Selective Activation of Protein Kinase C Isoforms by v-Src

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Abstract

Protein kinase C (PKC) is a gene family consisting of no less than 11 distinct isofoms. In both murine and rat fibroblasts, we detected expression of four PKC isofoms: the conventional PKC-α, the novel PKCs δ and e, and the atypical PKC-ζ. With the conventional and novel PKC isofoms, membrane association has been used to show PKC activation. In cells transformed by v-Src, there was a Ca2+-dependent increase in membrane association of the α isofom relative to the nontransformed parental cells. The ζ isofom had a slightly increased memmbrane association with murine fibroblasts transformed by v-Src but not in rat fibroblasts transformed by v-Src. However, since it is not clear whether cellular distribution of ζ isofom correlates with activation, the data are inconclusive with regard to this isofom. Interestingly, of the Ca2+-independent PKC isofoms δ and e, only the δ isofom was preferentially associated with membrane fractions in v-Src-transformed cells. The lack of PKC e activation was not due to lack of responsiveness to diacylglycerol (DG), since exogenously supplied DG and phorbol esters were both able to induce membrane association of PKC e. Thus, the differential activation of the δ and e isofoms by v-Src suggests a more complex mechanism for the activation of the novel Ca2+-independent PKC isofoms, involving more than simply elevating DG levels. Since PKC has been implicated in the intracellular signals activated by v-Src that lead to transformation, the selective activation of PKC α and δ suggests a role in mitogenesis and transformation for these PKC isofoms.

Introduction

PKC is a serine/threonine kinase gene family consisting of at least 11 distinct members. Generically, PKC has been implicated in a wide variety of signaling mechanisms (1). The PKC gene family has been divided into three major categories based on Ca2+ and lipid requirements. The conventional PKCs consist of four members (α, βI, βII, and γ) that require both Ca2+ and DG. A second class of PKC lacks a conserved Ca2+-binding domain (C2) and has been designated as nPKCs. This group includes the δ, e, η, and θ isofoms. Activation of the nPKCs is dependent upon DG but independent of Ca2+. The atypical PKCs ζ, λ, and μ, lack the C2 Ca2+-binding domain and possess only a single DG-binding site, which apparently renders them insensitive to both DG and phorbol esters that mimic DG. The existence of multiple PKCs with different requirements for activation suggests that the activation of the different PKC isofoms may be differentially regulated in the transduction of intracellular signals.

We and others have implicated PKC in the transduction of intracellular signals initiated by the protein-tyrosine kinase v-Src (2–8). Consistent with a PKC involvement in v-Src-induced intracellular signals, we found that v-Src stimulates a phospholipase D activity, which subsequently leads to an increase in DG, the physiological activator of most PKC isofoms (9). In cells depleted of PKC by prolonged treatment with phorbol esters, v-Src was unable to induce expression of either the 9E3 (2) or the TIS10 (3) genes in vivo or TPA response element-mediated gene expression in transient transfection assays (4). The activation of the TIS10 gene is not mediated by TPA response elements (10). Thus, there is a PKC requirement for the activation of at least two transcriptional control elements: the TPA-response element and the transcriptional control element(s) that regulate(s) v-Src-induced TIS10 gene expression. The existence of multiple isofoms of PKC in the same cell could contribute to the PKC-dependent signals that lead to the activation of different transcriptional control elements. In this report, we demonstrate that v-Src selectively activates the α and δ PKC isofoms in both rat and murine fibroblasts.

Results

PKC Isoform Expression in 3Y1 and v-Src-Transformed 3Y1 Cells. To determine which PKC isofoms were present in 3Y1 cells and 3Y1 cells expressing the NY-72 ts mutant of v-Src (3Y1-NY72ts cells; Ref. 11), whole-cell lysates from 3Y1 and 3Y1-NY72ts cells were examined by Western blot analysis for the presence of PKC isofoms. Using antibodies specific for the α, β, γ, δ, e, η, θ, and ζ isofoms, we determined that the α, δ, e, and ζ were present in both the 3Y1 and 3Y1-NY72ts cells; we were unable to detect the β, γ, η or θ isofoms (data not shown). These data are consistent with previous reports where the α, δ, e, and ζ isofoms were shown to be expressed in rat (12) and murine fibroblasts (13). The α, δ, e, and ζ isofoms were present in approximately equal amounts in both the 3Y1 and 3Y1-NY72ts cells, as determined by Western analysis (Fig. 1).

