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The dual-specificity kinases, TOPK and DYRK1A, are critical for oocyte maturation induced by wild-type-but not by oncogenic- ras-p21 protein

Yongxia Qu[1], Victor Adler[2,3], Lara Izotova[4], Sidney Pestka[4], Wilbur Bowne[5], Josef Michl[6], Mohamed Boutjdir[1], Fred K. Friedman[7], Matthew R. Pincus[2,3]

[1]Research and Development Service and Program in Molecular Cardiology, New York Harbor VA Medical Center, 800 Poly Place, Brooklyn, NY 11209, [2]Department of Pathology and Laboratory Medicine (113), New York Harbor VA Medical Center, 800 Poly Place, Brooklyn, NY 11209, [3]Department of Pathology, SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203, [4]Department of Microbiology and Human Genetics, Robert Wood Johnson Medical Center, University of Medicine and Dentistry of New Jersey, 675 Hoes Lane – Room 727, Piscataway, NJ 08854 and Cancer Institute of New Jersey, New Brunswick, NJ 08901, [5]Departments of Surgery, SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203 and VA Medical Center, 800 Poly Place Brooklyn, NY 11209, [6]Departments of Pathology, Microbiology and Anatomy and Cell Biology, SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203, [7]Laboratory of Experimental and Computational Biology Building 12B, Room, B103, National Cancer Institute, Bethesda, MD 20892

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1. ABSTRACT

We have previously found that oncogenic ras-p21 and insulin, which activates wild-type ras-21 protein, both induce *Xenopus laevis* oocyte maturation that is dependent on activation of raf. However, oncogenic ras-p21 utilizes raf-dependent activation of the two classic raf targets, MEK and MAP kinase (MAPK or ERK) while insulin-activated wild-type ras-p21 does not depend on activation of these two kinases. Utilizing a microarray containing the entire *Xenopus* genome, we discovered two dual specificity kinases, T-Cell Origin Protein Kinase (TOPK), known to bind to raf and the nuclear kinase, DYRK1A, that are expressed at much higher levels in insulin-matured oocytes. Using SiRNA's directed against expression of both of these proteins, we now show that each inhibits insulin-but not oncogenic ras-p21-induced oocyte maturation. Control siRNA's have no effect on either agent in induction of maturation. We find that each SiRNA "knocks down" expression of its target protein while not affecting expression of the other protein. These results suggest that both proteins are required for maturation induced by wild-type, but not oncogenic, ras-p21. They also suggest that oncogenic and wild-type ras-p21 utilize pathways that become divergent downstream of raf. On the basis of these findings, we propose a model for two signal transduction pathways by oncogenic and activated wild-type ras-p21 showing points of overlap and divergence.

2. INTRODUCTION

In a series of studies, we have found that oncogenic and wild-type ras-p21 induce mitogenesis utilizing overlapping but distinct pathways (1,2). Small molecule agents and peptides from ras-p21 and some of its targets selectively inhibit oocyte maturation induced by injected oncogenic ras-p21 protein but have only minimal effects on insulin-activated wild-type ras-p21-induced maturation (1,2). In investigating possible differences in the signal transduction pathways induced by these two proteins, we found that oncogenic ras-p21 interacts directly with jun-N-terminal kinase (JNK) and its substrate, jun protein, while the wild-type protein does not require these interactions (2-5). Surprisingly, while both proteins require activation of raf, levels of phosphorylated MAP kinase (MAPK), or ERK-1 and ERK-2, are much higher in oocytes induced to mature with oncogenic p21 than in those induced to mature using insulin that activates wild-type ras-p21 protein (6). Since raf is known to activate MEK that, in turn, activates MAPK, these results suggested that wild-type p21 may interact with raf in a manner that results in activation of alternate target proteins on its signal transduction pathway. This conclusion was further supported by our finding that the MAPK inhibitor, MAPK phosphatase, MKP-1T4, completely blocks oocyte maturation induced by oncogenic ras-p21 but only partially blocks insulin-induced maturation (7).

In order to determine possible alternate raf-dependent but MAPK-independent pathways utilized by wild-type p21 in mitogenic signaling, we utilized a *Xenopus* microarray using RNA recovered from oocytes induced to mature with oncogenic p21 and with insulin (8). We found a number of proteins that are preferentially expressed in one or the other system. Of these, two proteins, both dual function kinases, T-Cell Origin Protein Kinase (TOPK) and the nuclear kinase, DYRK1A, were preferentially expressed in oocytes induced to mature with insulin (8). Neither of these kinases activates or is activated by MAPK (9-12), and TOPK is known to interact directly with raf from studies employing the two-hybrid yeast system (13), making it an attractive candidate for an alternate target of raf. We found that blots of lysates from oocytes, induced to mature with oncogenic ras-p21 and insulin, with anti-TOPK and anti-DYRK1A antibodies, show much higher protein expression in the lysates from the insulin-matured oocytes (8).

