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Cytohesin-1 Regulates the Arf6-Phospholipase D Signaling Axis in Human Neutrophils: Impact on Superoxide Anion Production and Secretion

Mohammed-Amine El Azreq,* Valérie Garceau,* Danielle Harbour,* Christophe Pivot-Pajot,* and Sylvain G. Bourgoin*†

Polymorphonuclear neutrophil (PMN) stimulation with fMLP stimulates small G proteins such as ADP-ribosylation factors (Arfs) Arf1 and Arf6, leading to phospholipase D (PLD) activation and functions such as degranulation and the oxidative burst. However, the molecular links between fMLF receptors and PLD remain unclear. PMNs express cytohesin-1, an Arf-guanine exchange factor that activates Arfs, and its expression is strongly induced during the acquisition of the neutrophilic phenotype by neutrophil-like cells. The role of cytohesin-1 in the activation of the fMLF-Arf-PLD signaling axis, and the accomplishment of superoxide anion production, and degranulation was investigated in PMNs using the selective inhibitor of cytohesin, Sec 7 inhibitor H3 (secinH3). Cytohesin-1 inhibition with secinH3 leads to Arf6 but not Arf1 inhibition, demonstrating the specificity for Arf6, and fMLF-mediated activation of PLD and of the oxidative burst as well. We observed a decrease in fMLF-mediated protein secretion and expression of cell surface markers corresponding to primary (CD63/myeloperoxidase), secondary (CD66/lactoferrin), and tertiary (matrix metalloproteinase-9) granules in PMNs incubated with secinH3. Similarly, silencing cytohesin-1 or Arf6 in PLB-985 cells negatively affected fMLF-induced activation of PLD, superoxide production, and expression of granule markers on the cell surface. In contrast, stable overexpression of cytohesin-1 in PLB-985 cells enhanced fMLF-induced activation of Arf6, PLD, and NADPH oxidase. The results of this study provide evidence for an involvement of cytohesin-1 in the regulation of the functional responses of human PMNs and link these events, in part at least, to the activation of Arf6. The Journal of Immunology, 2010, 184: 637–649.

Polymorphonuclear neutrophils (PMNs) are the most abundant human blood leukocytes, and are considered the first nonspecific defense line against infectious agents. PMN responses depend on expression of their various surface markers, of which many are receptors for endogenous molecules, mediating inflammation (like IL-8 or PGs), or for foreign molecules like fMLF, which is a bacterial-derived peptide. The fMLF receptor is a 7 transmembrane domain receptor coupled to heterotrimeric G proteins. Ligand binding leads to an increase in intracellular calcium levels and activates several signaling pathways. In human PMNs, fMLF stimulation leads to phospholipase D (PLD) activation, which catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (PA) (1, 2). Several PMN functions are regulated by PLD-derived PA levels, such as Fc receptor-mediated phagocytosis (3), activation of the oxidative burst (4), and degranulation (5). The regulation of PLD activity depends on different effectors such as small G proteins of the Rho, Ras, and ADP-ribosylation factor (Arf) families (6). Arf, a small G protein family having six isoforms (Arf1–Arf6), was the first direct regulator of PLD to be recognized. All human Arf mRNA species have been detected in the promyelocytic cell line HL60 (7), and several of these Arf isoforms (Arf 1, 5, and 6) appear to activate PLD in vitro (8).

In human PMNs and PMN-like cell lines, Arf1 and Arf6 are expressed and are recruited to the membrane fraction after fMLF stimulation (4, 9, 10), and both of them control PLD activity (11, 12). The N-terminal domain of PLD is necessary for activation by Arfs (13, 14). Arf activity is regulated by guanine exchange factors (GEFs), which stimulate the exchange of GDP with GTP leading to the activated form, and GTPase activating proteins, which stimulate the hydrolysis of GTP to GDP leading to the inactivated form (15). Arf-GEFs are divided into two classes: those that are sensitive to inhibition by brefeldin A (BFA) and those that are not (16, 17). BFA has no inhibitory effect on fMLF-stimulated PLD activity in granulocytes, demonstrating that BFA-sensitive Arf-GEF are not implicated in PLD activation (18). BFA-insensitive Arf-GEF is a family of four members, cytohesin-1, ARF nucleotide-binding site opener (ARNO)/cytohesin-2, GRP1/cytohesin-3, and cytohesin-4, that activate preferentially Arf1 and Arf6 (19). Cytohesins share a common domain organization consisting of an N-terminal coiled-coil domain, a central Sec7 domain (Arf-GEF activity), a phosphoinositide-binding pleckstrin homology (PH) domain, and a short C-terminal extension rich in positively charged amino acids (19). Cytohesin-1 but not ARNO expression increases with granulocytic differentiation (10, 20), suggesting a potential role of cytohesin-1 in the acquisition of a granulocytic phenotype. In lymphocytes, cytohesin-1 regulates cell adhesion via β2 integrin activation (21, 22). Furthermore, a number of reports have...
implicated ARNO/cytohesin-1 in two major PMN functions: degranulation and superoxide anion production.

The aims of the current study were to investigate the potential involvement of cytohesin-1 in the regulation of the functional responsiveness of human PMNs. To address this point we have used Sec 7 inhibitor H3 (secinH3), a cytohesin inhibitor recently described by Hafner et al. (28) to inhibit cytohesin-1 in human blood PMNs. We demonstrate secinH3 inhibition of IML-induced cytohesin-1 recruitment to PMN membranes and regulation of the Arf-PLD signaling axis by cytohesin-1. The specificity of cytohesin-1 for Arf6 but not Arf1 in PMNs and PLB-985 cells is highlighted. Our study provides the first evidence for implication of cytohesin-1 in two major PMN functions: degranulation and superoxide anion production.

