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Phospholipase C-β2 interacts with mitogen-activated protein kinase kinase 3☆

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Abstract

Phospholipase C (PLC)-β enzymes (isoenzymes β1–β4) are activated by G protein subunits, leading to the generation of intracellular messengers which mobilize calcium and activate protein kinase C. It has recently been recognized that these enzymes interact with and are regulated by proteins other than G proteins. Using the yeast two-hybrid technique to screen a leukocyte library we identified mitogen-activated protein kinase kinase 3 (MKK3) as a partner of PLC-β2. The interaction was confirmed by co-immunoprecipitation assays which indicated that MKK3 interacts with PLC-β2, but not with other PLC-βs. PLC-β2 interacted weakly with MKK6, which is related to MKK3, but not with the other MKK3 tested. The region of PLC-β2 involved in the interaction with MKK3 was mapped to the C-terminus of PLC-β2. p38MAPK also co-immunoprecipitated with PLC-β2. The data suggest that PLC-β2 serves an unappreciated role assembling components of the p38MAPK signaling module. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Phospholipase C-β; Yeast two-hybrid; Mitogen-activated protein kinase kinase 3; p38MAPK; Leukocyte; Signaling; G protein; Calcium

A range of extracellular mediators initiate cellular responses by binding to G protein-coupled receptors which in turn regulate effectors such as phosphatidylinositol-specific phospholipase C (PLC) [1–3]. PLC hydrolyzes membrane inositol containing phospholipids to generate two important second messengers which mobilize calcium from intracellular stores and activate the protein kinase C. Three classes of PLC exist β, γ, and δ and within the PLC-β class there are four isoenzymes (β1–β4). PLC-β enzymes are activated to varying extents by subunits of heterotrimeric guanine nucleotide-binding proteins (G proteins), either Gαq or Gβγ. PLC-β2 is found primarily in hematopoietic cells and together with PLC-β3, these PLC-βs are regarded as the sole isoenzymes activated by chemoattractants in leukocytes. Studies with mice lacking PLC-β2 and PLC-β3 revealed that these PLC pathways are important in chemoattractant-mediated superoxide production and regulation of protein kinases; however, they are not required for chemotaxis [4,5].

Recently it has been recognized that PLC-β isoenzymes interact with proteins other than heterotrimeric G protein subunits. In the *Drosophila* phototransduction pathway, PLC-β interacts with a scaffold protein termed InaD, which in turn interacts with the eye-specific protein kinase C and a light-activated calcium channel thereby organizing components of the signaling cascade to enhance the efficiency and speed of signaling [6]. The association of PLC-β with InaD occurs via a motif at the C-terminus of PLC which interacts with a PDZ domain (postsynaptic density-95, disks large, ZO-1) of InaD. All four mammalian PLC-β isoenzymes have a consensus PDZ-binding motif at their C-terminus [7]. Recently Hwang et al. [8] reported, using the yeast two-hybrid system, that PLC-β3 interacts with Na/H exchanger regulatory factor 2 (NHERF2) via a C-terminal motif. It has also been demonstrated that PLC-β2 interacts directly with and is activated by the low-molecular-weight G proteins Cdc42 and Rac1, as well as by heterotrimeric

☆ *Abbreviations*: PLC, phospholipase C; PDZ, postsynaptic density-95, disks large, ZO-1; NHERF2, Na/H exchanger regulatory factor 2; MKK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase.

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G protein subunits [9,10]. In addition, fluorescence resonance energy transfer studies using purified PLC-β1 suggest that this enzyme interacts with regulator of G protein signaling 4 (RGS4); although this interaction is significantly weaker than that observed with Gαq [11]. The yeast hormone receptor-like protein Gpr1p interacts with phosphatidylinositol-specific PLC (Plc1p) as shown by two-hybrid and co-immunoprecipitation assays [12].