Because, therefore, these two proteins may be important components of a signal transduction pathway unique to the insulin-stimulated activated wild-type ras-p21 pathway, we now study the effects of inhibiting expression of each protein in the oocyte system. For this purpose, we have designed several inhibitory RNA (SiRNA) oligonucleotides that block expression of each of these two proteins. We explore the effects of each of these SiRNA's on oocyte maturation induced by oncogenic ras-p21 and by insulin and on the effects of each SiRNA on the expression of each of the two putative critical proteins.

3. MATERIALS AND METHODS

3.1. Materials

Val 12-Ha-*ras*-p21 protein was overexpressed in *E. coli* using the pGH-L9 expression vector containing the chemically synthesized Ha-ras gene, as previously described (14). Insulin was purchased from Sigma (St. Louis, MO) and was used directly. Silencing RNA (SiRNA) Nonadeca-Oligonucleotides to TOPK and DYRK-1A. We obtained four oligonucleotides (19-mers) for each gene from Dharmacon RNA Technologies (Lafayette, CO) that were optimized using SMARTpool with respect to 66 different parameters including sequence uniqueness. Each was based on the recently determined gene sequence for *Xenopus tropicalis* that has high identity and homology to that for *Xenopus laevis* (15). The oligonucleotide sequences from TOPK were taken from the gene accession number NM_001011346 and those for DYRK-1A were taken from gene accession number BC044104. We employed the primary oligonucleotide, i.e. the one with the most unique sequence, in each set. The following SiRNA sequences for each gene were used: TOPK: GGACGAAGGCGGAGTCATA, DYRK1A: TGAAATAGATTCCCTCATT. We also employed a negative control non-targeting SiRNA sequence optimized by D'harmacon RNA Technologies (e.g., at least four mismatches to any human, mouse or rat gene, Product No. D-001210-01-05). The sense strand sequence is: 5'-UAGCGACUAAACACAUCAAUU-3'.

3.2. Methods

3.2.1. Oocyte microinjection and incubation

Oocytes were obtained from *Xenopus laevis* frogs (Connecticut Valley Biological, Southhampton, MA) as described previously (1,23). Surgically removed ovarian lobes were dissected and treated for 1.5 hours with 1.5 mg/ml collagenase B dissolved in Ca-free ND96 medium (mmol/L: NaCl 96, KCl 2, $MgCl_2$ 2, HEPES 5, pH 7.4). Approximately 100 Stage VI oocytes were then harvested for each of eight sets of triplicate experiments, four involving injection of Val 12-p21 into oocytes and four in which the oocytes were incubated with insulin. In all experiments with RNA's, each RNA was injected into oocytes at a concentration of 300 pg/ml.

In the experiments with Val 12-p21, injected at a concentration of 100 ug/ml, 50 nl per oocyte in all experiments, triplicate experiments were performed in which this protein was injected alone; was co-injected with SiRNA to TOPK; was co-injected with SiRNA to DYRK1A; and was co-injected with control RNA. Identical experiments were performed using insulin, present at a concentration of 10 ug/ml in all experiments. In these experiments, the following conditions were employed: insulin alone; oocytes were injected with SiRNA to TOPK, and 1 hour later, insulin (10ug/ml) was added to the incubation medium; oocytes were injected with SiRNA to DYRK1A, and 1 hour later, insulin (10ug/ml) was added to the incubation medium; and oocytes were injected with control RNA, and 1 hour later, insulin (10ug/ml) was added to the incubation medium.

