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Activation of *SRC* family kinases in human neutrophils. Evidence that $p58^{C-FGR}$ and $p53/56^{LYN}$ redistributed to a Triton X-100-insoluble cytoskeletal fraction, also enriched in the caveolar protein *Caveolin*, display an enhanced kinase activity

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Abstract Protein tyrosine phosphorylation is one of the signals involved in stimulation of neutrophil (PMN) functions. We found that phorbol myristate acetate (PMA) activates the *src* family tyrosine kinases $p58^{c-fgr}$ and $p53/56^{lyn}$ in suspended PMNs. Moreover, we found that up to about 20% of $p58^{c-fgr}$ and $p53/56^{lyn}$ redistribute to a Triton X-100-insoluble fraction after PMA stimulation, and it is this fraction of the two kinases which displays an increased activity. These changes of $p58^{c-fgr}$ and $p53/56^{lyn}$ distribution and activity correlate with tyrosine phosphorylation of endogenous substrates. In fact, in PMA-stimulated PMNs tyrosine phosphorylated proteins are mostly recovered in a Triton-insoluble cell fraction. To separate cytoskeletal from caveolar structures, which both display Triton X-100-insolubility, we used the detergent *n*-octyl β-D-glucopyranoside (OGP) which solubilises components of caveolae. We found that the caveolae marker protein, caveolin, as well as the cytoskeletal protein α-actinin and $p58^{c-fgr}$ and $p53/56^{lyn}$, is insoluble in OGP. These findings suggest that PMA stimulation promotes the formation of multimolecular complexes containing cytoskeletal proteins, caveolin-containing structures and *src* family protein tyrosine kinases. Moreover, they show that $p58^{c-fgr}$ and $p53/56^{lyn}$ associated with this multimolecular complex display an enhanced kinase activity.

Key words: 58^{c-fgr}; $p53/56^{lyn}$; Tyrosine phosphorylation; Neutrophil; Cytoskeleton; Caveolae

1. Introduction

Evidence has been accumulated in the last few years that protein tyrosine phosphorylation represents one of the signal triggered by a wide array of unrelated and distinct molecules able to stimulate selective neutrophil (PMN) functions. For example, increased phosphorylation of proteins in tyrosine residues has been reported to occur in response to chemotactic agents [1–11] and chemokines [10,12], ligands for Fc receptors [13–17], cytokines [7,18–21], inflammatory microcrystals [22,23], and phorbol myristate acetate (PMA) [7,11,24]. Kinases possibly involved in tyrosine phosphorylation of PMN proteins have just started to be identified; in particular, recent reports showed that *src* family protein tyrosine kinases might play a major role in this phenomenon. For example, β2 integrin-dependent adhesion and stimulation of PMN functions are accompanied by activation of the protein tyrosine kinase $p58^{c-fgr}$ [21], as well as $p53/56^{lyn}$ [25]. In addition, ligation of FcγRII and FcγRIII were shown to lead to activation of $p58^{c-fgr}$ and $p59/61^{hck}$ respectively [26,27].

Studies on signal transduction by integrins in platelets, as well as other cell types, established a strict relationship between *src* family tyrosine kinases and the cytoskeleton (reviewed in [28]). Interestingly, ligation of PMN FcγRII and FcγRIII leads to Triton-insolubility of $p58^{c-fgr}$ and $p59/61^{hck}$ respectively [27]. In addition, we found that PMN adhesion redistributes $p58^{c-fgr}$ [25,29], as well as $p53/56^{lyn}$ [29] to a cell fraction insoluble in Triton X-100.

The studies described in this paper were performed to obtain evidence that the redistribution of the protein tyrosine kinases $p58^{c-fgr}$ and $p53/56^{lyn}$ to a Triton-insoluble cell fraction is essential for activation of the phosphorylating activities of the two kinases. The results of these studies show that PMA activates $p58^{c-fgr}$ and $p53/56^{lyn}$ in suspended PMNs. Analysis of the levels of the proteins and the kinase activities demonstrated that it is the fraction of the two kinases which is redistributed to the Triton-insoluble fraction upon PMA treatment which displays a higher kinase activity. Moreover, we found that this Triton-insoluble fraction also contains both the cytoskeletal proteins α-actinin and the caveolar protein caveolin, thus suggesting that PMN stimulation promotes the formation of a multimolecular complex containing cytoskeletal proteins, caveolin-containing structures and *src* family tyrosine kinases.

