Tyrosine phosphorylation and subcellular redistribution of p125 ras guanosine triphosphatase-activating protein in human neutrophils stimulated with FMLP

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Abstract In this paper, we show that the p125 ras guanosine triphosphatase-activating protein (p125 GAP) is present in the cytosol of human neutrophils and is transiently tyrosine phosphorylated and translocated to the membranes upon cell activation with formyl-methionyl-leucyl-phenylalanine (FMLP). When concanavalin A (ConA) or phorbol 12-myristate 13-acetate (PMA), which both induced a long-lasting respiratory burst, were used as stimuli, tyrosine phosphorylation and translocation of p125 GAP did not occur.

Key words: Neutrophils; NADPH oxidase; GTPase-activating proteins; Tyrosine phosphorylation

1. Introduction

The activation of the ras-related small GTP-binding proteins (G-proteins) results from the exchange of bound GDP for GTP [1,2]. The intrinsic GTPase activity of such G-proteins is regulated by the guanosine triphosphatase-activating proteins (GAPs) [3–5]. p125 GAP is a cytosolic polypeptide that catalyzes the GTPase activity of p21 ras [6,7]. In growth factor-stimulated and tyrosine kinase-transformed rat fibroblasts, p125 GAP becomes tyrosine phosphorylated and is translocated from the cytosol to cell membranes, where it interacts with its biological target, p21 ras, as well as with activated growth factor receptors [8–18]. It has been reported that p21 ras is responsible for the activation of mitogen-activated protein (MAP) kinases cascade [19], and that MAP kinases are involved in the mechanisms of activation of NADPH oxidase [20–22], the enzymatic system responsible for the generation of reactive oxygen metabolites [23–30]. For this reason we investigated whether p125 GAP is expressed by neutrophils and the effects of different agonists of NADPH oxidase on its subcellular localization and tyrosine phosphorylation.

2 Materials and methods

2 1. Reagents

PY20 anti-phosphotyrosine was purchased from Transduction Laboratories, Lexington, Kentucky, USA; anti-human p125 GAP monoclonal antibody, anti-p125 GAP rabbit polyclonal antibody, and IgG2bk anti-phosphotyrosine mouse monoclonal antibody from hybridoma 4G10, were from UBI, Lake Placid, NY, USA.

2 2. Neutrophil preparation and metabolic studies

Human neutrophils were prepared and oxygen consumption was measured as previously reported [31].

2.3. Neutrophil activation and fractionation

Neutrophils (3×10^7/ml) were activated and fractionated as previously reported [22,31].

2.4. Electrophoresis and immunoblotting

Aliquots of samples from resting and activated neutrophil cytosol (35 μg) and membranes (50 μg) were subjected to SDS/PAGE on 10% gels, according to Laemmli [32]. Proteins were electroblotted as previously described [22,33]. The blots were incubated overnight with 2 μg/ml anti-phosphotyrosine mAbs 4G10 or 2 μg/ml anti-125 GAP polyclonal antibody in blocking buffer [31]. All the subsequent steps have been performed as in [31]. When required, blots were stripped as previously described [33].

2.5. Immunoprecipitation

For immunoprecipitation experiments, cytosol and membranes (3×10^7 cell equivalent) were incubated for 30 min on ice with solubilization buffer [22] and then centrifuged at $15000\times g$ for 10 min. The supernatants were incubated with 4 μg of anti-phosphotyrosine PY20 or anti-125 GAP monoclonal antibody conjugated to trisacryl-protein A (Pierce). All the subsequent steps were performed as described in [22].

3. Results

3.1. Subcellular localization of p125 GAP in human neutrophils

Cytosolic and membrane fractions from resting and activated neutrophils were subjected to SDS/PAGE and immunoblotting with specific anti-p125 GAP polyclonal antibody. Fig. 1 shows that this antibody recognized a 125 kDa band in the cytosol but not in membranes of resting neutrophils. Upon neutrophil stimulation with FMLP, p125 GAP translocated from the cytosol to the membranes (Fig. 1). This translocation took place as early as 20 s after FMLP stimulation, being maximal at 1 min. After this time, the 125 kDa band decreased, disappearing from the membranes after 5–10 min. Fig. 1 also shows that the FMLP-induced respiratory burst paralleled the association of p125 GAP to the membranes.

