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TITLE: The Regulation of Nuclear Receptor Coactivator SRC-3 Activity Through Membrane Receptor Mediated Signaling Pathways

PRINCIPAL INVESTIGATOR: Ping Yi, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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14. ABSTRACT SRC-3/AIB1 is a steroid receptor coactivator with potent growth promoting activity. It is overexpressed in a number of cancers and has been shown to have oncogenic potential. An important mechanism for the regulation of SRC-3 coactivator 'activity' is its site-specific phosphorylation status, which is controlled by extracellular signals that stimulate specific protein kinase signaling pathways. It is well known that protein kinases such as aPKC are frequently overexpressed in cancers and that phosphorylation of SRC-3 can contribute to its oncogenic potential. However, it is unclear whether protein kinases can directly regulate SRC-3/AIB1 cellular protein levels. In the present study, we showed that atypical protein kinase C (aPKC) phosphorylates and stabilizes SRC-3; increased intracellular levels of the coactivator result in significant enhancement of steroid receptor transcription activity. The aPKC stabilization effect requires the presence of estrogen receptor. Using a variety of estrogen receptor mutants we found that this stabilization occurs primarily in the nucleus and is dependent on a direct interaction between SRC-3 and estrogen receptor. Furthermore, we showed that aPKC-phosphorylated SRC-3 becomes resistant to degradation by the 26s proteasome in a cell-free degradation assay using all purified components of SRC-3, ER, and 26s proteasome. Taken together, our results suggest that, in addition to its role in activation of this important SRC-3 growth coactivator, phosphorylation also plays a role in regulating SRC-3 stability and turnover. Thus, aberrant levels of select protein kinases such as aPKC in cancer cells might contribute to the high levels of SRC-3 seen in endocrine cancers.					
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INTRODUCTION:

Steroid receptor coactivator-3 (SRC-3/AIB1) is a member of the p160 coactivator family. It activates nuclear receptor mediated transcription through recruiting proteins such as CBP/p300 and CARM1 that have capability to modify histone. SRC-3 is overexpressed and amplified in a number of cancers including breast cancer (1). Recently, accumulated evidence also indicates an important role of SRC-3 in cancer development. Transgenic mice with SRC-3 overexpression were found to have extremely high tumor incidence (17). In contrast, SRC-3 knockout mice have significantly lower incidence of ras-induced mammary gland tumorigenesis (6). It appears that the level of SRC-3 protein is critical for tumorigenesis. A number of extracellular signals affect SRC-3 coactivator activity through activating downstream protein kinases, which eventually leads to SRC-3 phosphorylation. This post-translational modification was reported to contribute the oncogenic potential of SRC-3 (20). Protein kinases are often overexpressed in breast cancers and some kinases also serve as prognostic factors for breast cancer (7, 9, 11, 16). Protein stability is an important factor regulating protein function. Phosphorylation is often a way to regulate protein stability. Recently, it was shown that phosphorylation of SRC-3 by p38MAP kinase is associated with its degradation in a RAR-dependent manner (5). However, it is still not clear whether kinase mediated SRC-3 phosphorylation could also modulate its protein stabilization since many kinases and SRC-3 are overexpressed in breast cancer. Here we demonstrate that atypical protein kinase C phosphorylates SRC-3 and this phosphorylation changes its conformation into a form that is more resistant to proteasome degradation, which eventually leads to protein stabilization.

BODY:

Atypical PKC stabilizes SRC-3 protein

To determine whether protein kinases have any effect on SRC-3 protein level, we co-transfected flag-SRC-3 with PKC ζ , PKA catalytic subunit, IKK α or IKK β into HeLa cells. Flag-ER was also co-transfected as a control. Shown in Fig.1A, PKC ζ significantly increased SRC-3 protein level while other kinases have minor effects. PKC ζ belongs to atypical protein kinase C subfamily of kinases. Another member of atypical PKC is PKC ι . There are highly homologous with overall 72% amino acid identity (12). Unlike conventional and novel PKCs, atypical PKC is insensitive to phorbol ester, DAG or calcium stimulation (12, 13, 19). Atypical PKCs were found overexpressed in ovarian, prostate, lung cancers and liver, urine bladder carcinomas (3, 4, 14, 15, 18). We found that the effect of PKC ζ on SRC-3 protein level requires its kinase activity since the kinase dead mutant of PKC ζ does not have any effect on SRC-3 (Fig.1B). Similar results were also obtained with another atypical PKC, PKC ι . RT-PCR results (Fig.1C) showed that transfected flag-SRC-3 mRNA level did not

increase when PKC ζ was co-transfected, suggesting that the increased SRC-3 protein level is caused by change of SRC-3 protein stability instead of mRNA transcription. To examine whether PKC ζ also affects endogenous SRC-3 protein level, ER positive breast cancer cells, MCF7 cell, were treated with siRNA against PKC ζ . As shown in Fig.1D, PKC ζ protein level was significantly reduced by the siRNA. SRC-3 protein level also decreased with reduced PKC ζ level. Real time RT-PCR was also carried out to measure SRC-3 mRNA level when PKC ζ was knocked-down. Similar to the results shown in Fig.1C, the endogenous SRC-3 mRNA level was not significantly altered by siRNA against PKC ζ (Fig.1E). Since PKC ζ does not affect SRC-3 transcription, we then examine whether PKC ζ changes proteasome mediated SRC-3 degradation. As shown in Fig.1F, the proteasome inhibitor MG132 significantly increased flag-SRC-3 protein level and it abolished the effect of PKC ζ on SRC-3. All these results suggest that atypical PKC stabilizes SRC-3 protein by preventing the proteasome mediated protein degradation.