DG Levels Are Elevated in v-Src-Transformed Cells. The conventional PKCs and nPKCs are activated by the lipid second messenger DG, which is generated by the hydrolysis of membrane phospholipids (reviewed in Ref. 14). We demonstrated previously that v-Src induces increased DG levels by the phospholipase D/phosphatidic acid phosphohydrolyase pathway (9, 15). The phospholipase D activated

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3 The abbreviations used are: PKC, protein kinase C; nPKC, novel PKC; DG, diacylglycerol; ts, temperature sensitive; DIBC, 1,2-di-octanoyl-sn-glycerol; PC, phosphatidylycholine; TPA, 12-O-tetradecanoylphorbol 13-acetate; BAPTA/AM, bis-(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl)-ester.
by v-Src is specific for PC species lacking arachidonic acid (15). Therefore, we compared DG levels in 3Y1 cells and 3Y1-NY72\textsuperscript{ts} cells maintained at either the permissive or nonpermissive temperatures for v-Src. Cells were prelabeled with either \textsuperscript{1}H\textsuperscript{-}myristate or \textsuperscript{1}H\textsuperscript{-}arachidionate: \textsuperscript{1}H\textsuperscript{-}myristate is incorporated almost exclusively into PC, whereas \textsuperscript{1}H\textsuperscript{-}arachidionate is incorporated into all of the major membrane phospholipids, including PC (15). As shown in Fig. 2, there were elevated levels of DG in 3Y1-NY72\textsuperscript{ts} cells maintained at the permissive temperature for v-Src (35°C) relative to the nonpermissive temperature for v-Src (39.5°C); however, there was an even greater difference when the DG levels were compared between the 3Y1-NY72\textsuperscript{ts} cells maintained at the permissive temperature for v-Src and the parental 3Y1 cells. If the cells were labeled with \textsuperscript{1}H\textsuperscript{-}arachidionate, no difference between the two cell types was detected. Thus, there is an elevation of DG in 3Y1-NY72\textsuperscript{ts} cells that is derived from PC, the source of v-Src-induced increases in DG (9). The elevated levels of DG observed at the nonpermissive temperature for v-Src relative to the parental 3Y1 cells is likely due to a leaky phenotype of the NY72\textsuperscript{ts} mutant, as discussed below and shown in Fig. 4.

PKC Isoform Activation in 3Y1 and 3Y1-NY72\textsuperscript{ts} Cells. The activation of most, but not all, PKC isoforms correlates with a translocation from a cytosolic to a membrane distribution (1). This translocation has been widely used as a measure of PKC activation for the PKC isoforms that respond to activating stimuli in this way (12, 13, 16–18).

![Fig. 1. Expression of the α, δ, ε, and ζ PKC isoforms in 3Y1 and 3Y1-NY72\textsuperscript{ts} cells. Whole-cell extracts from the 3Y1-NY72\textsuperscript{ts} and the parental 3Y1 cells were normalized for total protein and subjected to Western analysis using antibodies to specific PKC isoforms as shown.](image-url)

![Fig. 2. DG levels in 3Y1 and 3Y1-NY72\textsuperscript{ts} cells. 3Y1 and 3Y1-NY72\textsuperscript{ts} cells were prelabeled with either \textsuperscript{1}H\textsuperscript{-}myristate (MA) or \textsuperscript{1}H\textsuperscript{-}arachidionate (AA) as shown, and DG levels were determined at the permissive (35°C) and nonpermissive (39.5°C) temperature for v-Src in 3Y1-NY72\textsuperscript{ts} cells and at 35°C for the 3Y1 cells as shown. Data are presented as the percentage DG of the total cpm incorporated into the 3Y1 and 3Y1-NY72\textsuperscript{ts} cells using the \textsuperscript{1}H\textsuperscript{-}myristate or \textsuperscript{1}H\textsuperscript{-}arachidionate labels as shown. Data are the average of duplicates from a representative experiment that was repeated at least twice.](image-url)

