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Expression of the Protein Kinase C Substrate Pleckstrin in Macrophages: Association with Phagosomal Membranes

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Despite evidence suggesting that protein kinase C (PKC) isoforms are important in phagocytosis by Fcγ receptors, the mechanisms by which the substrates of these kinases act are largely unknown. We have investigated the role of one PKC substrate, pleckstrin, in cells of the monocyte/macrophage lineage. Pleckstrin expression in mouse macrophages was induced severalfold in response to bacterial LPS and IFN-γ. In unstimulated cells, the protein was largely confined to the cytosol. Upon ingestion of IgG-opsonized zymosan particles (OPZ), however, pleckstrin accumulated on the phagosomal membrane. This association was transient, being maximal after 15 min and declining thereafter. Similar kinetics of association was also seen for both filamentous actin and the δ isoform of PKC. Ingestion of OPZ was found to induce phosphorylation of pleckstrin. To examine whether phosphorylation was required for phagosomal association, pleckstrin was expressed in CHO-IIIA cells that stably express the FcγRIIA receptor and are competent for phagocytosis of OPZ. In these cells, both wild-type pleckstrin and mutants in which the phosphoacceptor sites had been mutated to either alanine (nonphosphorylatable) or glutamine (pseudophosphorylated) were found to accumulate on OPZ phagosomes. Thus, association of pleckstrin with phagosomes is independent of its phosphorylation. Our findings suggest that pleckstrin may serve as an intracellular adaptor/targeting protein in response to particulate stimuli. By targeting interacting ligands to the phagosomal compartment, pleckstrin may serve to regulate phagocytosis and/or early steps during maturation of the phagosome. The Journal of Immunology, 1999, 163: 3388–3395.
Pleckstrin, also known as p47, was identified as the major PKC substrate in platelets and is expressed in all cells of the hemopoietic system (13–15). The protein consists of two pleckstrin homology (PH) domains at its N and C termini, bridged by three PKC phosphorylation sites (16, 17). Also intervening the two PH domains is a region of 80 amino acids that shares homology with the disheveled and egl-10 gene products, the so-called DEP domain for which very little is known (18). Although pleckstrin has not been found to possess an enzymatic function to date, its high expression in differentiated hemopoietic cells suggests an important function for this protein in mediating PKC signaling (19). For example, we have estimated the intracellular concentration of pleckstrin in neutrophils to be 15 μM (20). In these cells, IMLP led to rapid phosphorylation of the protein, with a time course similar to early PKC-dependent responses to chemotacticant. Cellular activation induced a subcellular redistribution of pleckstrin, including translocation from the cytosol to the plasma membrane. Although the mechanism that controls membrane association was not determined, phosphorylation by PKC was required. Membrane association of pleckstrin has also been demonstrated to occur in platelets and transfected COS-1 cells (21). These findings, in addition to reports that document the ability of PH domains to interact with various protein and/or lipid ligands, suggest that pleckstrin may serve as an intracellular adaptor/targeting protein.

In addition to the soluble agonist IMLP, IgG-opsonized zymosan (OPZ) particles were also found to induce pleckstrin phosphorylation in neutrophils (20). It was not determined, however, whether this particulate stimulus was also capable of inducing a subcellular redistribution of the protein. In the present study, we describe the expression and subcellular distribution of pleckstrin in cells of the monocyte/macrophage lineage, cells that absolutely require phagocytosis for antimicrobial functions. Despite the importance of PKC in mediating FcγR signaling, the role of pleckstrin has not been addressed in these cells. We report that pleckstrin is inducibly expressed in macrophages and associates with the phagosomal membrane upon ingestion of IgG-opsonized particles.

Materials and Methods

Primary cells

Circulating neutrophils (20), lymphocytes (14), monocytes (14), and platelets (22) were purified as described elsewhere. Bone marrow–derived macrophages were isolated from C57Bl/6J mice, as described (23). Briefly, bone marrow cells (6 × 10^7) were seeded on plastic petri dishes in macrophage medium (DMEM (Life Technologies, Gaithersburg, MD) supplemented with 15% L929 cell-conditioned supernatant, 10% FCS, 2 mM glutamine, and 0.5 mM 2-ME). The medium was replenished after 4 days, and after 8 days the culture represented confluent cells on glass coverslips and incubated for 3 h at 37°C. Cells were then shocked for 1 min with 10% DMSO in PBS, washed in PBS, and incubated in complete medium for 2 days to allow for expression of recombiant protein.