This study sought to identify proteins interacting with PLC-β2 of relevance to chemoattractant receptor signal transduction by using the yeast two-hybrid system to screen a leukocyte library. It is reported herein that mitogen-activated protein kinase kinase 3 (MKK3) interacts with PLC-β2 in the yeast two-hybrid system and in mammalian cells by co-immunoprecipitation assays. The interaction is selective and the region of PLC-β2 involved in the interaction has been mapped. The data also demonstrate that the "downstream" kinase in the signaling module, p38MAPK, co-immunoprecipitates with PLC-β2.

Materials and methods

Materials. Expression plasmids for epitope tagged MKK3, MKK4, MKK6, MEK-1, and p38MAPK were provided by Dr. R. Davis and Dr. J. Kyriakis [13,14]. PLC-β1, PLC-β2 plasmids were provided by Dr. D. Wu and the PLC-β3 plasmid was from Dr. G. Weber [15].

Construction of bait plasmids. Human PLC-β2 as a bait for yeast two-hybrid screens was constructed in the Gal4–DNA-binding domain vector pAS2 (Clontech). The N-terminus of PLC-β2 was amplified by PCR using a 5′ primer containing an *Nhe*I site and reverse primer corresponding to an internal sequence and digested with *Nhe*I and DraIII. This fragment and a PLC-β2 fragment from *Dra*III–*Xho*I were ligated in frame into the *Nhe*I and *Sal*I sites of pAS2, creating a plasmid encoding a Gal4-DBD–PLC-β2 fusion protein.

Yeast two-hybrid assays. Two-hybrid screening was performed as described (Clontech Manual). The yeast strain Y190 was transformed with the pAS2–PLC-β2 by the lithium acetate method and expression of the bait was confirmed by Western blotting. A human leukocyte cDNA library in the pACT2 vector (Clontech) was transformed into the yeast strain expressing the bait protein. Transformants expressing both the bait and interacting prey proteins were selected on medium lacking tryptophan, leucine, and histidine, containing 2.5 mM 3-amino-1,2,4-triazole (Sigma). Plates were incubated at 30 °C for 5–7 days and tested for β-galactosidase activity using the filter lift assay. Approximately 1.5×10^6 colonies were screened and six positive clones were identified. The library plasmids were isolated from positive colonies, amplified in *Escherichia coli*, and sequenced by the Duke DNA analysis facility. Library plasmids were co-transformed into yeast strain AH109 together with irrelevant bait proteins lamin C, p53, and SNF1 and grown on selective plates lacking tryptophan, leucine, histidine, and adenine to test the specificity of interactions.

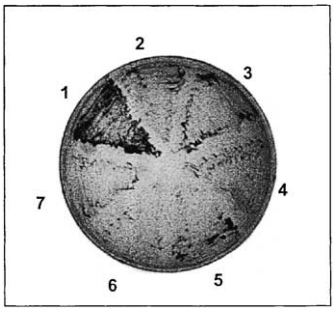
Co-immunoprecipitation and immunoblotting. PLC-β expression plasmids (2 μg) were transiently co-transfected with Flag-MKK3 or other MKKs (2 μg) in 1×10^6 Cos-7 cells using the Fugene-6 procedure (Roche). Cells were harvested from plates 48 h after transfection by scraping in PBS and pelleted. Cells were solubilized in 100 μl lysis buffer, 25 mM Tris–HCl (pH 7.6), 2 mM DTT, 2 mM EDTA, 10% glycerol, 1% Triton X-100, and (1:500) protease inhibitor cocktail (Sigma). The lysate was then diluted with 1 ml buffer A, 50 mM Hepes (pH 7.6), 250 mM NaCl, 0.1% Nonidet P-40, and 5 mM EDTA, mixed and the insoluble material was removed by centrifuging for 5 min at 14,000*g* at 4 °C. The lysate was immunoprecipitated with 1 μg PLC-β antibodies (Santa Cruz Biotech) and protein A–agarose beads (Roche) for 2 h. The beads were then washed with 3× 1 ml buffer A and 1 ml PBS. The immunoprecipitates were resolved by 10% SDS–PAGE, transferred to nitrocellulose membrane, and probed with (1:1000) Flag-M2 monoclonal antibody. Antibody was detected with HRP conjugated sheep anti-mouse antibody (Amersham) and ECL.

