c-Ki-ras oncogene amplification and FGF2 signaling pathways in the mouse Y1 adrenocortical cell line

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ABSTRACT

The mouse Y1 adrenocortical tumor cell line is highly responsive to FGF2-(Fibroblast Growth Factor 2) and possesses amplified and over-expressed c-Ki-ras proto-oncogene. We previously reported that this genetic lesion leads to high constitutive levels of activation of the c-Ki-Ras-GTP→PI3K→Akt signaling pathway (Forti et al. 2002). On the other hand, activation levels of another important pathway downstream of c-Ki-Ras-GTP, namely, Raf→MEK→ ERK, remain strictly dependent on FGF2 stimulation (Rocha et al. 2003). Here we show that, first, FGF2 transiently up-regulates the c-Ki-Ras-GTP→PI3K→Akt pathway, in spite of its high basal levels. Second, c-Ki-Ras-GTP transient up-regulation likely underlies activation of the ERK1/2 pathway by FGF2. Third, c-Ki-Ras-GTP high basal levels suppress activation of the c-H-Ras onco-protein. But, Y1 cells, expressing dominant negative mutant RasN17, display a rapid and transient up-regulation of c-H-Ras-GTP upon FGF2 treatment. Elucidation of FGF2-signaling pathways in Y1 tumor cells can uncover new targets for drug development of interest in cancer therapy.

Key words: FGF2, adrenocortical tumor cells, c-ki-ras, c-h-ras, ERK.

INTRODUCTION

Fifty years ago, Pavan and collaborators (Breuer and Pavan 1955, Ficq and Pavan 1957) described differential replication of DNA in the giant polytene chromosomes of salivary glands from the diptera *Rhynchosciara americana*, a phenomenon thereafter denominated DNA puffing. In the following twenty and five years, molecular biology studies from Francisco Lara's laboratory proved that DNA puffs were, in fact, end products of DNA or gene amplification (Meneghini et al. 1971, Glover et al. 1982). Thus, in a pioneering series of publications, the laboratories of Pavan and Lara concurred to discover, experimentally demonstrate and conceptualize gene amplification as a basic biological phenomenon.

During the eighties, amplification of oncogenes became progressively recognized as an important part of the genetic bases underlying both carcinogenesis and resistance of tumor cells to drug therapy (Schwab 1999). c-Ki-Ras was the first oncogene reported amplified, over-expressed and located in abnormal chromosomes of the mouse Y1 adrenocortical tumor cell line (Schwab et al. 1983). Y1, isolated by Yasumura et al. (1966), is a phenotyp-

ically stable, ACTH-responsive, steroid-secreting, clonal cell line that expresses high levels of ACTH-Receptors, closely mimicking fasciculate cells from the normal adrenal cortex. Y1, along of the last forty years, have remained an adrenocortical cell line singularly appropriate for studies of steroidogenesis and ACTH regulatory pathways (Schimmer 1979, Rainey et al. 2004). More recently, we have been investigating control of cell cycle in Y1 adrenocortical cells, aiming to unravel molecular mechanisms underlying $G0 \rightarrow G1 \rightarrow S$ transition of adrenal cell cycle (Armelin et al. 1996, Rocha et al. 2003). Although the malignant phenotype of Y1 cells includes rapid tumor growth in Nude mice, evasion from apoptosis and limitless replicative potential, this cell line retains a remarkably tight control of the $G0 \rightarrow G1 \rightarrow S$ transition, being highly permissive to mitogenic stimulation by FGF2. In addition, from a cancer biology viewpoint, it is timely to analyze FGF2 signaling pathways in a tumor cell line displaying over-expression of the c-Ki-ras oncogene.