**Materials and Methods**

**Reagents**

SecinH3 was synthesized according to Hafner et al. (28) at the Centre de Recherche du Centre Hospitalier de l’Université Laval (Québec, Canada). FBS, HBSS, PBS, RPMI-1640, penicillin-streptomycin-L-glutamine, and glycerol were purchased from Wisent (St. Bruno, Quebec, Canada); INDO-1 was from Molecular Probes (Eugene, OR); 1-[(3-Hyl)k-2-lyso-phosphatidylcholine and glutathione-Sepharose beads were obtained from Amersham Biosciences Canada (Baie d’Urfe, Quebec, Canada). Adenosine deaminase (ADA) was from Roche Diagnostics (Basel, Switzerland). Acetone was from Fisher Scientific (Ottawa, Ontario, Canada). Diisopropylfluorophosphate (DFP) was from Serva (Heidelberg, Germany). Cytochalasin B (CB), dibutyryl cAMP (dbcAMP), TCA solution, IMEL, PMA, PMSF, and DMSO were obtained from Sigma-Aldrich (St. Louis, MO). Dextran T-500 was purchased from GE Healthcare (Piscataway, NJ). Aprotinin and leupeptin were from Boehringer Ingelheim (Ridgefield, CT). Annexin V-FITC/propidium iodide (PI) kit was purchased from BD Biosciences (San Jose, CA).

**Abs**

Lactoferrin Ab was purchased from Sigma-Aldrich. Matrix metalloproteinase-9 (MMP9) Ab was obtained from Abcam (Cambridge, MA), and myeloperoxidase (MPO) Ab was from DakoCytomation (Carpinteria, CA). Flotil1in mAb was purchased from BD Biosciences, FITC-anti-CD63 and FITC-anti-CD66 Abs were from Beckman Coulter (Mississauga, Ontario, Canada). HRP-labeled donkey anti-rabbit and sheep anti-mouse IgGs were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and from GE Healthcare, respectively. Monoclonal anti-cytohesin-1 (2E11) Abs were generated in mice as previously described (20). Polyclonal Arf1 and cytohesin-1 Abs were produced in rabbits as described previously (20). Rabbit anti-human p47phox Ab was obtained from Upstate Biotechnology (Lake Poydi, NY).

**Isolation of human blood PMNs**

Venous blood was collected from healthy adult volunteers in isotrate anticoagulant solution. PMNs were isolated as described previously (29). In brief, whole blood was centrifuged at 180 × g for 10 min and the resulting platelet-rich plasma was discarded. Leukocytes were obtained after erythrocyte sedimentation in 2% Dextran T-500. Mononuclear cells were removed by centrifugation on Ficoll-Paque cushions and contaminating erythrocytes in the PMN pellets were removed by a 30-s hypotonic lysis in water. PMNs were resuspended in HBSS, pH 7.4, containing 1.6 mM CaCl₂ but no MgCl₂, at desired concentration.

**PLB-985 cell culture and differentiation**

PLB-985 cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO₂, at 37˚C. Differentiation was induced by the addition of 0.3 μM dbcAMP for 3 d. Before use, cells were washed with warmed PBS and resuspended in RPMI-1640.

**PLB-985 stable transfectants**

The cytohesin-1 coding sequence was subcloned in frame with EGFP in the pEGFP-C1 vector between the restriction sites EcoRI/XbaI, as described previously (20). Empty pEGFP-C1 vector was used as control for transfections.

Undifferentiated PLB-985 were electroporated (0.26 kV, 960 μF) in the presence of 20 μM plasmid DNA using a Bio-Rad GenePulser I (Bio-Rad, Hercules, CA). Cells were allowed to recover for 72 h at 37°C and grown for 1–2 wk in complete RPMI-1640 medium containing 1 mg/ml G418. The fluorescent-positive cells were sorted on a Beckman Coulter Epics Elite ESP FACS sorter using GFP setting. Cells were grown in complete RPMI-1640 medium containing 0.5 lg/ml G418 and differentiated as described previously.

**Cell treatment**

PMNs (10^7 cells/ml) in HBSS or dbcAMP-differentiated PLB-985 (10^7 cells/ml) in RPMI-1640 supplemented with 10 mM NaCl were incubated with 20 or 5 μM secinH3 or an equal volume of DMSO for 1 h at 37°C with gentle agitation prior to treatment with 1 mM DFP (used only for translocation and pull-down assays) for 10 min at room temperature. The cells were warmed for 5 min at 37°C and treated with 10 μg CB and 0.1 U/ml AOA for 5 min. Cells were stimulated with 100 nM IL-1β for an equal volume of DMSO at 37°C for 2 min. Incubations were stopped by diluting the cells 5-fold with ice-cold HBSS, and then centrifuging (7 min, 700 × g, 4°C).

**Cytohesin-1 translocation assay**

The cell pellets (80 × 10^6 cells) were resuspended in 1 ml relaxation buffer A, containing 100 mM KCl, 5 mM NaCl, 50 mM HEPES, 0.5 mM EGTA, 3.5 mM MgCl₂, 1 mg/ml aprotinin-leupeptin, and 200 mM PMSF, and sonicated for 20 ×. The lysates were then centrifuged (7 min, 700 × g at 4°C). To monitor the translocation of cytohesin-1 in PMNs, the samples were ultracentrifuged at 65,000 × g for 45 min at 4°C. Membrane pellets were resuspended in 100 μl lysis buffer, containing, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1.5 mM MgCl₂, 10% Triton X-100, 10% glycerol, 5 mM EGTA, 1 mM PMSF, and 10 nM aprotinin-leupeptin. The volume was adjusted to 2 ml, and the samples were mixed with the polycyonal cytohesin Ab 139 immobilized to protein A-Sepharose beads (20). To elute bound proteins, the beads were washed, mixed with 50 μl Laemmli sample buffer, and heated for 1 h at 37°C with gentle agitation. For PLB-985 cells, the 700 × g supernatants were centrifuged at 13,000 × g at 4°C for 45 min. Pellets were resuspended in 0.25 mM NaPO₄, pH 6.8, 0.3 mM NaCl, 2.5% SDS, 0.25 mM PMSF, and 0.5 mg/ml aprotinin-leupeptin. Samples were mixed with 50 μl Laemmli sample buffer and boiled for 10 min. Western blots were performed using monoclonal cytohesin-1 (1/1500) and flotillin (1/500) Abs.

**Western blotting**

Samples were resolved on a 7.5–20% gradient SDS-PAGE (translocation and Arf pull-down assays) or a 12.5% SDS-PAGE (degradation assay) and transferred to Immobilon polivinyldene difluoride membranes (Millipore, Bedford, MA). Proteins were revealed with HRP-conjugated anti-mouse or anti-rabbit secondary Ab (1/20000) or anti-mouse Ab (1/15000) and with Western Lightning detection system (PerkinElmer Life and Analytical Sciences, Waltham, MA).

