JFC1, a Novel Tandem C2 Domain-containing Protein Associated with the Leukocyte NADPH Oxidase*

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We have employed a yeast two-hybrid system to screen a B lymphoblast-derived cDNA library, searching for regulatory components of the NADPH oxidase. Using as bait the C-terminal half of p67phox, which contains both Src homology 3 domains, we have cloned JFC1, a novel human 62-kDa protein. JFC1 possesses two C2 domains in tandem. The C2A domain shows homology with the C2B domain of synaptotagmins. JFC1 mRNA was abundantly expressed in bone marrow and leukocytes. The expression of JFC1 in neutrophils was restricted to the plasma membrane/secretory vesicle fraction. We confirmed JFC1-p67phox association by affinity chromatography. JFC1-containing beads pulled down both p67phox and p47phox subunits from neutrophil cytosol, but when the recombinant proteins were used, only p67phox bound to JFC1, indicating that JFC1 binds to the cytosolic complex via p67phox without affecting the interaction between p67phox and p47phox. In contrast to synaptotagmins, JFC1 was unable to bind to inositol 1,3,4,5-tetrakisphosphate but did bind to phosphatidylinositol 3,4,5-trisphosphate and to a lesser extent to phosphatidylinositol 3,4-diphosphate. From the data presented here, it is proposed that JFC1 is acting as an adaptor protein between phosphatidylinositol 3-kinase products and the oxidase cytosolic complex.

The NADPH oxidase, a multisubunit enzymatic complex that is present in neutrophils and B lymphocytes, is responsible for the monoelectronic reduction of oxygen to produce superoxide anion (O2·−) at the expense of NADPH (1, 2). Free radical production is directly related to the bactericidal capacity of the cell, since patients with chronic granulomatous disease, whose NADPH oxidase is inactive (3, 4), suffer recurrent bacterial infections.

In resting cells, the components of the oxidase are distributed between different subcellular compartments and thus main unassembled while the oxidase is inactive. The main membrane component is cytochrome b558, an integral membrane protein containing one subunit of gp91phox and one of p22phox that is located in the secretory vesicles and specific granules (5). Meanwhile, the p47phox and p67phox, components that are known to be essential for the oxidase activation in vivo, remain in the cytosol in a complex that also includes p40phox (6, 7), a protein that is reported to regulate the activity of the oxidase. Two other factors, the small GTPases Rac2 (8) and Rap1a (9, 10), are also known to participate in the regulation of the oxidase. The exact mechanism of the activation process, however, remains obscure. In the presence of adequate stimuli, the cytosolic factor p47phox is phosphorylated and translocated, together with p67phox (11, 12), to the particulate fraction, where the cytosolic complex interacts with cytochrome b558. The subunit gp91phox is a flavohemoprotein that contains two hemes necessary to transfer electrons from NADPH to molecular oxygen (13, 14). The C terminus of p22phox binds to tandem SH3 domains present in p47phox (15). Because p47phox binds to p67phox (16, 17), it is considered to be responsible for assembling the oxidase in vivo. On the other hand, recent studies showed that p47phox is not required for the reconstitution of NADPH oxidase in a cell-free system when high concentrations of p67phox and Rac are present (18, 19). Therefore, these studies suggest that p67phox is the essential cytosolic factor for enzyme activity (19, 20).

Major structural features of p67phox are an acidic C terminus, two SH3 domains located in the middle (residues 245–295) and C-terminal region (residues 462–512), a Rac binding domain in its amino-terminal fragment, and two proline-rich regions in residues 226–234 and 317–329. Our group has previously reported that p67phox catalyzes pyridine nucleotide dehydrogenation, suggesting an active role of this factor during electron transfer (21). Moreover, we have recently shown that p67phox has a NADPH binding site located in its amino-terminal fragment (22). In agreement with this, it has been previously reported that the amino-terminal portion of p67phox lacking both SH3 domains was active in a cell-free system (23). However, it has been shown in the same study that complete restoration of NADPH oxidase in p67phox-deficient Epstein-Barr virus-B cells derived from chronic granulomatous disease patients was only achieved with full-length p67phox cDNA ex-
pression. Moreover deletions of either SH3 domain dramatically reduced NADPH oxidase activity in this system, findings that correlated with decreased membrane binding (23). It is well known that SH3 domains play an important role in protein-protein interactions regulating cellular localization of interacting factors, and, although the importance of these domains in the p67phox-p47phox interaction has been described (16, 17), it is not unlikely that p67phox-SH3 domains interact with other accessory proteins that could take part in the still unclear process of NADPH oxidase assembly or activation.