The ras oncogenes (H-, Ki-and N-ras) encode regulated GTPases switches of 21kDa that mediate, upstream signals initiated in membrane TKR (Tyr-kinase receptors), and, downstream signaling pathways that modulate gene transcription (Malumbres and Barbacid 2003). The ras gene family has been extensively studied over the last twenty years, mainly due to its growing importance in normal cell cycle control and cancer biology. The ras signaling pathways are now understood in great detail at the molecular levels, but this signaling network is not simple, with multiple points of bifurcation and feedbacks. Besides, Ras proteins have been described as the point of convergence of different pathways involved in diverse biological responses like: proliferation, survival, differentiation and tumorigenesis. Mutations and/or amplifications of c-Ki-ras are among the genetic lesions found in 90% of pancreatic, 50% of colon and 35% of lung human tumors (Bos 1990, Bardeesy and DePinho 2002). Presently, components of signaling pathways upstream and downstream of Ras oncoproteins are important targets in drug development

for cancer therapy (Downward 2003). However, this strategy has recognized limitations. Ras-signaling pathways are physiologically important for all normal cells and drugs that block Ras signaling are likely to have strong deleterious side effects in cancer patients.

In Y1 adrenocortical cells c-Ki-ras oncogene amplification (Schwab et al. 1983, Kimura and Armelin 1988) causes over-expression of both c-Ki-ras mRNA and protein, leading to high basal levels of c-Ki-Ras-GTP (Forti et al. 2002) and, consequently, partial deregulation of cell cycle control. Downstream of chronic c-Ki-Ras-GTP high levels the PI3K/Akt pathway is kept constitutively active, suppressing the expression of the CDK-inhibitor $p27^{kip1}$ protein (Forti and Armelin 2000, Forti et al. 2002) and partially deregulating transcription of the c-myc gene (Lepique et al. 2004). Attenuation of the constitutive levels of c-Ki-Ras-GTP in G_0/G_1-arrested Y1 cells, by enforced expression of the dominant negative mutant HaRasN17, renders the PI3K/Akt pathway stringently dependent on mitogen activation (Forti et al. 2002). In spite of the chronic high levels of c-Ki-Ras-GTP, Y1 cells retain stringent dependency of the ERK1/2 pathway on mitogens, accounting for the remarkable cell cycle control displayed by these cells upon serum step down and serum step up (Armelin et al. 1977, 1996, Lotfi and Armelin 2001, Rocha et al. 2003).

In G_0/G_1 cell cycle-arrested Y1 cells, FGF2 elicits a strong mitogenic response, involving: rapid ERK-MAPK activation (2-5min) and late S phase entry (8-9h), detected by BrdU labeling (Armelin et al. 1996, Lotfi et al. 1997, Lotfi and Armelin 2001, Schwindt et al. 2003, Rocha et al. 2003). But, FGF2, in addition to its classical mitogenic activity, concomitantly triggers a non-apoptotic cell death and/or senescence, by a process dependent on the high chronic levels of c-Ki-Ras-GTP found in Y1 cells (Costa et al. 2004, E.T. Costa and H.A Armelin, unpublished results). To elucidate the molecular mechanisms underlying this novel FGF2 death-effect, we are carefully analyzing FGF2 signaling in Y1 cells, particularly in respect to connec-

tions between FGF2-receptors (FGF2Rs) and Ras-initiated pathways. In this paper we report on the earliest activation steps of FGF2 signaling, namely, activation of Ras onco-proteins and of both ERK and PI3K/Akt pathways.

MATERIALS AND METHODS

Cell Line cultivation: Cells from the mouse Y1 adrenocortical tumor cell line (Yasumura et al. 1966) and Y1-RasN17 transfectant clonal lines carrying dominant negative mutant genes ras N17, were grown in 10% FCS-DME minus or plus 100 μg/mL G418. To arrest the cell cycle at G0/G1 boundary, we incubated cells growing exponentially in 10% FCS-DME for 48 h in SFM (serum-free medium). In SFM, Y1 cells are viable and fully responsive to FGF2, AVP and/or ACTH.