**Production of GST-fusion proteins**

BL21(DE3) bacteria transformed with the pGEX-Golgi associated, y adaptin car containing, ARF binding protein 3 (GGA3) plasmid were grown in Magic Media (Invitrogen, Carlsbad, CA) at 30°C overnight, and centrifuged. The bacterial pellets were resuspended in 1 ml PBS, and sonicated (3 × 20 s). Empigene BB (Calbiochem, San Diego, CA) was added (0.25% final) and the bacterial lysates were incubated for 30 min on ice prior to centrifugation (10 min, 12,000 × g, 4°C). Pellets were resuspended in a buffer containing 50 mM Tris pH 7.4, 1 mM EDTA, 10 mM NaCl, 10% glycerol, 1 mM DTT, and 1 mM PMSF and the samples centrifuged (10 min, 12,000 × g, 4°C). This step was repeated three times. Collected supernatants were pooled and stored at –20°C prior to their use in GST-GGA3 pull-down assays.

BL21(DE3) bacteria transformed with the pGEX-metallothionein 2 (MT2) plasmid, and protein expression induced with 1 mM isopropyl-β-d-thiogalactopyranoside. Bacteria were centrifuged and resuspended in 50 mM Tris pH 7.4, 1 mM EDTA, 10 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM PMSF, 0.5 mg/ml lysozyme, and kept on ice for 20 min. The samples were sonicated (3 × 20 s), centrifuged (15 min, 12,000 × g, 4°C), and the supernatant was collected and mixed with glutathione-Sepharose beads for 45 min at room temperature. Beads were washed twice with 10 volumes of PBS with 0.5 mM NaCl, and once with 10 volumes of 200 mM NaCl and 50 mM Tris pH 8. The amounts of GST-MT2 (or GST-GGA3) bound to the beads were estimated using Coomassie blue staining of SDS-PAGE gels.

**Arf1 and Arf6 pull-down assays**

PMNs or dbcAMP-differentiated PLB-985 cells (4 × 10⁷ cells) were stimulated as described previously, and lysed in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1.5 mM MgCl₂, 0.3% Empigen BB, 10% glycerol, 5 mM EGTA,
1 mM PMSE, and 10 mM aprotinin-leupeptin. Cell lysates were clarified by centrifugation (14,000 rpm, 6 min, 4˚C). Aliquots of each lysate (5%) were saved, and normalized for total amount of Arf1 or Arf6 protein in each sample (input). Samples were then incubated with 40 µg of GST-GGA3 immobilized on glutathione-Sepharose for 1 h at 4˚C for monitoring GTP-ARF or with 40 µg of GST-MT2 in the presence of 2 mM ZnCl2 for monitoring GTP-ARF (30). The pellets were washed three times with appropriate lysis buffer. Proteins were eluted by boiling in 50 µl Laemmli sample buffer for 10 min. Western blots were performed as previously described, using the polyclonal Arf1 (1/2500) and the monoclonal Arf6 Ab (1/500).

**Arf1 and Arf6 translocation assay**

Cell pellets (4 × 10⁷ PMNs or dbcAMP-differentiated PLB-985) were resuspended in 1 ml relaxation buffer A and sonicated for 20 s. The samples were centrifuged (7 min, 700 × g, 4˚C) and the resulting supernatants were then centrifuged (45 min, 65,000 × g, 4˚C). The pellets were resuspended in 0.25 M Na2PO4, pH 6.8, 0.3 M NaCl, 2.5% SDS, 0.25 mM PMSE, and 0.5 mg/ml aprotinin-leupeptin. Western blots were performed using polyclonal Arf1 (1/2500), monoclonal Arf6 (1/500), and monoclonal anti-floatillin1 (1/500) Abs. Floatillin1, an integral membrane-associated protein, that remains constant through all the experiments, was used as a control for loading of membrane proteins.

**Measurements of PLD activity**

PMNs or dbcAMP-differentiated PLB-985 cells were labeled with 1-O-[3H]alkyl-2-lyso-phosphatidylcholine (2 µCi/10⁶ cells) for 90 min. The cells were washed and resuspended at 5 × 10⁵ cells/ml in HBSS, and pretreated with secinH3 at different concentrations, or DMSO. Cell suspensions (0.5 ml) were warmed for 5 min at 37˚C and treated for 5 min with 10 µM CB and 0.1 U/ml ADA. PMNs were stimulated with 100 nM IMLF or PMA for 10 min in the presence of 1% ethanol to monitor the formation of labeled phosphatidylethanol (PEt) catalyzed by PLD. Incubations were stopped by adding 1.8 ml chloroform/methanol/HCl (50:100:1, v/v/v) and unlabeled PEt. Lipids were extracted and the levels of [3H]PEt were quantified as described previously (29).

**Superoxide measurements**

Superoxide production was measured using the reduction of cytochrome c assay as described previously (31). PMNs (10⁶ cells/ml) or dbcAMP-differentiated PLB-985 (5 × 10⁵ cells/ml) were preincubated for 1 h at 37˚C with secinH3 or an equal volume of DMSO. The cells were then incubated in the presence of 130 µM cytochrome c, 10 µM CB, and 0.1 U/ml ADA for 5 min at 37˚C before being stimulated for 10 min with 100 nM IMLF or PMA. The amounts of superoxide anions were calculated from differences between the OD readings at 550 and 540 nm. The absorbance was transformed into the amounts of superoxide anions. The amounts of superoxide anions were calculated from differences between the OD readings at 550 and 540 nm.