We used the yeast two-hybrid system to search for additional components of the phagocyte antimicrobial machinery. Using as bait the C-terminal half of p67phox, including both SH3 domains, we isolated a protein that interacts with oxidase components. In this communication, we describe the cloning and some of the properties of this protein, which we have designated JFC1.

**EXPERIMENTAL PROCEDURES**

**Materials—**Reagents for the yeast two-hybrid assay, including vectors, yeast strains, the yeast two-hybrid cDNA library, and control vectors, were provided by the generous gifts of Dr. Plummer (Baylor College of Medicine, Houston, TX). The α-ZAP cDNA library was kindly provided by Dr. Ernest Beutler (The Scripps Research Institute, La Jolla, CA). Phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and phosphatidylinositol 4-phosphate (PtdIns(4)P) were purchased from Sigma; phosphatidylinositol 4,5-diphosphate (PtdIns(4,5)P2) was obtained from CalBiochem; and phosphatidylinositol 3-phosphate (PtdIns(3)P), phosphatidylinositol 3,4-diphosphate (PtdIns(3,4)P2), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) were obtained from Matreya, Inc. (Pleasant Gap, PA). Inositol 1,3,4,5-tetrakisphosphate (InsP4) was purchased from Sigma, and [3H]InsP4 was obtained from Matreya, Inc. (Pleasant Gap, PA). InsP3 was purchased from Matreya, Inc. (Pleasant Gap, PA). Inositol 1,3,4,5-tetrakisphosphate (InsP4) was supplied by the manufacturer. Las Vegas, NV. For standard filter hybridization methods, the yeast two-hybrid assay “bait” constructs, the C-terminal portion of p67phox, including the two SH3 domains and the intervening sequence (residues 245–512), was amplified from cDNA by polymerase chain reaction using a 5′ primer (GAATTCCGCTACCGGTGTTACATT) that contained an EcoRI site (underlined) and a sequence that annealed to nucleotides 730–747 and a 3′ antisense primer (GAATTCCTCCCTCAACAAACCCTGCGA) that also contained an EcoRI site (underlined) and a sequence that annealed to nucleotides 1516–1536. The resulting fragment was then ligated into pAS1 so as to be in frame with Saccharomyces cerevisiae strain Y190, and the screening was performed following procedures and using negative controls that have been described previously (25, 26). From 4.2 million transformants, 46 positive colonies were detected. Twenty-four of these were selected for sequencing. Of these 24 clones, four corresponded to a single protein that showed a strong homology to C2 domains in synaptotagmins (27) and rabphilin 3 (28), two proteins that are involved in lipid binding, secretion, and protein-protein interactions. One of these clones was chosen for further study.

To obtain a full-length clone, a peripheral blood leukocyte cDNA library in α-ZAP was screened by standard filter hybridization methods (29), using the full-length 3′-end of the yeast two-hybrid clone as a probe. After isolation, the λ-ZAP clone containing the full-length JFC1 insert was circularized to the plasmid pBK-CMV-JFC1 by Cre-lox-mediated recombination using the ExAssist helper plasmid and the Escherichia coli strain XLO LR (Stratagene, La Jolla, CA). In vitro—The sequences of both the yeast two-hybrid cDNA fragment in pACTII and the full-length JFC1 cDNA in λ-ZAP were determined in The Scripps Research Institute molecular biology facility, using an automated fluorescent dye terminator sequencer. Overlapping oligonucleotide primers (Life Technologies, Inc.) were designed so as to obtain complete sequence information from both strands of the cDNA.

**RNA Blot Analysis—**Leukocyte RNA was obtained as previously described (29). The probe was labeled with 32P[dCTP (PerkinElmer Life Sciences) using the Prime-It RnT random primer labeling kit from Stratagene (La Jolla, CA) and probed as described elsewhere (29). Multiple tissue Northern blots (CLONTECH, Palo Alto, CA) were probed with the full-length EcoRI fragment of the JFC1 cDNA, and the corresponding tissue Northern blots were probed as recommended by the manufacturer.