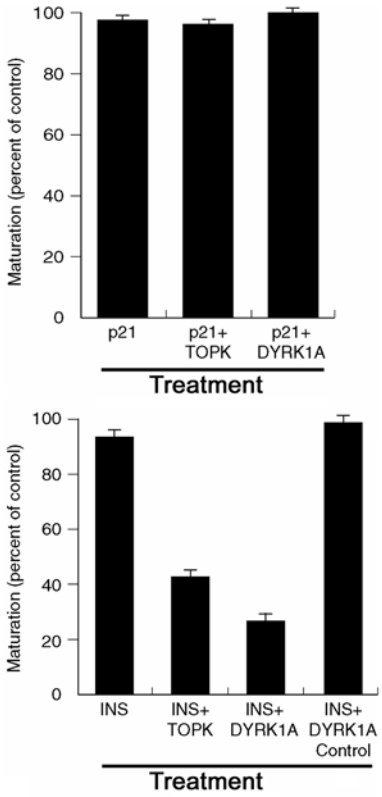

Figure 1. A. Effects of various RNA's on oocyte maturation, as measured by GVBD (1,8) described in the Methods section, co-injected into oocytes with Ha-ras-Val 12-p21. Condition 1 shows the extent of maturation when ras-p21 is injected alone; conditions 2 and 3 show extent of maturation when Val 12-ras-p21 is co-injected with SiRNA against TOPK (condition 2) and SiRNA against DYRK1A (condition 3). All maturation results are expressed as percent of control that yielded the highest level of maturation i.e., injection of Val 12-ras-p21 + DYRK1A SiRNA. All experiments were performed in triplicate (N=3). B. Effects of various RNA's, as also measured by GVBD, on the ability of insulin to induce oocyte maturation. In each condition, a particular RNA was injected into oocytes at least 1 hour prior to incubating the oocytes with insulin. Condition 1 shows results for insulin alone; 2, when SiRNA against TOPK is injected; 3, when SiRNA against DYRK1A is injected; and 4, when negative control RNA is injected. All maturation results are expressed as percent of control, i.e., incubation of oocytes with insulin + control (condition 4). All experiments were performed in triplicate (N=3).

Each set of approximately 100 oocytes was then incubated in Barth's medium at 19^{o}C for 24 hours. Oocyte maturation was determined by observing germinal vesicle breakdown (GVBD) (1,8).

3.2.2. Lysis of oocytes

The matured oocytes were subjected to lysis using a glass tissue homogenizer in modified Group 6 lysis buffer (80 mM beta-glycerophosphate, 20 mM EGTA, 20 mM HEPES, pH 7.5, 1 mM PMSF, 2 uG/ul pepstatin, 1 mM leupeptin, 2 µg/µl Aprotinin, 1 mM Na_3VO_4 and 1 percent Triton X-100) as described previously (24).

3.2.3. Western blotting

These were performed as described in a previous publication (8). Briefly, the lysate was centrifuged for 15 min at 17000 X g at 4^{0}C, and the supernatant was either used directly or frozen at –78^{o}C until used. About 43 ug of lysate protein was loaded onto a 12 percent resolving gel and subjected to SDS PAGE, and the proteins then electrophoretically transferred onto nitrocellulose membranes overnight at 4^{o}C as described previously (12); the membranes were then blocked with non-fat dry milk in Tris-buffered saline with 1 percent Tween-20 (TBS-T, pH 7.6) and were then incubated with the appropriate antibody to either TOPK (Cell Signaling, Beverely, Mass) or DYRK1 (Abcam, Cambridge, Mass). For expression of endogenous control protein, we further blotted for total JNK using a polyclonal antibody [Sigma], which recognizes both JNK-1 and JNK-2, diluted 1:1000 prior to use (6). Prior to incubation, each of these antibodies was diluted 1:2000 in an aqueous solution containing 0.25% bovine serum albumin (Sigma). All incubations were performed for 12 hr at 4^{o}C, after which the membranes were washed three times with tris-buffered saline with Triton (TBS-T) and incubated with secondary antibody (Pierce, Rockford, IL) at 1:20000 dilution. Detection was accomplished using the ECL chemiluminescence detection kit (Pierce). Positive controls for both antibodies consisted of lysates from 3T3 AH3 cells (Upstate Biotechnology, Charlottesville, VA). Blots were scanned using densitometry on an MTX Lab Systems (Vienna, VA) Fluoreskan 2 reader at 755 nm. Total optical density readings for DYRK1A and TOPK proteins were then normalized to JNK levels determined in the same manner. The means and standard deviations were computed from the triplicate experiments that were performed.

Similar to the work presented in a number of prior studies (e.g., ref. 18), we have used quantitative Western blotting, and not RT-PCR, for determining levels of protein expression of the two dual specificity kinases. The latter method yields semi-quantitative estimation of transcript levels but does not reflect the actual levels of specific proteins in cells. Since these levels are affected not only by rates of protein synthesis but also by protein half-lives intracellularly, it was deemed more accurate to determine actual protein levels quantitatively as described above in this section.

4. RESULTS AND DISCUSSION

4.1. Effects of SiRNA's on oocyte maturation

As shown in Figure 1A, oncogenic (Val 12-) ras-p21 protein induces high levels of maturation (condition 1), as determined by GVBD (1,8), that are not affected by co-injection of SIRNA's against either TOPK (condition 2) or DYRK1A (condition 3). Injection of control RNA likewise had no effect on the ability of Val 12-p21 to induce oocyte maturation (not shown). These results are consistent with our prior results showing that oocytes induced to mature with Val 12-p21 express TOPK and DYRK1A only minimally (8). These results and our prior results on protein expression