**Recruitment of cytosolic p47phox to membranes**

PMNs (30 × 10⁶ cells/ml) in HBSS were incubated with 20 or 50 µM secinH3 or an equal volume of DMSO for 1 h at 37˚C with gentle agitation prior to treatment with 1 mM DFP for 10 min at room temperature. The cells were washed and resuspended at 5 × 10⁵ cells/ml in HBSS, and pretreated with secinH3 at different concentrations, or DMSO. Cell suspensions (0.5 ml) were warmed for 5 min at 37˚C and treated for 5 min with 10 µM CB and 0.1 U/ml ADA for 5 min prior to stimulation with 100 nM IMLF for 2 min. Incubations were stopped by diluting the cells 5-fold with ice-cold HBSS, and then centrifuging (7 min, 700 × g, 4˚C). The cells were resuspended in relaxation buffer A and sonicated for 20 s. Samples were centrifuged (7 min, 700 × g, 4˚C) and the resulting supernatants were centrifuged (45 min, 65,000 × g, 4˚C). Pellets were sonicated 20 s on ice and boiled in Laemmli sample buffer for 10 min. Samples were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking of nonspecific Ab-binding sites (5% BLOTTO) and incubation with p47phox Ab (1/500), p47phox was revealed by secondary peroxidase-conjugated goat anti-rabbit Ab (1/15000) and the Western Lightning detection system.

**Degranulation assay**

PMNs or dbcAMP-differentiated PLB-985 (10⁶/ml) were treated with secinH3 and incubated with 10 µM CB and 0.1 U/ml ADA for 5 min at 37˚C prior to stimulation with 100 nM IMLF. The reaction was stopped by cooling, and the suspension was centrifuged for 1 min at 10,000 × g at 4˚C. Primary and secondary granule exocytosis was analyzed by monitoring cell-surface expression of CD63 and CD66 using FITC-coupled anti-CD63 or anti-CD66 Abs as previously described (32). Cells were washed three times with cold PBS/0.2% BSA/0.1% azide, resuspended in PBS and analyzed by flow cytometry using an EPICS XL cytometer (Beckman Coulter).

Cell supernatants were used to monitor the effect of secinH3 on fMLF-induced release of MPO (primary granule marker), lactoferrin (secondary granule marker), and MMP9 (tertiary granule marker). Proteins were precipitated using TCA (17% final). Samples were then centrifuged 15 min at 13,000 rpm at 4˚C. The protein pellets were washed with 800 µl acetone, mixed with Laemmli sample buffer, and boiled 10 min. Proteins were detected by immunoblotting using MPO (1/200000), lactoferrin (1/100000), and MMP9 (1/10000) Abs.

**Annexin V-FITC/PI viability assay**

Differentiated PLB-985 (10⁶ cells) were washed twice in PBS and resuspended in 100 µl binding buffer. Annexin V-FITC (5 µl) and 10 µl PI were added and samples incubated at room temperature in the dark prior to analysis by flow cytometry.

**Cytosolic calcium measurements**

PMNs (10⁶ cells/ml) were loaded at 37˚C for 30 min with 1 µg/ml Indo-1 in RPMI 1640 containing 1% FBS and antibiotics. Cells were washed with HBSS, resuspended at 10⁶ cells/ml in fresh medium (RPMI 1640 + 1% FBS + antibiotics), and incubated with secinH3 or DMSO for 1 h at 37˚C. Flow 5’-GAGCAUAAGACGCAUGUUA, 5’-GUCUGAUUCAAUUCGCGU1-3’, and 5’-UUUCCCGAAGCGUGUCAGCUG-3’, respectively. After nuclease treatment, cells were immediately transferred to prewarmed complete medium containing 0.3 mM dbcAMP. Cell functions were monitored at 48 h posttransfection.

**Statistical analysis**

Statistical analysis was calculated using the unpaired Student t test. A value of p < 0.05 was considered statistically significant.

**Results**

The effect of secinH3 on cell viability and fMLF-mediated calcium mobilization

SecinH3 is a new cytohesin inhibitor recently described by Hafner et al. (28). It is selective for cytohesin-1, cytohesin-2/ARNO, and cytohesin-3/Grp-1 and inhibits nucleotide exchange on Arf small G protein family members, especially Arf1 and Arf6. SecinH3 competes with the M69 small RNA aptamer (an inhibitor of cytohesin-1) for binding to the cytohesin-1 sec7 domain. In this study, we have used the inhibitor to assess the role of cytohesin-1 in fMLF-induced signaling and functional responses of PMNs. On receptor occupancy, fMLF induces a rapid increase in intracellular calcium resulting from the mobilization of intracellular calcium stores by phospholipase Cβ-released inositol 3,4,5-triphosphate, that is followed by a sustained influx from the extracellular milieu. We first monitored whether secinH3 affects this early step in fMLF receptor coupling to calcium mobilization. Fig. 1A shows that a pretreatment of the cells with 50 µM secinH3, the highest dose tested, for 1.5 h does not impact the fMLF-mediated increase in intracellular calcium.
cytohesin-1 membrane recruitment

Our previous studies showed that cytohesin-1 is more abundantly expressed than cytohesin-2/ARNO in human PMNs, and established that granulocytic differentiation of HL60 cells with dbcAMP and DMSO increases cytohesin-1 but not ARNO expression (10, 20). Two splice variants of cytohesins that differ in the number of glycines in their PH domain can be expressed in cells. The triglycine variant binds inositol lipids (phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate) equally, whereas the diglycine motif confers selectivity for phosphatidylinositol 3,4,5-trisphosphate. Granulocytes express the 3G-splice variant of the PH domain of cytohesin-1 and this could explain why fMLF-induced recruitment of cytohesin-1 to membranes of DMSO-differentiated cells is insensitive to inhibitors of PI3K (10), induction of granulocyte differentiation with dbcAMP increased cytohesin-1 expression in PLB-985 cells (Fig. 9A and data not shown). As shown in Fig. 2B, cytohesin-1 was recruited to the membrane cytoskeleton fraction of PLB-985 granulocytes stimulated with fMLF for 2 min in the presence of CB and ADA. fMLF-mediated translocation of cytohesin-1 to membranes was inhibited in a concentration-dependent manner by a preincubation of the cells with secinH3 (Fig. 2B, 2C). fMLF-mediated cytohesin-1 translocation was reduced by 90% and 97% with 20 μM and 50 μM secinH3, respectively.

