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The eIF2α-specific protein kinase, PKR, has been implicated as a tumor suppressor gene because of its growth suppressive and translational inhibitory properties, as well as the ability of nonfunctional mutants of PKR to transform cells. We have sought to investigate the possibility that the aberrant regulation of cellular protein synthesis underlies the loss of growth control in breast carcinoma cells through dysfunction of the dsRNA activated, eIF2α-specific protein kinase, PKR. We have compared the expression and regulation of activity of PKR in normal breast and breast carcinoma cell lines and found unusually high levels of PKR, as well as evidence of an inhibitor of PKR activity in breast carcinoma cells. This PKR inhibitor could be involved in the establishment of the transformed state of breast carcinoma cells and could also be responsible for the resistance of breast carcinoma cells to interferon treatment. These data represent the first documentation of a defect in PKR associated with a human malignancy.

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INTRODUCTION

This grant was an innovative developmental and exploratory award to an investigator who had no previous background in human breast cancer, but who has a long history in the study of protein synthesis regulation and its role in the regulation of cell proliferation. The investigator sought to investigate the possibility that aberrant regulation of cellular protein synthesis could underlie the loss of growth control in breast carcinoma cells through dysfunction of the dsRNA activated, eIF-2α-specific protein kinase, PKR. This kinase has been implicated as a tumor suppressor gene, because of its growth suppressive and translational inhibitory properties, as well as the ability of nonfunctional mutants of PKR to transform cells. The start date for the grant was 07/01/94, with Olga Savinova, M.S. beginning work in October 1994. Using the IDEA award we began to investigate the role of PKR in the loss of growth control in breast cancer cells. We have compared the expression and regulation of activity of PKR in normal breast and breast carcinoma cell lines and found evidence of an inhibitor of PKR in breast carcinoma cells. Our results are consistent with those of the findings of Haines who compared PKR levels in normal breast tissue and a range of human breast carcinomas [1]. The putative PKR inhibitor could be involved in the establishment of the transformed state of breast carcinoma cells and could also be responsible for the resistance of breast carcinoma cells to interferon treatment [2].

BODY

Four tasks were outlined in the **Statement of Work** in the original application. These were:

1. Analysis of PKR expression in breast carcinoma tissue.
2. Analysis of PKR expression in breast carcinoma cell lines.
3. Analysis of the effect of estrogen antagonists on the regulation of PKR expression/activity in estrogen receptor positive breast carcinoma cell lines.
4. Analysis of PKR expression/activity in cell lines stably transformed with the human placental aromatase gene and effects of aromatase inhibitors.

Tasks 2 and 3 analyzing the characteristics of breast carcinoma cell lines have been completed and extended in view of the results. Over a range of cell densities, eIF-2α levels, PKR levels, PKR activation state and eIF-2α phosphorylation state has been monitored in two estrogen receptor positive cell lines, MCF7 and T47D, and two estrogen unresponsive cell lines, BT-20 and MDA-MB-468. These have been compared to the same parameters in a normal human breast cell line, MCF10A. The results with breast carcinoma cell lines have also been compared to those obtained in the human HeLa fibroblast cell line, since much is known about the regulation of PKR expression/regulation in this system. Since estrogen/tamoxifen were found to have no effects of PKR or eIF2α, Aim 4 was not pursued. Similarly, since Haines *et al.* [1] published data recently pertinent to Aim 1, we have not pursued that aim either.

Our initial studies were very simple. We looked at eIF2α levels, PKR levels and eIF2α phosphorylation state in normal breast cells in culture and compared the results with those obtained from two estrogen-receptor positive breast carcinoma cell lines, MCF7 and T47D, and one estrogen receptor-negative cell line, BT20. When our results suggested the presence of a PKR inhibitor in breast carcinoma cells, we began

exploratory biochemical studies and found evidence of a transdominant PKR inhibitor in breast carcinoma cells.

eIF2α levels in normal breast and breast carcinoma cells

Figure 1 shows the levels of the protein synthesis initiation factor, eIF2α, in MCF7, T47D and BT20 breast carcinoma cells compared to MCF10A. Equal amounts of protein were loaded for each sample. The level of eIF2α is compared to that of α-actin. The levels of eIF2α are 5-6 fold higher in the human breast carcinoma cell lines compared to the normal human breast cell line. To some extent this parallels the increased ribosome content (1.5-2 fold higher), although eIF2α:ribosome ratios are higher in breast carcinoma cells compared to normal human breast cell lines. This is consistent with the increased protein synthetic activity necessary to sustain higher proliferation rates. It is also consistent with the raised *c-myc* levels of breast carcinoma cells. Increased c-*myc* increases the expression of the eIF2α gene which contains a PAL element in its promoter region [3, 4]. Although the eIF2α levels in MCF7, T47D and BT20 cells are illustrated here, several additional breast carcinoma cells we have looked at show higher PKR levels compared with normal breast (results not shown).