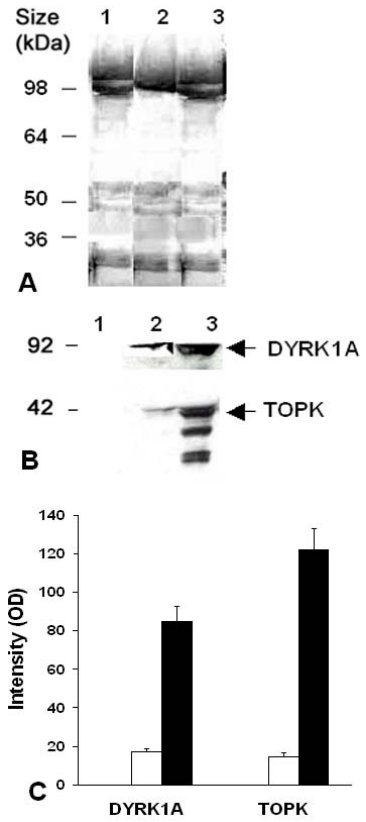

Figure 2. Expression of TOPK and DYRK1A, as measured by Western blotting, when either Val 12-ras-p21 is injected into oocytes or when oocytes are incubated with insulin. A. Coomassie blue-stained gels of whole cell lysates from resting oocytes (lane 1), Val 12-p21-matured oocytes (lane 2) and insulin-matured oocytes (lane 3) showing approximately equal amounts of protein present on gels for each condition. B. Western blots for TOPK (lower) and DYRK1A (upper). Lane 1, resting oocytes; lane 2, Val 12-p21-matured oocytes; lane 3, insulin-matured oocytes. C. Scans of the blots shown in Figure 2B normalized with JNK. The patterns are: open bars, ras-p21-matured oocytes; solid line bars, insulin-matured. Antibodies to specific protein are labeled on the X-axis. Experiments were performed in triplicate (N=3). Scans for DYRK1A and TOPK in lysates from resting (untreated) oocytes were negative for each protein.

suggest that neither protein is involved in the maturation/mitogenic signaling pathway induced by oncogenic ras-p21.

These results are in striking contrast to our results on insulin-induced maturation. As can be seen in Figure 1B, insulin induces oocyte maturation (condition 1) that is significantly inhibited by SiRNA against TOPK (condition 2). Furthermore, insulin-induced maturation is inhibited by SiRNA against DYRK1A (condition 3) but not by control RNA (condition 4). Thus both SiRNA's against the putative protein targets of insulin-activated wild-type ras-p21 block the insulin maturation signal. These results suggest that both TOPK and DYRK1A are critical to mitogenic signaling by wild-type, but not by oncogenic, ras-p21.

As can be seen in Figure 1B, while both siRNA's inhibit insulin-induced maturation, this inhibition is incomplete in that some maturation still occurs. In previous studies, we noted that certain agents, such as ras-p21 peptides, e.g., p21 35-47 sequence, that block oncogenic ras-p21-induced maturation, can block insulin-induced maturation to a small extent (1); we interpreted these results to signify that insulin-activated wild-type p21 can utilize the oncogenic ras pathway (e.g., JNK, MEK and MAPK) but mainly activates a non-oncogenic or wild-type pathway (1). Our results in Figure 1B suggest, conversely, that blockade of the wild-type pathway still allows for a low level of maturation signaling via the MEK-MAPK pathway that is not blocked by the two SiRNA's.

4.2. Effects of SiRNA's on expression of TOPK and DYRK1A proteins in oocytes injected with oncogenic ras-p21

Figure 2B shows the results of control experiments in which we blotted, with anti-TOPK and anti-DYRK1A, whole cell lysates from oocytes that were not treated (lane 1), induced to mature with Val 12-p21 (lane 2) and induced to mature with insulin (lane 3). As can be seen in this figure, resting oocytes contain little TOPK and DYRK1A (lane 1). As we found in our previous study (8), oncogenic ras-p21 induces a low level of expression of both of these proteins (lane 2) while insulin induces high levels of expression of these proteins (lane 3). These levels are quantitated in Figure 2C and normalized against JNK expression (8 and see below) where it can be seen that insulin induces much higher expression of TOPK and DYRK1A. Figure 2A is a Coomassie blue stain of whole cell oocyte lysates showing that the same amounts of protein were present for all three conditions.

To determine the efficacy of each SiRNA in blocking protein expression in oocytes injected with Val 12-ras-p21, we performed Western blots for TOPK and DYRK1A in oocytes that were co-injected with Val -21-p21 with one of the two SiRNA's. The results are shown in Figs. 3. Figure 3A shows overall Coomassie blue staining of whole cell lysates demonstrating that similar amounts of total protein were loaded onto the gels. Figure 3B shows the results of blots with anti-DYRK1A of whole cell lysates from oocytes injected with Val 12-ras-p21 alone (control, lane 1); co-injected with Val 12-ras-p21 + SiRNA against DYRK1A (lane 2) and with Val 12-ras-p21 +SiRNA against TOPK (lane 3). Lane 1 shows that ras-p21 induces a low level of expression of DYRK1A as in Figs. 2 above. This level of expression is unaffected by injection of control RNA (not shown). Lane 2 shows that DYRK1A expression is markedly reduced by the SiRNA against DYRK1A while lane 3 shows that DYRK1A expression is unaffected by SiRNA against TOPK. These results confirm that the SiRNA against DYRK1A is specific for inhibiting expression of this protein.