![Fig. 3. PKC isoform activation in 3Y1 and 3Y1-NY72\textsuperscript{ts} cells. Cytosolic (C) and membrane (M) fractions from 3Y1 and 3Y1-NY72\textsuperscript{ts} cells maintained at both the permissive (35°C) and nonpermissive (39.5°C) temperatures for v-Src were prepared and subjected to Western analysis using antibodies raised against the α, δ, ε, and ζ PKC isoforms as shown. As a positive control for activation, the 3Y1 cells were treated with TPA (200 nM; 30 min). The effect of prolonged exposure to TPA (800 nM; 24 h) on the PKC isoforms in 3Y1 cells is also shown.](image-url)

Therefore, we compared the subcellular distribution of the PKC isoforms in the 3Y1-NY72\textsuperscript{ts} and parental 3Y1 cells. PKC α is dependent upon both Ca\textsuperscript{2+} and DG for activation (1). TPA can substitute for DG to activate PKC α, even in the absence of Ca\textsuperscript{2+}, although higher levels of TPA were required for activation of the α isoform than were required for the Ca\textsuperscript{2+}-independent ε isoform (12). As a positive control for PKC α activation, we examined the effect of short-term TPA treatment on the subcellular distribution of PKC α. As shown in Fig. 3, short-term treatment with 200 nM TPA for
30 min caused the complete association of PKC α with the membrane fraction in 3Y1 cells. Prolonged treatment with 800 nM TPA for 24 h, which down-regulates most PKC isoforms, eliminated PKC α in these cells (Fig. 3). The distribution of PKC α between the membrane and the cytosol in 3Y1 and 3Y1-NY72 Δc cells, which were maintained in both permissive temperature (35°C) and nonpermissive temperature (39.5°C), is also shown in Fig. 3. The α isoform is almost completely associated with the cytosolic fraction in the 3Y1 cells, suggesting that PKC α is not activated in these cells. In the 3Y1-NY72 Δc cells, a substantial portion of PKC α is associated with the membrane fraction, suggesting that PKC α is activated in these cells. These data suggest that the α isoform of PKC is activated in cells that express v-Src.

As shown in Fig. 3, there was a higher degree of membrane association for the α isoform at the permissive temperature for v-Src relative to that observed at the nonpermissive temperature; however, there was still a substantial amount of PKC α associated with the membrane fraction of 3Y1-NY72 Δc cells maintained at the nonpermissive temperature for v-Src when compared to the parental 3Y1 cells. This was also true for the production of DG (Fig. 2). These cells have a strong temperature dependence for the transformed phenotype as determined morphologically (Fig. 4a). However, since the data in Fig. 3 suggested that a substantial amount of PKC α was membrane associated at the nonpermissive temperature for v-Src (39.5°C), we examined the extent of tyrosine phosphorylation in these cells at both the permissive and nonpermissive temperatures for v-Src and in the parental 3Y1 cells. As shown in Fig. 4b, there was a detectable increase in phosphotyrosine levels in the 3Y1-NY72 Δc cells upon shift from the nonpermissive to the permissive temperature for v-Src. However, as expected, there was a far greater difference in phosphotyrosine levels observed between the 3Y1-NY72 Δc cells and the parental 3Y1 cells. Thus, the increased membrane association of the α isoform observed at the nonpermissive temperature for v-Src can be explained by an elevated kinase activity in these cells. This observation is important because it demonstrates that the v-Src-induced increase in membrane association of PKC α is not an indirect effect of transformation since increased membrane association can be observed at the nonpermissive temperature for v-Src and the cells are not transformed at this temperature. Thus, the increased association of the α isoform with the membrane fraction is likely due to kinase activity of v-Src. Consistent with this conclusion, genistein, which inhibits tyrosine kinase activity, blocked the membrane association of PKC α in the 3Y1-NY72 Δc cells (Fig. 5).