2. Materials and methods

2.1. PMN isolation and stimulation

PMNs were isolated from buffy coats of healthy volunteers by standard procedures, as described in details before [21]. After isolation, cells were suspended in Hank's balanced salt solution supplemented with 1.0 mM $CaCl_2$ and 5.5 mM D-glucose at a density of 10×10^6/ml. 1 ml of the cell suspension was dispensed in polypropylene tubes, and after 5 min of incubation at 37°C in a shaking water bath, PMA (at the final concentration of 10 ng/ml), TNF (at the final concentration of 20 ng/ml) or PMA diluent (dimethyl sulfoxide) were added. The reaction was prolonged up to 15 or 30 min (see section 3) and then stopped by the addition of an equal volume of 2× RIPA buffer (see reference [21] for composition) or 2× cytoskeleton stabilisation buffer (CSK, see below for composition).

2.2. Isolation of Triton-soluble and -insoluble fractions

This was performed essentially as described [30] with minor modifications. PMNs incubated as above described were added with 2×CSK (2% Triton X-100, 100 mM Tris, pH 7.5, 10 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 2 mM PMSF, 2 mM diisopropyl fluorophosphate, 200 μM sodium orthovanadate, 20 μM phenylarsine oxide) and cell lysates transferred to microfuge tubes and immediately centrifuged at 4°C for 4 min in a Sorvall MC12V micro-

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fuge at 12,000 rpm. Supernatants were carefully collected with a Gilson pipette and further clarified by centrifugation for 10 min in a microfuge. The supernatant obtained after this second round of centrifugation was taken as the Triton-soluble fraction and accounted for about 70% of the total PMN proteins. The pellet obtained after recentrifugation of the Triton-soluble fraction accounted for less than 1% of the total proteins and was not analysed. Triton-soluble fractions were brought to the same composition of RIPA buffer by additions of detergents before analysis. The pellets obtained after centrifugation of PMN CSK lysates were resuspended in RIPA buffer (see reference [21] for composition) and after incubation at 4°C for 30 min under rotation, clarified by centrifugation for 10 min in a microfuge. Supernatants of this Triton-insoluble/RIPA-soluble fraction accounted for about 25% of total proteins, while the pellets of this fraction contained <8% of total proteins.

2.3. Analysis of $p58^{c-fgr}$ and $p53/56^{lyn}$ autophosphorylating kinase activities

This was done essentially as described before [21,25]. Routinely, 40 μg proteins of the Triton-soluble fraction and 80 μg proteins of the Triton-insoluble/RIPA-soluble fraction were used for immunoprecipitation experiments. Immunoprecipitates were washed and analysed as described before [21,25].

2.4. Analysis of $p58^{c-fgr}$ and $p53/56^{lyn}$ proteins

Triton-soluble and Triton-insoluble/RIPA-soluble fraction were solubilised in SDS-PAGE sample buffer and after electrophoresis in 10% acrylamide gels blotted to nitrocellulose membranes and analysed as described before, using a chemiluminescence detection kit (ECL, Amersham, Little Chalfont, UK) [21,25,29].

2.5. Solubilisation of proteins with n-octyl β-D-glucopyranoside (OGP) and analysis of distribution of caveolin

Triton-insoluble fractions were prepared as above described and resuspended in the same solubilisation buffer (CSK, see above) supplemented with 60 mM OGP. After incubation at 4°C for 30 minutes under rotation, samples were centrifuged for 10 min in a microfuge. The OGP-insoluble fraction recovered by centrifugation was solubilised in SDS-PAGE sample buffer. For analysis of caveolin, nitrocellulose blots were probed with rabbit anti-caveolin antibodies (Transduction Laboratories, Lexington, KY, USA), followed by HRP-labelled donkey anti-rabbit IgG. Distribution of α-actinin in the OGP-soluble and -insoluble fractions was analysed by western blotting as described before [29].