The stabilization effect of PKC ζ on SRC-3 requires its interaction with ER

The experiments shown in Fig.1 were always done in the presence of ER, either with transfected or endogenous ER. We also tested the effect of PKC ζ on SRC-3 in the absence of ER or in the presence of progesterone receptor. Surprisingly, we found that PKC ζ failed to stabilize SRC-3 in both cases (data not shown). It appears that ER is required for the stabilization effect of PKC ζ on SRC-3. We then tested whether estrogen has any effect on this stabilization (Fig.2A). In the absence of estrogen, PKC ζ wild type and its kinase dead mutant can not stabilize SRC-3 while the constitutive active mutant increased SRC-3 protein level. In the presence of estrogen, PKC ζ wild type stabilizes SRC-3 while the effect of its constitutive active mutant was further augmented. Interestingly, ER protein level was also increased by the wild type and active mutant of PKC ζ in the presence of estrogen. These results suggest that estrogen may have dual roles in the stabilization effect. First, it may activate PKC ζ activity since only active PKC ζ is able to stabilize SRC-3 in the absence of estrogen. Second, estrogen enhances the effect of PKC ζ on SRC-3.

It was reported that PKC ζ interacts with ER (2). Since ER is required in this stabilization, we then examined the interaction between PKC ζ and SRC-3 or ER in the co-immunoprecipitation experiment. As shown in Fig. 2B, Both SRC-3 and ER interact with PKC ζ and the interaction between SRC-3 and PKC ζ does not require ER. We then tested the effects of different ER mutants in the SRC-3 stabilization. ER wild type, the mutant with AF1 domain deletion (ER Δ AF1), the mutant with mutations in AF2 function (ERAF2m) or the mutant localized in the cytoplasm (ER(cyto)m) was co-transfected with flag-SRC-3 in the absence and presence of PKC ζ . As shown in Fig.2C, ER Δ AF1 mutant significantly reduced the stabilization effect while ERAF2m and ER(cyto)m abolished the stabilization. To determine whether the inability of ERAF2m and ER(cyto)m to support the PKC ζ stabilization effect is due to their defect in the interaction with PKC ζ or SRC-3, we did co-immunoprecipitation experiment (Fig.2D). V5- PKC ζ and different ER mutants were co-transfected into

HeLa cells. The cell lysates were immunoprecipitated by anti-ER antibody and immunoblotted with anti-V5 antibody for detection of bound PKC ζ . The results show that ERAF2m was still able to interact with PKC ζ while ER(cyto)m failed to interact. Since ERAF2m can not interact with SRC-3, these results suggest that the interaction between ER and SRC-3 is important for the PKC ζ stabilization effect and this stabilization occurs in the nucleus. To further confirm the importance of the ER-SRC-3 interaction. We also examined the effect of PKC ζ on SRC-3 AAA mutant which contains mutations in three LXXLL motifs that are important for the interaction with nuclear receptors. The stabilization effect is significantly reduced (Fig.2E).

Since SRC-3 was shown to be a phosphor protein and PKC ζ is a protein kinase, we then wanted to examine whether PKC ζ can phosphorylate SRC-3 and whether ER has any function in this phosphorylation. In vitro PKC ζ kinase assay was carried out using purified SRC-3 as a substrate. As shown in Fig.2F, PKC ζ is able to phosphorylate SRC-3. The presence of ER increased and estrogen further enhanced the phosphorylation, suggesting that the interaction between ER, SRC-3 and PKC ζ may put SRC-3 in an optimal conformation and facilitate the phosphorylation by PKC ζ .

PKC ζ protects SRC-3 from proteasome mediated degradation

SRC-3 can be degraded through both ubiquitin-dependent and independent pathways (8, 10). Since PKC ζ directly phosphorylates SRC-3, we reason that this phosphorylation may change the conformation of SRC-3 into a form that is more resistant to proteasome degradation. To test this hypothesis we co-transfected flag-SRC-3 with ER, PKC ζ or empty vectors into HeLa cells. Flag-SRC-3 purified from cell lysates by immunoprecipitation was then subjected to in vitro 26S proteasome degradation. As shown in Fig.3A, 26S proteasome efficiently degraded SRC-3 when either ER or PKC ζ was present. When both ER and PKC ζ were present, less SRC-3 was degraded. This protection effect requires PKC ζ kinase activity since the presence of kinase dead mutant of PKC ζ does not have this effect (Fig.3B). These results are consistent to the effect of PKC ζ and ER on SRC-3 protein level in cells. It was shown recently that SRC-3 can also be degraded through REG γ mediated proteasome pathway (8). We then wanted to examine whether this PKC ζ induced conformation of SRC-3 is also resistant to REG γ mediated degradation. Flag-SRC-3 purified from cell lysates was subjected to REG γ and 20S proteasome mediated degradation (Fig.3C). Similar to the results shown in Fig.3A, PKC ζ also protected SRC-3 from degradation in this case. Although we showed that PKC ζ can directly phosphorylates SRC-3, it does not necessary mean that the protection effect we observed is due to the direct phosphorylation of SRC-3 by PKC ζ . To test this, purified baculovirus expressed SRC-3 was phosphorylated by PKC ζ in vitro and the protein was then subjected to REG γ mediated degradation. In this assay PKC ζ is the only kinase. As shown in Fig.3D, PKC ζ still protected SRC-3 from degradation in this vitro purified system. These results suggest that PKC ζ phosphorylates SRC-3 and induces its conformational change which is more resistant to degradation mediated by both ubiquitin dependent and independent proteasome pathways.