Figure 3C shows the results of blots with anti-TOPK of whole cell lysates from oocytes injected with Val 12-ras-p21 alone (control, lane 1); co-injected with Val 12-

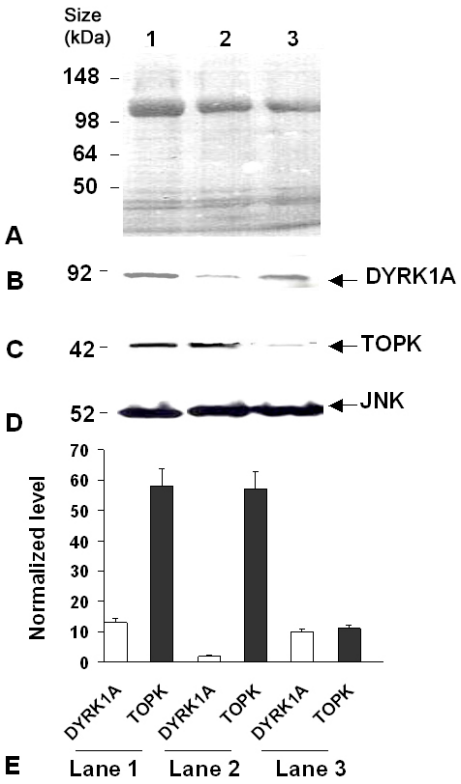

Figure 3. Western blots for DYRK1A and TOPK in Val 12-p21-matured oocytes. Panel A. Coomassie blue-stained gels of whole cell lysates from oocytes injected with Val 12-p21 alone (lane 1); with Val 12-p21 + SiRNA against DYRK1A (lane 2); and with Val 12-p21 + SiRNA against TOPK (lane 3), showing approximately equal amounts of protein present on gels for each condition. Panel B. Blots for DYRK1A in oocytes injected with: Val 12-p21 alone (lane 1); Val 12-p21 + SiRNA against DYRK1A (lane 2); and Val 12-p21 + SiRNA against TOPK (lane 3). Panel C. Blots for TOPK in oocytes injected with: Val 12-p21 alone (lane 1); Val 12-p21 + SiRNA against DYRK1A (lane 2); and Val 12-p21 + SiRNA against TOPK (lane 3). As explained in the Methods Section, blots were scanned using densitometry on an MTX Lab Systems (Vienna, VA) Fluoreskan 2 reader at 755 nm. Total optical density readings for DYRK1A and TOPK proteins were then normalized to JNK levels determined in the same manner (see Panel D). Panel D. Blots for JNK in each cell lysate. Quantitative scans of these blots reveal that JNK levels are virtually the same in each lane. Panel E. Quantitative scans of the blots in Figures 3B and C above, normalized for JNK expression. The results are presented, from left to right, for DYRK1A and TOPK, respectively, for each lane in the scans shown in blots B and C above. For example, "lane 1" in this panel scans refer to scans for DYRK1A and TOPK, respectively, in lane 1 from the control, i.e., levels of these proteins in oocytes injected with oncogenic ras-p21 and normalized to the JNK levels in these oocytes. The results shown in this figure represent means and standard deviations from experiments performed in triplicate (N=3).

ras-p21 + SiRNA against DYRK1A (lane 2) and with Val 12-ras-p21 +SiRNA against TOPK (lane 3). Lane 1 shows that ras-p21 induces a low level of expression of DYRK1A as in Figs. 2 above. As with DYRK1A, this level of expression is unaffected by injection of control RNA (not shown). Lane 2 shows that TOPK expression is unaffected by the SiRNA against DYRK1A while lane 3 shows that TOPK expression is markedly reduced by injection of SiRNA againstTOPK. These results confirm that the SiRNA against TOPK is specific for inhibiting expression of this protein.

As shown in Figure 3D, expression of total JNK is unaffected by injection of either SiRNA and, from scans of these bands (not shown), remains constant irrespective of the conditions, as we have found previously (6,8). We have scanned the blots in B and C above and normalized these to the amounts of JNK present for each lane in D. The results are shown in Figure 3E. As can be seen in this figure, oncogenic ras-p21 induces only low levels of DYRK1A protein (left graph for lane 1 in Figure 3-E) as also shown in Figure 2 and as found in a previous study (8). Expression of this protein is completely blocked by SiRNA against DYRK1A (left graph for lane 2). Oncogenic ras-p21 induces significantly higher amounts of TOPK protein intracellularly (right graph for lane 1) that are not affected by injection of SiRNA against DYRK1A (right graph for lane 2). Injection of SiRNA against TOPK, however, reduces TOPK levels dramatically (right graph for lane 3) but has no effect on the low level of ras-p21-induced expression of DYRK1A (left graph for lane 3, which has the same height as the graph [left, lane 1] for injection of ras-p21 alone).