We next examined the subcellular distribution of the Ca2+-independent PKC isoforms δ and ε in the 3Y1 and 3Y1-NY72 Δc cells. As demonstrated for the α isoform, short-term TPA treatment lead to a complete association of both the δ and ε isoforms with the membrane fractions, and long-term TPA treatment lead to down-regulation of both of these isoforms (Fig. 3). Analysis of the subcellular distribution of the δ isoform in 3Y1 and 3Y1-NY72 Δc cells revealed a substantially enhanced membrane association of PKC δ in the 3Y1-NY72 Δc cells relative to the parental 3Y1 cells (Fig. 3). In contrast, the ε isoform had the same subcellular distribution in both the 3Y1-NY72 Δc and 3Y1 cells, in which this isoform fractionated almost exclusively with the cytosol (Fig. 3). As with the α isoform, the membrane association of the δ isoform was blocked with genistein treatment (Fig. 5). The sensitivity of membrane association of both the α and

![Fig. 4](image_url) 3Y1-NY72 Δc cells have a leaky phenotype for protein-tyrosine kinase activity but not for transformation. α, morphology of 3Y1 and 3Y1-NY72 Δc cells maintained at 39.5°C or 35°C. β, phosphotyrosine levels were determined by Western analysis using an anti-phosphotyrosine antibody in 3Y1 and 3Y1-NY72 Δc cells maintained at 39.5°C and in cells shifted to 35°C for 16 h.
δ isoforms to genistein is consistent with our conclusion that the association of these PKC isoforms with the membrane at the nonpermissive temperature for v-Src is due to a leaky tyrosine kinase activity. These data demonstrate that for the Ca^{2+}-independent nPKCs δ and ε, there is a selective activation of the δ isoform relative to the ε isoform in response to the kinase activity of v-Src.

The ζ isoform of PKC is both Ca^{2+}- and phorbol ester independent (19). The ζ enzyme lacks the Ca^{2+}-binding and one of the DG-binding sites and has been designated an atypical PKC (1). Unlike the α, δ, and ε isoforms, a change in the subcellular distribution for the ζ isoform has not been demonstrated to correlate with or represent activation. The subcellular distribution of PKC ζ is not dramatically affected by either short-term or long-term TPA treatment in 3Y1 cells (Fig. 3). There were no significant changes in the subcellular distribution of the ζ isoform in the 3Y1 and 3Y1-NY72^r cells (Fig. 3).

Ca^{2+}-dependence of PKC α Membrane Association in 3Y1-NY72^r Cells. Activation of the α isoform of PKC is dependent upon both DG and Ca^{2+}, whereas the nPKC isoforms δ and ε lack Ca^{2+}-binding sites (1). A role for Ca^{2+} in the intracellular signals activated by v-Src is not well characterized. To establish a role for Ca^{2+} in the activation of PKC α by v-Src, we examined the sensitivity of PKC α activation to the membrane-permeant Ca^{2+} chelator BAPTA/AM and the extracellular Ca^{2+} chelator EGTA. As shown in Fig. 5, membrane association of the α isoform in 3Y1-NY72^r cells was sensitive to both BAPTA/AM and EGTA. As expected, neither BAPTA/AM nor EGTA had any effect on the subcellular distribution of the δ isoform in either 3Y1 or 3Y1-NY72^r cells. These data suggest that the increased membrane association of the α isoform of PKC in response to v-Src requires Ca^{2+}. Although the mechanism for generation of the Ca^{2+} needed for the v-Src-induced activation of PKC α requires further investigation, it is likely to involve an influx of extracellular Ca^{2+} since EGTA, which chelates only extracellular Ca^{2+}, inhibited the membrane association of PKC α in the 3Y1-NY72^r cells.

To confirm the requirement of both DG and Ca^{2+} for the α isoform in the 3Y1 and 3Y1-NY72^r cells, we examined the effects of DiC8, a soluble DG, and the Ca^{2+} ionophore, ionomycin, on the subcellular distribution of the α, δ, and ε isoforms. As shown in Fig. 6, incubation with DiC8 alone induced the translocation both PKC δ and ε but not α in both the 3Y1 and 3Y1-NY72^r cells. Ionomycin had no effect on the translocation of any of the PKC isoforms. However, DiC8 and ionomycin together induced translocation of PKC α in the 3Y1 and 3Y1-NY72^r cells. It is of interest that DiC8 is able to activate the ε isoform in both the 3Y1 and 3Y1-NY72^r cells. Therefore, the lack of PKC ε activation by v-Src is not due to a lack of responsiveness to DG. These data suggest a more complex mechanism for activation of the nPKC isoforms, since PKC ε can be activated by exogenously provided DG but not by the DG generated by v-Src.

