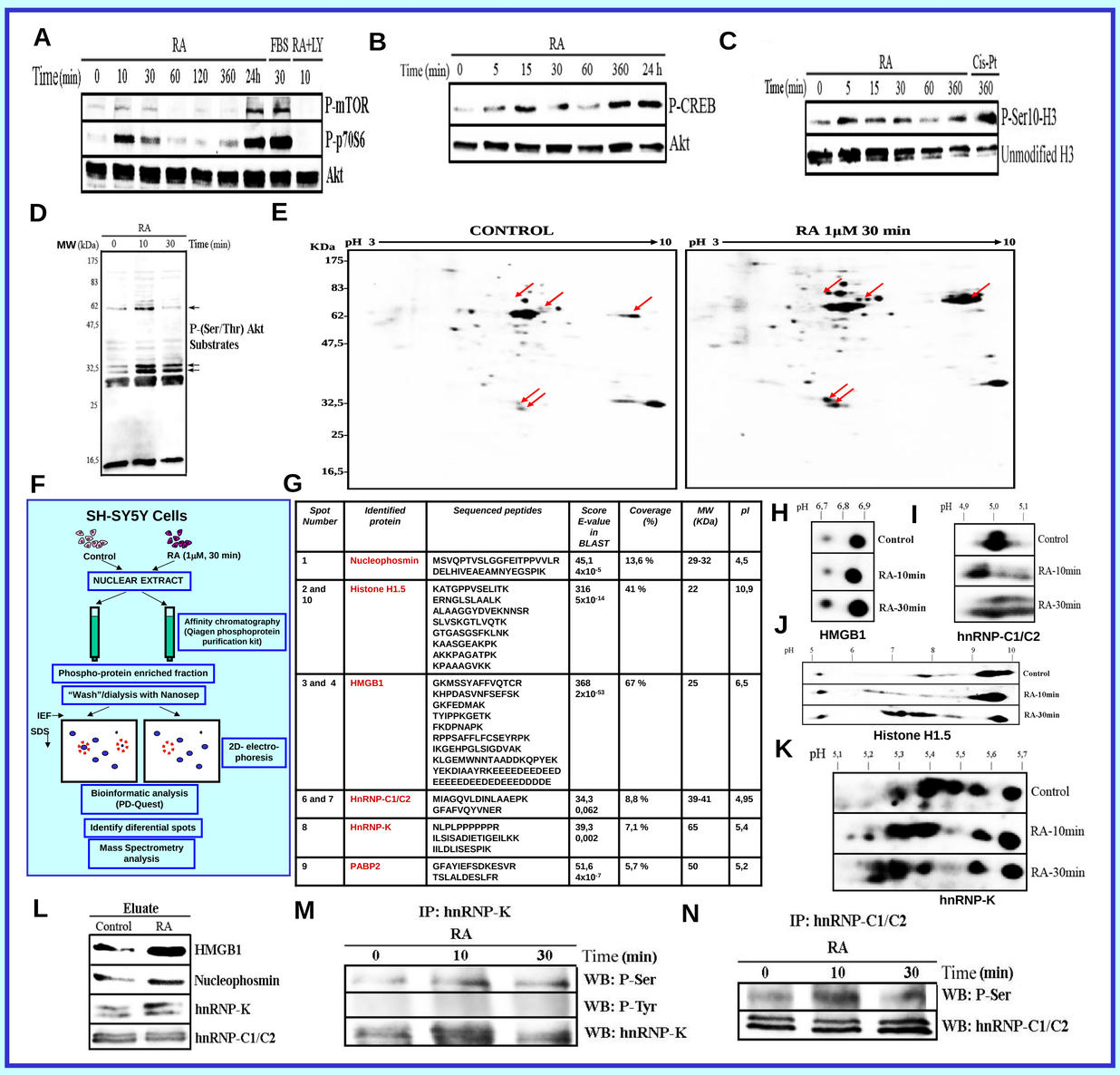
4. *RA induces rapid phosphorylation of nuclear proteins*



Spot Number	Identified protein	Sequenced peptides	Score E-value in BLAST	Coverage (%)	MW (KDa)	pI
1	Nucleophosmin	MSVQPTVSLGGFEITPPVVLR DELHIVEAEAMNYEGSPIK	45,1 4x10^{-5}	13,6 %	29-32	4,5
2 and 10	Histone H1.5	KATGPPVSELITK ERNGLSLAALK ALAAGGYDVEKNNSR SLVSKGTLVQTK GTGASGSFKLNK KAASGEAKPK AKKPAGATPK KPAAAGVKK	316 5x10^{-14}	41 %	22	10,9
3 and 4	HMGB1	GKMSSYAFFVQTCR KHPDASVNFSEFSK GKFEDMAK TYIPPKGETK FKDPNAPK RPPSAFFLFCSEYRPK IKGEHPGLSIGDVAK KLGEMWNNTAADDKQPYEK YEKDIAAYRKEEEEDEEDEED EEEEEDEEDEEDEEEDDDDE	368 2x10^{-53}	67 %	25	6,5
6 and 7	HnRNP-C1/C2	MIAGQVLDINLAAEPK GFAFVQYVNER	34,3 0,062	8,8 %	39-41	4,95
8	HnRNP-K	NLPLPPPPPPR ILSISADIETIGEILKK IILDLISESPIK	39,3 0,002	7,1 %	65	5,4
9	PABP2	GFAYIEFSDKESVR TSLALDESLFR	51,6 4x10^{-7}	5,7 %	50	5,2

Short-time RA treatment of neuroblastoma cells leads to rapid phosphorylation of PI3K/Akt downstream substrates, as mTOR and p70S6 kinases (Fig. 4A). The phosphorylation levels of other likely targets for that signalling pathway, as transcription factor CREB (Fig. 4B) and Histone H3 (Fig. 4C) are also increased by RA administration. RA-induced phosphorylation of several nuclear Akt substrates was detected in 1D- (Fig. 4D) and 2D- Western Blots (Fig. 4E) with specific antibodies against phosphorylated Akt kinase motif. To identify the targets of RA-induced phosphorylation in the nucleus, we have set a proteomic approach (Fig. 4F), combining affinity purification of phosphoproteins, 2D-electrophoretic separation, and mass spectrometry analysis of the differential spots. This approach leaded to the identification of 6 nuclear proteins: Nucleophosmin, Histone H1.5, HMGB1, hnRNP-C1/C2, hnRNP-K, and PABP2 (Fig. 4G and 4L). Validation of those identified targets is being carried out by their shift towards acidic pI on 2D-Western blots (Fig. 4H-K) and by immunoprecipitation with antibodies against phosphorylated amino acids (Fig. 4M and 4N). These results suggest that RA-induced rapid phosphorylation may be involved in chromatin remodelling and mRNA processing, transport and/or translation.