PKC ζ increases nuclear SRC-3 protein level and enhances its coactivator activity

As shown above the cytoplasm localized ER mutant failed to support the stabilization effect of PKC ζ on SRC-3, suggesting that this stabilization takes place in the nucleus. To confirm that nuclear SRC-3 protein really increased, we separated HeLa cell lysates into cytoplasmic and nuclear fractions. The successful separation of cytoplasmic and nuclear compartments was shown by detecting nuclear protein PARP and the protein β -tubulin, most of which are localized in cytoplasm (Fig.4A). Under normal condition SRC-3 was localized in HeLa nucleus. When PKC ζ was overexpressed in cells, nuclear SRC-3 protein level was dramatically increased while small amount of SRC-3 was also found in the cytoplasm. This may be due to the nucleus-cytoplasm shuttling of SRC-3. To confirm these results immunostaining of V5- PKC ζ and HA-SRC-3 in HeLa cells was performed (Fig.4B). PKC ζ is localized in both cytoplasm and nucleus. Consistent with the results shown in Fig.4A, SRC-3 was found to have increased protein level in the nucleus and it also showed cytoplasmic staining.

We then wanted to ask whether these increased SRC-3 protein in the nucleus are functionally active or not. To answer this question the transcription of endogenous ER target gene, pS2, was measured when cells were co-transfected with SRC-3 and PKC ζ . The addition of PKC ζ did not increase ER activated pS2 transcription without the co-transfection of SRC-3. When SRC-3 was transfected, PKC ζ wild type significantly enhanced pS2 transcription in the presence of estrogen and the constitutively active mutant of PKC ζ further augmented the transcription while the kinase dead mutant did not have any effect. These results suggest that the stabilized SRC-3 protein by PKC ζ is transcriptionally active. PKC ζ appears to boost SRC-3 activity by preventing its degradation.

In summary, we found that PKC ζ phosphorylates and stabilizes SRC-3 protein. This effect requires the interaction between ER and SRC-3. ER increases the phosphorylation level of SRC-3 and the phosphorylation appears to change the conformation of SRC-3 into a form that is less degradable by proteasome. Furthermore, we showed that this stabilization occurs in the nucleus and PKC ζ enhances ER mediated transcriptional activation by increasing the coactivator SRC-3 protein level.

KEY RESEARCH ACCOMPLISHMENTS:

Identify the kinase that leads to SRC-3 protein stabilization.

Determine the interaction between ER and SRC-3 is an important factor for the stabilization effect of atypical PKC.

Determine that the mechanism of atypical PKC stabilizing SRC-3 is to changing its conformation which is more resistant to proteasome mediated degradation.

Determine that the stabilization occurs in cell nucleus and the functional effect of this stabilization is to enhance SRC-3 coactivator activity.

REPORTABLE OUTCOMES:

Publications and Meeting Abstracts:

Yi, P., Wu, R. C., Sandquist, J., Wong, J., Tsai, S. Y., Tsai, M-J, Means, A. R., O'Malley, B.W. Peptidyl-prolyl isomerase 1 (Pin1) serves as a coactivator of steroid receptor by regulating the activity of phosphorylated steroid receptor coactivator 3 (SRC-3/AIB1). *Mol. Cell. Biol.* (2005) 25, 9687-9699.

Yi, P., Wong, J., Tsai, S.Y., Tsai, M-J, Sandquist, J., Means, A.R., and O'Malley B.W. Peptidyl-prolyl Isomerase 1 (Pin1) Serves as a Coactivator of Steroid Receptor-mediated Transcription by Regulating the Function of Phosphorylated Steroid Receptor Coactivator-3 The Program and Abstracts of the 87th Annual Meeting of the Endocrine Society (2005) p.616: P3-299.

Yi, P., Wong, J., Tsai, S.Y., Tsai, M-J, Sandquist, J., Means, A.R., and O'Malley B.W. Peptidyl-prolyl Isomerase 1 (Pin1) Serves as a Coactivator of Steroid Receptor-mediated Transcription by Regulating the Function of Phosphorylated Steroid Receptor Coactivator-3 Era of Hope Department of Defense Breast Cancer Research Program Meeting (2005) P35-21.