Phagocytosis of IgG-opsonized zymosan particles

Zymosan particles (Sigma) were washed extensively with PBS and opsonized by incubation with 2 mg human IgG/1 mg zymosan for 1 h at 37°C. After washing, a monolayer of particles was applied to cells plated on glass coverslips and allowed to bind for 10 min on ice. Unbound particles were removed by washing three times with ice-cold DMEM, and phagocytosis was initiated by addition of 37°C DMEM for the indicated time, after which cells were rapidly washed with cold PBS and then fixed for immunofluorescence analysis.

Immunofluorescence

Cells were fixed with 2% paraformaldehyde in PBS for 30 min and then permeabilized by treatment with a buffer containing 0.1% Triton X-100, 100 mM Pipes (pH 6.8), 5 mM EDTA, 100 mM KCl, 10 mM NaCl, 1 mM MgCl₂, for 15 min at room temperature. Fixed cells were preblocked with 5% donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBS (containing 1% BSA) for 2–4 h, washed twice with PBS, and incubated with affinity-purified Abs to pleckstrin for 2 h in PBS containing 1% BSA. After washing three times with PBS and incubation with Cy3-conjugated donkey anti-rabbit secondary Ab (Jackson ImmunoResearch), the samples were analyzed under three times PBS and then mounted with Slow Fade (Molecular Probes, Eugene, OR). Samples were analyzed using a cooled charge-coupled device camera attached to a Zeiss microscope (Zeiss, New York, NY) and captured using the Northern Exposure software (Northern Exposure, Toronto, Ontario, Canada). For costaining of filamentous-actin structures with pleckstrin, rhodamine-conjugated phalloidin (Sigma) was included with primary Ab, and a fluorescein-conjugated secondary Ab (Jackson ImmunoResearch) was used. Digitalized images were cropped in Adobe Photoshop (Mountain View, CA) and imported to Adobe Illustrator for assembly and labeling.

Isolation of phagosomal membranes from J774 macrophages

Latex bead-containing phagosomes were isolated from J774 macrophages using the method of Desjardins et al. (5). Briefly, phagosomes were formed by internalization of 0.8 μm blue dyed latex beads (Sigma) after mitogenic stimulation by overnight incubation at 4°C with human IgG (50 mg/ml) and washing four times with PBS. IgG-opsonized beads (1/50 dilution) were added to subconfluent J774 cell cultures (10–20 plates) for the indicated time at 37°C. Following internalization, cells were rapidly washed with ice-cold PBS and fixed for immunochemistry using the Northern Exposure software. Latex bead-containing phagosomes were collected from the interface of the 10 and 25% sucrose solutions and washed twice with ice-cold PBS before resuspension in 5 ml homogenization buffer. Cells were disrupted on ice (by 100 strokes) with a Dounce homogenizer. Light microscopy analysis confirmed that >90% of cells were disrupted without damage to cell nuclei. Lysates were briefly centrifuged to remove unbroken cells, and the remaining supernatant was centrifuged at 4°C, and the resulting supernatant was then centrifuged at 100,000 × g for 1 h at 4°C using an SW28 rotor (Beckman Instruments, Palo Alto, CA). The latex bead-containing phagosomes were collected from the interface of the 10 and 25% sucrose solutions and washed twice with ice-cold PBS before resuspension and boil in Laemmli sample buffer (25) and subjected to SDS-PAGE and immunoblotting. Protein concentrations of each sample were determined using the modified Lowry procedure.