Y1-RasN1-1.5 and Y1-RasN17-3.1 Clonal Lines: Plasmid pMMrasDN is a construct containing the rasN17 under the control of the MMTV promoter (Forti et al. 2002) obtained from Dr. L. Feig (Tufts University, Boston) that was transfected into Y1 cells and neutrally selected with G418 to generate clonal lines exhibiting dexamethasone-inducible RasN17 protein. Y1-RasN17-3.1 transfectant clone express RasN17 in a dexamethasone dose dependent manner: 10^{-10}M dexamethasone is sufficient to increase RasN17 protein levels by more than 10 fold.

Ras-GTP assay by specific binding to RBD-GST (Raf1 Binding Domain-Glutathione S Transferase) fusion protein, covalently linked to Sepharose beads: Cells were lysed in 50mM Tris-HCl pH 8.0, 0.5% Nonidet P-40, 150 mM NaCl, 1% SDS, 0.5% sodium deoxycholate, 10 μg/mL leupeptin, 10 μg/mL aprotinin. Lysates were collected by centrifugation (10^4 g for 10 min at 4°C) and kept frozen. Aliquots of cell lysates containing 500 μg protein, quantified by the Bradford assay, were incubated with RBD-GST beads for 60 min at 4°C to bind Ras-GTP. Beads, carrying bound Ras-GTP and recovered by centrifugation, were washed with lysis buffer at 4°C, suspended in SDS-PAGE sample buffer (100 mM Tris-HCl pH 7.5, 200 mM DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue), and loaded on 12% SDS-PAGE gels. After electroblotting onto Hybond-C nitrocellulose membranes, using a semidry Bio-Rad apparatus, activated c-Ki-Ras and c-H-Ras were detected with polyclonal antibodies specific for, respectively, c-Ki-Ras and c-H-Ras (Santa Cruz). Each sample of activated Ras, recovered by these procedures, was compared in Western blots to total Ras in 50 μg cell-protein aliquots of the respective lysate not incubated with RBD-GST beads.

Analysis of ERK and AKT Phosphorylation: Cells were lysed in cold 62.5 mM Tris-HCl pH 2% w/v SDS, 10% glycerol, 50 mM DTT, 10% w/v bromophenol blue. Lysates were sonicated for 2 min, boiled for 5 min, clarified by centrifugation (14 000 g, 5 min, 4°C), and 150 μg protein aliquots were loaded on 10% SDS-PAGE gels. After electroblotting onto Hybond-C nitrocellulose membranes, using a semidry Bio-Rad apparatus, the total or active (phosphorylated) proteins isoforms were detected with monospecific rabbit antibodies (New England Biolabs and Cell Signaling), followed by a secondary peroxidase-conjugated antirabbit polyclonal antibody for chemiluminescent detection (ECL, Amersham-Pharmacia).

RESULTS AND DISCUSSION

Y1 adrenocortical tumor cells display high basal levels of c-Ki-Ras-GTP independently of extracellular mitogenic signals (Forti et al. 2002, Rocha et al. 2003). Assays of c-Ki-Ras-GTP levels in G_0/G_1-arrested parental Y1 cells show that FGF2 triggers a very rapid transient increase in c-Ki-Ras-GTP above the high basal levels of c-Ki-RasGTP exhibited by these cells (0.5–1 min) [Fig. 1A], followed by sequential activation and nuclear migration of ERK1/2 kinases, that are prerequisites for a ERK1/2-dependent mitogenic response [Fig. 1B and C]. Thus, first, despite its high basal levels, c-Ki-Ras-GTP is further transiently up regulated by FGF2. Second, the very rapid transient up-regula-

tion of c-Ki-RasGTP (0.5–1 min) could mediate a transient activation of ERK1/2 (5–30 min) according to the canonical pathway Ras→Raf1→MEK1 →ERK1/2.

To analyze the phenotypic effects of the constitutive c-Ki-Ras-GTP levels, we developed transfectants of Y1 cells carrying the human dominant negative mutant Ha-rasN17 (Feig and Cooper 1988) under the control of the MMTV conditional promoter. In these Y1-RasN17-transfectants (R3.1 and R1.5 clones), dexamethasone induces the H-rasN17 transgene causing drastic reduction of c-Ki-Ras-GTP levels, but without completely blocking cell cycle, allowing analysis of both G1 progression and cell proliferation (Forti et al. 2002, Lepique et al. 2004).