**Preparation and Fractionation of Neutrophils—**Whole blood was obtained from the anonymous donor program at The Scripps Research Institute General Clinical Research Center. Neutrophils (polymorphonuclear leukocytes (PMNs)) were isolated by dextran sedimentation, hypotonic lysis, and Ficoll density centrifugation as previously described (30). Fractionation was carried out by a published protocol (31). Briefly, PMNs were resuspended in PIPES buffer (10 mm), pH 7.3 containing 100 mm KCl, 3 mm NaCl, 3.5 mm MgCl2, and 1 mm ATP (buffer A) supplemented with protease inhibitors (dipropylfluorophosphate, 0.5 mm (Sigma) and Complete protease inhibitor mixture (Roche Molecular Biochemicals)) and then disrupted by nitrogen cavitation for 400 p.s.i. EGTA was added to a final concentration of 1.25 mm, and nuclei and unbroken cells were removed by centrifugation at 500 × g. The supernatant from this step (postnuclear supernatant (PNS)) was either centrifuged at 200,000 × g for 30 min to separate cytosol from particulate matter or fractionated on a two-step discontinuous Percoll gradient (1 step, δ = 1.076 g/ml; 2 step, δ = 1.111 g/ml) with tonicity adjusted to 25% of standard EGTA. Eluted fractions were previously described (31). All particulate fractions were washed with buffer A containing 1.25 mm EGTA to remove Percoll.

**Stimulation of PMN with Phorbol 12-Myristate 13-Acetate (PMA)—**PMNs prepared as described above were washed once in Ca2+- and Mg2+-free phosphate-buffered saline (Life Technologies, Inc.) and then resuspended in PBS containing 0.5 mm CaCl2 and 1.5 mm MgCl2 and warmed to 37 °C for 10 min. PMA (Sigma) was added to a final concentration of 1 μg/ml and the cells were incubated for an additional 6 min at 37 °C. The cells were then washed once in ice-cold PBS and subjected to the cavitation/fractionation protocol.

**In Vitro Binding Studies—**Recombinant JFC1 was expressed in E. coli as the glutathione S-transferase fusion protein and purified using glutathione-Sepharose beads (Amersham Pharmacia Biotech) as recommended by the manufacturer. Sepharose beads (100 μl) containing 10 μg of the recombinant fusion protein or GST alone were washed twice in RIPA buffer (10 mm Tris/HC1, pH 7.5, 140 mm NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.025% NaN3) and incubated overnight at 4 °C in the presence of 1.8 × 105 or 9 × 105 cell equivalent of neutrophil-derived cytosol or buffer. Beads were washed five times in RIPA buffer (10 mm Tris/HCl, pH 7.5, 140 mm NaCl) and then boiled in Laemmli SDS sample buffer (31). Samples were resolved by SDS-PAGE, and proteins were transferred to nitrocellulose and probed with primary antibodies directed against p47phox or p67phox. Detection was performed using alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. The bound alkaline phosphatase activity was detected using the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium coupled colorimetric reagent (Bio-Rad). In vitro Translated JFC1 and p67phox—Wild type JFC1 cDNA and p67phox cDNA were cloned into pBR-CMV under the control of the T7 promoter. In vitro translated proteins labeled with [35S]methionine were produced using the TNT coupled transcription and translation system from Promega (Madison, WI) as recommended by the manufacturer. Translational grade [35S]methionine was purchased from Amer sham Pharmacia Biotech. In order to normalize the amount of radiocative protein added to the experiments, serial dilutions of each in vitro translation reaction were electrophoresed on SDS-PAGE, and the specific protein bands were quantified using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

**Binding of 35SJFC1 to p47phox or p67phox in Vitro—**The binding of [35S]JFC1 to GST-p47phox or GST-p67phox was evaluated as described above in “In Vitro Binding Studies” except that 5 μg of GST-p47phox, GST-p67phox, or GST were used in the assays. The reactions were performed either in RIPA buffer or in a buffer that contained 50 mm Tris/HCl, pH 7.5, 50 mm NaCl, and 0.1% Triton X-100. Serial dilutions of the in vitro translation reactions containing [35S]JFC1, [35S]p47phox as a positive control, or [35S]luciferase as a negative control were incubated in a reaction with a 200 μl final volume. Samples were resolved at 4 °C during 1 h, washed four times for 15 min with the reaction buffer, spun down, and boiled in Laemmli SDS sample buffer. Samples were resolved by SDS-PAGE, and the specific protein bands were quantified using a PhosphorImager (Molecular Dynamics).