SDS-PAGE and immunoblotting

Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed as described (26) using the following: affinity-purified polyclonal Abs to pleckstrin (20), polyethylene glycol (PEG)–conjugated monoclonal actin (Sigma), polyclonal Abs to the 39-kDa subunit of the H⁺-ATPase (27), and polyclonal Abs to both PKC-δ and the receptor for activated C kinase 1 (RACK1) from Transduction Laboratories (Lexington, KY).
Expression of pleckstrin in cells of the monocyte/macrophage lineage: induction with LPS/IFN-γ. A, Whole cell lysates of circulating human platelets, monocytes/macrophages, and lymphocytes (25 μg total protein) were subjected to SDS-PAGE and immunoblotting with affinity-purified polyclonal Abs to pleckstrin. Cell lysates (25 μg) from CHO-IIA cells transiently transfected with wild-type pleckstrin (+) or mock transfected (−) were also analyzed for pleckstrin expression. The mobility of pleckstrin is indicated with an arrow. Full-length recombinant GST-pleckstrin is indicated with an open arrowhead, its major breakdown product with a closed arrowhead. B, Bone marrow-derived mouse macrophages were cultured for 16 h in the absence (−) or presence (+) of 10 μg/ml LPS and 2 U/ml IFN-γ. Following treatment, expression of pleckstrin was determined as in A.

**Mobility shift assay of pleckstrin phosphorylation**

Peritoneum-elicited mouse macrophage suspensions (10⁶ cells) were bound to IgG-opsonized zymosan particles by cosedimentation in cold medium (4°C), achieved by addition of particles to the macrophage suspension and centrifugation for 10 s at 14,000 × g. Phagocytosis was then initiated by resuspension of the cell pellet in warm (37°C) medium and incubation at 37°C for the indicated times. Following incubation, cells were rapidly sedimented, the medium removed by aspiration, and the cells lysed by addition of boiling Laemmlli sample buffer and boiling for 5 min. Lysates were analyzed by SDS-PAGE and antipleckstrin immunoblotting, as described above. Using 18 × 20-cm gels, the mobility shift of phosphorylated pleckstrin could be easily observed. We have previously demonstrated that this shift is due to phosphorylation of pleckstrin at Ser113, Thr114, and Ser117, and not some other modification of the protein (20). To demonstrate complete phosphorylation of pleckstrin, cells were treated with 10−5 M 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma). The PKC inhibitor, bis-indolylmaleimide (BIM; also known as GF109203X) was purchased from Calbiochem (La Jolla, CA). Where indicated, cells were preincubated for 30 min at 37°C with 5 μM BIM (a concentration that selectively inhibits pleckstrin phosphorylation in platelets (28) and neutrophils (20), before addition of the indicated stimulus).

**Results**

**Expression of pleckstrin in cells of the monocyte/macrophage lineage**

**Induction with LPS/IFN-γ.** Expression of pleckstrin is exclusive to cells of the hemopoietic system (15). To demonstrate that cells of the monocyte/macrophage lineage express this protein, circulating leukocytes and platelets were isolated from human volunteers and subjected to immunoblotting with affinity-purified Abs to human pleckstrin (Fig. 1A). Isolated monocytes actually represent a mixed population of cells, both of which are separable from lymphocytes by their ability to bind plastic surfaces. As shown, this mixed population of cells expresses pleckstrin at levels comparable with other leukocytes and platelets. As a control for the immunoblotting procedure, pleckstrin was not detected in a fibroblast cell line (CHO-11A), except after transient transfection with a vector encoding wild-type pleckstrin. Furthermore, purified GST-pleckstrin fusion protein (and its breakdown product encountered during purification) was recognized efficiently by the Abs. Activation of macrophages by exposure to bacterial LPS and IFN-γ induces the expression of genes thought to be important for antimicrobial responses. These genes include the inducible nitric oxide synthase 2, which is crucial for killing pathogens within the phagosome (29). Induced expression of a PKC substrate, the myristoylated, alanine-rich C-kinase substrate, has also been described in response to LPS treatment of microglial cells (30). To determine whether pleckstrin expression is also regulated by inflammatory mediators, mouse bone marrow-derived macrophages were treated for 16 h with a combination of LPS and IFN-γ. Immunoblotting revealed that untreated cells express pleckstrin, which is recognized by Abs raised to the human protein (Fig. 1B). Importantly, treatment with LPS/IFN-γ induced expression of the protein several-fold. This suggests that pleckstrin plays an important role in mediating antimicrobial functions in response to infection.