3. Results and discussion

Increased phosphorylation of proteins in tyrosine residues has been repeatedly reported to occur as a consequence of PMN stimulation with different stimuli (see [1–24]). In studies aimed to elucidate signals generated by β2 integrins, we demonstrated that TNF-stimulated, β2 integrin-dependent adhesion and spreading of PMNs activate the protein tyrosine kinases $p58^{c-fgr}$ [21] and $p53/56^{lyn}$ [25]. Lysing PMNs adherent to fibrinogen with Triton X-100 we found that TNF-stimulated adhesion caused redistribution of $p58^{c-fgr}$ [25,29], as well as $p53/56^{lyn}$ [25], to a Triton-insoluble fraction also enriched in the cytoskeletal protein α-actinin [29]. A comparison between the amount of the $p58^{c-fgr}$ protein and its autophosphorylating kinase activity in the Triton-insoluble fraction, indicated that the fraction of $p58^{c-fgr}$ which became Triton-insoluble after PMNs were induced to spread with TNF, displayed an enhanced kinase activity [25].

Redistribution of *src* family tyrosine kinases to cytoskeletal, Triton X-100-insoluble fractions has been investigated in details in platelets stimulated to form aggregates [30,31]. In these studies, separation of a cytoskeletal fraction from cell lysates on the basis of its Triton X-100 insolubility was done with suspension of cells. In order to demonstrate that in PMNs $p58^{c-fgr}$ and $p53/56^{lyn}$ redistributed to a Triton-insoluble fraction do display an enhanced kinase activity, we decided to exploit protocols utilised to separate Triton-soluble and Triton-insoluble fractions from platelet suspensions [30,31].

As our previous studies demonstrated an adhesion-dependent activation of $p58^{c-fgr}$ and $p53/56^{lyn}$ [21,25], at first we performed experiments aimed to find conditions of PMN stimulation which caused an increase of $p58^{c-fgr}$ and $p53/56^{lyn}$ activities in cell suspensions. As shown in Fig. 1, the level of $p58^{c-fgr}$ and $p53/56^{lyn}$ activities immunoprecipitated from RIPA buffer lysates (see section 2) rapidly increased after PMA stimulation of suspended PMNs. Interestingly, TNF,

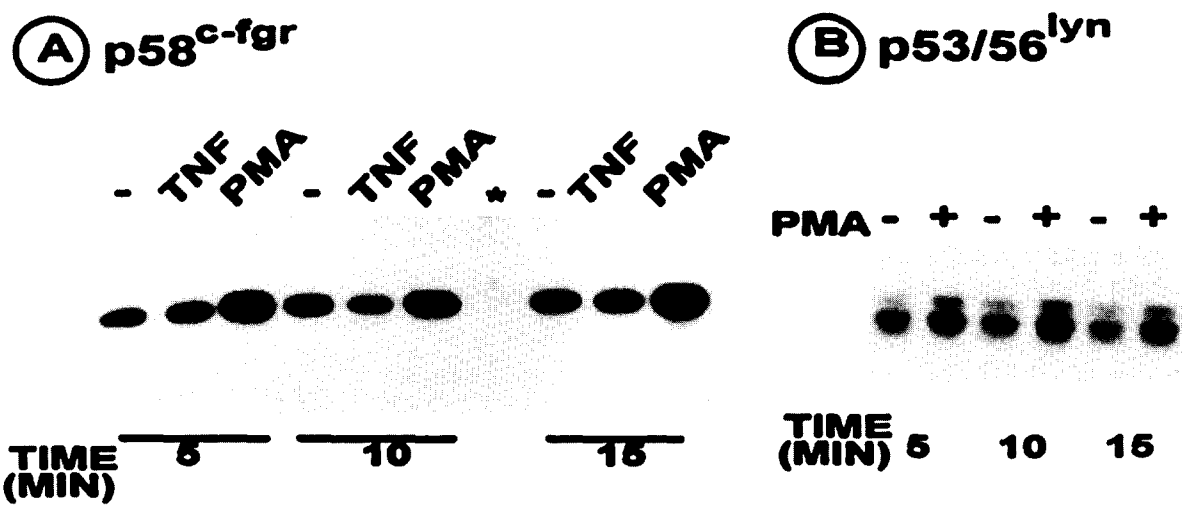

Fig. 1. PMA enhances $p58^{c-fgr}$ and p53/56lyn autophosphorylating kinase activities in suspended PMNs. PMNs were stimulated with 10 ng/ml PMA or 20 ng/ml TNF for different times and then lysed by addition of RIPA buffer (see section 2). $p58^{c-fgr}$ and $p53/56^{lyn}$ were immunoprecipitated from RIPA buffer lysates and immunocomplexes subjected to in vitro kinase assays. One autoradiogram from one of three trials is shown. The asterisk in Panel A indicate a lane loaded with molecular weight markers.