**Binding of JFC1 to Phosphoinositides: Dot Blot Assay—**The binding of in vitro translated [35S]JFC1 to several phospholipids and phospho
Functional NADPH oxidase. Among the library clones that were formed human B lymphoblasts, cells known to contain a fully screen a cDNA library derived from Epstein-Barr virus-trans-

Results

A C2 Domain-containing Protein Interacts with the C-terminal Half of p67phox—We were interested in identifying proteins that might be involved in the modification and regulation of the cytosolic subunits of the NADPH oxidase. For this purpose, we constructed a yeast two-hybrid system "bait" vector containing a fragment of p67phox comprising residues 245–512, spanning both SH3 domains and the intervening sequence, and used it to screen a cDNA library derived from Epstein-Barr virus-transformed human B lymphoblasts, cells known to contain a fully functional NADPH oxidase. Among the library clones that were found to interact strongly and specifically with the C-terminal half of p67phox was a sequence encoding p47phox, which served as a convenient internal control, and four copies of a sequence that encoded the C-terminal part of a previously undescribed protein. This protein fragment contained a region of homology to the C2 domains of a number of proteins. C2 domains have been found in proteins that function in protein phosphorylation, lipid modification, GTPase regulation, and membrane trafficking (40). These domains also have been found to interact specifically with keyhole limpet hemocyanin (Chiron Mimotopes, San Diego, CA). The antibody against p67phox was raised against recombinant p67phox purified from baculovirus and was the generous gift of Robert M. Smith (University of California, San Diego, La Jolla, CA). The anti-JFC1 antibody was raised by inoculating rabbits with the library clone as a potential regulator of oxidase activity. JFC1 was detected only in the particulate (membranes/organelles) fractions. JFC1 was de-

To identify the specific phospholipid-containing compartment that JFC1 colocalizes with, we also utilized a dot blot assay as previously described (32) with minor modifications. Phospholipids at 2 mg/ml in 1:1 chloroform/methanol solution containing 0.1% HCl were spotted (4 µg) onto nitrocellulose sheets. After drying, nitrocellulose was blocked overnight at 4 °C in Tris-buffered saline containing 1% bovine serum albumin and 0.3% HCl. After incubating in Tris-buffered saline containing 1.5% bovine serum albumin and 300 µM L-methionine, JFC1 was then used to probe the phosphoinositide-containing nitrocellulose for 30 min at room temperature. Filters were washed five times with Tris-buffered saline and dried, and bound radioactivity was visualized by autoradiography. Samples were eluted from the nitrocellulose spots containing the radio-

Subcellular Localization of JFC1—Many C2 domain-containing proteins are associated with membranous subcellular structures. We examined the subcellular localization of JFC1 by immunoblotting (Fig. 4). An antibody raised against an N-terminal peptide from JFC1 recognized a specific doublet in whole neutrophils that had been lysed by boiling in SDS-PAGE sample buffer (Fig. 4A), probably representing either different translation starting points or just phosphorylated and unphosphorylated forms of JFC1. JFC1 has been successfully phosphorlated in vitro by protein kinase C, mitogen-activated protein kinase, and Ca2+/calmodulin kinase II.2 When neutrophils were lysed by nitrigen cattivation, JFC1 segregated to the PNS. Ultrastructural JFC1 in pancreas, trachea, stomach, salivary gland, and prostate. Northern blot analysis of leukocyte mRNA using the library cDNA as a probe identified a single band at~1.8 kilo-

JFC1, a NADPH Oxidase-related, C2 Domain-containing Protein

JFC-1 fragment (Fig. 2). As expected, the clone was abundantly expressed in bone marrow and lymphoid tissues, which have a high leukocyte content. There was also significant expression of JFC1 in pancreas, trachea, stomach, salivary gland, and prostate. The antibody against p67phox was raised against recombinant p67phox purified from baculovirus and was the generous gift of Robert M. Smith (University of California, San Diego, La Jolla, CA). The anti-JFC1 antibody was raised by inoculating rabbits with the library clone as a potential regulator of oxidase activity. JFC1 was detected only in the particulate (membranes/organelles) fractions. JFC1 was de-