**Subcellular localization of pleckstrin in macrophages**

**Association with the phagosomal membrane.** The subcellular localization of pleckstrin in peritoneum-elicited mouse macrophages was explored by immunofluorescence. Fixed cells were costained with rhodamine-phalloidin to visualize filamentous-actin. As shown in Fig. 2, pleckstrin is predominantly cytosolic in unstimulated, glass-adherent cells (0 min). The membrane cytoskeleton can be seen at the periphery of these cells. Following exposure to OPZ particles, pleckstrin and F-actin underwent subcellular redistribution. Both proteins were observed at the phagosomal membrane, with maximal accumulation occurring 15 min after initiation of phagocytosis. Some nuclear-associated pleckstrin can be witnessed at this time point, the significance of which is unknown. In accordance with previous studies, F-actin was rapidly removed from phagosomes and could not be detected by 60 min. Pleckstrin redistribution was also transient and returned to the cytosol with very similar kinetics to actin.

To further characterize subcellular redistribution of pleckstrin during phagocytosis, we isolated purified phagosomal membranes from J774 macrophages using a method described by Desjardins et al. (5). Macrophage cultures were allowed to ingest IgG-coated latex beads for different times, after which cells were homogenized. Phagosomes were then isolated by sucrose gradient centrifugation, taking advantage of the low density of the latex beads. Isolated membranes were analyzed by SDS-PAGE and immunoblotting.

As demonstrated in Fig. 3, pleckstrin associates with phagosomal membranes isolated 15 min after exposure to the IgG-coated beads. In accordance with our findings for peritoneum-elicited mouse macrophages (Fig. 2), this accumulation was transient. The
amount of phagosomal-associated pleckstrin decreased 60 min after ingestion, and at 120 min was difficult to detect. Thus, pleckstrin associates with nascent phagosomes, but is released from the phagosomal membrane as maturation of the phagosome progresses. mAbs to actin revealed that this protein also associates transiently with the phagosomal membrane, with kinetics of association/dissociation very similar to those of pleckstrin. To confirm that maturation of the phagosomes was occurring, purified membranes were immunoblotted with Abs raised to the 39-kDa subunit of the vacuolar-type H^+-ATPase, or proton pump. Delivery of this ATPase causes acidification of the phagosome, an important prerequisite for killing ingested pathogens. As shown, phagosomes rapidly accumulated the 39-kDa subunit over the 120-min time course, indicating fusion with proton pump-bearing vesicles during the maturation process.

Conventional (calcium-dependent) isoforms of the PKC family have been found to associate with phagosomes (10, 31). The possibility that nonconventional, calcium-independent isoforms may also associate with phagosomes has not, however, been explored. This is an important issue since our previous study of pleckstrin in neutrophils implicated these isoforms of PKC in mediating its function. A recombinant pleckstrin mutant having glutamate at Ser113, Thr114, and Ser117. To examine pleckstrin phosphorylation following phagocytosis of IgG-opsonized zymosan particles.

Phosphorylation of pleckstrin following phagocytosis of IgG-opsonized zymosan particles

Phosphorylation of pleckstrin is induced in neutrophils, platelets, and transfected COS-1 cells in response to a variety of soluble agonists (16, 17, 20). In all cases, phosphorylation occurs exclusively at Ser113, Thr114, and Ser117. To examine pleckstrin phosphorylation in response to particulate stimuli, peritoneum-elicited mouse macrophages were exposed to OPZ and then subjected to SDS-PAGE and immunoblotting. By using optimized conditions for SDS-PAGE (see Materials and Methods), a shift in the electrophoretic mobility of pleckstrin could be visualized. We have previously demonstrated that this change in mobility is due to phosphorylation of the protein (20). Shown in Fig. 4, phagocytosis of OPZ induced a rapid appearance of the upper (phosphorylated) form of pleckstrin (indicated by open arrowhead). This band comigrated with pleckstrin from cells treated with phorbol ester (TPA), indicating that it was fully phosphorylated on all three phosphorylation sites. Maximal phosphorylation was witnessed 15 min after OPZ phagocytosis had occurred and persisted after 60 min. The PKC inhibitor BIM blocked pleckstrin phosphorylation in response to both stimuli, as previously described (20). The phosphorylation-dependent mobility shift of pleckstrin is thought to be induced by a conformational change in the protein that persists under the denaturing conditions of SDS-PAGE. This conformational change may serve as a switch that regulates pleckstrin function. A recombinant pleckstrin mutant having glutamate...
residues in the place of its three phosphoacceptor sites (the so-called pseudophosphorylated mutant, 3 Phos-Glu) was found to display a slower electrophoretic mobility on SDS-PAGE, mimicking the phosphorylated form of pleckstrin (Fig. 4, right panel). This suggests that the conformational changes associated with phosphorylation are most likely present in the pseudophosphorylated mutant of pleckstrin. That this mutation has functional consequences has previously been suggested (16, 34).