CONCLUSION:

We found that PKC ζ phosphorylates and stabilizes SRC-3 protein. This effect requires the interaction between ER and SRC-3. ER increases the phosphorylation level of SRC-3 and the phosphorylation appears to change the conformation of SRC-3 into a form that is less degradable by proteasome. Furthermore, we showed that this stabilization occurs in the nucleus and PKC ζ enhances ER mediated transcriptional activation by increasing the coactivator SRC-3 protein level. These findings provide one of the mechanisms that SRC-3 is overexpressed in cancers. They suggest that aberrant expression or abnormal activity of atypical PKC in cancers could result in highly concentrated and highly active SRC-3 protein in cells, which in turn activate a number of transcription factors and downstream target genes and eventually lead to tumor development. Our findings also provide a potential therapeutic target, atypical PKC, for the control of breast cancer development.

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APPENDICES:

Figure legends:

Fig.1 Atypical PKC stabilizes SRC-3 protein. (A) Flag-SRC-3 and ER expression vecotors were co-transfected with PKC ζ , PKA, IKK α or IKK β expression vectors into HeLa cells. The expression of flag-SRC-3 or ER was detected by western blot using anti-flag or anti-ER antibody. β -actin expression was included as a control. (B) Western blot analysis of flag-SRC-3 protein level when cells were co-transfected with constitutively active mutant of PKC ζ or PKC ι , or their kinase dead mutants. (C) RNA was extracted from cells transfected with flag-SRC-3 with or without co-transfection of PKC ζ . RT-PCR was performed using flag-specific primer and SRC-3-specific primer to detect the mRNA level of transfected flag-SRC-3. A RT-PCR experiment without the addition of reverse transcriptase (RT) was included as a control. (D) Western blot analysis of endogenous SRC-3, ER and PKC ζ protein level in MCF-7 cells treated with siRNA against PKC ζ and scramble control siRNA. (E) real time RT-PCR was carried out to detect the mRNA level of endogenous SRC-3 in MCF-7 with or without PKC ζ knocking-down. (F) PKC ζ stabilizes SRC-3 protein in proteasome-mediated degradation pathway. HeLa cells transfected with flag-SRC-3

and PKC ζ or its kinase dead mutant (DM) were treated with proteasome inhibitor MG132 or vehicle DMSO.

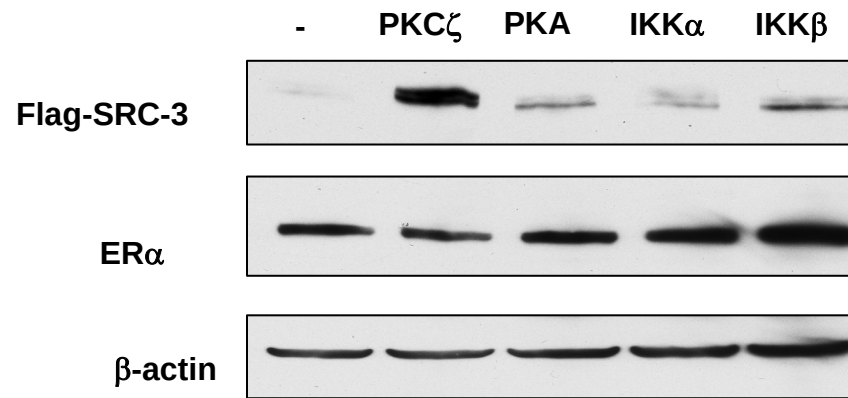
Fig.2 The stabilization effect of PKC ζ on SRC-3 requires its interaction with ER. (A) Estrogen increases the PKC ζ mediated stabilization effect. WT: wild type PKC ζ , CAT: constitutively active mutant, DM: kinase dead mutant. (B) Co-immunoprecipitation experiment. Cells were transfected with V5- PKC ζ and flag-SRC-3 or flag-ER. Cell lysates were immunoprecipitated by anti-flag antibody or normal IgG and western blot analysis was performed using anti-V5 antibody. (C) The effects of different ER mutants on the PKC ζ mediated stabilization effect. (D) Co-immunoprecipitation experiment detecting the interaction between PKC ζ and different ER mutants. (E) PKC ζ has significantly reduced stabilization effect on SRC-3 mutant which has mutations in three LXXLL motifs. (F) Autoradiograph of in vitro PKC ζ kinase assay detecting phosphorylation of SRC-3 in the absence and presence of ER and estrogen.

Fig.3 PKC ζ protects SRC-3 from proteasome mediated degradation. (A) in vitro 26S proteasome degradation assay. HeLa cells were transfected with flag-SRC-3 with or without co-transfection of either ER or PKC ζ . Flag-SRC-3 was purified by immunoprecipitated from cell lysates and eluted by flag peptides. The purified flag-SRC-3 was then subjected to 26S proteasome degradation. (B) PKC ζ kinase activity is required for its protection effect on SRC-3. The procedure was the same as described in panel A. (C) PKC ζ protects SRC-3 from REG γ -mediated proteasome degradation. Similar procedure as described in panel A except that purified REG γ and 20S proteasome were used to degrade SRC-3. (D) PKC ζ protects SRC-3 from REG γ -mediated proteasome degradation in in vitro purified system. Purified baculovirus expressed recombinant SRC-3 was phosphorylated by PKC ζ in vitro. Purified REG γ and 20S proteasome were then added into the reaction and were incubated for indicated time period.