Generation of chimeras. Chimera#1, PLC-β2 (M^1–H^{774}) with PLC-β3 (Y^{822}–L^{1234}) was generated by cutting out the N-terminus of the PLC-β2 with *Eco*RI and *Kpn*1 and ligating this fragment to a PCR product corresponding to the nucleotide sequence of PLC-β3 from Y^{822}–L^{1234}. Chimera#2, PLC-β2 (M^1–Q^{316}) with PLC-β3 (P^{322}–L^{1234}) was generated by cutting out the N-terminus of PLC-β3 with *Eco*RI and *Dra*III and ligating in a PCR product corresponding to the nucleotide sequence of PLC-β2 from M^1–Q^{316}. Chimera#3, PLC-β3 (M^1–S^{645}) with PLC-β2 (R^{598}–L^{1181}) was generated by cutting out the N-terminus of PLC-β3 with *Eco*RI and *Blp*I and ligating this sequence to a PCR product corresponding to the nucleotide sequence of PLC-β2 from R^{598}–L^{1181}. Constructs were sequenced by the Duke DNA Sequencing Facility.

Results

PLC-β2 interacts with MKK3

The yeast two-hybrid system was used to identify proteins that interact with PLC-β2. Screening of 1.5×10^6 clones in a human leukocyte library resulted in the identification of six positive clones. Sequencing the cDNAs revealed that three clones encoded the same polypeptide, residues 15–318 of the dual specificity MKK3 [13]. Control experiments confirmed that neither PLC-β2 nor MKK3 had autoactivating properties. The specificity of the observed interaction was tested by co-transforming the yeast strain AH109 with the prey plasmid encoding MKK3 and irrelevant bait proteins and either PLC-β2 or PLC-β3 as a bait fusion protein. The transformants were plated on selective plates lacking tryptophan, leucine, histidine, and adenine. Co-expression of PLC-β2 and MKK3 enabled strong growth



Bait	Prey
1 PLC-β2	MKK3
2 p53	MKK3
3 PLC-β2	Gal4-AD
4 PLC-β3	MKK3
5 Gal4-DBD	MKK3
6 Lamin C	MKK3
7 SNF1	MKK3

Fig. 1. PLC-β2 interacts specifically with MKK3 in the yeast two-hybrid system. Yeast strain AH109 was co-transformed with plasmids encoding fusion proteins with either the Gal4–DNA-binding domain (bait) or the Gal4 activation domain (prey). The ability of the two proteins to interact was assessed by monitoring the growth of transformants after plating on selective medium (SC–Trp/–Leu/–His/–Ade).

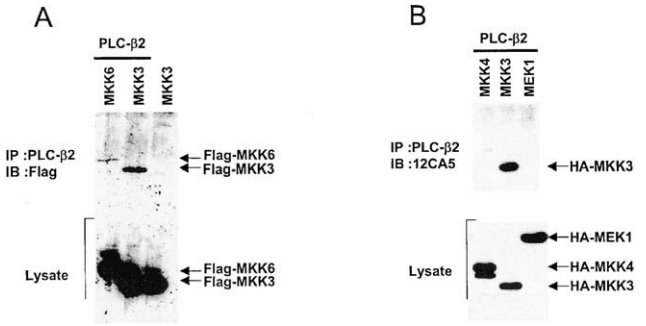


Fig. 2. Co-immunoprecipitation of PLC-β2 and MKK3 from transfected Cos-7 cells. PLC-β2 and MKK3, MKK4, MEK1, or MKK6 were co-expressed in Cos-7 cells. PLC-β2 was immunoprecipitated from cell lysates with a rabbit polyclonal antibody as described under "Materials and methods." Immunoprecipitates and whole cell lysate were resolved by SDS–PAGE and immunoblotted with the Flag-M2 antibody (A) or 12CA5 anti-HA antibody (B).

on the selective plates (Fig. 1) while expression of the empty bait vector pAS2 (Gal4-DBD), PLC-β3, or irrelevant bait proteins lamin C, p53, SNF1 together with the MKK3 prey protein did not enable yeast growth.

