Two dual specificity kinases are preferentially induced by wild-type rather than by oncogenic RAS-P21 in *Xenopus* oocytes

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1. ABSTRACT

In prior studies, we have found that oncogenic ras-p21 protein induces oocyte maturation using pathways that differ from those activated by insulin-induced wild-type ras-p21. Both oncogenic and wild-type ras-p21 require interactions with raf, but unlike oncogenic ras-p21, insulin-activated wild-type ras-p21 does not depend completely on activation of MEK and MAP kinase (MAPK or ERK) on the raf kinase pathway. To determine what raf-dependent but MAPK-independent pathway is activated by wild-type ras-p21, we have analyzed gene expression in oocytes induced to mature either with oncogenic ras-p21 or with insulin using a newly available *Xenopus* gene array. We find 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, are preferentially expressed in the insulin system.

Confirming this finding, blots of lysates of oocytes, induced to mature with oncogenic ras-p21 and insulin, with anti-TOPK and anti-DYRK1A show much higher protein expression in the lysates from the insulin-matured oocytes. Neither of these kinases activates or is activated by MAPK and is therefore an attractive candidate for being on a signal transduction pathway that is unique to insulin-activated wild-type ras-p21-induced oocyte maturation.

2. INTRODUCTION

Microinjection of oncogenic (G12V) ras-p21 protein, but not the wild-type protein, into stage VI metaphase-arrested *Xenopus* laevis oocytes in the second meiotic division induces progression of the cell cycle through metaphase and completion of cell division, resulting in oocyte

maturation (1). In this way, oocytes are a good prototype for studying the effects of oncogenic ras-p21 since injection of this protein, but, likewise, not its wild-type counterpart protein, into mammalian cell lines induces cell transformation (2). Insulin also induces maturation of oocytes (oocytes have insulin receptors) and requires activation of endogenous wild-type ras-p21 to achieve this effect (3).

We have found that specific peptides, corresponding to segments of ras-p21 and some of its targets, e.g., raf and SOS guanine nucleotide exchange protein, designed from molecular modeling studies, and some small molecules, selectively inhibit Val 12-p21-induced oocyte maturation but have only minimal effects on insulin-induced maturation (4). These results suggested to us that, since insulin requires activation of wild-type p21, induction of mitogenesis in cells by oncogenic and wild-type ras-p21 might proceed by different signal transduction pathways.

In studies designed to identify possible signal transduction pathway differences between these two proteins, we have found that oncogenic ras-p21 interacts directly with jun-N-terminal kinase (JNK) and its target, jun, that forms the transcriptionally active AP1 complex with fos (4-7). Agents that are specific inhibitors of JNK block oncogenic p21 but not insulin-induced oocyte maturation suggesting that, unlike oncogenic p21, activated wild-type p21 protein does not require direct interaction with and activation of the JNK/jun system (8,9).

Activated ras-p21 is known to interact directly with the raf kinase protein resulting in its activation (10). Activated raf activates a kinase cascade by activating the downstream MEK kinase protein that then, in turn, activates MAP kinase (MAPK or ERK), that ultimately activates fos (10). We have found that a kinase dead dominant negative mutant raf (dn-raf) strongly inhibits both Val 12-p21- and insulin-induced oocyte maturation (11). On the other hand, the selective MAPK phosphatase (MKP-1T4) blocks Val 12-p21-induced oocyte maturation to a significantly greater extent than it does insulin-induced maturation, suggesting that oncogenic p21 requires raf-induced MAPK activation while activated wild-type p21 has a lesser dependence on this pathway (11).

This observation has been corroborated in studies in which we have blotted lysates of oocytes induced to mature either by oncogenic ras-p21 or insulin with anti phospho-JNK and MAPK (12). We have found that both kinases undergo an early and progressive increase in phosphorylation in a time-dependent manner that correlates with extent of maturation in oncogenic p21-injected oocytes. In contrast, we found much lower levels of phosphorylation of JNK and MAPK in oocytes induced to mature with insulin (12). These results suggested that oncogenic ras-p21 requires activation of raf and of both JNK and MAPK while insulin requires activation of raf but not the latter two kinases and that activated wild-type ras-p21 activates raf such that it promotes mitogenesis by pathways that are not fully dependent on MEK and MAPK activation.

Recent studies suggest that *raf* can in fact induce MAPK-independent mitogenic signaling pathways (13-19).