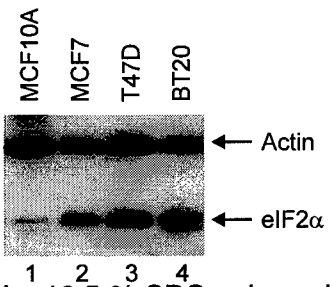


Figure 1: eIF2α levels in normal breast and breast carcinoma cells : Extracts (10,000 xg supernatants) were prepared from MCF10A, (lane 1); MCF7, (lane 2); T47D (lane 3); and BT20 cells (lane 4), after lysis in 20 mM HEPES-KOH, pH 7.2; 5 mM EDTA; 100 mM KCl; 0.005 % SDS; 0.5 % Elugent; 10 % glycerol; 20 μg/ml chymostatin; 1 mM DTT. This figure shows a western blot of eIF2α and α-actin from these extracts using a sample load of 50 μg separated by 12.5 % SDS-polyacrylamide gel electrophoresis.

PKR levels in normal breast and breast carcinoma cells

There are several reports in the literature demonstrating an inverse relationship between PKR levels and the rate of cell proliferation [5-9]. Consequently, we expected to see lower PKR levels in breast carcinoma cells compared with normal breast cells. However, Figure 2 shows that this is not the case, and that PKR levels are 4-5 fold higher in breast carcinoma cells compared to normal breast.

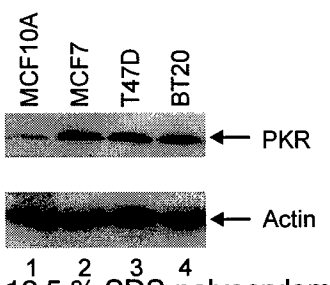


Figure 2: PKR levels in normal breast and breast carcinoma cells: Extracts (10,000 xg supernatants) were prepared from MCF10A, (lane 1); MCF7, (lane 2); T47D (lane 3); and BT20 cells (lane 4), after lysis in 20 mM HEPES-KOH, pH 7.2; 5 mM EDTA; 100 mM KCl; 0.005 % SDS; 0.5 % Elugent; 10 % glycerol; 20 μg/ml chymostatin; 1 mM DTT. This figure shows a western blot of PKR and α-actin from these extracts using a sample load of 50 μg separated by 12.5 % SDS-polyacrylamide gel electrophoresis.

Although the PKR levels in MCF7, T47D and BT20 cells are illustrated here, several additional breast carcinoma cells we have looked at show higher PKR levels compared with normal breast (results not shown). This same relationship is also found in SV40 large T-antigen transformed rat embryo fibroblasts compared to normal rat embryo fibroblasts (Jagus & Freund labs, unpublished results). However, it is not a

feature of all transformed cells (see Haines and Beretta refs above). PKR levels in breast carcinoma cells are approximately equimolar with ribosomes, whereas in normal human fibroblasts, WI38 cells, the equivalent level is 1 molecule of PKR for every 4-5 ribosomes [[10]. Consistent with these findings in breast carcinoma cells in culture, invasive ductal human breast tumors show high levels of PKR compared with normal lobular and luminal ductal epithelial cells [1]. All these data suggest that the ability of PKR to inhibit cell proliferation is impaired in some way in breast carcinoma cells.

eIF2α phosphorylation state in normal breast and breast carcinoma cells

In contrast to the high levels of PKR, the phosphorylation state of eIF2α is very low in the breast carcinoma cell lines compared to the normal human breast cell line MCF10A. As shown in Figure 3, comparing cells at similar densities, the phosphorylation of eIF2α in MCF10A cells is significantly higher than eIF2α phosphorylation in MCF7, or BT20 cells.

PKR activity, as measured by the phosphorylation state of eIF2α, increases with increasing cell density in most cell types [11]. No such increase is observed in MCF7 cells as cell density increases (data not shown). The high levels of PKR in breast carcinoma cells, in conjuction with low eIF2α phosphorylation levels, suggests an impairment of PKR activity in the breast carcinoma cell lines.