In G_0/G_1-arrested cells of the Y1-RasN17, clone3.1, transfectant line, c-Ki-Ras-GTP levels are maintained constitutively high, but, upon dexamethasone treatment, c-Ki-Ras-GTP decreases to negligible levels [Fig. 2]. However, dexamethasone induction of the H-RasN17 dominant negative transgene is not sufficient to interrupt activation of the Ras→Raf→MEK→ERK pathway by signals initiated in FGF2Rs, as shown by the pattern of ERK1/2 phosphorylation displayed in Fig. 2.

In parental Y1 cells and Y1-RasN17 transfectants in the absence of dexamethasone, mouse endogenous c-H-Ras-GTP is maintained at negligible levels, irrespective of FGF2 stimulation (not shown). However, in G_0/G_1-arrested Y1-RasN17-3.1 transfectants treated with dexamethasone, FGF2 causes a very rapid transient increase in c-H-Ras-GTP levels (Fig. 3). Therefore, high basal levels of c-Ki-Ras-GTP suppress c-H-Ras activation, whereas under minimal basal levels of c-Ki-Ras-GTP Y1 cells respond to signals initiated in FGF2Rs with rapid and concomitant activation of both c-Ki-Ras and c-H-Ras proteins, resembling normal cells.

We previously reported that Y1 cells exhibit high basal levels of activated Akt dependent on chronically elevated c-Ki-Ras-GTP levels and PI3K activity (Forti et al. 2002). In Y1-RasN17 transfectant lines dexamethasone causes severe reduction

of both c-Ki-Ras-GTP levels and phosphorylated-Akt, which are transiently restored by FGF2 treatments. But, PI3K inhibitors block phosphorylation of Akt promoted by FGF2 (Forti et al. 2002), indicating that the whole pathway is comprised by FGF2R→c-Ki-Ras-GTP→PI3K→Akt. Thus, the genetic lesion of Y1 cells, namely, amplification of the c-Ki-ras gene, leads to high basal levels of c-Ki-Ras-GTP, keeping the PI3K→Akt pathway constitutively activated. Here we confirm and extend these observations showing that, under low or high basal levels of c-Ki-Ras-GTP, signals initiated in FGF2Rs trigger a sequential wave of transient activations, first, with c-Ki-Ras-GTP by 0.5 min [Fig. 1A], second, with ERK-phosphorylation by 5 min [Figs. 1C and 2] and third, Akt-phosphorylation by 5 min (Figs. 4A and 4B). However we cannot completely rule out the possibility that signals initiated in FGFR, via adaptor proteins FRS2→Grb2→Gab1, might directly activate PI3K, bypassing SOS→Ras-GTP (Ong et al. 2001).

We also had previously shown that, in Y1 cells, FGF2 mitogenic activity is antagonized by ACTH (Armelin et al. 1996, Lepique et al. 2004) and AVP (arginine-vasopressin) (Schwindt et al. 2003). These inhibitory effects of ACTH and AVP follow different molecular mechanisms (Lepique et al. 2004, Schwindt et al. 2003), however both converge to PI3K→Akt pathway, as presented in Fig. 5. ACTH, via cAMP/PKA, promotes dephosphorylation of basal activated-Akt, but cannot block transient activation of Akt elicited by FGF2 [Fig. 5]. Therefore ACTH does not block signals initiated in FGF2-Receptors, however it inactivates one specific downstream effector of c-Ki-Ras-GTP chronic high levels, namely, basal activated-Akt. On the other hand, AVP antagonizes FGF2-Receptor signals, preventing the transient activation of Akt by FGF2, but without any effect on basal levels of phosphorylated Akt [Fig. 5].

