PROTEOMIC ANALYSIS OF PHOSPHORYLATED NUCLEAR PROTEINS
UNDERSCORES NOVEL ROLES FOR RAPID ACTIONS OF RETINOIC ACID IN
THE REGULATION OF mRNA SPlicing AND TRANSLATION

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Retinoic Acid (RA) signaling is mediated by the Retinoid Acid Receptor (RAR), belonging to the Nuclear Hormone Receptor superfamily. In addition to its classical transcriptional actions, RAR also mediates rapid transcription-independent actions (also called non-genomic), consisting in the activation of signal transduction pathways, as the phosphatidylinositol-3-kinase (PI3K) or the ERK MAP-kinase signaling pathways. RA-induced rapid actions play roles in different physiological contexts. As an effort towards understanding the functions of those rapid actions on signaling elicited by RA, we have identified nuclear proteins whose phosphorylation state is rapidly modified by RA treatment in neuroblastoma cells, using a proteomic approach. Our results show that RA treatment led to changes in the phosphorylation patterns in two families of proteins: (i) those related to chromatin dynamics in relation to transcriptional activation, and (ii) those related to mRNA processing and in particular mRNA splicing. We show that treatment of neuroblastoma cells with RA leads to alteration of the regulation of pre-mRNA splicing and mRNA translation, via the activation of signaling pathways. Thus, our results support the idea of the non-genomic signaling elicited by RAR in the regulation of mRNA processing, as part of a cellular response orchestrated by the nuclear receptor RAR. The results shown here contribute to the idea that transcriptional and transcription-independent actions elicited by nuclear hormone receptors are integrated and converge at multiple levels in the regulation of gene expression.

1. Short RA treatments lead to modifications in the phosphorylation patterns of nuclear proteins

Short RA treatments induce the activation of the PI3K signaling pathway and its downstream components mTOR and p70S6 Kinase (Fig. 1A). Western blot with an antibody against phospho-serine/threonine residues within Akt kinase phosphorylation consensus site (RRKXXS/T), revealed that RA treatment increased rapidly the phosphorylation of several Akt substrate proteins, detectable in whole cell extracts (Fig. 1B), as well as in nuclear extract (Fig. 1C). 2-D western blots demonstrate that RA treatment leads to an increase in the number and intensity of the spots corresponding to phosphorylated Akt substrates in nuclear extracts from neuroblastoma cells (Fig. 1D).

The ITRAQ method is based in the labeling of peptide fractions with reporter tags of different masses. We run in parallel the affinity-purified nuclear phospho-proteins from cells treated during 0, 15 and 30 min with RA. After denaturation and trypsin digestion, the samples were labeled and mixed. The peptides were separated by multi-dimensional nano-liquid chromatography, and different fractions analyzed by tandem Mass Spec. The proteins are identified on the basis of peptide fragments, and a relative quantification is obtained from the relative levels of each reporter tag.

The ITRAQ assay resulted in the identification of 63 proteins whose phosphorylation changed as effect of RA treatment.

3. Analysis of RA-induced differentially phosphorylated proteins through the ITRAQ assay

Among the proteins whose phosphorylation is modified by RA, the proteins involved in RNA metabolism are majority. The phosphorylation of proteins involved in mRNA splicing is remarkable. Therefore we have postulated the following hypothesis:

HYPOTHESIS:
RA, through the rapid activation of signaling pathways could regulate mRNA processing/splicing as part of a cellular response orchestrated by the nuclear receptor RAR

4. ITRAQ: Gene Ontology analysis of RA-induced differentially phosphorylated proteins

Using a splicing reporter minigene based on E1A (Fig. 5A) we could show that RA treatment alters the pattern of alternative splicing. This regulation is sensitive to PI3K inhibitors, but not to MAP kinase inhibitors. The interaction between the SR proteins and the 5’ UTR of the E1A minigene is highly dependent on the presence of a specific SR protein binding site. RA increases the phosphorylation of 4E-BP1, an inhibitor of cap-binding eIF4E, through the activation of PI3K (Fig. 6C). However additional mechanisms must exist to explain the effects of MEK inhibitors such as U0126, that does not interfere with the activation of PI3K pathway (Fig. 6D).

5. RA influences the regulation of alternative splicing via activation of signaling pathways

6. RA regulates translation through the activation of signaling pathways

7. RA treatment modifies the alternative splicing pattern on endogenous genes in neuroblastoma cells

Preliminary data obtained with Affymetrix Human Gene 1.0 ST microarray (exon-specific probes) showed that RA treatment affects the expression of many genes involved in alternative splicing. These results open new perspectives for understanding the mechanisms that underlie the regulation of alternative splicing in response to RA treatment.

8. Conclusions

We provide the first evidence that RA, through the activation of signaling pathways could regulate mRNA splicing and translation. The results shown here contribute to the idea that transcriptional and transcription-independent actions elicited by nuclear hormone receptors are integrated and converge at multiple levels in the regulation of gene expression.

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