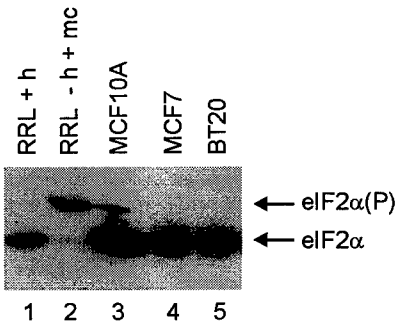


Figure 3: eIF2α phosphorylation state in normal breast and breast carcinoma cells: Cell lysate equivalent to 250 μg of protein (usually 20-200 μl extract), from MCF10A (lane 3), MCF7 (lane 4), and BT20 cells (lane 5) were brought up to an equal volume with lysis buffer and 400 μl binding buffer (100 mM KCl; 20 mM HEPES-KOH, pH 7.2; 1 mM EDTA; 10 % glycerol; 20 μg/ml chymostatin; 1 μM microcystin), along with 20 μl anti-eIF2α-MAb-beads. The mixture was rocked at 4 °C for 6 h. The beads were collected by microcentrifugation at 10,000 xg at 4 °C for 5 min. The supernatant was discarded and the beads rinsed with 1 ml of rinsing buffer (100 mM KCl; 20 mM HEPES-KOH, pH 7.2; 10 % glycerol) prior to a final microcentrifugation at 10,000 xg at 4 °C for 5 min. 70 μl of VSIEF sample buffer (9.5 M urea; 1 % Pharmalyte, pH 4.5-5.4; 1 % Pharmalyte, pH 5-6, 0.15 % SDS, 50 mM DTT) were added which were incubated at 30 °C for 5 min to dissociate eIF2α from the beads. The samples were subjected to isoelectric focusing, at 2 mA per gel, with an 1,200 voltage limit for 18 h (11,000 volt hours) at 18 °C. An immunoblot is shown here. Lanes 1 and 2 are control samples of reticulocyte lysate incubated to give totally dephosphorylated (lane 1) or totally phosphorylated eIF2α (lane 2).

Effects of interferon on PKR levels

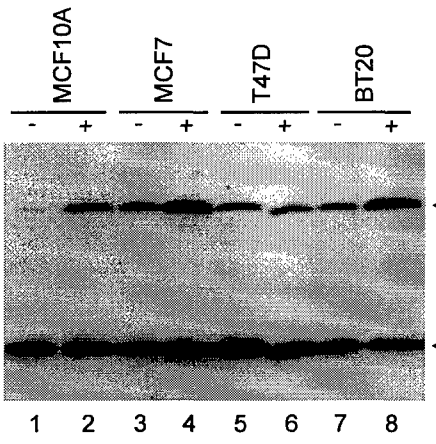


Figure 4: Effects of interferon on PKR levels: Extracts (10,000 xg supernatants) were prepared from MCF10A, (Lanes 1 & 2); MCF7, (lanes 3 &4); T47D (lanes 5 & 6); and BT20 cells (lanes 7 & 8) treated without (lanes 1,3,5,7) or with (Lanes 2,4,6,8) 100 units/ml β-interferon for 20 h, by lysis in 20 mM HEPES-KOH, pH 7.2; 5 mM EDTA; 100 mM KCl; 0.005 % SDS; 0.5 % Elugent; 10 % glycerol; 20 μg/ml chymostatin; 0.5 μM microcystin; 1 mM DTT. This figure shows a western blot of PKR and α-actin from these extracts using a sample load of 50 μg separated by 12.5 % SDS-polyacrylamide gel electrophoresis.

Consistent with the already high levels of PKR in breast carcinoma cells, treatment of MCF7, T47D and BT20 cells with β-interferon (100 units/ml) increased PKR levels only slightly, or not at all, as shown in Figure 4. This is in striking contrast to the effect of interferon on PKR levels in MCF10A cells which exhibited a 5-10 fold increase in PKR levels.

The stimulatory effects of estrogen in the estrogen-responsive cell lines do not work through effects on PKR levels or activities. In the estrogen receptor positive cell lines, MCF-7 or T47D, estrogen removal or re-addition had no effects on PKR levels/activities (results not shown). Furthermore, estrogen removal or re-addition had no effects on eIF2α phosphorylation state (results not shown).