The interaction of PLC-β2 and MKK3 was examined in mammalian cells transiently expressing both proteins. Flag epitope tagged MKK3 was transiently co-expressed in Cos-7 cells together with PLC-β2 and the cell lysate was then immunoprecipitated with an anti-PLC-β2 polyclonal antibody. Immunoprecipitated proteins were resolved by SDS–PAGE and subjected to Western blot analysis with the Flag-M2 antibody (Fig. 2A). Flag-MKK3 was present in immunoprecipitates from PLC-β2 transfected cells but not in immunoprecipitates from cells transfected with empty vector and Flag-MKK3 plasmid. The selectivity of the interaction of PLC-β2 with MKK3 was examined in the co-immunoprecipitation assays by using other mitogen-activated protein kinase kinase (MKK) related to MKK3. MKK6 has significant homology to MKK3 and also interacted with PLC-β2 although the interaction was significantly weaker than for MKK3. In contrast, HA epitope tagged forms of MKK4 and MEK-1 did not co-immunoprecipitate with PLC-β2 although expressed to similar levels as HA-MKK3 (Fig. 2B).

The examination of the selectivity of observed interaction was extended to other PLC-β isoenzymes. Comparison of PLC-β1, PLC-β2, and PLC-β3 demonstrated that MKK3 co-immunoprecipitates only with PLC-β2 and not with other PLC-β isoenzymes (Fig. 3).

Identification of a PLC-β2 region that interacts with MKK3

To delineate the region of PLC-β2 that is required for interaction with MKK3, chimeras of PLC-β2 and PLC-β3 were generated and used in co-immunoprecipitation

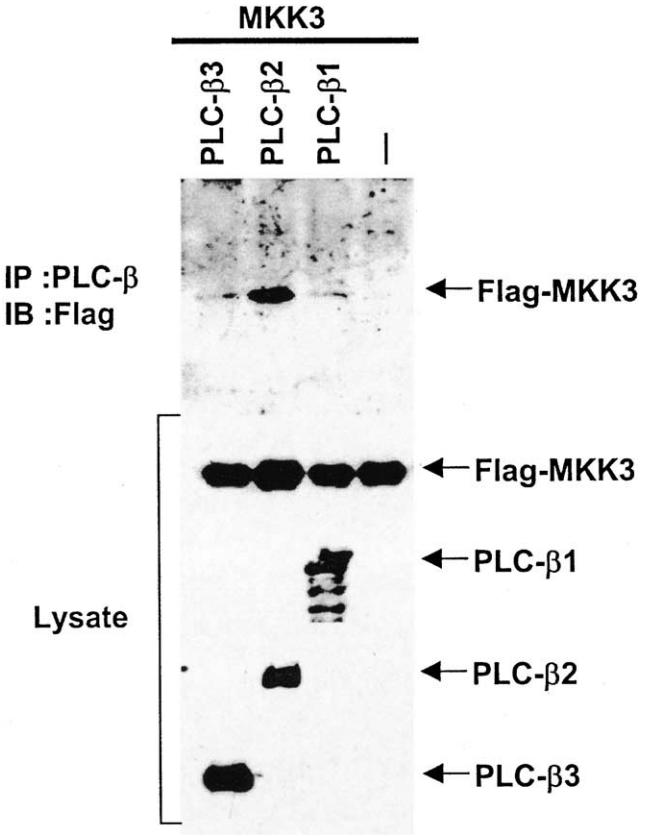


Fig. 3. MKK3 co-IPs with PLC-β2 but not with other PLC-β isoenzymes. Either PLC-β1, PLC-β2, PLC-β3, or control plasmids were transfected into Cos-7 cells together with Flag-MKK3. PLC-β enzymes were immunoprecipitated from cell lysates with rabbit polyclonal antibodies as described under "Materials and methods". Immunoprecipitates or whole cell lysate were resolved by SDS–PAGE and immunoblotted with the Flag-M2 antibody and the PLC-β antibodies to confirm the expression of transfected proteins.