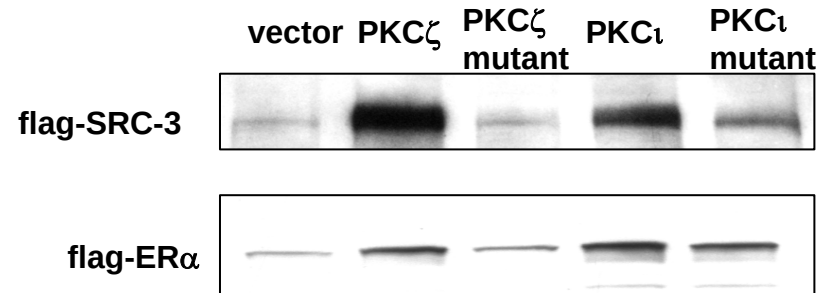
Fig.4 PKC ζ increases nuclear SRC-3 protein level and enhances its coactivator activity. (A) Majority of increased SRC-3 protein by PKC ζ are localized in the nucleus. HeLa cells transfected with flag-SRC-3 and PKC ζ constitutively active mutant, its kinase dead mutant or empty vector were separated into cytoplasmic and nuclear fractions. Nuclear protein PARP and the protein β -tubulin whose majority is in cytoplasm were included in the western blot as controls. (B) Immunostaining of HA-SRC-3 in the absence or presence of exogenous expressed constitutively active PKC ζ and its kinase dead mutant. (C) PKC ζ enhances SRC-3 activated ER targeted gene transcription. HeLa cells were transfected with ER, SRC-3, PKC ζ or its mutants in the absence or presence of estrogen. The transcription of pS2 gene was measured by real time RT-PCR on pS2 mRNA level.