PKR activity in breast carcinoma cells

In Figure 3, PKR activity is assessed indirectly by looking eIF2α phosphorylation. PKR activity was also assessed by lysing the cells and measuring the ability of PKR in the lysate to autophosphorylate. PKR becomes autophosphorylated as part of its activation, so this assay gives an indication of PKR activation state. Figure 5 shows the autophosphorylation of PKR affinity-purified or immunopurified from extracts of MCF10A, MCF7, T47D and BT20 cells as measured by the incorporation of γ-^{32}P ATP. PKR from interferon-treated 293 extracts is also included as a comparison of PKR activity from a different tumor-derived cell line. PKR from MCF10A, either affinity-purified using poly(I):poly(C)-agarose, or immunopurified using monoclonal antibody to PKR linked to CL-Sepharose beads shows significantly higher γ-^{32}P ATP incorporation than PKR affinity-purified or immunopurified from MCF7, T47D, or BT20 cells. This difference does not reflect intrinsic differences in the ability of PKR from these different cell types to bind to the purification resins. Greater than 90 % of PKR in the cell extracts bound to the purification resins and the level of PKR in the assays was the same for all cell extracts (data not shown). The reduced activity of PKR in breast carcinoma cells is not a common characteristic of transformed cell lines since HeLa, Daudi and the adenovirus E1A-transformed cell line, 293, all show normal PKR activity [12-14].

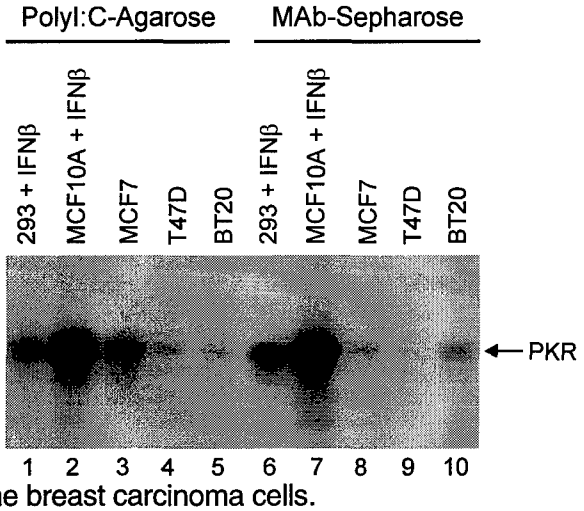


Figure 5: PKR activity in breast carcinoma cells Extracts were prepared from 293 (lanes 1 & 6), MCF10A (lanes 2 & 7), MCF7 (lanes 3 &9), T47D (lanes 4 & 9), and BT20 cells (lanes 5 & 10), as described in the legend to Figure 1. PKR was purified from each by affinity-purification on poly(I):poly(C)-agarose (lanes 1-5), or immunopurification using PKR/Mab-Sepharose (lanes 6-10) and used in a kinase assay in 100 mM KCl, 25 mM HEPES-KOH, pH 7.2, 10 % glycerol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, 3 μg/ml acetylated BSA, and 5 μM γ-^{32}P ATP (50-100 Ci/mmol). Following incubation at 30 °C for 20 min, the reaction is stopped by the addition of one volume of a two-fold concentration of SDS-PAGE sample buffer, and analyzed by 12.5 % SDS-polyacrylamide gel electrophoresis and autoradiography. Equal levels of PKR were used in each reaction. Note that the 293 and MCF10A cells were treated with β-interferon (100 units/ml) for 20 h prior to harvest to increase PKR levels to those seen in the breast carcinoma cells.

PKR can be activated in extracts of breast carcinoma cells

The reduced activity of PKR in breast carcinoma cells could reflect the production of a variant, inactive PKR from a mutated gene, or it could indicate an impairment of PKR activity in breast carcinoma cells. Figure 6 suggests the latter, since inclusion of poly(I):poly(C) (0.5 μg/ml) in the incubation increased PKR autophosphorylation in extracts of the breast carcinoma cells, MCF7, T47D and BT20, to a level equivalent to that seen in the normal breast cells, MCF10A. These data also suggest that the ability of PKR to bind dsRNA is not intrinsically impaired in breast carcinoma cells. The substrate, eIF2, was also included in these incubations and serves to indicate that the increased autophosphorylation of PKR was indeed reflective of increased activity. PKR from interferon-treated HeLa cells is again given as a comparison. Overall the results are consistent with the presence of a PKR inhibitor in breast carcinoma cells. As in the experiments Figure 5, equal levels of PKR was present in all samples.