SecinH3 inhibits fMLF-mediated Arf6 but not Arf1 activation

The GEF cytohesin-1 is active on Arf1 and Arf6 in vitro assays (10). Given that Arf6 is a substrate of ARNO and cytohesin-1 in various cell lines (24, 37, 38), and that Arf1 is recruited to membranes after stimulation of PMNs with fMLF (39), we tested whether secinH3 affects the fMLF-mediated activation of Arf in these cells. The activation of Arf1 and Arf6 was monitored using GST-GGA3 (23) and GST-MT2 (30) immobilized on glutathione-Sepharose beads, respectively. As shown in Fig. 3A, fMLF increased the level of Arf6-GTP that was pulled down with GST-MT2. fMLF-mediated activation of Arf6 was significantly inhibited by a 1-h pretreatment of PMNs with secinH3. Denstometry analyses of the Arf6-GTP/Arf6 ratios revealed that secinH3 reduced the amounts of Arf6-GTP 10-fold (Fig. 3C, left panel). fMLF also increased the amount of Arf1-GTP that was pulled down with GST-GGA3, which, in contrast to Arf6, was not inhibited by secinH3 (Fig. 3B, 3C, right panel). Together, the data suggest that the inhibition of cytohesin-1 by secinH3 interferes with fMLF-mediated activation of Arf6 but not Arf1 in PMNs and differentiated PLB-985 (not shown).

FIGURE 1. Effects of secinH3 on fMLF-induced calcium mobilization and on the viability of PMNs. A, PMNs were incubated with secinH3 or DMSO for 1 h and stimulated with fMLF. Fluorescence was measured by fluorometry as described in Materials and Methods. Representative responses are shown in A. B, Quantification using INDO-1 as fluorescent probe of calcium mobilization in response to stimulation with fMLF. C, After treatment with secinH3 or DMSO for 1 h, cell suspensions were incubated with Annexin V-FITC/PI for 15 min. Apoptosis and necrosis were monitored by FACS. Data are the means ± SD from three independent experiments.
Effect of secinH3 on fMLF-mediated Arf6 and Arf1 membrane translocation

Redistribution of small GTPases from cytosol to plasma membranes is generally thought to reflect their activation status. Given that the redistribution of Arf1 and Arf6 from cytosol to membranes has been reported in PMNs (39) and HL60 cells (40), we monitored the impact of secinH3 on fMLF-induced Arf6 and Arf1 recruitment to membranes in PMNs. Fig. 4A shows that fMLF stimulation increased the amounts of Arf1 and Arf6 bound to membranes, which were reduced in secinH3-treated PMNs to near basal levels. Densitometry analyses indicate that 20 μM secinH3 reduced the amounts of membrane-bound Arf1 and Arf6 by 80% and 90%, respectively (Fig. 4B). No further inhibition of fMLF-induced Arf1 and Arf6 translocation to membranes was achieved when the concentration of secinH3 was increased to 50 μM. Similar results were obtained using dbcAMP-differentiated PLB-985 cells (not shown).

SecinH3 inhibits PLD activation

The activation of PLD is regulated by small GTPases of the Arf and Rho families (41). Although both Arf1 (42) and Arf6 from cytosol to membranes has been reported in PMNs (39) and HL60 cells (40), we monitored the impact of secinH3 on fMLF-induced Arf6 and Arf1 recruitment to membranes in PMNs. Fig. 4A shows that fMLF stimulation increased the amounts of Arf1 and Arf6 bound to membranes, which were reduced in secinH3-treated PMNs to near basal levels. Densitometry analyses indicate that 20 μM secinH3 reduced the amounts of membrane-bound Arf1 and Arf6 by 80% and 90%, respectively (Fig. 4B). No further inhibition of fMLF-induced Arf1 and Arf6 translocation to membranes was achieved when the concentration of secinH3 was increased to 50 μM. Similar results were obtained using dbcAMP-differentiated PLB-985 cells (not shown).

Effect of secinH3 on superoxide anion production

Because PLD activation and Arf6 were associated with superoxide anion production (4, 43, 44), we next examined the impact of a pretreatment of PMNs and PLB-985 cells with secinH3 on the oxidative burst. As shown in Fig. 6A (left panel), incubation of PMNs with 20 μM and 50 μM secinH3 decreased fMLF-induced superoxide anion production by 42% and 63%, respectively. The amount of superoxide anion produced by dbcAMP-differentiated PLB-985 cells was 3-fold less than that released by PMNs stimulated with fMLF (11 nmol O₂⁻/10 min/10⁶ cells versus 35 nmol O₂⁻/10 min/10⁶ cells). SecinH3 also inhibited in a concentration-dependent manner the oxidative burst in PLB-985 granulocytes. Superoxide anion production was reduced by 42% and 63% with 20 μM and 50 μM secinH3, respectively. When PMA was used as a PMN stimulus, no inhibition of superoxide anion production by secinH3 was observed (Fig. 6A, right panel).

Activation of the NADPH oxidase complex in PMNs has been associated with phosphorylation of p47phox, p67phox, and p40phox subunits and their association with PMN plasma membranes (45). Therefore, we monitored the impact of secinH3 on fMLF-induced p47phox recruitment to membranes in PMNs. Fig. 6C shows that fMLF stimulation increased the amount of p47phox bound to membranes, which was reduced in secinH3-treated PMNs to near basal levels.
SecinH3 inhibits secretion from primary, secondary, and tertiary granules in PMNs

Secretion is a primordial PMN function that has been shown to be regulated by PLD (46). To examine PMN degranulation we monitored the expression of granule markers at the cell surface by flow cytometry and quantified the release of specific granule molecules in the extracellular medium. In the first series of experiments, we examined the effect of secinH3 on cell surface expression of CD63 and CD66, which are markers of primary and secondary (gelatinase) granules, respectively (47). As expected,

**FIGURE 3.** Effects of secinH3 on Arf1 and Arf6 activation in fMLF-stimulated PMNs. Cells were incubated with secinH3 or DMSO for 1 h at 37°C prior to stimulation with fMLF for 2 min. A, GTP-bound Arf6 was precipitated with GST-MT2 beads as described in Materials and Methods. The amounts of GTP-bound Arf6 and total Arf6 (input) were detected with the monoclonal Arf6 Ab. B, GTP-bound Arf1 was precipitated with GST-GGA3 beads as described in Materials and Methods. The amounts of GTP-bound Arf1 and total Arf1 (input) were detected with the polyclonal Arf1 Ab. C, Data represent the ratio of Arf6-GTP/Arf6 (A) or Arf1-GTP/Arf1 (B) from three independent experiments. *p < 0.05 versus control fMLF.