Fig. 1

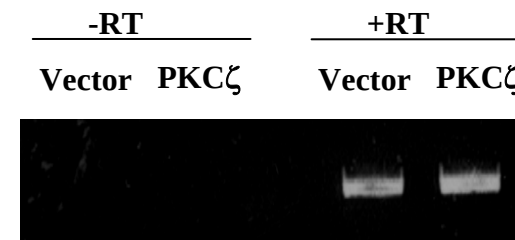
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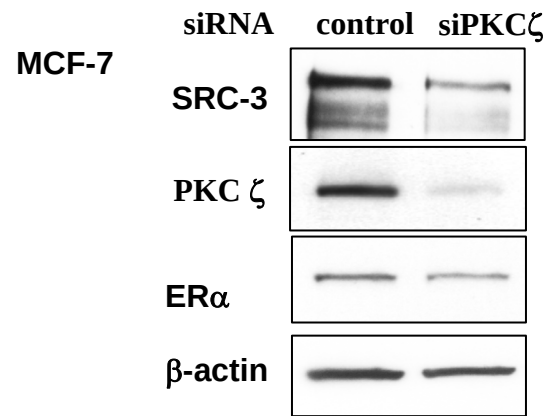
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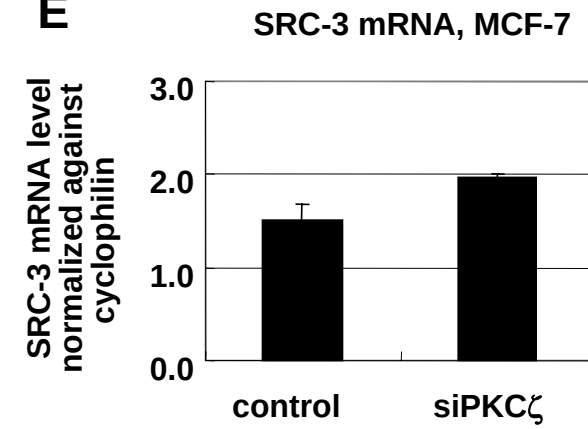
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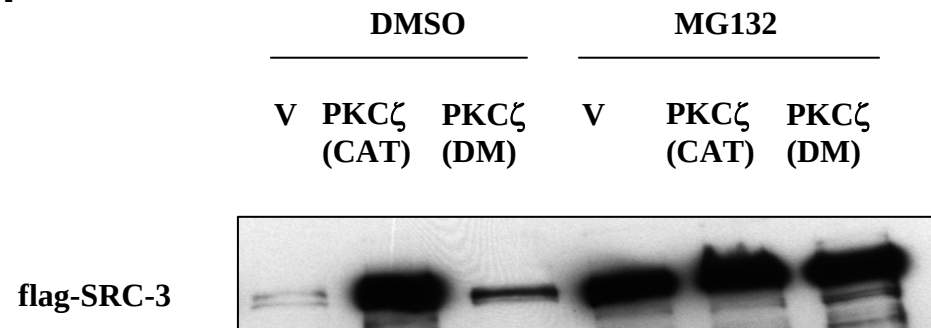
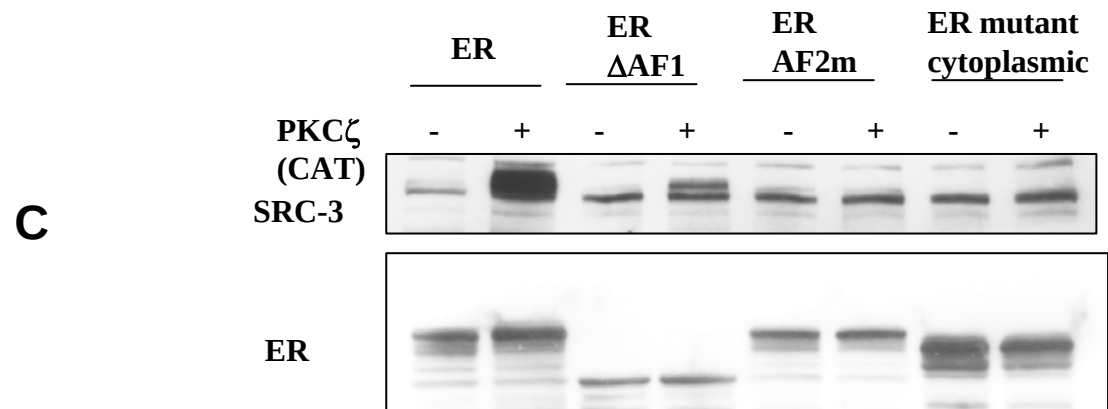
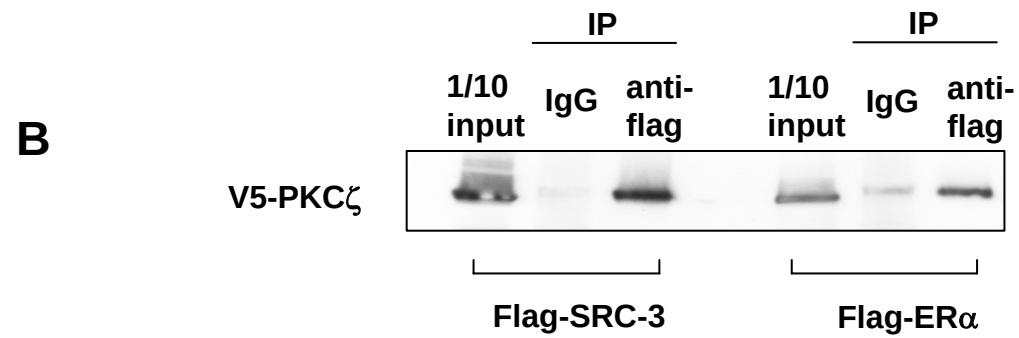
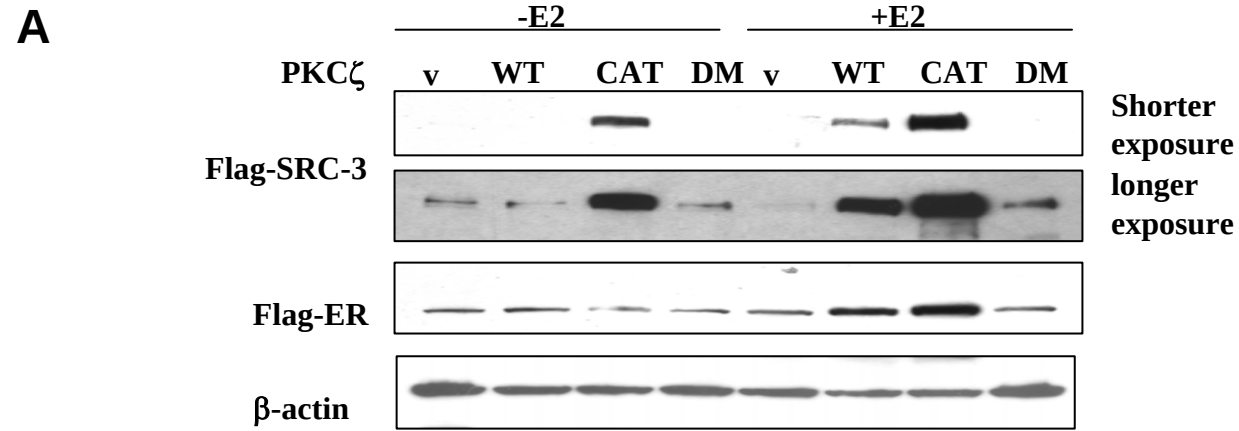
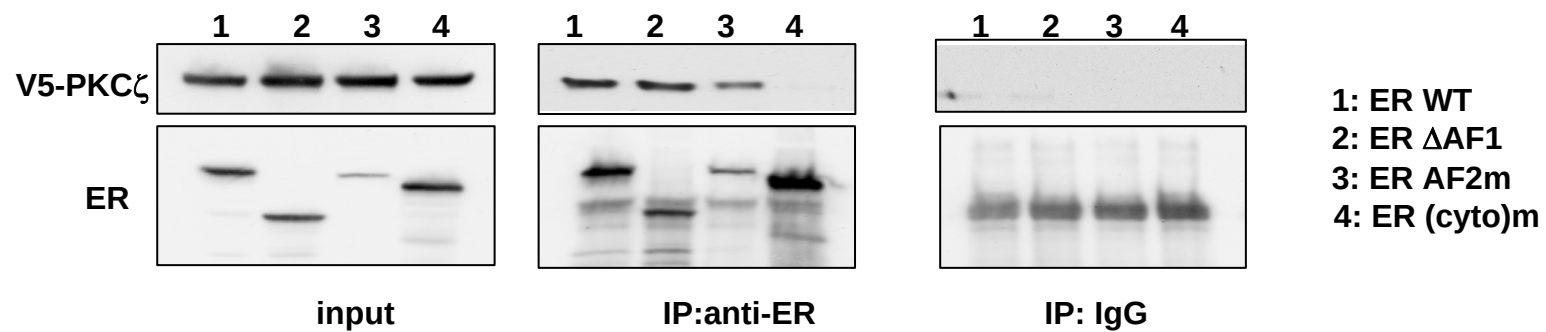
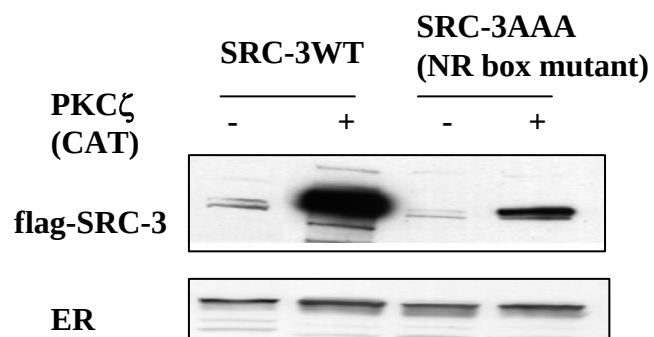
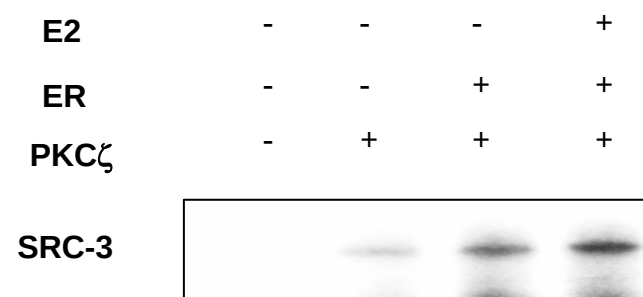