These include direct interaction with the pro-apoptotic protein, apoptosis signal regulating kinase (ASK-1), in such a way as to inactivate it (14), and with the mitogenic proteins p21-activated protein kinase-3 (PAK-3) (13), that induces activating phosphorylations in *raf* on Ser 338 (17) and connector enhancer of kinase suppressor of *ras* (CNK) protein (15) and stimulation of the anti-apoptotic protein, BCL-2 (19). In an exhaustive two-hybrid yeast system study of proteins that can bind to raf, it was found that *raf* directly interacts with the pro-mitotic non-MEK/MAPK-utilizing kinase TOPK (lymphokine-activated killer **T** cell-**O**riginated **P**rotein **K**inase)(16,20).

In order to determine the proteins that may be critical to signal transduction induced by activated wild-type p21 but which may not be critical to the oncogenic forms of this protein, we have studied, using a *Xenopus* gene array that has recently become available, gene expression in oocytes induced to mature with insulin as compared with gene expression in oocytes induced to mature with oncogenic (Val 12-containing) p21. We have obtained a set of proteins that are preferentially expressed in one or the other set of matured oocytes. Of the proteins that are predicted to have higher levels of expression in the insulin-matured oocytes, we have further examined which of these may be candidates for interacting with raf.

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 (21). Insulin was purchased from Sigma (St. Louis, MO).

3.2. Methods
3.2.1. Oocyte Microinjection

Oocytes were obtained from *Xenopus laevis* frogs (Connecticut Valley Biological, Southhampton, MA) as described previously (22-24). 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 six experiments (three sets in which Val 12-p21 protein was injected and three for oocytes incubated with insulin). Each set of oocytes was then incubated in Barth's medium at 19°C for 12 hours. Oncogenic Val 12-Ha-ras-p21 protein was microinjected into oocytes at a concentration of 100 μg/ml (50 nl per oocyte) that were then incubated in Barth's medium for 36 hr at 19°C; oocytes treated with insulin were incubated in Barth's medium containing insulin, present at a concentration of 10 ug/ml, for 36 hours at 19°C. Oocyte maturation was determined by observing germinal vesicle breakdown (GVBD) (1,22).

3.2.1.1. Lysis of Oocytes

The matured oocytes were subjected to lysis using a glass tissue homogenizer in a lysis buffer system followed by centrifugation (12). Lysis buffer for RNA extraction was as

supplied with the RNeasy® Lipid Tissue kit from Qiagen (Valencia, CA) used for the RNA extraction as discussed in the next paragraph. Lysis buffer for Western blotting was the modified Group VI lysing buffer (12) described in section 6 below.

3.2.2. RNA Extraction

Total RNA was extracted from the lysed, matured oocytes using RNeasy® Lipid Tissue kit from Qiagen according to the manufacture's instruction. RNA was quantified by spectrophotometry at 260 nm and the ratio of absorbance at 260 nm to that of 280 nm was >1.8 for all samples. Degradation of RNA samples was monitored by the observation of appropriate 28S to 18S ribosomal RNA ratios as determined by ethidium bromide (EtBr) staining on agarose gels as described previously (23,24).

3.2.3. Gene Microarray

We have employed a newly prepared gene microarray, called the *Xenopus* Array-Ready Oligo Set, from Qiagen. This array contains 10,898 70-mer single-stranded deoxynucleotides that are unique gene probes from *Xenopus tropicalis* to which *Xenopus laevis* genes have high homology (25). Each of these 70-mer gene probes has been selected as being unique for each gene in the *Xenopus* genome on the basis of sequence and cluster analysis by Professor J. Smith of the Wellcome Trust/Cancer Research UK Gurdon Institute of Cancer and Developmental Biology in Cambridge, UK. All probes are provided in a 384-well plate format with 600 pmol of probe per well. A control plate with 24 (12 positive and 12 negative) controls in 16 replicates is also included.

These oligonucleotide probes were then imprinted onto Codelink glass slides (Amersham) in a 32-pin-array format by Microarrays, Inc. (Nashville, TN). Coupling efficiency was verified by using the Microarrays Veriprobe assay (Microarrays, Inc., Nashville, TN).