**FIGURE 4.** Effects of secinH3 on Arf1 and Arf6 membrane translocation in fMLF-stimulated PMNs. A, Cells were incubated with secinH3 or DMSO for 1 h at 37°C prior to stimulation with fMLF for 2 min. Membrane proteins (80 µg) were resolved by SDS-PAGE (7.5–20% gradient gel) and analyzed by Western blot using monoclonal Arf6, and polyclonal Arf1 and flotillin1 Abs as described in Materials and Methods. B, Densitometry ratio of Arf1 (right panel) or Arf6 (left panel) versus flotillin of three independent experiments. *p < 0.05 versus control fMLF.
stimulation with fMLF strongly enhanced the cell surface expression of CD63 and CD66 (Fig. 7A). Incubation of the cells with 20 μM and 50 μM secinH3 reduced fMLF-induced cell surface expression of CD63 by 43% and 65%, respectively. CD66 expression at the cell surface was inhibited by ~50% with 20 μM and 50 μM secinH3.

In the next series of experiments, we quantified the release into the extracellular milieu of MPO, lactoferrin, and MMP9, which are markers of primary, secondary, and tertiary PMN granules, respectively (47). Fig. 7B shows that the antagonist of cytohesins inhibited the release of MPO, lactoferrin, and MMP9 from fMLF-stimulated PMNs. Densitometry analyses from three independent experiments indicate that 20 μM and 50 μM secinH3 inhibited fMLF-induced MPO release by ~65% (Fig. 7C). Lactoferrin secretion was inhibited by 50% and 65% with 20 μM and 50 μM secinH3, respectively. The release of MMP9 from gelatinase granules was less sensitive to inhibition by secinH3 when compared with MPO (primary granule) and lactoferrin (secondary granule). FMLF-mediated MMP9 secretion was reduced by 20% and 35% in PMNs treated with 20 μM and 50 μM secinH3, respectively (Fig. 7C).

Knockdown of cytohesin-1 and Arf6 with specific siRNAs inhibits PLD activation, superoxide anion production, and degranulation in dbcAMP-differentiated PLB-985

To bypass the impossibility of using the siRNA strategy in PMNs, we explored the effect of overexpressing cytohesin-1 in PLB-985 cells. Endogenous cytohesin-1, but not cytohesin-1-GFP expression, was strongly induced after differentiation with dbcAMP for 3 d. As shown in Fig. 9A, the level of overexpressed cytohesin-1-GFP in dbcAMP-differentiated PLB-985 was equivalent or slightly superior to that of endogenous cytohesin-1. Furthermore, the amounts of endogenous cytohesin-1 are identical in PLB-985 cells stably overexpressing GFP or cytohesin-1-GFP. Overexpression of cytohesin-1 significantly increased fMLF-induced membrane translocation of Arf6 by 42% but had no effect on the recruitment of Arf1 (Fig. 9B) confirming its specificity for Arf6 as observed with secinH3 in PMNs (Fig. 3). As expected, overexpression of cytohesin-1 significantly enhanced the amounts Arf6-GTP in fMLF-stimulated cells (Fig. 9C). Furthermore, cytohesin-1 overexpression enhanced fMLF-induced PLD activation by 29% (Fig. 9D) and superoxide anion production by 75% (Fig. 9E) when compared with PLB-985 cells overexpressing GFP. In contrast, cytohesin-1

**FIGURE 5.** Effect of secinH3 on PLD activation in fMLF-stimulated granulocytes. PMNs or dbcAMP-differentiated PLB-985 cells were labeled with 1-O-[3H]alkyl-2-lyso-phosphatidylcholine for 90 min at 37°C, and incubated with secinH3 or DMSO for 1 h at 37°C. Cells were then stimulated with fMLF in the presence of 1% ethanol. The reactions were stopped and the amounts of [3H]PEt were quantified as described in Materials and Methods. The data or the normalized data are the means ± SD from three independent experiments. *p < 0.01 versus control fMLF.
overexpression has no effect on PMA-induced PLD activation (Fig. 9D) and superoxide anion production (Fig. 9E).

**Discussion**

In this study, we examined the contribution of cytohesin-1, a GTP exchange factor for the Arf family of small GTPases, to fMLF-induced signaling and functional responses in PMNs and dbcAMP-differentiated PLB-985 cells. Because PMNs are terminally differentiated cells, short lived in culture, and poorly amenable to transfection, we took advantage of the recently described inhibitor of cytohesins, secinH3, to monitor the role of this family of Arf GEFs in the fMLF-induced response. We found that a pretreatment of the cells with secinH3 inhibited, in a concentration-dependent manner, fMLF-induced recruitment of cytohesin-1, Arf6, and Arf1 from cytosol to membranes. Although secinH3 totally abolished fMLF-induced activation of Arf6, that of Arf1 was unaffected. PMN functional responses such as activation of PLD, oxidative burst, and exocytosis of granules were also inhibited by secinH3. Gene silencing with specific siRNA knockdown and PLB-985 cells stably expressing cytohesin-1 were used as a model to confirm the results obtained with secinH3.

The cytohesin family of Arf GEFs is a subfamily of BFA-insensitive proteins that includes cytohesin-1, ARNO/cytohesin-2, Grp1/cytohesin-3 and cytohesin-4 (49). The expression of cytohesin-1 but not ARNO in HL60 cells is dramatically increased during dbcAMP- or DMSO-induced differentiation in HL60 cells (10, 20) or PLB-985 cells (unpublished observation), thereby suggesting an important role for cytohesin-1 in the activation of Arf small GTPases in these cells. Cytohesins possess a sec7 domain that catalyses nucleotide exchange on Arf proteins in vitro, a PH domain and a polybasic motif (50, 51). Targeting of cytohesin-1 and ARNO diglycine constructs to membranes requires the PH domain and is regulated by the C-terminal polybasic region (50, 52–54). We previously reported that cytohesin-1 triglycine is the major splice variant expressed in differentiated HL60 cells and that fMLF-induced translocation of cytohesin-1 to membranes, but not that of ARNO, was insensitive to inhibition by inhibitors of PI3K (10).