The subcellular localization of p125 GAP as well as the kinetics of its translocation, were confirmed by immunoprecipitation experiments performed with an anti-125 GAP monoclonal antibody (Fig. 2).

3.2. Tyrosine phosphorylation of p125 GAP in human neutrophils

To investigate whether p125 GAP is tyrosine phosphorylated in human neutrophils, cytosol and membrane fractions from resting and FMLP-stimulated cells used in the experiments reported in Figs. 1 and 2 were immunoprecipitated with anti-phosphotyrosine monoclonal antibody (PY20). Fig. 3 illustrates that PY20 immunoprecipitated p125 GAP from cytosol and membranes of FMLP-stimulated, but not resting neutrophils. The time course of tyrosine phosphorylation of

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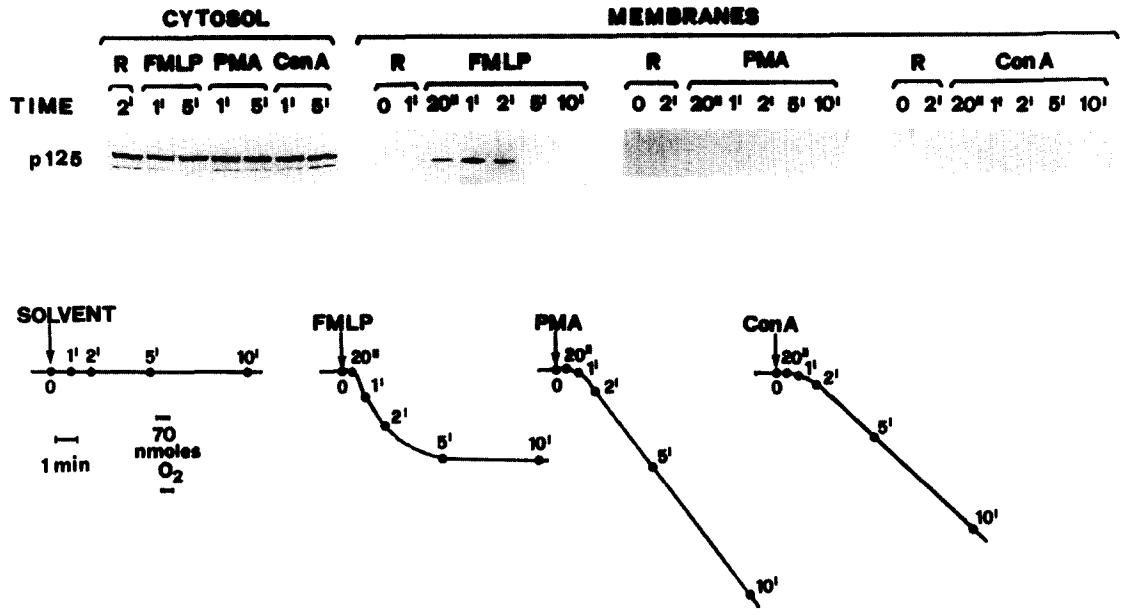

Fig. 1. Kinetics of p125 GAP translocation to the membranes of human neutrophils. Neutrophils (2×10^7/ml) were incubated at 37°C under agitation with FMLP (1 μM), ConA (300 μg/ml), PMA (100 ng/ml) or the solvent alone plus cytochalasin B (R); the reactions were stopped at the indicated times and the cells were fractionated as described under Methods. The membrane and cytosol fractions were subjected to SDS/PAGE on a 10% gel, and immunoblotted with anti-p125 GAP polyclonal antibody. Values for oxygen consumption, which were determined in parallel assays, are also reported (numbers indicate the time points at which protein translocations were investigated). Abbreviations; R, resting neutrophils. The data are from one experiment representative of six.

p125 GAP (Fig. 3) paralleled the kinetics of the translocation of this protein (Figs. 1 and 2).