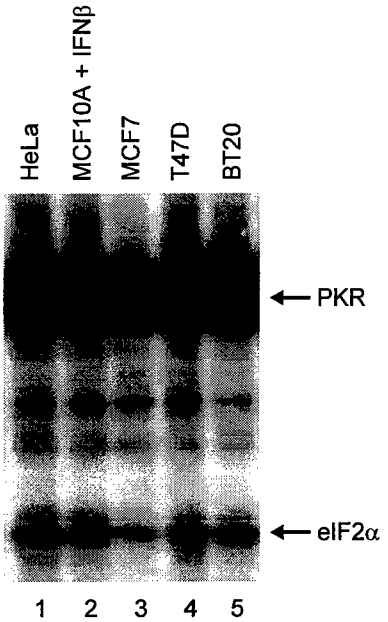


Figure 6: PKR can be activated in extracts of breast carcinoma cells Extracts were prepared from HeLa (lane 1), MCF10A (lane 2), MCF7 (lane 3), T47D (lanes 4), and BT20 cells (lane 5), as described in the legend to Figure 1. PKR was immunopurified from each using Mab-Sepharose and used in a kinase assay in 100 mM KCl, 25 mM HEPES-KOH, pH 7.2, 10 % glycerol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, 3 μg/ml acetylated BSA, and 5 μM γ-^{32}P ATP (50-100 Ci/mmol), along with 0.5 μg/ml poly(I):poly(C) and eIF2α. Following incubation at 30 °C for 20 min, the reaction was stopped by the addition of one volume of a two-fold concentration of SDS-PAGE sample buffer, and analyzed by 12.5 % SDS-polyacrylamide gel electrophoresis and autoradiography. Equal levels of PKR were used in each reaction. Note that the HeLa and MCF10A cells were treated with β-interferon (100 units/ml) for 20 h prior to harvest to increase PKR levels to those seen in the breast carcinoma cells.

Cytoplasmic extracts of MCF7 cells contain a transdominant PKR inhibitory activity

Mixing experiments were performed to determine the ability of the putative PKR inhibitor to act in *trans*. Equal volumes of extracts from MCF10A cells were mixed with increasing volumes of extract from MCF7 cells to give ratios of 1:0.5, and 1:1 MCF10A: MCF7 cell extract respectively, prior to immunopurification and incubation with γ-^{32}P ATP.

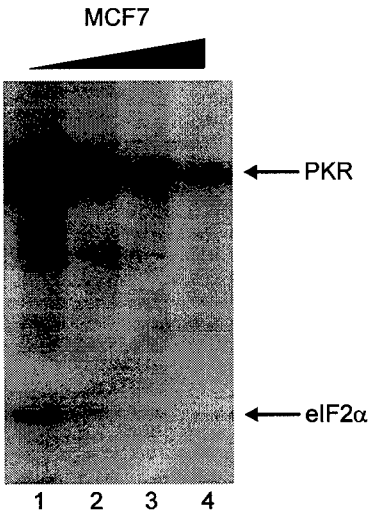


Figure 7: Cytoplasmic extracts of MCF7 cells contain a transdominant PKR inhibitory activity Equal volumes of extracts from MCF10A cells were mixed with increasing amounts of extract from MCF7 cells to give ratios of MCF10A:MCF7 of 1:0 (lane 1), 1:0.5 (lane 2), or 1:1.5 (Lane 3), or 1:2 (lane 4). PKR was immunopurified from each using Mab-Sepharose and used in a kinase assay in 100 mM KCl, 25 mM HEPES-KOH, pH 7.2, 10 % glycerol, 2 mM $MgCl_2$, 2 mM $MnCl_2$,1 mM dithiothreitol, 3 μg/ml acetylated BSA, and 5 μM γ-^{32}P ATP (50-100 Ci/mmol), along with eIF2α. Following incubation at 30 °C for 20 min, the reaction was stopped by the addition of one volume of a two-fold concentration of SDS-PAGE sample buffer, and analyzed by 12.5 % SDS-polyacrylamide gel electrophoresis and autoradiography. Equal levels of PKR were used in each reaction.