As previously reported for dbcAMP-differentiated HL60 cells, stimulation of dbcAMP-differentiated PLB-985 and PMNs with fMLF redistributed cytohesin-1 from cytosol to membranes. Stimulation of PMNs or differentiated PLB-985 cells with fMLF also redistributed cytosolic Arf1 and Arf6 to membranes (4, 9). Moreover, we showed that cytohesin-1 has a clear preference for Arf1 over Arf6 in vitro (10), and other biochemical assays also indicate that the preferred substrate of ARNO (55, 56), cytohesin-1 (57), and Grp1 (58) is Arf1. However, the selectivity of cytohesins for Arfs is ambiguous in vivo because transfection with the Arf GEF ARNO and Grp1 has been reported to stimulate Arf6 (23, 56, 59) as well as Arf1 (35).

Small inhibitory molecules that target the BFA-insensitive Arf pathways in cells have been discovered (28, 60). Among them is LM11, which recognizes motifs in both ARNO and Arf at the interface between the two proteins, and inhibits the activation of Arf1 but not of Arf6 in vitro (60). Using an aptamer displacement screen Hafner et al. (28) have identified secinH3, a small molecule that competes with the RNA aptamer M69 cytohesin-1 (an inhibitor of
CD66 was analyzed by FACS as described in Materials and Methods. Cohen et al. (35) have proposed a model in which Arf6 activation and recruitment, but an indirect effect on Arf1, istoylated arm, our data suggest a direct impact of secinH3 on the membranes through reorientation of their N-terminal myristoylated arm, which is assumed to be a good indicator of Arf activation. As we show in this study, secinH3 inhibited fMLF-mediated translocation of Arf6 to the plasma membrane and led to hepatic insulin resistance when administered to mice (28). Although cytohesin-1 is recruited to PMN membranes in response to stimulation with fMLF, its presence in this compartment could barely be detected after immunoprecipitation from the membrane fractions derived from 60 million activated PMNs. However, in dbcAMP-differentiated PLB-985 cells fMLF-mediated translocation of cytohesin-1 to membranes was more easily detected and additional immunoprecipitation steps were not required for protein detection from membrane fraction by Western blotting. The reason for this discrepancy is unclear, but we cannot exclude the possibility of high levels of plasma membrane adaptors or docking sites that serve for cytohesin-1 recruitment of cytohesin GEFs to sites at the plasma membrane leading to Arf1 activation. Another interesting possibility is that activation of Arf6 by cytohesin-1 at the plasma membrane may locally recruit proteins that serve as docking sites for activated Arf1 in PMNs. Though the activation and the translocation of Arf6 to membranes were enhanced in cells stably overexpressing cytohesin-1 which were stimulated with fMLF, cytohesin-1 overexpression had no impact on fMLF-mediated Arf1 redistribution from cytosol to membranes. These results confirm the involvement of cytohesin-1 in the activation of Arf6 in human granulocytes, but suggest that Arf1 membrane translocations may be independent from Arf6 activation and translocation in PLB-985 cells.

There are several reports of G protein-coupled receptor activation of Arf6 because of the ability of GTP-bound Arf1 or Arf6 to directly bind to the activated receptors (64, 65) or to interact with activated Gq and ARNO (66). Arf6 has also been shown to stimulate PLD through activation of fMLF receptors in differentiated PLB-985 cells (4), whereas the addition of myristoylated Arf1 or Arf6 and GTPyS in permeabilized HL60 cells stimulates PLD activity (67). Stimulation of PMNs or PMN-like cells with fMLF leads to activation of PLD and correlates with membrane recruitment of several phospholipase coactivators, including Arf1, Arf6, Rho GTpases, and protein kinase Cα (9). In this study, we took advantage of the inhibitor of cytohesins to evaluate the contribution of the cytohesin-1–Arf signaling pathway in fMLF-mediated activation of PLD. As expected secinH3 reduced fMLF-mediated activation of PLD in PMNs and PLB-985 cells. Inhibition of PLD was more pronounced in dbcAMP-differentiated PLB-985 than in PMNs, which can be explained by a 3- to 4-fold higher level of Arf6 expression in PLB-985 cells in comparison with PMNs (unpublished observations). This result was validated using siRNA knockdown of Arf6 or cytohesin-1, leading also to inhibition of fMLF-induced PLD activation, and cytohesin-1 overexpression that had the opposite effect in dbcAMP-differentiated PLB-985.
Cells. Nonspecific inhibition of PLD activity by secinH3 is unlikely because this compound does not inhibit PMA-mediated PLD activation. Indeed, studies on the mechanism of PMA-mediated activation of PLD have provided evidence of a role for protein kinase C (68). Inhibition of the recruitment and activation of both cytohesin-1 and Arf6 at the membranes suggests that secinH3 specifically targets the cytohesin-1-Arf6-PLD signaling axis in PMNs, an observation that is confirmed by the inhibitory effect of cytohesin-1 and Arf6 siRNA, and the stimulatory effect of cytohesin-1 overexpression, on fMLF-induced PLD activation.

PLD activation is associated with the modulation of several PMN functions such as chemotaxis (69), adhesion (70), phagocytosis (71), superoxide production (72), and degranulation (46). We monitored the impact of inhibition of the cytohesin-1-Arf6 signaling pathway by secinH3 on fMLF-induced superoxide anion production and exocytosis. We found that secinH3 inhibited in a concentration-dependent manner, the oxidative burst induced by fMLF in PMNs. As reported for PLD activation, the inhibition of NADPH activity by secinH3 was partial, supporting a model in which fMLF receptors signal through cytohesin-1 and Arf6 to stimulate PLD and the activation of the NADPH oxidase by PLD-derived PA (4). We also confirmed, using cytohesin-1 and Arf6 siRNA in PLB-985 cells, a role for the cytohesin-1-Arf6 signaling axis in superoxide anion production. This study highlights the implication of a BFA-insensitive Arf GEF in fMLF-mediated activation of PLD and NADPH oxidase. Similar to secinH3, BFA has been shown to inhibit fMLF-stimulated superoxide production in PMNs (44). The role of BFA-insensitive Arf GEFs in the regulation of NADPH oxidase activity is intriguing given the ability of various Arfs to regulate PLD and lipid metabolism in cells (73).