Figures 3B, 3C and 3E show that we have successfully suppressed expression of TOPK and DYRK1A selectively with SiRNA's to each of these proteins. However, the fact that neither SiRNA suppresses oocyte maturation induced by oncogenic ras-p21 suggests that neither protein is an important component of oncogenic ras-p21 signaling.

4.3. Effects of SiRNA's on expression of TOPK and DYRK1A proteins in oocytes incubated with insulin

We have performed a similar set of experiments on oocytes incubated with insulin. We have blotted whole cell lysates from oocytes matured with insulin with antibody to DYRK1A as shown in lane 1 of Figure 4B. As in Figure 2, this protein is expressed significantly in these lysates. This level of expression is unaffected by injection of control RNA (not shown). Lane 2 of this figure shows that the DYRK1A level is significantly reduced by anti-DYRK1A but, as shown in lane 3, is unaffected by SiRNA against TOPK. As can be seen in Figure 4A, Coomassie blue stains for each lysate sample, the amount of protein in each sample was the same.

Figure 4C shows the results of blotting each of the lysates for TOPK. Lane 1 shows strong expression of TOPK induced by insulin. As with DYRK1A, this level of expression is unaffected by injection of control RNA (not shown) .Lane 2 shows that SiRNA against DYRK1A has no effect on TOPK expression. In contrast, lane 3 shows that SiRNA against TOPK strongly reduces expression of this protein.

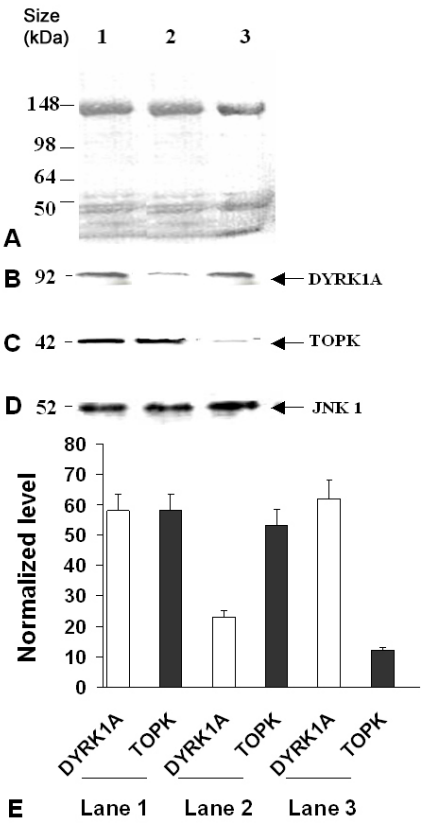

Figure 4. Effects of injected SiRNA's on the expression of DYRK1A and TOPK proteins in oocytes incubated with insulin, as measured from Western blots. Panel A. Coomassie blue-stained gels for whole cell lysates from oocytes incubated with insulin plus: no injected agent (lane 2); SiRNA against DYRK1A (lane 3); and SiRNA against TOPK (lane 4), showing approximately equal amounts of protein present on gels for each condition. Lane 1 shows molecular mass markers. Panel B. Blots for DYRK1A from oocytes incubated with insulin plus: no injected agent (lane 1); SiRNA against DYRK1A (lane 2); and SiRNA against TOPK (lane 3). Panel C. Blots for TOPK from oocytes incubated with insulin plus: no injected agent (lane 1); SiRNA against DYRK1A (lane 2); and SiRNA against TOPK (lane 3). Panel D. Blots for JNK in each cell lysate. Quantitative scans of these blots reveal that JNK levels are virtually the same in each lane. Panel E. Quantitative scans of the blots in Figures 4B and C above, normalized for JNK expression. The results are presented, from left to right, for DYRK1A and TOPK, respectively, for each lane in the scans shown in blots B and C above and as explained in the legend to Fig. 3E. The results shown in this figure represent means and standard deviations from experiments performed in triplicate (N=3).

The results in Figs. 4B and 4C suggest that anti-DYRK1A SiRNA specifically suppresses DYRK1A but not TOPK expression while anti-TOPK SiRNA suppresses TOPK but not DYRK1A expression.