A full-length cDNA was retrieved from a peripheral blood leukocyte library by conventional plaque screening. The sequence of this clone is shown in Fig. 1. Translation of the open reading frame disclosed a 562-amino acid protein (starting from the first methionine) that contained a second C2 domain just upstream of the one found in the original yeast library clone (Fig. 1). According to a previously described classification (40), both C2 domains present in JFC1 correspond to topology I. Comparison of the C2 domains from JFC1 with other C2 domain-containing proteins by sequence alignment identified that the C2A domain has structural homology with the synaptotagmin-C2B domains and with rabphilin-3-C2B domain (27, 41).

Tissue-specific expression of JFC1 mRNA—We examined the tissue-specific expression of JFC1 by probing a semiquantitative mRNA dot blot with the radiolabeled full-length cDNA JFC-1 fragment (Fig. 2). As expected, the clone was abundantly expressed in bone marrow and lymphoid tissues, which have a high leukocyte content. There was also significant expression of JFC1 in pancreas, trachea, stomach, salivary gland, and prostate. Northern blot analysis of leukocyte mRNA using the library cDNA as a probe identified a single band at~1.8 kilo-

2 S. D. Catz and B. M. Babior, unpublished observation.
was achieved using an affinity adsorption technique that tests the ability of proteins from neutrophil cytosol to bind to immobilized GST-JFC1. As described earlier, JFC1 was identified through the ability of its C-terminal region to associate with \( \text{p67}^{\text{phox}} \) in a yeast two-hybrid system. Fig. 5 demonstrates that affinity chromatography confirms this association. \( \text{p67}^{\text{phox}} \) was pulled down from neutrophil cytosol by JFC1-containing beads (Fig. 5A, lower panel, lanes 3 and 5), while GST alone was not effective (Fig. 5A, lower panel, lanes 1 and 2). It is noteworthy that \( \text{p47}^{\text{phox}} \), another cytosolic factor essential for the oxidase activity in vivo, was also pulled down by JFC1 in the same reaction (Fig. 5A, upper panel, lanes 3 and 5). It is very well established that \( \text{p67}^{\text{phox}} \) exists as a stable complex with \( \text{p47}^{\text{phox}} \) in the cytosol of nonstimulated neutrophils (6), raising the question whether JFC1 associated independently with both \( \text{p47}^{\text{phox}} \) and \( \text{p67}^{\text{phox}} \) or whether it bound to \( \text{p67}^{\text{phox}} \) pari passu as a component of the complex. The fact that we were unable to detect binding of GST-\( \text{p47}^{\text{phox}} \) attached to glutathione Sepharose beads to in vitro translated \( [35S] \text{JFC1} \) (Fig. 5B, upper panel) although \( [35S] \text{JFC1} \) was pulled down by GST-\( \text{p67}^{\text{phox}} \) (Fig. 5B, lower panel) supports the idea that JFC1 binds to \( \text{p67}^{\text{phox}} \) but not to \( \text{p47}^{\text{phox}} \). Binding of the latter occurs because it is complexed to \( \text{p67}^{\text{phox}} \). Moreover, increasing the concentration of cytosol in the reaction augmented the binding of \( \text{p67}^{\text{phox}} \) and \( \text{p47}^{\text{phox}} \) to JFC1 (Fig. 5A, lanes 3–5), again suggesting that they bind as a complex and indicating that JFC1 binds to a \( \text{p67}^{\text{phox}} \) site that is different from that involved in the recognition of \( \text{p47}^{\text{phox}} \).

**JFC1 Binds to PtdIns(3,4,5)P 3—**

As described above, JFC1 contains two tandem C2 domains that resemble those present in JFC1.
in synaptotagmins and rabphilin 3. The C2A domain of JFC1 is highly homologous to the synaptotagmin C2B domain (Fig. 6), suggesting that a functional correlation may exist. The synaptotagmin C2B domain is known to bind inositol polyphosphates, mainly InsP$_4$, in the absence of calcium (43). In Fig. 6, we show a comparison of putative inositol polyphosphate binding domains of various proteins. It is of special interest that, despite the homology observed between the JFC1-C2A domain and the polyinositol binding domain of synaptotagmins, the former was unable to bind $^{[3]H}$InsP$_4$ when evaluated either by a previously described precipitation assay (33) or by nitrocellulose filter binding assays (Fig. 7B).