In Y1 adrenocortical tumor cells, the results reported here imply that: a) activated constitutive levels of c-Ki-Ras-GTP→PI3K→Akt do not preclude transient up-regulation of this pathway by
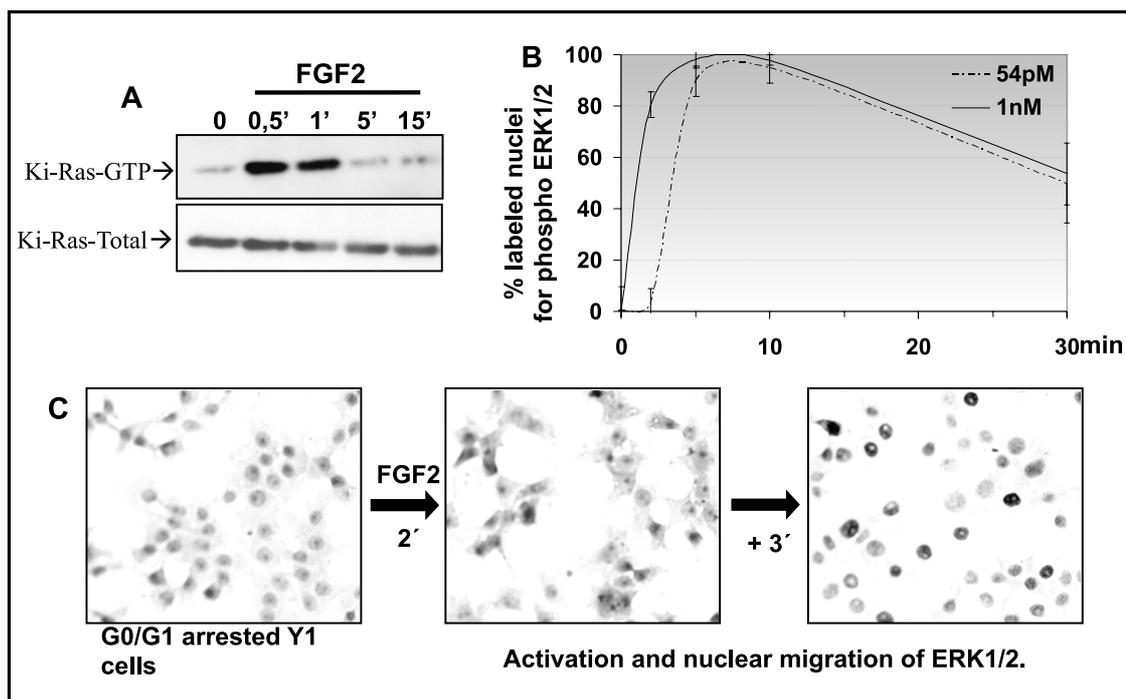
Fig. 1 – Initial signals discharged by FGF2R activation in G0/G1-arrested Y1 adrenocortical cells: (A) SDS-PAGE Western Blot assays show a rapid and transient c-Ki-Ras activation induced by FGF2 (1 nM). These results are representative of two independent experiments. (B) Kinetics of ERK1/2 activation in G0/G1 arrested Y1 cells treated with 54 pM or 1 nM FGF2. Results from eight independent immunocytochemistry experiments were pooled to derive these curves. (C) Micrographs of an immunocytochemistry experiment showing rapid nuclear labeling with an anti-phospho-Erk1/2 antibody.
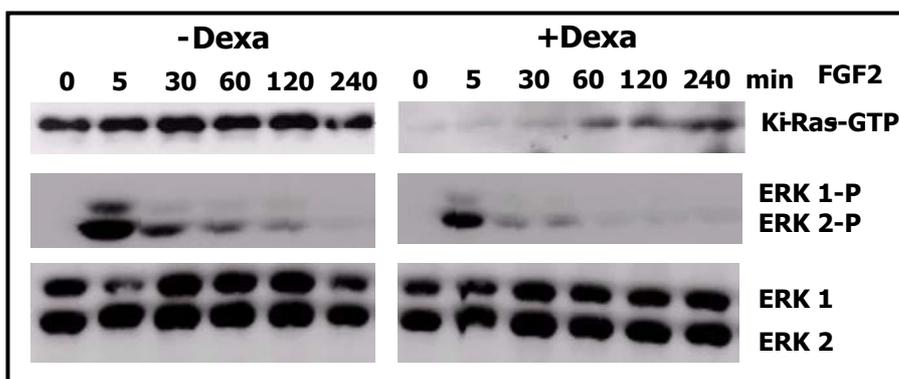
Fig. 2 – SDS-PAGE/Western Blot showing activation of both c-Ki-Ras and ERK by FGF2, in Y1-rasN17-3.1 clonal transfectant treated and untreated with dexamethasone. Cultures of G0/G1-arrested Y1-rasN17-3.1 cells were treated with FGF2 and