As previously reported for BFA (44), secinH3 had no effect on PMA-induced oxidative burst, ruling out an indirect effect of the inhibitor on NADPH oxidase activity. Inhibition by secinH3 of fMLF-induced NADPH activity is likely linked to inhibition of the cytohesin-1-Arf6-PLD signaling axis. Indeed, a previous study has shown that 2',5'-dihydroxy-2-furfurylchalcone inhibits several

![Image](http://www.jimmunol.org/DownloadedFrom/)

**FIGURE 8.** Effect of cytohesin-1 and Arf6 knockdown on PLD activity, degranulation, and superoxide anion production. A, Cytohesin-1 and Arf6 knockdown in differentiated PLB-985 cells. Cytohesin-1 expression was analyzed 48 h posttransfection with cytohesin-1 siRNA by immunoprecipitation with polyclonal anticytohesin-1 Ab from 10^7 cells, and immunoblotting with monoclonal anticytohesin-1 Ab (left panel). The amounts of Arf6 expression were analyzed 24 h and 48 h posttransfection with Arf6 siRNA by immunoblotting with anti-Arf6 and antiactin (loading control) Abs (right panel). Functional responses were monitored as described in Materials and Methods 48 h posttransfection with cytohesin-1, Arf6, or control siRNA. B, Impact of cytohesin-1 (left panel) and Arf6 knockdown (right panel) on fMLF-induced PLD activity. Cell suspensions were treated and stimulated as described in Fig. 7. C, Impact of cytohesin-1 and Arf6 knockdown on fMLF-mediated superoxide anion production. D, Impact of cytohesin-1 and Arf6 knockdown on fMLF-induced CD63 expression at the cell surface. Data are the means ± SEM of at least 3 separate experiments. \( p < 0.05 \) using the paired Student t test.
molecules, among them PLD, and Arf proteins, which leads to inhibition of NADPH oxidase (74). The inhibitory effect of secinH3 on the NADPH oxidase subunit p47phox translocation to membranes in response to fMLF is associated with a significant reduction of fMLF-induced PLD activity. Because PA is known to regulate p47phox, phosphorylation and membrane translocation in PMNs (75, 76), we suggest that inhibition by secinH3 of fMLF-induced NADPH oxidase activity is, at least in part, secondary to reduced production of PLD-derived PA. The positive impact of cytohesin-1 overexpression on fMLF-mediated superoxide anion production provides additional evidence for the involvement of cytohesin-1 in the regulation of the functional responsiveness of PMNs and links this event, in part at least, to the activation of Arf6 and PLD.

Arf6 and ARNO were reported to regulate exocytosis in chromaffin cells (25), and PLD was shown to regulate degranulation of primary (46), secondary, and tertiary (43) granules in PMNs. Taken together, these observations led us to test the involvement of cytohesin-1 in fMLF-mediated mobilization of various PMN granules. Inhibition of cytohesin-1 by secinH3 markedly decreased fMLF-mediated expression of CD63 and CD66 at the cell surface and markers of primary and secondary granule fusion with the plasma membrane, respectively. We have already reported that in fMLF-stimulated PLB-985 cells, only CD63 expression was augmented, but not CD66 (48). For this reason, we monitored the effect of cytohesin-1 or Arf6 siRNA only on CD63 expression, confirming the results obtained with secinH3. This result is also in agreement with the inhibition by secinH3 of MPO, lactoferrin, and MMP9 secretion, which are selective markers of primary, secondary, and tertiary granules, respectively. The molecular mechanism by which cytohesin-1 regulates granule secretion remains to be studied, but a potential explanation may be provided by inhibition of the production of fusogenic lipids, such as PLD-derived PA (77) and/or the remodeling of the actin cytoskeleton (61). Indeed, the actin cytoskeleton regulates the secretion of several PMN granules (78), and a direct association of cytohesin-1 with the actin cytoskeleton has already been reported (54).

In conclusion, secinH3, for the first time, allowed us to demonstrate in PMNs the involvement of cytohesin-1 in the fMLF receptor signaling pathways. Taken together our observations suggest that the recruitment of cytohesin-1 to the plasma membrane is essential for the activation of Arf6 but not Arf1. Though cytohesin-1 does not activate Arf1 in living cells, we propose that a pathway downstream of cytohesin-1 and Arf6 is required to recruit activated Arf1 to membranes. Our findings with secinH3 provide evidence for a cytohesin-1-Arf6-PLD signaling cascade in fMLF receptor-mediated activation of the oxidative burst and exocytosis. The role

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**FIGURE 9.** Impact of cytohesin-1 overexpression on fMLF-mediated activation of Arf6, PLD, and NADPH oxidase. A, Expression of cytohesin-1 and cytohesin-1-GFP in undifferentiated and dbcAMP-differentiated cells overexpressing cytohesin-1-GFP or GFP. Proteins were immunoprecipitated using polyclonal anticytohesin-1 (CTH139), and detected by Western blot using monoclonal anticytohesin-1 (mAb 2E11). B, fMLF-induced translocation of Arf1 and Arf6 to membranes in dbcAMP-differentiated PLB-985 stably expressing cytohesin-1-GFP (cytohesin-1) or GFP (control). Flotillin is used as loading control. C, Arf6 GST-MT2 pull-down from dbcAMP-differentiated PLB-985 stably expressing cytohesin-1-GFP (cytohesin-1) or GFP (control) stimulated with fMLF (left panel) and densitometric quantification of the Arf6-GTP/Arf6 ratio (right panel). D, PLD activation in dbcAMP-differentiated PLB-985 stably expressing cytohesin-1-GFP (cytohesin-1) or GFP (control) stimulated with fMLF (left panel) or PMA (right panel). E, Superoxide anion production in dbcAMP-differentiated PLB-985 stably expressing cytohesin-1-GFP (cytohesin-1) or GFP (control) after stimulation with fMLF (left panel) or PMA (right panel). Data are the means ± SEM of at least 3 separate experiments. *p < 0.05 using the paired Student t test.
of cytohesin-1 or Arf6 activation and their importance in PLD activity, superoxide anion production and degranulation were validated by siRNAs and cytohesin-1 overexpression in the PLB-985 PMN-like cell model. Further studies will be required to assess the effect of secinH3 on other PMN functional responses and to determine whether cytohesin-1 could be a therapeutic target to reduce neutrophil function.

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Disclosures

The authors have no financial conflicts of interest.

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