To evaluate the ability of JFC1 to bind phospholipids and polyphosphoinositides, we performed dot blot assays as described under “Experimental Procedures” (SDS-PAGE analyses of samples eluted from the nitrocellulose spots are shown here). Fig. 7A shows that in vitro translated $^{[35}S]$JFC1 bound specifically to PtdIns(3,4,5)P$_3$ in these assays. JFC1 binding to PtdIns(3,4)P$_2$, although still significant, was 48% lower than that detected with PtdIns(3,4,5)P$_3$, indicating that the fully
phosphorylated head group is necessary for maximum binding. JFC1 binding to PtdIns(4,5)P₂ was only 16.6% of the maximum observed with PtdIns(3,4,5)P₃, suggesting that 3-phosphorylated phosphoinositides play an important role in JFC1 recognition. In the same way, JFC1 bound to PtdIns(3)P to the same extent as that detected for PtdIns(4,5)P₂. No significant binding of JFC1 to phosphatidylinositol, phosphatidylserine, or PtdIns was detected under these assay conditions.

**DISCUSSION**

Using a yeast two-hybrid system, we have identified a protein that interacts with p67phox N-terminal domain and the p47phox SH3 domain (17), although association between C-terminal SH3 domain of p67phox and a proline-rich C-terminal sequence in p47phox has also been shown by the same group (16). We observed that JFC1 did not bind to p47phox, although p47phox and JFC1 interacted in the presence of cytosol, which implies that their association is through an intermediary factor, presumably p67phox. The fact that JFC1 binds to p67phox without altering the association of this cytosolic factor with p47phox has physiological significance and suggests that JFC1 could have an important role in vivo. It should be taken into account that p47phox is present in molar excess over p67phox in the cytoplasm and that a large percentage of the protein actually exists in a dissociated form (6). In this work, we show that augmenting the cytosol concentration in the reaction medium increased the binding of p67phox to JFC1 and, consequently, the detection of p47phox, supporting the idea that free p47phox does not interfere with the recognition of the complex by JFC1 and that JFC1 recognizes p67phox at a molecular site different from that involved in the p67phox-p47phox interaction.

Herein we have demonstrated that JFC1 is associated with a subset of membranes in the phagocyte, although by our method we were not able to distinguish whether it localized to the secretory vesicles, the plasma membrane, or both. Therefore, it is unlikely that JFC1 interacts with the cytosolic complex in unstimulated cells, the components remaining in different intracellular compartments. Although little is known about the molecular basis for the up-regulation of the oxidase in the phagocytic vesicles or at the phagocyte surface, it is general knowledge that after stimulation, the cytosolic factors translocate to the particular fraction, suggesting that, in vivo, the interaction between JFC1 and the cytosolic factors would take place at some point after cell activation. In the presence of an appropriate stimulus, the cytosolic component p47phox becomes sequentially phosphorylated on serines Ser³⁵⁹ and Ser³⁷⁰ followed by Ser³⁰³ and Ser³⁰⁴, and the cytosolic complex migrates to the particulate fraction where it is known to interact with cytochrome b₅₅₈ (44). p67phox also becomes phosphorylated (45), although the significance of this phosphorylation in the activation of the oxidase remains unknown. Although there are still aspects of the oxidase assembly that are unclear, several studies have shown that p⁴⁷phox interacts with the p₂₂phox subunit of the cytochrome b₅₅₈ (46). Moreover, it has been previously described that the cytosolic factors fail to translocate in the absence of cytochrome b₅₅₈ (47). Since JFC1 localizes in the particular fraction where the assembly of the oxidase takes place and interacts with the cytosolic subunits of the oxidase, it is conceivable that this protein could play such a role in vivo.