3.3. *Effect of PMA and ConA on translocation and tyrosine phosphorylation of p125 GAP*

To understand whether the translocation of p125 GAP is involved in the activation of NADPH oxidase, we investigated the effect of various NADPH oxidase agonists on the subcellular localization of this protein. Fig. 1 shows that p125 GAP was not detected by specific antibodies in membranes from PMA- or ConA-stimulated neutrophils. These findings were confirmed by experiments of immunoprecipitation of this protein (Fig. 2). Fig. 3 shows that PMA and ConA did not induce any tyrosine phosphorylation of p125 GAP both in cytosol and membrane fractions. However, upon cell stimulation with PMA, the respiratory burst started at 20 s, reached a maximum between 1 and 2 min and then was linear up to 10 min. In the case of ConA the oxygen consumption started at 20 s, reached a maximum at 2 min, and then remained linear up to 10 min (Fig. 1).

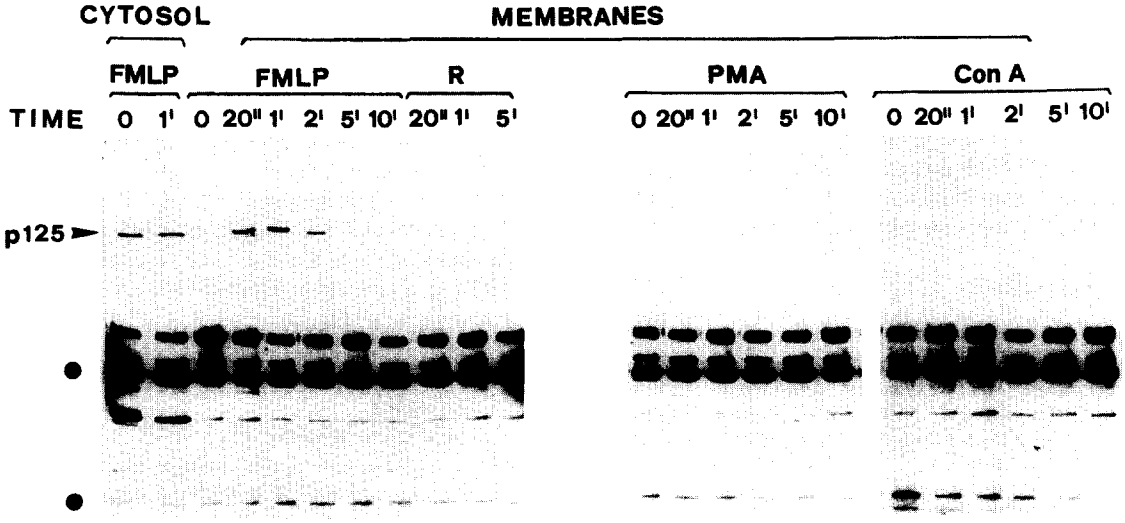

Fig. 2. Immunoprecipitation of p125 GAP from cytosol and membranes of human neutrophils. Neutrophils were stimulated and fractionated as described in Fig. 1. Cytosol and membrane fractions were extracted in solubilization buffer (see section 2) and immunoprecipitated with anti-p125 GAP monoclonal antibody. The immunoprecipitated material was subjected to SDS/PAGE on a 10% gel and immunoblotted with anti-p125 GAP polyclonal antibody. Immunoglobulin molecules are indicated by a dot. Abbreviations; R, resting neutrophils. The data are from one experiment representative of five.