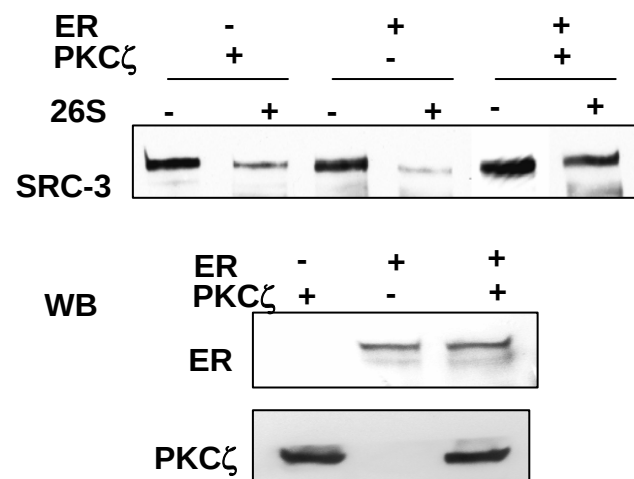
Fig. 1 (Continued)

Fig. 2

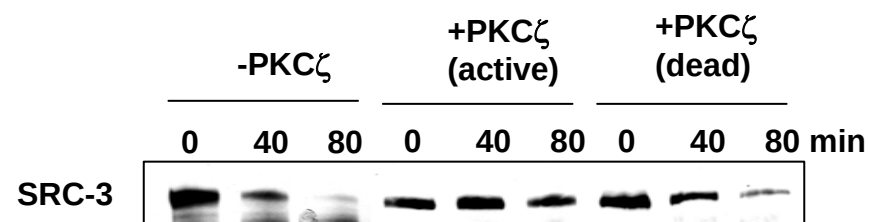


D**E****F****Fig. 2 (Continued)**

A



B



C



D

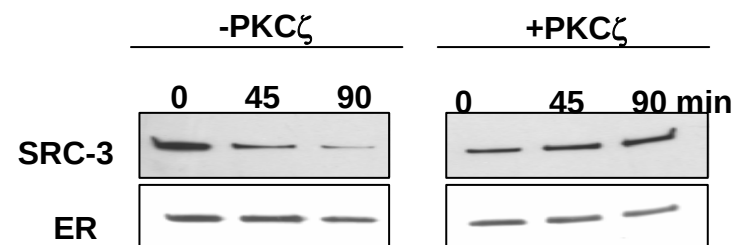
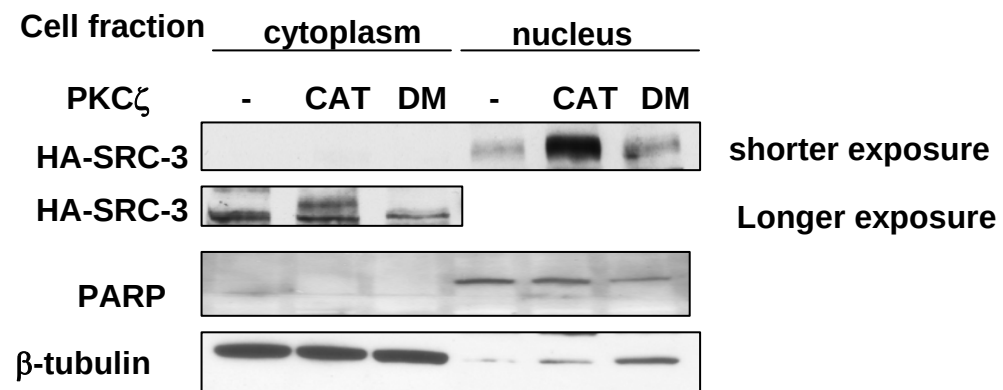