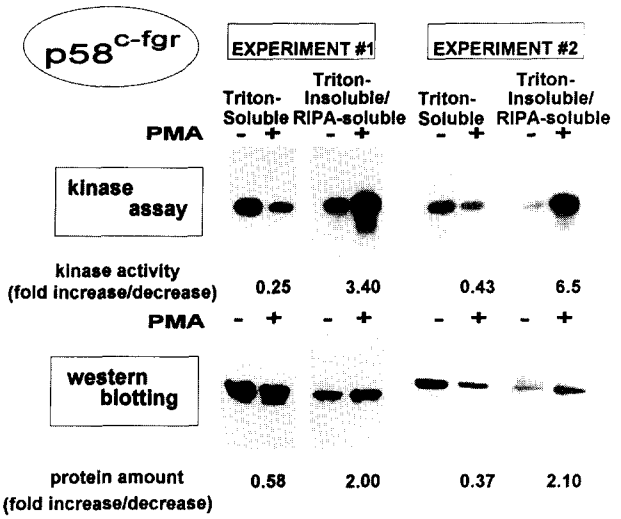

Fig. 2. PMA redistributes $p58^{c-fgr}$ to a Triton X-100-insoluble fraction in suspended PMNs and Triton X-100-insoluble $p58^{c-fgr}$ displays a higher autophosphorylating kinase activity. PMNs were incubated for 15 min in the absence or the presence of 10 ng/ml PMA and then lysed in buffer containing 1% Triton X-100 (see section 2). 40 μg of proteins from the Triton-soluble fraction were used to immunoprecipitate $p58^{c-fgr}$ and perform in vitro kinase assays, or dissolved in SDS-PAGE sample buffer to analyse the $p58^{c-fgr}$ protein by immunoblot assays. The Triton-insoluble fraction was further extracted with RIPA buffer (see section 2) and 80 μg of proteins from the Triton-insoluble/RIPA-soluble fraction were used to immunoprecipitate $p58^{c-fgr}$ and perform in vitro kinase assays, or dissolved in SDS-PAGE sample buffer to analyse the $p58^{c-fgr}$ protein by immunoblot assays. The autoradiograms were scanned with a BioRad-LKB Ultrascan XL laser densitometer to quantify intensities of the bands and to calculate fold increase or decrease of these intensities in the Triton-soluble or Triton-insoluble/RIPA-soluble fractions from PMA-treated versus unstimulated PMNs.

which increased $p58^{c-fgr}$ and $p53/56^{lyn}$ activities in PMNs adherent to fibrinogen [21,25], had no effect on suspended cells at least up to 15 minutes of incubation (Panel A, and data not shown). In some experiments we found that TNF had an effect also on suspended PMNs but only if the time of incubation was prolonged; moreover, in these experiments we observed that TNF induced the formation of PMN aggregates. We then analysed $p58^{c-fgr}$ and $p53/56^{lyn}$ proteins and activities in the soluble and insoluble fractions obtained after PMN lysis with Triton X-100. Suspended PMNs were incubated for 15 minutes at 37°C in the absence or the presence of PMA and lysed with Triton X-100; a soluble and an insoluble fraction were then recovered by centrifugation of the total cell lysate (see section 2). To examine the level of $p58^{c-fgr}$ and $p53/56^{lyn}$ activities in the Triton-insoluble fraction, we incubated this fraction with RIPA buffer to further solubilise the cytoskeletal associated proteins (see [31], and section 2).