As discussed above, JFC1 possesses tandem type I C2 domains in its C-terminal end. Comparison of the C2A domain of JFC1 with the sequence alignment of other C2-containing proteins showed that it is highly homologous to the C2B domains present in synaptotagmins (Fig. 6). Several functions have been attributed to the synaptotagmin C2B domains including dimerization, interaction with clathrin assembly protein-2 (48), a process involved in endocytosis of synaptic vesicles, and binding to β SNAP (49) and SNAP25 (50). Notably, several members of the synaptotagmin family have been shown to bind to the second messenger InsP₄ by an InsP₄-binding site present in

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**Fig. 5. Affinity adsorption assays.** A, GST-JFC1 fusion protein was immobilized on glutathione-agarose beads. Beads (100 μl) containing 10 μg of the recombinant fusion protein or GST alone were washed twice in RIPA buffer and incubated overnight at 4 °C in the presence of neutrophil-derived cytosol or buffer (lanes 1 and 2) or 9 × 10⁵ (lane 5) cell equivalents of neutrophil-derived cytosol or buffer (lanes 1 and 4). Beads were washed five times during 10 min with RIPA buffer at 4 °C, spun down, and boiled in Laemmli SDS sample buffer. Samples were resolved by SDS-PAGE, and proteins were transferred to nitrocellulose and probed with primary antibodies directed against p47phox (top panel) or p67phox (bottom panel). The identity of the immobilized protein in each assay is indicated above the lanes. B, the binding of in vitro translated [³⁵S]JFC1 to GST-p47phox (upper panel) or to GST-p67phox (lower panel) was evaluated as described under “Experimental Procedures.” In vitro translated [³⁵S]p67phox was used as positive control, and in vitro translated [³⁵S]luciferase (Luc) was used as negative control.
in vitro translated [35S]JFC1 to several phospholipids and phosphoinositides and 2 nM [3H]InsP4 (10,000 cpm/assay) were carried out during 30 min at 30 °C. The final volume was 200 μl. Free and bound InsP4 was evaluated as described under "Experimental Procedures." The reactivity was visualized by autoradiography. Samples were eluted from the nitrocellulose spots containing the radioactive probe and were further resolved by SDS-PAGE. Radioactivity was visualized using a PhosphorImager (Molecular Dynamics).

In vitro translated [35S]JFC1 (0.1–0.5 μg) was then used to probe the phosphoinositide-containing nitrocellulose for 30 min at room temperature. Filters were washed and dried, and bound radioactivity was visualized by autoradiography. Samples were eluted from the nitrocellulose spots containing the radioactive probe and were further resolved by SDS-PAGE. Radioactivity was visualized using a PhosphorImager (Molecular Dynamics). PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol. Results shown are representative of three different experiments.

FIG. 6. Sequences alignments of C2 domains containing a "lysine stretch." Sequence similarity of the C2B domains of synaptotagmin I (residues 303–352) (SytI; NP-005630); GTPase-activating protein 1m (residues 181–233) (GAP1m; AAD98281); Doble C2a (residues 281–330) (DocC2a; NP-003577); rabphilin 3A (residues 590–639) (Rab3A, A8697); and the C2A domain of JFC1 (residues 205–342). Lysines described to be essential for InsP4 in synaptotagmins (43) are indicated by asterisks. Residues that are identical in the five sequences are darkly shaded; three or more matches are lightly shaded.
PMA-activated neutrophils, leading to the suggestion that PtdIns 3-kinase is not necessarily upstream of protein kinase C in the oxidase signaling pathway (63). Thus, other events might be involved downstream of PtdIns 3-kinase in the signaling pathway that lead to the activation of the oxidase. In this way, it has been indicated that members of the undefined “renaturable kinases” take action downstream of PtdIns 3-kinase during human neutrophil oxidase activation, while mitogen-activated protein kinase and mitogen-activated protein kinase/extracellular signal-regulated kinase cascade would be involved in the secretory pathway, also downstream of PtdIns(3,4,5)P3 formation (59). In conclusion, while there is no doubt about the essential participation of PtdIns 3-kinase in the secretory pathway, also activated protein kinase/extracellular signal-regulated kinase is restricted to leukocytes, PtdIns 3-kinase-related events taking place in activated neutrophils, leading to the suggestion that JFC1 plays a more general role, namely as an adaptor protein, presumably interacting with other SH3 domain-containing proteins. This function is currently under investigation in our laboratory.

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