periodically lysed according to the time points indicated in the top of the figure. The results presented in the figure are representative of two independent experiments.
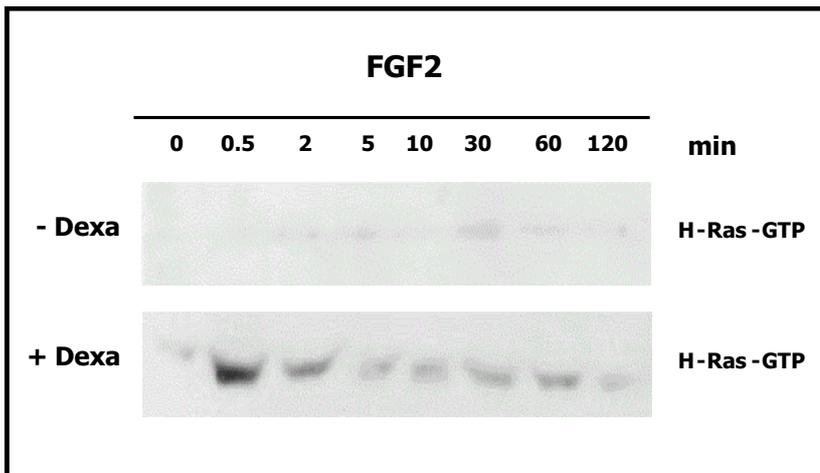
Fig. 3 – c-H-Ras activation by FGF2 in G_0/G_1-arrested Y1-RasN17-3.1 transfectants treated with dexamethasone. Protocol as in Fig. 2, except for the Western blot developed with a rabbit monoclonal antibody specific for c-H-Ras protein. Results representative of two independent experiments.
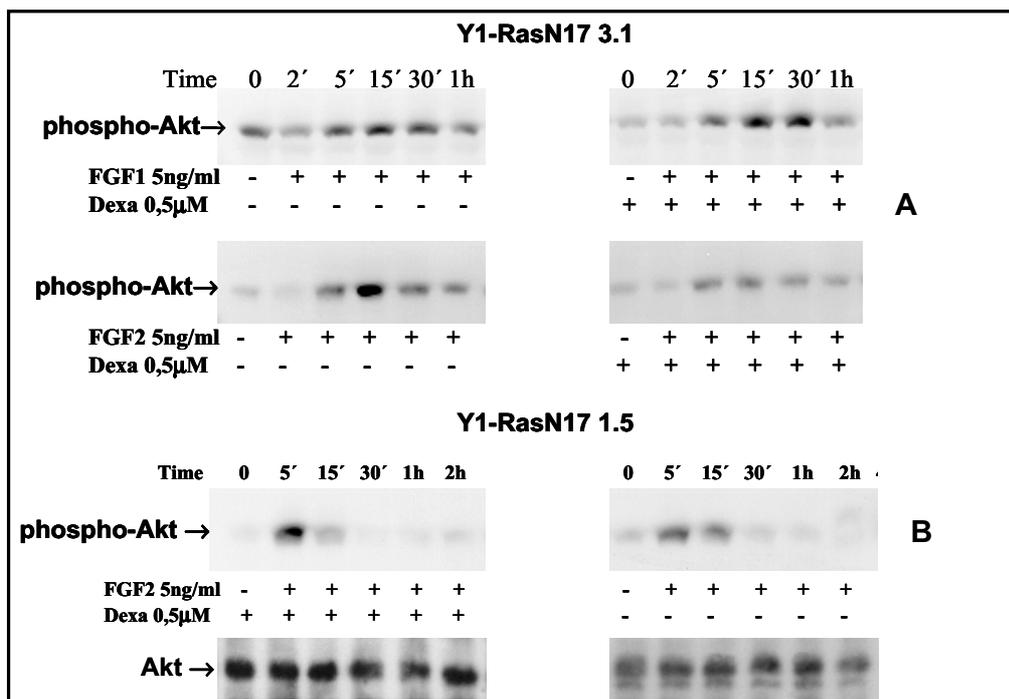
Fig. 4 – Kinetics of Akt activation by both, FGF2 and FGF1, in Y1-RasN17 transfectants. G0/G1-arrested Y1-rasN17-3.1 and Y1-rasN17-1.5 cells, ±dexamethasone for 24 h, were treated with FGF2 or FGF1 and periodically lysed according to the time points indicated in the top of the figure. Lysate aliquots containing 100-150 μg total protein were used for Western blot analysis with rabbit polyclonal antibodies against, respectively, phospho-Akt (Ser-473) and total-Akt. These results are representative of three independent experiments.