Fig. 2 shows the results of analysis of $p58^{c-fgr}$ autophosphorylating kinase activity and protein in the Triton-soluble and Triton-insoluble/RIPA-soluble fractions. As shown in Fig. 2, the level of $p58^{c-fgr}$ kinase activity immunoprecipitated from the Triton-soluble fraction decreased after PMA stimulation of PMNs. This decrease reflected a parallel decrease in the amount of the $p58^{c-fgr}$ protein detected in this Triton-soluble fraction. On the contrary, the level of $p58^{c-fgr}$ kinase activity immunoprecipitated from the Triton-insoluble/RIPA-soluble fraction, as well the level of the $p58^{c-fgr}$ protein in this fraction, increased after PMA stimulation. Interestingly, comparing the increases of the $p58^{c-fgr}$ activity and protein in this Triton-insoluble/RIPA-soluble fraction in unstimulated and PMA-stimulated PMNs, we found that the levels of the $p58^{c-fgr}$ activity increased consistently more than those of the $p58^{c-fgr}$ protein after PMA stimulation (see Fig. 2). Comparable results were obtained by analysing distribution and activity of the $p53/56^{lyn}$ kinase (Fig. 3). In fact, also $p53/56^{lyn}$ kinase activity and protein decreased in the Triton-soluble and increased in the Triton-insoluble fraction after PMA treatment of PMNs. In addition, also for $p53/56^{lyn}$ the relative increase of its kinase activity in the Triton-insoluble fraction after PMA treatment was consistently higher than the increase in the protein amount (see Fig. 3).

The results of the experiments illustrated in Figs. 2 and 3, allowed us to demonstrate that $p58^{c-fgr}$ and $p53/56^{lyn}$ redistribute to a Triton-insoluble cell fraction after PMA stimulation of PMNs. From the intensities of the $p58^{c-fgr}$ and $p53/56^{lyn}$ bands (Figs. 2 and 3) and the total amount of proteins in the Triton-soluble and insoluble fraction, we calculated that the per cent of $p58^{c-fgr}$ and $p53/56^{lyn}$ in the Triton-insoluble fraction increased, after PMA stimulation, from 12.5±3.5 (S.D.) to 23.5±6.3 and from 4.0±1.4 to 20.0±4.5, respectively; per cent of total proteins recovered in the Triton-insoluble fraction was 23.5±2.1 in unstimulated and 29.0±2.9 in PMA stimulated PMNs. To understand whether this redistribution was possibly implicated in phosphorylation of endogenous substrates, we analysed distribution of tyrosine phosphorylated proteins in the Triton-soluble and insoluble fraction. As shown in Fig. 4, treatment with PMA did not cause any major change in tyrosine phosphorylation of proteins recovered in the Triton-soluble fraction (compare lane 1 and 2). However, several proteins which redistributed to the Triton-insoluble fraction after PMA treatment were stained with anti-phosphotyrosine antibodies (compare lane 5 and 6). As shown in Fig. 4, stimulation of suspended PMNs with TNF did not cause any consistent alteration in the tyrosine phosphorylation of proteins recovered in the Triton-soluble (lanes 3 and 4) or insoluble (lanes 7 and 8) fraction. The inability of TNF to trigger protein tyrosine phosphorylation in suspended PMNs is in accord with previous findings which

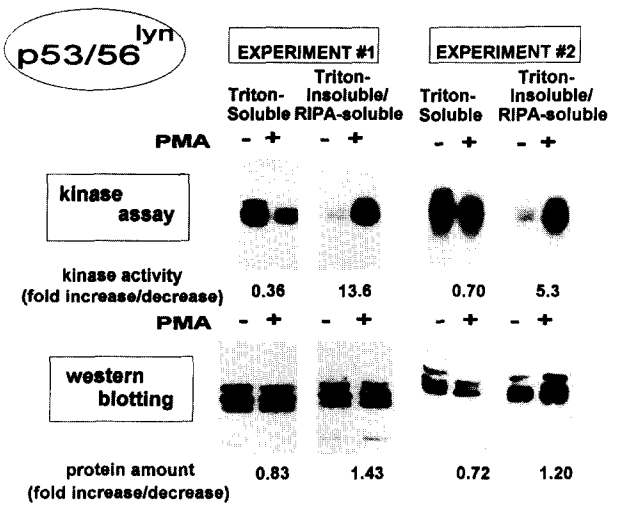

Fig. 3. PMA redistributes $p53/56^{lyn}$ to a Triton X-100-insoluble fraction in suspended PMNs and Triton X-100-insoluble $p53/56^{lyn}$ displays a higher autophosphorylating kinase activity. The experiments reported were performed exactly as those described in Fig. 2 legend.