PKC Isoform Activation in v-Src-transformed BALB/c 3T3 Cells. We next asked whether the phenomena of selective activation of PKC isoforms in response to v-Src observed in the 3Y1 rat fibroblasts could also be observed in another cell line transformed by v-Src. Therefore, we compared the subcellular distribution of the PKC isoforms in v-Src-transformed and parental murine BALB/c 3T3 cells. The v-Src-transformed and parental BALB/c 3T3 cells express the same PKC isoforms as the 3Y1 cells (data not shown). As shown in Fig. 7, there were substantial increases in the membrane association of the α and δ isoforms in the v-Src-transformed cells relative to the BALB/c 3T3 cells, whereas there was no difference in the membrane association of PKC ε in the two cell lines. Thus, qualitatively, v-Src induces the same pattern of PKC isoform activation in the murine BALB/c 3T3 cells as that observed in the rat 3Y1 cells. A small but reproducible increase in the membrane association of the ζ isoform was observed in the v-Src-transformed BALB/c 3T3 cells. However, as discussed above, it is not clear that such changes in membrane distribution correlate with activation of this PKC isoform.

Discussion

The role of PKC in the transduction of many diverse intracellular signals is well established (1); however, the molecular mechanisms for the activation of the different PKC isoforms are more complex than originally proposed. In this report, we have shown that in cells expressing the oncogenic protein-tyrosine kinase v-Src, there is a selective activation of the α and δ isoforms of PKC. The ε isoform is not
activated by v-Src, despite the production of DG. We were not able to detect substantial differences in the cellular distribution of the ε isoform in v-Src-transformed 3T3 cells, but there was a small increase in membrane association of the ε isoform in v-Src-transformed BALB/c 3T3 cells. It is not known whether membrane association represents activation of the ε isoform; however, since PKC ε has been implicated in mitogenic signaling (20), a role for PKC ε in v-Src-induced signaling cannot be ruled out.

It was shown previously that the nPKC isoforms δ and ε are coactivated in response to bombesin and platelet-derived growth factor (13). Additionally, the δ and ε isoforms were shown to be coactivated in response to both serum and epidermal growth factor in 3Y1 rat fibroblasts (21). In this report, we have demonstrated that the δ and ε isoforms are not coactivated in response to v-Src. One possible explanation for the selective activation of the δ isoform in response to v-Src is the generation of unique DG species. It was shown recently that a differential activation of the α and ε PKC isoforms could be explained simply by the presence or absence of Ca\(^{2+}\) and not by different DG species that might be generated (17). However, the preferential activation of the δ over the ε isoform observed in v-Src-transformed cells cannot be explained by a difference in Ca\(^{2+}\) production since both of these isoforms are independent of Ca\(^{2+}\). Thus, it is possible that the DG species generated in response to v-Src might preferentially activate the δ isoform. We demonstrated previously that v-Src generates DG via a phospholipase D that is specific for a subpopulation of PC lacking arachidonic acid (15). Although the basis for this apparent aliphatic specificity is not understood, it is possible that specific DG species are generated to activate specific PKC isoforms. Several reports have demonstrated that different DG species are differentially phosphorylated by DG kinase (22–24). Additionally, it has been demonstrated that DG production does not always lead to PKC activation (10, 25, 26) and that different stimuli generate different DG species (27). Thus, there is a precedent for biological specificity at the level of the aliphatic composition in the generation, metabolism, and effector function of DG that could contribute to the differential activation of the δ and ε PKC isoforms observed here.

Another possible explanation for the selective activation of the δ isoform over the ε isoform is that these PKC isoforms may be differentially modified in v-Src-transformed cells. In this regard, it is of interest that the δ isoform has been reported to be phosphorylated on tyrosine concurrent with its activation (28) and that Src can phosphorylate PKC δ in vitro (29). Thus, phosphorylation of PKC δ by v-Src itself could contribute to the selective activation of PKC δ over PKC ε. Phosphorylation of the α isoform on threonine is essential for the catalytic activity of PKC α (30). Thus, phosphorylation may be an important component of PKC activation that could provide additional mechanisms for selective activation of PKC isoforms.