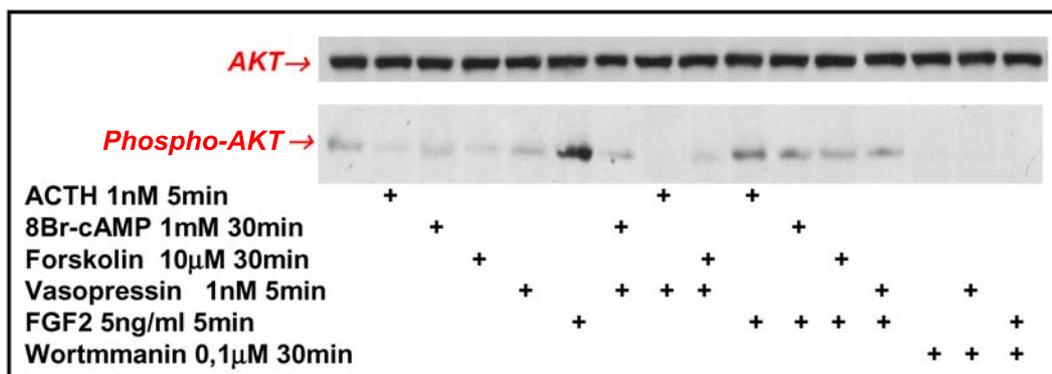
Fig. 5 – ACTH and AVP inhibition of Akt activation by FGF2, in Y1 parental cells. ACTH promotes dephosphorylation of basal phospho-Akt, but does not inhibit FGF2-induced Akt phosphorylation, AVP, on the other hand, blocks FGF2-induced Akt phosphorylation, but does not interfere with basal levels of phospho-Akt. Protocols as described in Fig. 2 and Methods. Results representative of three independent experiments.
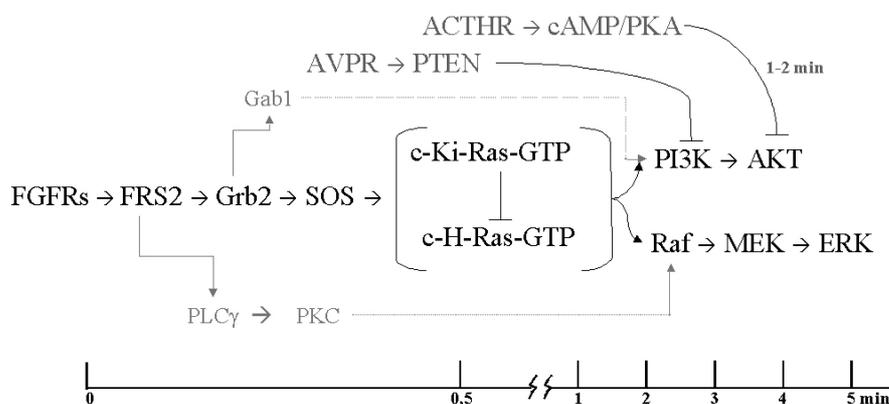
Fig. 6 – Scheme of the early steps of Ras-signaling pathways activation by FGF2 receptors in Y1 adrenocortical cells. The organization of elements and steps displayed in this Figure follows, nowadays, largely accepted diagrams of FGFR signaling pathways (Schlessinger 2004). This scheme overlooks the recognized fact that Ki-Ras and H-Ras proteins do not co-localize in membranes of normal cells (Hancock 2003). In the scheme PTEN mediates the inhibitory effects of AVPR over PI3K activity, this is a tentative conclusion based on recent observations (F.L. Forti and H.A. Armelin, unpublished results).