experiments. Either PLC-β2, PLC-β3 or these chimeras was transiently expressed in Cos-7 cells together with Flag-MKK3 (Fig. 4). The PLC-β proteins were immunoprecipitated from cell lysates and the immunoprecipitates were analyzed for co-immunoprecipitated Flag-MKK3 by Western blotting. Flag-MKK3 did not co-immunoprecipitate to a significant extent with either chimera#1 or chimera#2. Flag-MKK3 did co-immunoprecipitate with chimera#3 indicating that the C-terminus of PLC-β2 is required for the interaction (Fig. 4). The amount of Flag-MKK3 detected in the immunoprecipitate was less than that in immunoprecipitates from cells expressing PLC-β2; however, this may be related to differences in the expression levels of PLC-β2 and the chimeras.

PLC-β2 and p38MAPK

It has recently been recognized that the individual kinases of MAPK signaling modules are often assembled by scaffold proteins; therefore the hypothesis

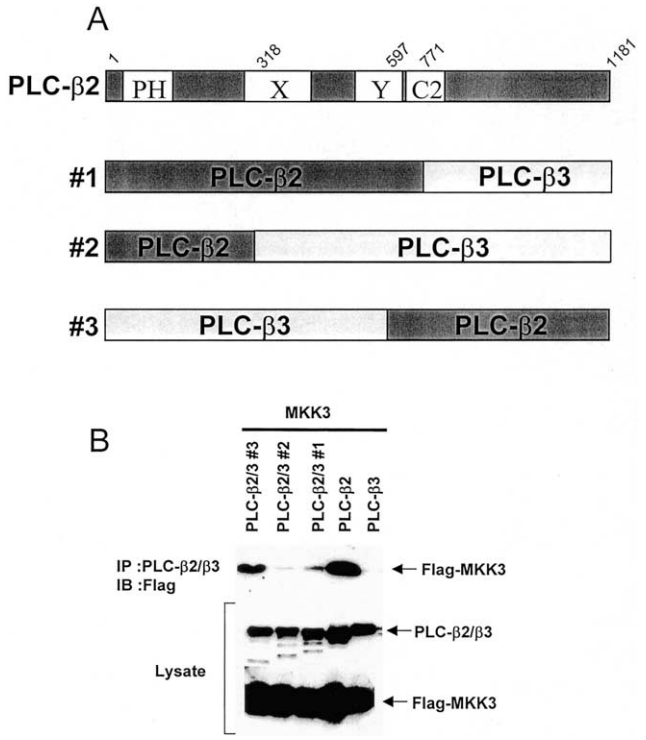


Fig. 4. Analysis of MKK3 interaction with PLC-β2/β3 chimeras. (A) Domain structure of PLC-β2 and a schematic representation of the chimeras. (B) Cos-7 cells were transfected with PLC-β3, PLC-β2 or PLC-β chimera expression plasmids together with Flag-MKK3. PLC-β enzymes were immunoprecipitated and immunoprecipitates or whole cell lysate were resolved by SDS–PAGE and immunoblotted with the Flag-M2 antibody and the PLC-β antibodies to confirm the expression of transfected proteins.