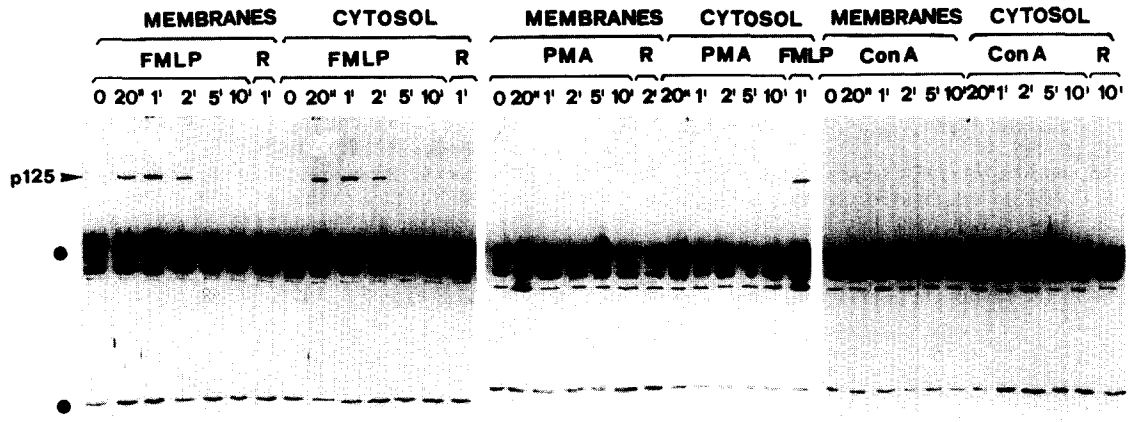

Fig. 3. Time course of tyrosine phosphorylation of p125 GAP in neutrophils. Neutrophils were stimulated and fractionated as described in Fig. 1. Cytosol and membrane fractions were extracted in solubilization buffer and immunoprecipitated with anti-phosphotyrosine PY20 monoclonal antibody. The immunoprecipitated proteins were subjected to SDS/PAGE on a 10% gel and immunoblotted with anti-p125 GAP antibody. The dots indicate the position of the immunoglobulin molecules. Abbreviations; R, resting neutrophils. The data are from one experiment representative of five.

4. Discussion

In this paper we report for the first time that p125 GAP is present in the cytosol of human neutrophils, and that upon neutrophil stimulation with FMLP, its transient translocation to the membrane correlates with its tyrosine phosphorylation. It is possible that this phosphorylation can mediate the binding of p125 GAP to the membrane, and its dephosphorylation is responsible for the release from the membrane. Alternatively, the tyrosine phosphorylation of p125 GAP might serve to regulate its interaction with other proteins, such as p190rhoGAP [11,13,14]. Attempts to coimmunoprecipitate p125 GAP together with p190rhoGAP have not been successful.

It is worth noting that the kinetics of translocation of p125 GAP paralleled that of oxygen consumption in response to FMLP. This observation might suggest that p125 GAP plays a role in the activation of NADPH oxidase. This role is also suggested by the previous finding that p125 GAP regulates the activity of p21 ras [6,7], which activates mitogen-activated protein kinases [19]. Evidence was presented that mitogen-activated protein kinases could be involved in the activation of the respiratory burst by FMLP [20–22]. However, the finding that the activation of the respiratory burst by PMA or ConA is not accompanied by tyrosine phosphorylation and translocation of p125 GAP and the previous reports that the in vitro reconstitution of active NADPH oxidase does not require p125 GAP [34,35] are in contrast with this hypothesis. Therefore, the role of p125 GAP in the mechanisms of NADPH oxidase activation remains to be clarified.

In previous studies it has been shown that p21 ras is activated by different stimuli, including phorbol esters [36]. Interestingly, the data presented in this paper show that at least in neutrophils, the ras regulator p125 GAP is affected only by FMLP, whose receptors act through trimeric GTP-binding proteins. In fact FMLP induced the tyrosine phosphorylation and translocation of p125 GAP. In contrast, PMA and ConA, which activate transmembrane signalling bypassing the GTP-binding proteins [23], were unable to induce the tyrosine phosphorylation and translocation of p125 GAP. This finding suggests that in neutrophils p125 GAP may be a target of signals arising exclusively from trimeric GTP-binding proteins.

In conclusion, our findings might indicate that, in human neutrophils, tyrosine phosphorylation and translocation of p125 GAP are related to signalling pathways selectively triggered by FMLP and resulting in the activation of NADPH oxidase and/or other functions activated by this agonist.

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