FGF2, b) c-Ki-Ras-GTP transient up-regulation likely underlies activation of ERK1/2 by FGF2, c) c-Ki-Ras-GTP high chronic levels suppress c-H-Ras activation by FGF2. These results and respective interpretations are summarized in the scheme of Fig. 6 depicting the early steps of activation of Ras-signaling pathways in Y1 adrenocortical cells.

In G0/G1-cell cycle-arrested Y1 cells, FGF2 triggers a canonical mitogenic response, involving G1 progression and S phase entry (Lotfi and Armelin 2001, Rocha et al. 2003), and, a nonapoptotic cell death and/or senescence (Costa et al. 2004, E.T. Costa and H.A. Armelin, unpublished results). We have seen that reduction of c-Ki-Ras-GTP basal lev-

els by enforced expression of the dominant negative mutant RasN17 recovers activation of both c-Ki-Ras and c-H-Ras by FGF2 (this paper) and, also, renders Y1 cells resistant to this novel FGF2 cell death-effect (Costa et al. 2004, E.T. Costa and H.A. Armelin, unpublished results). These observations suggest that a FGF2-triggered cell death-process, independent of the well-known FGF2-mitogenic activity, might exist as a natural unsuspected mechanism specific for restraining oncogene-induced proliferation of tumor cells. Hence, full elucidation of the signaling pathways activated by FGF2 in Y1 adrenocortical cells promises to uncover novel targets for drug development of interest in cancer therapy.

RESUMO

A linhagem tumoral Y1, originada de adrenocórtex de camundongo responde a FGF2 (Fator de Crescimento de Fibroblasto), possui o proto-oncogene c-ki-ras amplificado e a proteína c-Ki-Ras super-expressa e ativa (c-Ki-Ras-GTP). Em trabalhos anteriores mostramos que esta lesão genética causa ativação constitutiva da via de sinalização: c-Ki-Ras-GTP→PI3K→Akt (Forti et al. 2002). Por outro lado, a ativação da via de Raf→MEK→ERK, permanece estritamente dependente de estímulos de FGF2 (Rocha et al. 2003). Neste trabalho mostramos, primeiro, que estímulos de FGF2 ativam transientemente a via c-Ki-Ras-GTP→PI3K→Akt para níveis superiores aos expressos constitutivamente. Segundo, a ativação transiente de c-Ki-Ras-GTP por FGF2 permite a ativação da via de ERK1/2. Terceiro, os níveis basais elevados de c-Ki-Ras-GTP inibem a ativação da proteína c-H-Ras, pois células Y1 expressando o mutante negativo RasN17 apresentam uma rápida e transiente ativação de c-H-Ras-GTP após tratamentos de FGF2. Estes estudos das vias de sinalização acionadas por FGF2 em células adrenais tumorais Y1 podem fornecer novos alvos para o desenvolvimento de drogas de interesse para terapia oncogênica.

Palavras-chave: FGF2, células adrenocorticais tumorais, c-ki-ras, c-h-ras, ERK.

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