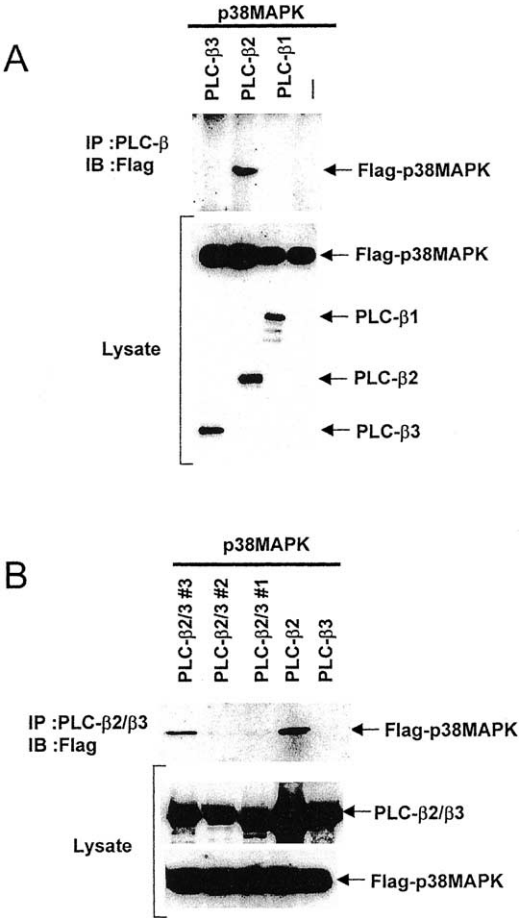


Fig. 5. p38MAPK selectively co-IPs with PLC-β2 via an interaction at the C-terminus. (A) Cos-7 cells were transfected with PLC-β isoenzymes together with Flag-p38MAPK. PLC-β enzymes were immunoprecipitated and immunoprecipitates or whole cell lysate were resolved by SDS–PAGE and immunoblotted with the Flag-M2 antibody and the PLC-β antibodies to confirm expression of transfected proteins. (B) Analysis of p38MAPK interaction with PLC-β2/β3 chimeras by co-immunoprecipitation from transiently transfected Cos cells.

that the kinase downstream of MKK3, p38MAPK, co-immunoprecipitates with PLC-β2 was examined. Cos cells were co-transfected with PLC-β1, PLC-β2, PLC-β3 or the three PLC-β2/β3 chimeras together with p38MAPK (Fig. 5). Similar to MKK3, P38MAPK co-immunoprecipitated selectively with PLC-β2 and chimera#3, but not with PLC-β1, PLC-β3 or chimera#1 or chimera#2.

Discussion

These data indicate that PLC-β2 interacts with MKK3 as determined by yeast two-hybrid and co-immunoprecipitation studies. This suggests a functional relationship between these proteins. To corroborate the interaction between PLC-β2 and MKK3 we performed co-immunoprecipitation experiments from Cos cells transiently expressing the proteins. MKK3, and to a small extent the closely related MKK6, selectively co-immunoprecipitated with PLC-β2 and our results show that of the PLC-β isozymes tested only PLC-β2 interacts with MKK3. To examine whether the endogenously expressed proteins interact, we attempted to co-immunoprecipitate PLC-β2 and a splice variant of MKK3, MKK3b, from the human myeloid cell line PLB-985 which expresses both proteins [16]. However, we did not detect MKK3b in immunoprecipitates with the PLC-β2 antibody. A possible explanation for this is that the significantly lower expression level of the endogenous proteins relative to the transfected cells may make the interaction difficult to detect. Alternatively, the interaction may be relatively weak, transient in nature or be present only in specific cell types. Indirect immunofluorescence microscopy in HEK-293 cells, transfected with both PLC-β2 and Flag-MKK3 plasmids, demonstrated significant co-localization of both proteins at the plasma membrane (data not shown). PLC-β2 was detected with a rabbit PLC-β2 antibody followed by Alexa Fluor conjugated goat anti-rabbit IgG and Flag-MKK3 was detected using a mouse anti-Flag antibody followed by Oregon Green goat anti-mouse IgG. Studies of the subcellular distribution of

MAP kinases have indicated specialized cellular localization with the cytoskeleton, membrane regions, and microtubules. This may play a role in determining which signals are transmitted or received and may restrict kinase substrates (reviewed in [17]).