3.2.4. Labeling of RNA and Hybridization Procedure

An amount of 2 ug of total RNA, isolated as described above, was used for target preparation using a Low RNA input fluorescent linear amplification kit (Agilent, Foster City, CA) following the manufacturer's protocols. Briefly, input total RNAs were reversed transcribed using oligo dT-T7 primers and further converted into double-stranded cDNAs, which were then being used as templates in *in vitro* transcription reactions containing cy-3 or cy-5-labeled CTP to generate fluorescent-labeled cRNAs. The labeled cRNAs were purified with an RNeasy micro kit (Qiagen) and eluted in 15 ul of nuclease-free water supplied by the manufacturer. One ul of the purified cRNAs were used to determine the fluorescence-incorporation of the target using a micro spectrophotometer (Nanodrop, Nyxor Biotech, Paris, France). Equal amounts of incorporated cy-3 and cy-5 cRNAs of paired samples were combined and fragmented in the fragmentation buffer (Agilent) at 60°C for 30 min. The fragmented cRNA mixture was resuspended into a total of 50 ul of hybridization solution (4x SSC, 0.1% SDS), which was denatured at 99°C for 2 min, cooled on ice and centrifuged. The supernatant was applied onto each

of the imprinted glass slides covered by a lifterSlip (Erie Scientific, Portsmouth, NH) and then incubated at 50°C for 18 hr. The slides were then washed twice with 2X SSC-0.1% SDS at 50°C for 5 min each in a stirred waterbath, once with 0.2X SSC at room temperature for 1 min and once with 0.1 X SSC at room temperature for 1 min. Finally, the slides were spin-dried at 1000rpm for 5 min.

3.2.5. Analysis of Fluorescent Patterns

The glass slides were scanned at 10 um resolution using a ScanArray HT scanner (Perkin Elmer, Boston, Mass). The image was analyzed and converted into spreadsheet data format using ImaGene 6.0 (BioDiscovery, El Segundo, CA). Empty, poor and negative spots were flagged according to the default settings. The signal was corrected by subtracting the local median background value and log base 2 transformed followed by Lowess (subgrid) normalization excluding the flagged spots. A pair of dye-swap experiments was performed to avoid dye preference. Genes that show at least two-fold change with opposite change direction in the dye-swap pair are selected as candidate differentiated genes.

3.2.6. Western Blotting for Specific Proteins Predicted from the Gene Array Results To Be Expressed Preferentially in Insulin-Matured Oocytes

Approximately 100 oocytes, induced to mature either with oncogenic ras-p21 or with insulin, were lysed as described in part 1 above and subjected to lysis in buffer consisting of 0.35 M LiCl, 50 mM HEPES, pH 7.6, 1 mM EGTA, 1 mM dithiothreitol (DDT), 2 mM MgCl, 50 mM NPP, 1 mM sodium vanadate, and an inhibitor 'cocktail' consisting of 1 microgram/ml each of the protease inhibitors: pepstatin, leupeptin and aprotinin; and the phosphatase inhibitors: 1 mM sodium orthovanadate and 5 mM sodium fluoride). The lysate was centrifuged for 15 min at 17000 X g at 4°C, and the supernatant was either used directly or frozen at -78°C until used. An amount of 43 microgram of lysate protein (protein determined by the Coomassie blue assay [12]) was then loaded onto a 12 percent resolving gel and subjected to SDS PAGE, and the proteins then electrophoretically transferred onto nitrocellulose membranes overnight at 4°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 anti-kinase antibody against one or the other of two dual specificity kinase proteins: T-cell-Originated Protein Kinase (TOPK, also termed PDZ Domain-Binding Protein or PBK)(Cell Signaling, Beverely, Mass) and DYRK1A (Abcam, Cambridge, Mass). 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°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). Virtually identical procedures were employed in blotting for two control proteins in oocyte

Preferential Gene Expression by Oncogenic ras-p21

Table 1. Genes Expressed Preferentially in Oocytes Induced to Mature with Insulin