Fig. 3

A



B

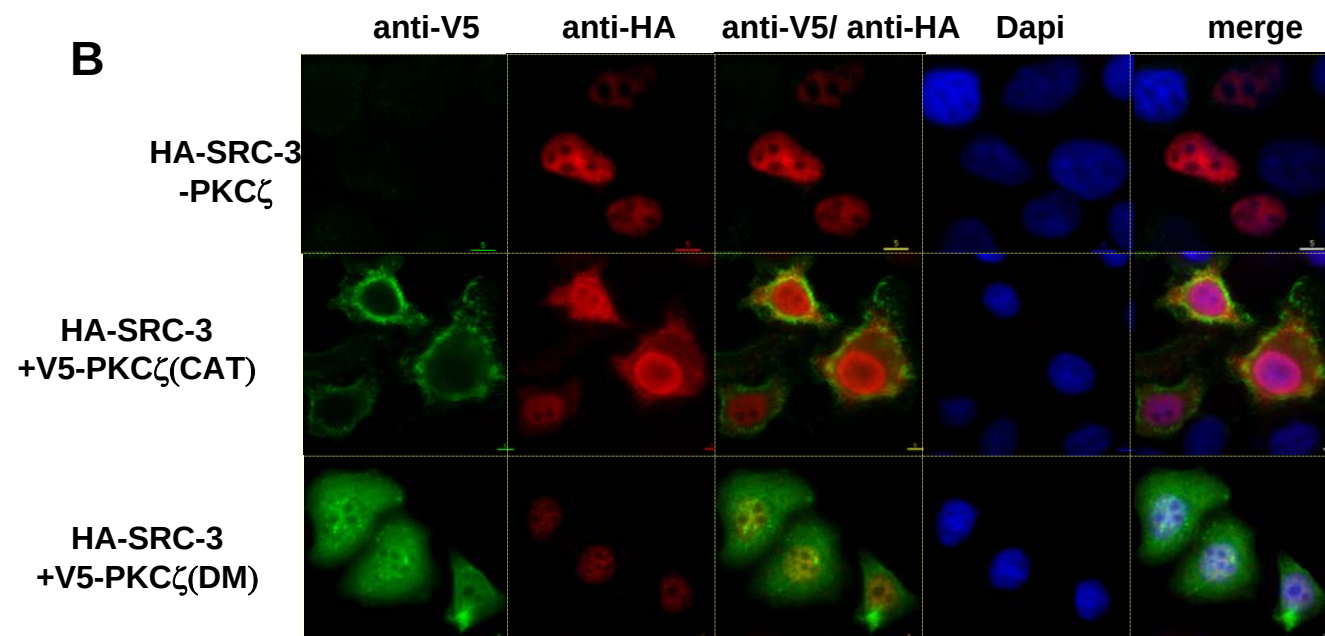


Fig. 4

C

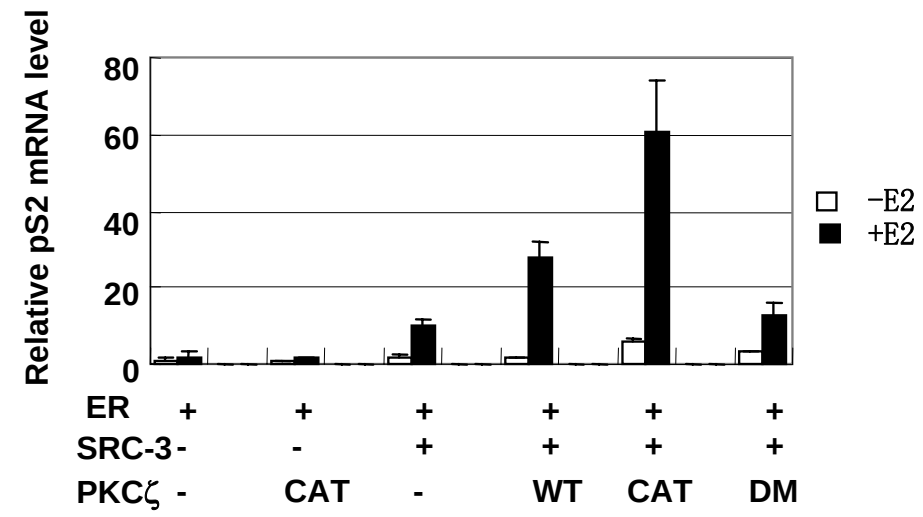


Fig. 4 (Continued)