The region of PLC-β2 which interacts with MKK3 was delineated by generating PLC-β2/PLC-β3 chimeras. These two enzymes have a high degree of overall sequence identity (46%) and a very high degree of identity within the catalytic region (~63%) [18]; however, only PLC-β2 interacts with MKK3. Co-immunoprecipitation experiments from transiently transfected Cos cells indicated that the C-terminus of PLC-β2 (amino acids 774–1181) is required for the interaction since the PLC-β3 chimera containing this region of PLC-β2 did interact with MKK3. The C-terminal region of PLC-β isoenzymes is recognized to be involved in membrane association and interaction with Gq, while Gβγ interacts with a distinct region near the N-terminus [19,20]. Sequence comparisons of the C-terminal region of PLC-β2 with PLC-β1 and PLC-β3 indicate that there is a high degree of sequence divergence in this region (26–36%), which is consistent with the additional role for this region of PLC-β2 interacting with MKK3.

P38MAPK also co-immunoprecipitated with PLC-β2 and as with MKK3 there was a requirement for the C-terminal region of PLC-β2. Cos cells contain endogenous MKK3 and it is possible that p38 is interacting with MKK3 via an N-terminal docking motif in MKK3 [21] and is consequently pulled down indirectly. However, since levels of endogenous MKK3 relative to transfected protein are very low, the co-immunoprecipitation of p38 with PLC-β2 most likely occurs, at least in part, via a direct interaction.

The functional consequences of the observed interactions of PLC-β2 with MKK3 and p38MAPK are unclear. Three MAPK signaling modules, ERK, JNK, and p38MAPK, have been characterized in mammalian cells [17]. The modules are composed of a series of at least three kinases that phosphorylate and activate each other sequentially. Typically MAPK-kinase-kinases (MKKKs) activate MAPK-kinases (MKKs) which in turn activate MAPKs, such as p38MAPK. MKK3 phosphorylates and activates p38MAPK in response to a range of environmental stresses, inflammatory cytokines, and activation of GPCRs. There is incomplete information on how signals from GPCRs are linked to activation of these kinases and multiple mechanisms involving heterotrimeric G protein subunits, protein kinase C, calcium mobilization, tyrosine kinases, and Rac and Cdc42 have been invoked [22,23]. Multiple MKKKs, including MEKK1, MEKK4, ASK1, TAK1, and MLK3, induce activation of MKK3 and the p38MAPK pathway [22]. A recently emerging property of MAPK signaling modules is that they are often assembled into signaling complexes by scaffold or adaptor proteins [24–27]. These signaling complexes serve several functions such as localizing their binding partners to specific subcellular compartments, increasing signaling efficiency and insulation from inappropriate activation. The scaffold proteins are diverse in nature and may have multiple roles; for example, the β-arrestin 2 protein functions as a scaffold for the JNK signaling module and also functions in GPCR desensitization [28].

Experiments investigating whether the function of the interactions is to enhance activation of p38MAPK by MKK3 were carried out by measuring kinase activation using a phospho-p38-specific antibody. However, comparisons of kinase activation in cells over-expressing either PLC-β3 or PLC-β2 suggested that PLC-β·2MKK3 interaction does not lead to enhanced p38MAPK activation (data not shown). Nonetheless, there is strong evidence for the importance of PLC in the MKK3–p38MAPK pathway. Yamauchi and co-workers [29] reported that a PLC inhibitor inhibits the activation of MKK3 by Gαq but not Gβγ. Also the use of protein kinase C inhibitors and intracellular calcium chelators has demonstrated that activation of p38MAPK by the chemoattractant fMLP in neutrophils is dependent on PLC activation [30,31]. A functional role of the PLC-β2MKK3 interaction may relate to localized cellular activation of MKK3 following PLC-β2 activation. In addition, the physiological role of the observed interactions may relate to the low-molecular-weight G proteins, Rac and Cdc42, which interact with PLC-β2 and certain MKKKs involved in activation of the p38MAPK pathway [9,10,32].

In conclusion, these data demonstrate that PLC-β2 serves a novel and unappreciated role as an interaction partner with MKK3 and p38MAPK.

Acknowledgments

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