Genbank ID Number	Protein Accession #	Putative Encoded Protein
TTpA048m24	NP_000055	Complement C-3 precursor; acylation-stimulating protein cleavage product
TNeu006j18	NP_001387	*Dual-specificity tyrosine kinase1A-Drosophila mini-brain; dyrk-1A[1]*
TGas116k08	NP_003195	Erythroid-derived nuclear transcription factor
IMAGE:6998085	NP_002023	Ferritin heavy polypeptide
TTpA057c07	NP_002023	Ferritin polypeptide 1
TTpA044g06	NP_055984	Homologue of yeast ribosome biogenesis regulatory protein RRS1
TEgg054e20	NP_077016	Hypothetical Protein
EC0CBA004BE09	NP_115700	Hypothetical Protein
IMAGE:7007563	NP_000575	Interleukin 8 precursor; monocyte-derived neutrophil-activating protein
Tegg041f04	NP_060962	*PDB/TOPK Protein[1]*
TGas059i19	NP_506824	Putative nuclear protein-with coiled-coil domain
TTpA014o02	NP_690608	Regucalcin
TTpA025g05	NP_005618	Serum glucocorticoid-regulated kinase in humans
TTpA077n12	NP_689481	Similar to tRNA synthetase in humans
TNeu044g15	NP_114104	Tektin 3; testicular microtubules-related protein
TNeu062c23	NA	Unknown Sequence
TGas065l13	NA	Unknown Sequence
TNeu100o03	NA	Unknown Sequence
EC2BBA12DE11	NA	Unknown Sequence
TGas054l22	NA	Unknown Sequence
TTpA068h07	NA	Unknown Sequence
IMAGE:6977051	NA	Unknown Sequence

[1]Dual Specificity Kinase

lysates from oncogenic ras-p21- and insulin-matured oocytes, JNK (polyclonal antibody [Sigma], which recognizes both JNK-1 and JNK-2, diluted 1:1000) and NF-kappa-B (Cell Signaling, dilution 1:1000) as described previously (6, 12).

4. RESULTS AND DISCUSSION

4.1. Oncogenic ras-p21- and Insulin-Matured Oocytes Express a Number of Different Proteins

Tables I and II summarize the genes that are expressed preferentially in oocytes induced to mature by incubation with insulin or by injection of oncogenic (Val 12-) ras-p21, respectively. Several of the predicted protein sequences in both sets of oocytes are based on homologies with known protein sequences in Genbank. Some of these sequences can only be inferred by homology to correspond to proteins of known function such as putative nuclear protein-with coiled-coil domain in insulin-matured oocytes (Table 1) or the carbonic anhydrase sequence in Val 12-p21-matured oocytes (Table 2). In addition, nine predicted protein sequences in insulin- and six predicted protein sequences in Val 12-p21-matured oocytes are hypothetical and do not correspond even by homology to any known protein sequence. Thus conclusions concerning possible proteins that may induce oocyte maturation unique to activated wild-type ras-p21 must be limited by these considerations.

On the other hand, many of the proteins of known function probably do not contribute to mitogenic signal transduction. These include such proteins as ferritin, regucalcin, yeast RRS1 protein, complement and interleukin 8 precursor proteion in Table 1. In addition, from Table 1, in the insulin-matured oocytes, increased

expression of serum glucocorticoid-regulated kinase may be specific to metabolic but not mitogenic effects of insulin.

In addition to these studies, we have further examined the levels of expression of RNA's encoding proteins that we have found are expressed at the same levels in oocytes induced to mature with oncogenic ras-p21 and insulin. For example, in prior studies (12), we have found that the levels of JNK and MAPK are approximately the same. We find that JNK and ERK gene expression on the arrays is the same in oocytes treated with insulin and with oncogenic ras-p21, correlating with the results of our prior Western blots.

4.2. Two Dual Specificity Kinases Are Expressed at Higher Levels in Insulin-Matured Oocytes

In the list of the predicted known protein sequences preferentially expressed in insulin-matured oocytes, principally two appear to have obvious functions in regulating the cell cycle as highlighted in Table 1: killer T-cell originated protein kinase (TOPK) and the dual specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), as shown in Figure 1. The former of these has been determined to be in the MEK (or MAPKK) superfamily although it does not activate MAPK or ERK but rather induces phosphorylation of p38 protein (20,26). In addition, it has recently been implicated in spindle formation and stabilization in metaphase (27). Importantly, TOPK has been found to associate with raf (16). If activated raf interacts with TOPK and induces its activation, the possibility exists that TOPK could stimulate mitotic or meiotic signal transduction without involving MAPK. This could explain why raf is essential for insulin