Expression of pleckstrin in CHO-IIA cells

Association with the phagosomal membrane. In both neutrophils and transfected COS-1 cells, phosphorylation is required for pleckstrin translocation to the plasma membrane (20, 21). To determine whether phosphorylation also regulates association of pleckstrin with phagosomes, we utilized an epithelial-derived cell line stably expressing the monomeric Ig receptor FcγRIIA (CHO-IIA cells). These cells possess the machinery required for ingestion of IgG-coated particles in a manner similar to phagocytosis by leukocytes (4). CHO-IIA cells were transiently transfected with an expression vector encoding wild-type human pleckstrin and then analyzed by immunofluorescence. As shown in Fig. 5, transfected cells express pleckstrin that is predominantly localized in the cytosol (Unstimulated). Some transfected cells displayed ruffling of the membrane.
at sites of adhesion to the coverslip. At these ruffled regions, pleckstrin was found to accumulate (arrows). Extensive ruffling of the membrane was induced by exposure to phorbol ester, causing a marked redistribution of pleckstrin to these regions (+TPA). Following ingestion of OPZ, pleckstrin was found to associate with the phagosomal membrane, mimicking our results in macrophages (arrows).

**Phosphorylation does not regulate pleckstrin association with phagosomes**

Having established the utility of the CHO-IIA model system for the analysis of pleckstrin association with phagosomes, we transiently transfected these cells with phosphorylation site mutants of the protein. Both the pseudophosphorylated (described above) and a nonphosphorylatable mutant, in which the three phosphoacceptor sites were replaced with alanine residues (3 Phos-Ala), were used. Following ingestion of OPZ, cells were fixed and the ability of each mutant to associate with phagosomes was analyzed by immunofluorescence. As shown in Fig. 6, neither mutation had an effect on the subcellular redistribution of pleckstrin. Arrows indicate the accumulation of each pleckstrin mutant with phagosomes. Thus, pleckstrin association with phagosomes appears not to be regulated by phosphorylation.

**Discussion**

The precise mechanisms by which PKC isoforms influence FcγRII mediated phagocytosis remain to be determined. In this study, we describe the expression and subcellular localization of the PKC substrate pleckstrin in macrophages, cells that absolutely require phagocytosis for antimicrobial activities. Exposure of these cells to inflammatory mediators (LPS/IFN-γ) induced expression of pleckstrin, suggesting that this protein plays an important role in killing pathogens. Association with the nascent phagosomal membrane indicates that this role may include the regulation of phagocytosis and/or early maturation processes.

Ingestion of OPZ was found to induce phosphorylation of pleckstrin. Phosphorylation causes a shift in its electrophoretic mobility on SDS-PAGE gels, possibly due to a conformational change in the protein. This conformational change may regulate its association with the plasma membrane in fMLP-stimulated neutrophils as PKC inhibitors block the translocation of pleckstrin in these cells (20). Interestingly, phagosomal association in macrophages does not appear to be regulated by phosphorylation. This is suggested by the following: 1) pleckstrin remains phosphorylated 60 min after phagocytosis, when association with the phagosome is not observed, and 2) both phosphorylation site mutants of pleckstrin retain the ability to associate with phagosomes in CHO-IIA cells. Thus, translocation of pleckstrin to different subcellular compartments may be regulated by different intra- and/or intermolecular interactions. In this way, pleckstrin may serve many unique roles as an intracellular adaptor/targeting protein in response to both soluble and particulate stimuli. At present, the mechanisms that underlie pleckstrin redistribution are unknown.

Pleckstrin contains two PH domains, conserved modular structures found in many proteins (35, 36). PH domains have been referred to as universal membrane adaptors, although the ligands that tether these modules to the membrane surface remain controversial (37). Protein ligands for PH domains include the bg subunits of heterotrimeric G proteins (38) and PKC isoforms (39). With respect to the latter, it is noteworthy that PKC-δ was highly enriched in phagosomal membranes from J774 cells. Thus, interaction with this kinase may direct pleckstrin to the phagosome, allowing efficient phosphorylation to occur.