As shown in Figure 4D, expression of JNK for each condition in this figure is the same, as further revealed by scans (not shown), as we found previously (6,8) indicating that injection of control RNA and either DYRK1A or TOPK SiRNA has no effect on its expression. Results of quantitative scans of the blots shown in Figures 4B and 4C, that were then normalized to the JNK levels in each lane, are shown in Figure 4E. In contrast to oncogenic ras-p21, insulin is seen to induce a high level of expression of DYRK1A (left graph, lane 1); it also induces a high level of expression of TOPK. Injection of anti-DYRK1A siRNA into oocytes incubated with insulin results in a three-fold reduction of DYRK1A expression (left graph, lane 2) but has no effect on expression of TOPK (right graph, lane 2). Injection of anti-TOPK SiRNA has no effect on DYRK1A expression (left graph, lane 3) but induces a four-fold reduction in TOPK expression (right graph, lane 3). These results again suggest that each SiRNA specifically blocks expression of its target protein uniquely and has no effect on the expression of other proteins. In contrast to our results with oncogenic ras-p21, insulin-induced maturation is blocked when SiRNA's against either DYRK1A or TOPK are injected. We conclude that reduced expression of either of these two proteins blocks insulin-induced oocyte maturation.

4.4. Implications of SiRNA studies for ras signaling pathways

The above studies were prompted by our prior finding that both oncogenic and wild-type ras-p21 proteins require activation of raf (2). However, oncogenic ras-p21 requires the critical proteins on the raf-induced phosphorylation cascade, specifically MEK and MAPK, while insulin-activated wild-type ras-p21 depends on these downstream proteins to a much lesser extent (2). Therefore, to attempt to infer possible alternate raf-stimulated pathways, we utilized a microarray of the entire genome for Xenopus, using RNA harvested from oocytes induced to mature with oncogenic ras-p21 and with insulin (8). We specifically sought dual specificity kinases that were similar but not identical to MEK and MAPK that would be candidates for possible targets of activated raf and that would be preferentially expressed in insulin-matured oocytes.

We found RNA's for 22 proteins that were expressed at much higher levels in insulin-matured oocytes. Of these, seven were unknown sequences (8). Of the remaining 15, two were dual-specificity kinases, i.e., TOPK and DYRK1A. TOPK, a member of the MEK/MAPKK superfamily, is known to bind to raf directly and therefore was an attractive protein for possibly being involved on an alternate raf-induced pathway (8). This protein does not activate MAPK or ERK but rather induces phosphorylation of p38 protein (9,10). In addition, it has recently been implicated in spindle formation and stabilization in metaphase (10). DYRK1A has been implicated in mini-brain development in Drosophila (11,12) and has been found to be a nuclear protein that interacts uniquely with nuclear cyclin L2, suggesting it as a possible further target of insulin action (12).

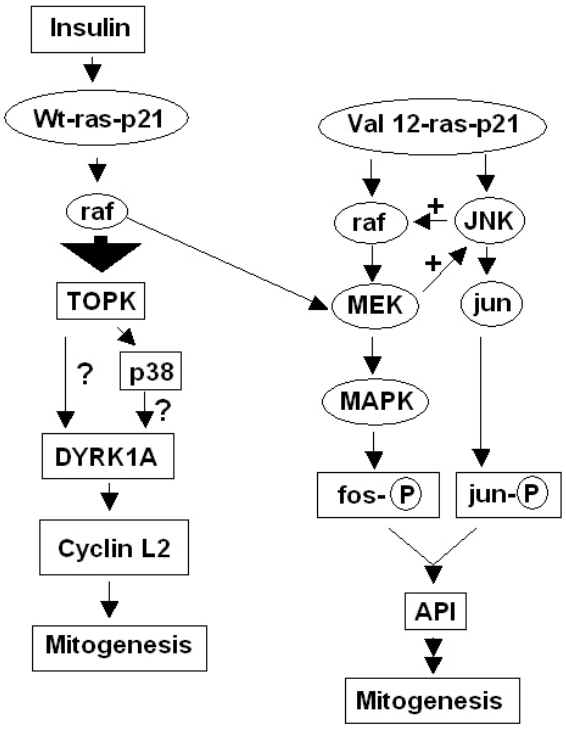

Figure 5. Proposed scheme for the overlapping but distinct upstream signal transduction pathways for mitosis in oocytes incubated with insulin that induces endogenous wild-type ras-p21 (left) and injected oncogenic Val 12-ras-p21 protein (right). Within each pathway, pathway elements that we found to co-immunoprecipitate with wild-type ras-p21 (left) or injected Val 12-p21 (right) from oocytes induced to mature with insulin or oncogenic ras-p21, respectively, are shown in circles indicating that they are components of complexes with ras-p21. All other target proteins on each pathway are shown in boxes. The figure shows that, in oocytes, insulin-activated wild-type p21 induces activation of raf as does oncogenic ras-p21. But then the pathways diverge, the wild-type pathway activating mainly TOPK and DYRK1A as the major pathway (large arrow) and activation of the MEK-MAPK pathway as more minor pathway (smaller arrow). In contrast, the oncogenic pathway involves activation of JNK and jun proteins and the raf-MEK-MAPK pathway and no activation of TOPK and DYRK1A. Activation of jun (phosphorylated jun or jun-P) and fos (phosphorylated fos or fos-P) by JNK and MAPK, respectively, leads to formation of the transcriptionally active AP1 complex that results in expression of mitogenic proteins. Also, as indicated by the "+" signs on the arrows of the right side of this figure, from prior results, JNK can activate raf that activates MEK that can then activate JNK in a cyclic positive feedback pathway (17).