Table 2. Genes Preferentially Expressed in Oocytes Induced to Mature with Val 12-ras-p21 Protein

| GenBank ID Number	Protein Accession #	Putative Encoded Protein
TEgg036o16	NP_726796	*D. melanogaster* wings apart-like-CG3707 protein
TNeu105h16	NP_002058	Guanine nucleotide-binding protein alpha 11 (Gq class)
IMAGE: 6997993	NP_789795	Human sulfotransferase family 1C1-b isoform
TGas066e18	NP_542387	Hypothetical Protein
TGas090a11	NP_612426	Hypothetical Protein
IMAGE: 6982858	NP_498083	Predicted carbonic anhydrase from *C. elegans*
TEgg034b16	AAH20022	Rik protein in *Mus muscularis*
TGas089a22	NP_666186	Transcription factor B1, mitochondrial *Mus muscularis*
TTpA012e14	NA	Unknown Sequence
TGas081i11	NA	Unknown Sequence
TGas016g13	NA	Unknown Sequence
EC0CBA003CA10	NA	Unknown Sequence
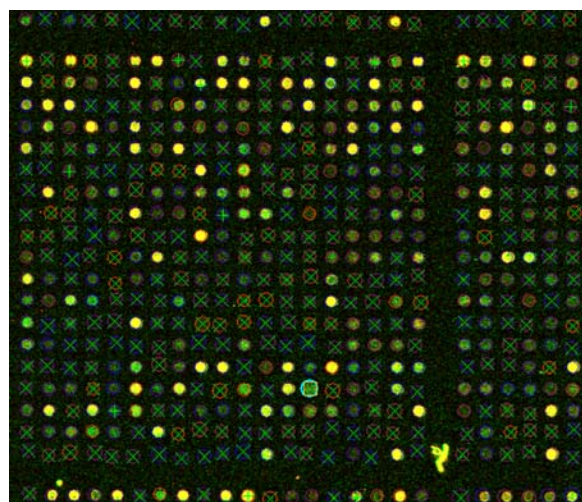
Figure 1. Gene array result showing increased expression of TOPK, circled in blue, in oocytes induced to mature with insulin compared with its expression in oocytes induced to mature by microinjection of Val 12-p21. In this array, green dye color at any locus represents increased expression in insulin-matured oocytes whereas red color loci represent increased expression in Val 12-p21-matured oocytes. Yellow color represents equal expression in both systems whereas no color represents no expression in either system.

stimulation of maturation that can occur independently of MAPK (ERK) activation.

DYRK1A has recently been cloned and found to be important in mini-brain development in *Drosophila* (28). Through photobleaching studies it 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 (29).

4.3. Both TOPK and DYRK1A Proteins Are Expressed at Much higher Levels in Oocytes Treated with Insulin than in Those Treated with Oncogenic ras-p21

To confirm that these two dual specificity kinases are expressed at higher levels in oocytes induced to mature with insulin as opposed to oocytes induced to mature by injection of oncogenic Val 12-ras-p21, we have blotted whole cell lysates from oocytes induced to mature with each agent with antibodies specific to each of these two proteins. The results are summarized in Figure 2. In this figure, in A, lanes 1-3 are blots using anti-TOPK. The blot in lane 1 shows the result of a positive control for 3T3 AH3 cell lysate. This result shows two bands of Mr 45 (arrow) and 40 kDa corresponding to known isoforms of TOPK. Lane 2 shows that in oocytes matured with Val 12-p21, there is only a minimal amount of either TOPK isoform expressed. On the other hand, in lane 3, lysate from oocytes induced to mature with insulin, there is a large band, corresponding to the 45 kda isoform of TOPK (45 kDa, arrow) indicating a substantially higher level of expression of TOPK in these oocytes. As shown in Figure 2B, densitometric scans of these blots show that there is at least an eight-fold increase in the level of TOPK induced in insulin-matured oocytes compared with that induced in oncogenic ras-p21-matured oocytes. As shown in Figure 2C, blots of the oocyte lysates for control proteins, JNK and NF-kappa-B, show that these protein levels are similar in oocytes induced to mature with oncogenic ras-p21 (lanes 1 and 3, respectively) and with insulin (lanes 3 and 4 , respectively). In previous work, we have found that JNK levels in oocytes matured with oncogenic ras-p21 and with insulin have similar levels of protein expression of total JNK , although phospho-JNK is much higher in oncogenic ras-p21-matured oocytes (12).