Whether activation of either PKC α or δ plays any role in the transformed phenotype induced by v-Src has not been established. It was reported recently that overexpression of the δ isoform in NIH 3T3 cells caused cells to grow more slowly and to lower cell densities, whereas the ε isoform caused opposite effects (31). The data here would tend to support a role for PKC δ in cell growth since v-Src can transform cells. Expression of PKC δ has been correlated with anchorage-independent growth in rat embryo fibroblasts (32), and it was reported recently that PKC δ can be phosphorylated on tyrosine (28). Thus, PKC δ may play a role in the mitogenic signals where tyrosine phosphorylation is involved. Overexpression of the α isoform has been reported to enhance cell growth potential (32, 33). Thus, PKC α has also been implicated in the regulation of cell proliferation. The data presented here support roles for PKC α and PKC δ in cell proliferation and transformation. Specific roles for PKC α and δ in the signals initiated by v-Src remain to be determined.

Materials and Methods
Cells and Cell Culture Conditions. Rat fibroblasts 3Y1 and 3Y1-NY72\(^{2+}\), which express the NY72 mutant of v-Src in a Moloney murine leukemia virus vector (11) and BALB/c 3T3 cells transformed with the Schmidt-Rupin D (SRD) strain of Rous sarcoma virus (34, 35), were maintained in DMEM supplemented with 10% newborn calf serum (HyClone). The 3Y1-NY72\(^{2+}\) cells were maintained at the permissive temperature for transformation (35°C). In some cases, TPA was added in the culture at 200 nM for 30 min to activate PKC or 800 nM for 24 h to down-regulate PKC. Ca\(^{2+}\) was depleted by incubation with 25 μM BAPTA/AM (CalBiochem) or 10 mM EGTA (Sigma Chemical Co.) for 30
min. In some cases, the cells were treated with 100 μg/ml DiC8 (Sigma) and/or 5 μM ionomycin (Sigma) for 5 min. Genistein (Sigma) treatment, when used, was at 300 μM for 4 h prior to harvesting of cells.

Antibodies. mAbs to PKC α and PKC β were obtained from Upstate Biotechnology and Sekagaku, respectively. Polyclonal antibodies to PKC δ, PKC ε, and PKC ζ were obtained from Gibco-BRL. Polyclonal antibodies to PKC γ, PKC θ, and PKC η were obtained from Santa Cruz Biotechnology. The anti-phosphotyrosine mAb (PY20) was obtained from Transduction Laboratories.

Determination of DG Levels. Confluent 60-mm culture dishes were preloaded for overnight in 2 ml of media containing 0.5% newborn calf serum. Isotopes were added to the culture media as follows: [3H]myristate, 2 μCi (40 Ci/mmol); [3H]arachidonate, 2 μCi (240 Ci/mmol); [3H]myristate (NET-830) and [3H]arachidonate (NET-2982) were obtained from New England Nuclear. The extraction of lipids was performed as described previously (9). DG levels were characterized by TLC (silica gel 60A plates) using the following solvent system: hexane/diethylether:methanol:glacial acetic acid (90:20:3:2; v/v). Lipid standards were visualized by treating TLC plates with iodine vapor. To quantitate metabolically labeled DG, the TLC plate was subject to autoradiography, and the intensity of DG bands was determined using a Molecular Dynamics densitometer.

Subcellular Fractionation. To examine the subcellular localization of PKC isoforms, cultures were allowed to get to approximately 85% confluency and then shifted to 0.5% DMEM for 24 h. For subcellular fractionation, cells from 150-mm culture dishes were washed three times with ice-cold isotonic buffer (PBS; 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH₂PO₄, and 4.2 mM Na₂HPO₄, pH 7.2) and then scraped in 4 ml homogenization buffer [20 mM Tris-HCl (pH 7.5), 5 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 2 mM DTT, 200 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. The cells were disrupted with 20 strokes in a Dounce homogenizer (type B pestle), and the lysate was centrifuged at 100,000 × g for 1 h. The supernatant was collected and considered to be the cytosolic fraction. The membrane pellet was suspended in the same volume of homogenization buffer with 1% Triton-100. After incubation for 30 min at 4°C, the suspension was centrifuged at 100,000 × g for 1 h. The supernatant was collected as the membrane fraction.

Western Analysis. Equal amounts of protein from both cytosolic and membrane fractions or total cell lysates were subjected to SDS-PAGE using an 8% acrylamide separating gel. Transfer to nitrocellulose was performed as described previously (34). After blocking at 4°C overnight with 5% non-fat dry milk in PBS, the nitrocellulose filters were incubated with appropriate antibodies. Depending on the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG was used for detection using the ECL system (Amersham).

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