We found that both of these proteins were expressed at significantly lower levels in oncogenic ras-p21-matured oocytes than in insulin-matured oocytes (8), confirming the microarray results. In this study we have sought to test the functionality of these proteins in signal transduction by oncogenic and insulin-activated wild-type ras-p21 proteins. Our results clearly suggest that SiRNA directed against TOPK does not block oncogenic ras-p21-induced oocyte maturation but strongly inhibits insulin-induced maturation. Since insulin-induced maturation requires activation of wild-type ras-p21 (18), these results suggest that TOPK is critical for signaling by wild-type but not oncogenic ras-p21. We have obtained identical results using SiRNA against DYRK1A. These results therefore suggest that both proteins may be unique to the insulin-activated wild-type ras-p21 mitogenic pathway.

Significantly, in demonstrating that our SiRNA's are specific to each protein against which it was designed, we found that, while the SiRNA against TOPK blocks expression of this protein, it has no effect on the expression of DYRK1A in oocytes treated either with oncogenic ras-p21 or insulin. Conversely, while the SiRNA to DYRK1A blocks expression of DYRK1A, it has no effect on expression of TOPK under the same conditions. These results strongly suggest that each SiRNA is specific to its target and does not induce non-specific lowering of protein expression in oocytes. This conclusion is further supported by our findings that injection of control RNA or either SiRNA has no effect on the level of expression of JNK or on the level of total protein shown on the Coomassie blue stains.

Since both SiRNA's block insulin-induced oocyte maturation, it appears that both proteins are necessary to allow insulin-induced maturation. Since TOPK, but not DYRK1A, has been found to bind to raf in the two-hybrid yeast system (13), it is tempting to hypothesize that insulin-activated wild-type ras-p21 binds to raf in such a way as to result in activation of TOPK. This protein would promote mitogenesis by activating p38 and spindle formation and may also activate DYRK1A as a downstream target. These events would result in activation of nuclear cyclins that would be critical to maturation. The elements of this proposed pathway are shown in Figure 5.

In this figure, wild-type p21 is shown to be activated by insulin, resulting in the binding of p21 to raf. The latter protein is a branch point on the wild-type p21 pathway. When bound to wild-type p21, it activates TOPK and DYRK1A predominantly (large arrow at raf) but can also induce activation of the MEK-MAPK pathway to a lesser extent (thinner arrow at raf in the figure). This feature of the figure is based on our prior finding that inhibitors of MAPK weakly block insulin-induced maturation and on our current finding that SiRNA's against the "alternate" path strongly but do not completely block insulin-induced maturation (Figure 1B). These findings suggest that insulin-activated ras-p21 may utilize the MEK-MAPK pathway though to a lessor extent.

On the wild-type pathway, TOPK is shown to be activated first since it is known to bind directly to raf. When activated, it is known to activate, in turn, the p38 protein; we therefore show this as a target of TOPK. TOPK and/or activated p38 (question marks at TOPK and p38) may then activate the nuclear DYRK1A protein, that is known to stimulate nuclear cyclins such as cyclin L2, which, in turn, may promote mitosis.

In contrast, oncogenic (Val 12-containing) ras-p21 protein interacts with JNK/jun and with raf. In a recent study, we found that Val 12-p21 injected into oocytes forms a large complex that contains raf, JNK, MEK and MAPK (17). In the same study, we found that JNK promotes raf activation; raf then activates MEK (17). Besides activating MAPK, activated MEK also activates JNK (17). These features are shown on the right side of Figure 5. Since MAPK inhibitors totally block oncogenic ras-p21-induced maturation and the SiRNA's against TOPK and DYRK-1A have no effect on the ability of oncogenic p21 to induce maturation, we conclude that oncogenic p21 does not utilize the alternate TOPK-DYRK1A pathway. Also shown in Figure 5, we surmise that activation of both fos by activated MAPK and jun by activated JNK leads to enhanced formation of the transcriptionally active AP1 complex that is known to induce expression of mitogenic proteins. Although not explicitly shown, it is possible that the TOPK-DYRK1A-cyclin pathway may also activate AP1.

5. ACKNOWLEDGEMENTS

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Send correspondence to: Dr Matthew R. Pincus, Chief, Department of Pathology and Laboratory Medicine (113), New York Harbor VA Medical Center, 800 Poly Place, Brooklyn, NY 11209, Tel: 718-630-3688, Fax: 718-630-2960, E-mail: Matthew.Pincus2@med.va.gov

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