Similarly, we have blotted whole cell oocyte lysate with antibody to DYRK1A as shown in lanes 4-6 of Figure 2. Shown in lane 4 is the result of a blot of positive control 3T3 AH3 cell lysate with this antibody. Only one strong band for DYRK1A (90kDa, arrow) was observed in this control although a faint band for one other form of DYRK1A (Mr 50kDa) was observed in this positive control (not shown).

Lanes 5 and 6 show the results of blots for DYRK1A in lysates from oocytes induced to mature with oncogenic ras-p21 and with insulin, respectively. These blots show that there is a substantially increased level of DYRK1A in the insulin-treated oocytes (lane 6) as compared with that in Val 12-ras-p21-matured oocytes (lane 5). As can be seen from the densitometric scans of lanes 5 and 6 for lysates of ras-p21- and insulin-matured oocytes, respectively, in Figure 2B, this increase in the insulin-matured oocytes is almost threefold.
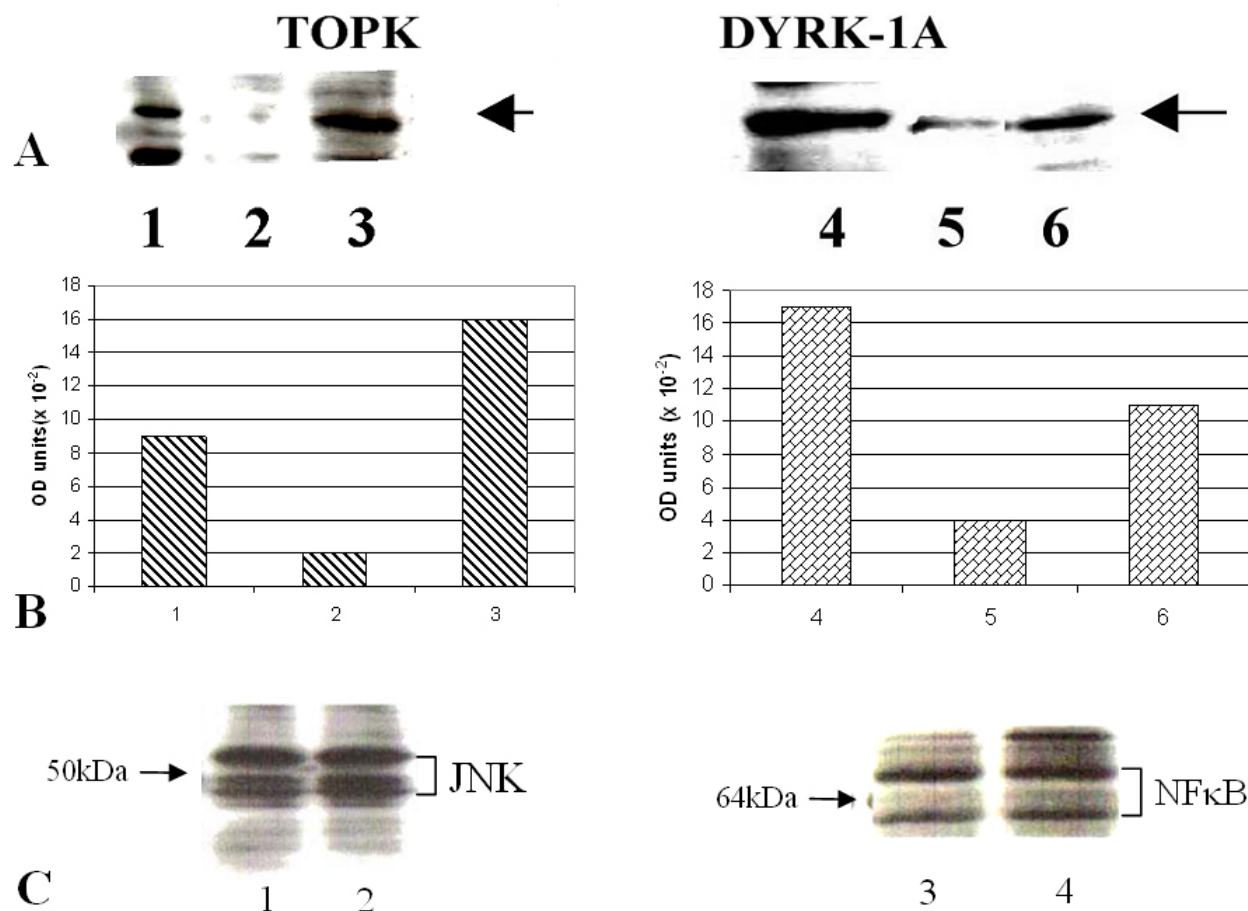
Figure 2. TOPK and DYRK1A are expressed in insulin-matured oocytes more highly than in oocytes induced to mature with oncogenic ras-p21. In these experiments 43 μg protein was loaded onto each lane. A. Blots of lysates of *Xenopus laevis* oocytes induced to mature either from microinjection of Val 12-p21 or incubation with insulin with antibodies against TOPK (PBK) and DYRK1A. Lanes 1-3 are blots using anti-TOPK: lane 1, positive control; lane 2, lysate from oocytes induced to mature with Val 12-p21; lane 3, lysate from oocytes induced to mature with insulin. The arrow to the right of the TOPK blots designates the position of migration of the major isozyme of TOPK (45 kDa). Lanes 4-6 are blots using anti-DYRK1A: lane 4, positive control; lane 5, lysate from oocytes induced to mature with Val 12-p21; lane 6, lysate from oocytes induced to mature with insulin. The arrow to the right of the DYRK1A blots designates the position of migration of the major isozyme of DYRK1A (90kDa). B. Densitometric scans of the blots in A. Each numbered lane corresponds to the numbered lane in A. C. Results of blots for control proteins, JNK (lanes 1 and 2) and NF-kappa B (lanes 3 and 4), in the lysates of oocytes induced to mature with oncogenic ras-p21 (lanes 1 and 3) and insulin (lanes 2 and 4).