Figure 7 shows that mixing with MCF7 inhibited both PKR autophosphorylation and eIF2α phosphorylation even at the lowest ratio. Several types of activities could act as *trans*-inhibitors of PKR autophosphorylation, such as a PKR-specific protease, a phosphatase, a tightly binding protein, or a dsRNA-binding protein that sequesters

activating RNAs. Since PKR levels are not reduced by the mixing experiment (data not shown) and since PKR levels are so high in extracts of breast carcinoma cells, a protease seems unlikely. Similarly, a phosphatase seems unlikely since the inclusion of the phosphatase inhibitor, microcystin (1 μM) in the reaction had no effect on the *trans*-inhibition (data not shown).

PKR inhibitory activity found in 25-40 % ammonium sulfate fraction

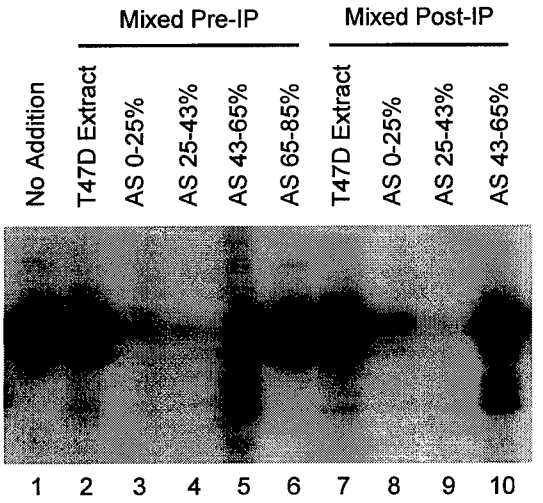


Figure 8: PKR inhibitory activity found in 25-40 % ammonium sulfate fraction Equal volumes of extracts from HeLa cells were mixed with nothing (lane 1), or crude extract (lanes 2 & 7), 0-25 % (lanes 3 &8), 25-40 % (lanes 4 & 9), 40-60 % (lanes 5 & 9), 60-80 % (lane 6) ammonium sulfate fractions from T47D cells, either prior to (Lanes 1-6) or after (lanes 7-10) immunopurification. Kinase assays were performed as described in previous legends with the inclusion of 0.5 μg/ml poly(I):poly(C). Following incubation at 30 °C for 20 min, the reaction was stopped by the addition of one volume of a two-fold concentration of SDS-PAGE sample buffer, and analyzed by 12.5 % SDS-polyacrylamide gel electrophoresis and autoradiography. Equal levels of HeLa PKR were used in each reaction.

Figure 8 shows the PKR inhibitory activity in ammonium sulfate fractions of MCF7 cells. PKR inhibitory activity was measured by the ability to prevent autophosphorylation of PKR immunopurified from interferon-treated HeLa extracts. In panel A, HeLa extracts were mixed with the ammonium sulfate fractions prior to immunopurification. In panel B, the ammonium sulfate fractions were added to the phosphorylation reaction after immunopurification of PKR. In each case, the PKR inhibitory activity is found predominantly in the 25 - 40 % ammonium sulfate fraction. However, a significant inhibitory activity is also found in the 0 - 25 % fraction, which contains significantly less protein. Narrower ammonium sulfate fractions over the 0 - 40 % range will be taken to further the initial purification. The PKR inhibitory protein, p58 is found in the 40 - 60 % ammonium sulfate fraction, making it unlikely that p58 is the activity involved here. Similarly, p58 levels in the MCF7 cells are very low, consistent with an inhibitory activity distinct from p58.

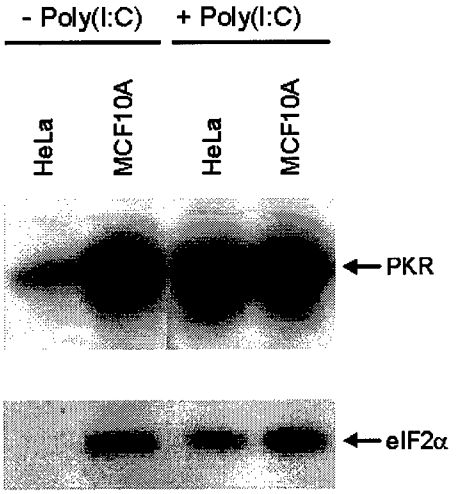
The PKR inhibitory activity is stable to this initial purification and should therefore be amenable to further purification. Preliminary studies have shown that the inhibitory activity does not function as a protease, nor does it function as an eIF2α-protease (data not shown).