PH domains also interact with lipid ligands, most notably phosphoinositides (36, 40). The N-terminal PH domain of pleckstrin binds to phosphatidylinositol 4,5-bisphosphate (PIP₂), the significance of which is discussed below (41). Another important phosphoinositide, phosphatidylinositol 3,4,5-trisphosphate (PIP₃), has also been shown to bind, albeit more selectively, to certain PH domains. Using a novel dot-blot assay for binding of individual PH
domains to various anionic lipids, Kavran and others have suggested that pleckstrin’s N-terminal PH domain can bind PIP2. Cantley and colleagues determined that PIP2 binding by Btk was mediated by positively charged amino acids within a discreet region of its PH domain (43). A correlation was found between the number of positively charged residues in this region and the ability of a given PH domain to bind PIP2. The C-terminal PH domain of pleckstrin contains a relatively high number of positively charged amino acids within this region, including an arginine residue at the same position as Arg28 of Btk. This residue was found to be absolutely required for PIP3 binding by Btk (43). It is tempting to speculate that pleckstrin’s C-terminal PH domain can also bind PIP2, although careful binding analysis has yet to be performed. A requirement for pleckstrin binding to PIP2 might explain the ability of phosphatidylinositol 3-kinase inhibitors to block pleckstrin phosphorylation in both platelets (44, 45) and neutrophils (20).

Although the function of pleckstrin is not clear, recent evidence suggests it may serve as a down-modulator of phosphoinositide/Ca2+ signaling (34). When expressed in COS-1 cells, pleckstrin inhibits phosphoinositide hydrolysis by phospholipase C, an effect attributed to its ability to bind PIP2 (46). Interestingly, the pseudophosphorylated mutant had a greater inhibitory effect, and the nonphosphorylatable mutant a lesser effect (16). Thus, phosphorylation-induced conformational changes may affect PIP2 binding of the protein. PIP2 metabolism by phospholipase C generates two important signaling intermediates: inositol-4,5-bisphosphate (IP3), which causes release of Ca2+ from intracellular stores (47), and diacylglycerol, a coactivator of many PKC isoforms (48). Although not directly measured, pleckstrin’s inhibition of IP3 production is presumed to block Ca2+ release from internal stores. Further inhibition of the Ca2+-mobilizing signal may come from pleckstrin’s association with an inositol polyphosphate 5-phatase, which catalyzes degradation of IP3 (49). Blocking generation of diacylglycerol would impair the activation of PKC isoforms. That pleckstrin associates with the phagosomal membrane suggests that it may be regulating phosphoinositide/Ca2+ signaling and PKC activation at this intracellular compartment.

The kinetics of pleckstrin accumulation correlated with actin polymerization at phagosomes. Pleckstrin association with the cytoskeleton has also been witnessed in IMLP-stimulated neutrophils (20). These findings indicate a role for pleckstrin in regulating the actin cytoskeleton. A number of actin-regulating proteins are modulated by PIP2 binding, including profilin (50), cofilin (51), gelsolin (52), and α-actinin (53). Competing for PIP2 binding might allow pleckstrin to indirectly regulate the actin cytoskeleton.

Early studies utilizing biochemically purified protein from platelets suggested that pleckstrin could inhibit actin polymerization in vitro (54), although these results could not be replicated by others using purified recombinant pleckstrin (17). A possible role for pleckstrin in regulating the actin cytoskeleton remains to be demonstrated.

In summary, our data establish that pleckstrin is inducibly expressed in macrophages and is phosphorylated in response to FcγR-mediated phagocytosis. Association with the phagosomal membrane suggests that pleckstrin acts as an intramolecular adapter that targets interacting molecules to this compartment. Although the role of pleckstrin remains unclear, these results extend our understanding of this PKC substrate by demonstrating that its function(s) is most likely relevant to several subcellular compartments. That phosphorylation does not regulate phosphatase activity while it has been reported to affect interaction with the plasmalemma in response to soluble stimuli (20) indicates localization to each compartment may be regulated by different mechanisms.

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References