4.4. Implications of the Results Showing Higher TOPK and DYRK1A Levels of Protein Expression in Insulin-Matured Oocytes

These results confirm that the level of expression of both TOPK and DYRK1A is significantly higher in oocytes induced to mature in insulin than in oocytes that are induced to mature by injection of Val 12-ras-p21. Since TOPK has been found to bind to raf in the two-hybrid yeast system (16), there is the possibility that insulin-activated endogenous ras-p21 binds to raf which may then interact preferentially with TOPK allowing for activation of downstream targets such as p38, ultimately resulting in mitogenesis. Since TOPK does not activate MAPK (ERK), this pathway would circumvent the MEK-MAPK pathway. Possibly, signals from activated TOPK, the ras-raf complex

or from other insulin-activated targets may result in promoting the synthesis and activation of DYRK1A.

If this pathway does exist, it would explain our prior findings that insulin-induced oocyte maturation is blocked by dominant negative raf (dn-raf) but is only partially blocked by MAPK-specific phosphatase (MKP-1T4) (11) and why insulin-induced phosphorylation of MAPK in oocytes is much lower than in oocytes induced to mature with oncogenic ras-p21 (12).

This putative pathway would also explain why certain agents, like the ras-p21 35-47 and 96-110 peptides, block oncogenic ras-p21-induced oocyte maturation while only minimally affecting insulin-induced maturation (4).

Since insulin activates wild-type ras-p21 and the latter stimulates raf so that it can activate TOPK, the TOPK pathway can circumvent the inhibition of specific anti-oncogenic ras-p21 agents that block JNK and MEK and MAPK activation. If this circumventing pathway exists in mammalian cells, it may allow for normal cell growth. We have introduced both ras-p21 35-47 and 96-110 peptides that specifically inhibit oncogenic ras-p21 into ras-transformed pancreatic cancer cells and found that they both induce phenotypic reversion of the cells to the untransformed phenotype. Yet neither of these peptides affects the normal growth of a corresponding untransformed pancreatic acinar cell line (4,30). These findings are compatible with the existence of a circumventing growth pathway that allows regulated normal cell growth.

This formulation is based directly on our finding that the two dual function kinases are expressed at much higher levels in the insulin-matured oocytes. However, these results do not indicate the level of activation, i.e., phosphorylation states and activities, of these kinases. In our prior studies of JNK and MAPK expression and activation in oocytes matured with insulin and oncogenic ras-p21, we found that, while the levels of expression of these two kinases were about the same in the two sets of oocytes, the level of phosphorylation of both proteins was much higher in the oncogenic ras-p21-matured oocytes (12). Nonetheless, since both TOPK and DYRK1A are expressed at disproportionately higher levels in the insulin-matured oocytes, it is more likely that both proteins are involved in maturation pathways that are uniquely stimulated by insulin.

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