PKR in the normal breast cell line, MCF10A, is is highly active

Figure 9 shows that PKR immunopurified from MCF10A cells is highly active and does not need the addition of dsRNA to show maximal activity. PKR activity was measured by autophosphorylation as well as by its ability to phosphorylate eIF2α. The results using PKR from MCF10A cells were in contrast to PKR immunopurified from HeLa cells which requires the addition of dsRNA {(poly(I):poly(C)}, to show measurable activity. These data suggest that MCF10A cells contain high levels of endogenous activators. Preliminary

experiments demonstrate that RNA species can be co-immunoprecipitated with PKR in extracts from ^{32}P orthophosphate-labelled MCF10A and MCF7 cells (data not shown)

Figure 9: PKR activity is high in normal breast cells



Extracts were prepared from HeLa, and the normal breast cell line, MCF10A cells as described in the legend to Figure 1. PKR was purified from each by immunopurification using PKR/Mab-Sepharose and used in a kinase assay in 100 mM KCl, 25 mM HEPES-KOH, pH 7.2, 10 % glycerol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, 3 μg/ml acetylated BSA, 0.3 μg eIF2, and 5 μM γ-^{32}P ATP (50-100 Ci/mmol) in the presence or absence of poly(I):poly(C) (0.5 μg/ml) as described [15, 16]. Following incubation at 30 °C for 20 min, the reaction was stopped by the addition of one volume of a two-fold concentration of SDS-PAGE sample buffer, and analyzed by 12.5 % SDS-polyacrylamide gel electrophoresis and autoradiography. Equal levels of PKR were used in each reaction. Note that the HeLa and MCF10A cells were treated with β-interferon (100 units/ml) for 20 h prior to harvest to increase PKR levels to those found in breast carcinoma cells.

The role of PKR, in the loss of growth control in breast cancer cells has been investigated by a comparison of the expression and regulation of activity of PKR in normal breast and breast carcinoma cell lines. Furthermore, an evaluation of the role of PKR in the the estrogen responsiveness of breast carcinoma cell lines has been made by a comparison of PKR expression, and regulation of activity, in an estrogen responsive line treated with or without estrogen, or the estrogen antagonist, tamoxifen. In the estrogen receptor positive cell lines, estrogen removal or re-addition had no effects on PKR levels/activities. Furthermore, estrogen removal or re-addition had no effects on eIF-2α phosphorylation levels or phosphorylation state (data not shown).

CONCLUSIONS

The eIF2α-specific protein kinase, PKR, has been implicated as a tumor suppressor gene because of its growth suppressive and translational inhibitory properties, as well as the ability of nonfunctional mutants of PKR to transform cells. We have sought to investigate the possibility that the aberrant regulation of cellular protein synthesis underlies the loss of growth control in breast carcinoma cells through dysfunction of the dsRNA activated, eIF2α-specific protein kinase, PKR. We have compared the expression and regulation of activity of PKR in normal breast and breast carcinoma cell lines and found unusually high levels of PKR, as well as evidence of an inhibitor of PKR activity in breast carcinoma cells. This PKR inhibitor could be involved in the establishment of the transformed state of breast carcinoma cells and could also be responsible for the resistance of breast carcinoma cells to interferon treatment. The data suggest that an element of the PKR regulatory cascade is dysfunctional. We seek to identify the defects in PKR regulation in breast carcinoma cells and look for the downstream effects of PKR downregulation that could give rise to a transformed phenotype. In addition, we propose to determine whether these defects are causative for the development of the transformed phenotype, or correlative. Our major goals for

the future, if fundung can be secured, is the identification of the PKR inhibitor(s) in breast carcinoma cells, assessment of the downstream effects of deregulated PKR and determination of the relationship between the downregulation of PKR and the development of breast tumors.

Knowledge of PKR regulation in breast carcinoma cells will allow us to pose some different questions on the interactions between hormones, growth factors, and cytokines. Furthermore, an understanding of PKR regulation in breast carcinoma cells should suggest what therapeutic agents might be effective in suppressin/reducing the growth of breast carcinomas.

Personnel funded by award: Olga Savinova, M.S. began work in the laboratory October, 1994.

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Savinova O. and **Jagus, R.**: Deregulation of the interferon induced, dsRNA activated protein kinase, PKR, in breast carcinoma cells. J. Interferon and Cytokine Research, **15**, P4-3, S167, 1995.

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