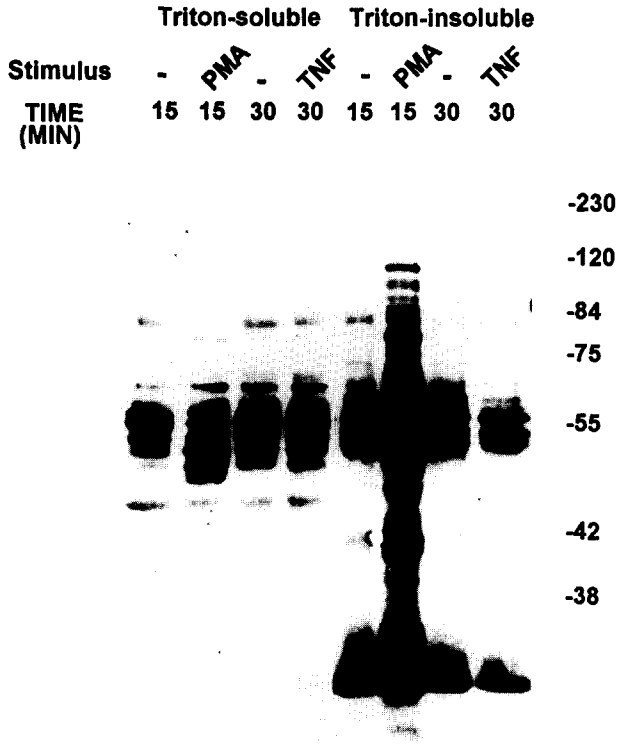

Fig. 4. Tyrosine phosphorylated proteins in suspended PMNs stimulated with PMA are present in a Triton X-100-insoluble fraction. PMNs were stimulated as described in Fig. 1 legend, and Triton X-100-soluble and insoluble fractions were obtained as described in Fig. 2 legend and section 2. 60 μg proteins of each fraction were dissolved in heated (95°C) SDS-PAGE sample buffer and analysed by immunoblot assays with the anti-phosphotyrosine Ab 4G10. One representative experiment of three performed is reported.

demonstrated that TNF acts as a stimulus of protein tyrosine phosphorylation only in adherent PMNs [20].

Triton X-100 insolubility has been used as the most feasible approach to separate interconnected cytoskeletal proteins and associated plasmamembrane and cytosolic proteins [32]. However, in the last few years it has been clearly shown that another subcellular structure, known as caveolae and enriched in glycolipids, glycosylphosphatidylinositol-linked (GPI-linked) proteins and cytoplasmic signalling proteins, displays the classical Triton X-100 insolubility of cytoskeleton-based structures (reviewed in reference [33]). We therefore designed experiments aimed to separate from the Triton-insoluble fraction we found to be enriched in protein tyrosine kinases and tyrosine phosphorylated proteins after PMA treatment, a truly cytoskeletal from a caveolar structure. To this purpose we exploited the evidence that the 22 kDa integral membrane protein named caveolin is a well recognised marker of caveolae and that *n*-octyl β-D-glucopyranoside (OGP) solubilises GPI-linked proteins, as well as caveolin from different cell types [34–37]. We then asked whether OGP could solubilise caveolin from the Triton-insoluble fraction. As shown in Fig. 5, OGP extracted a small fraction of α-actinin and $p58^{c-fgr}$ from the Triton-insoluble fraction. However, caveolin resisted extraction with OGP and was recovered exclusively in the Triton-insoluble/OGP-insoluble fraction which was solubilised with SDS-PAGE sample buffer. Results comparable to those obtained with $p58^{c-fgr}$ were obtained analysing the distribution of $p53/56^{lyn}$ (data not shown). On the basis of the amount of protein recovered in the Triton-soluble, the Triton-insoluble/OGP-soluble and the Triton-insoluble/OGP-insoluble fraction, and the densitometric intensity of the α-actinin and $p58^{c-fgr}$ bands we could make an approximate estimation of the distribution of the two proteins in the different fractions (see Fig. 5). This estimation shows that PMA increases the per cent of the two proteins present in the Triton-insoluble fraction, independently of their solubility in OGP. To our knowledge this is the first evidence that caveolin is contained in PMN. In addition, our findings suggest that in PMN, caveolin-containing structure can interact with cytoskeletal proteins, as well as signalling proteins such as $p58^{c-fgr}$ and $p53/56^{lyn}$. Interaction of caveolin-containing structures with the cytoskeleton has been demonstrated in other cell type (see [33]). We do not know the reason of the OGP-insolubility of caveolin in PMNs. However, it is tempting to

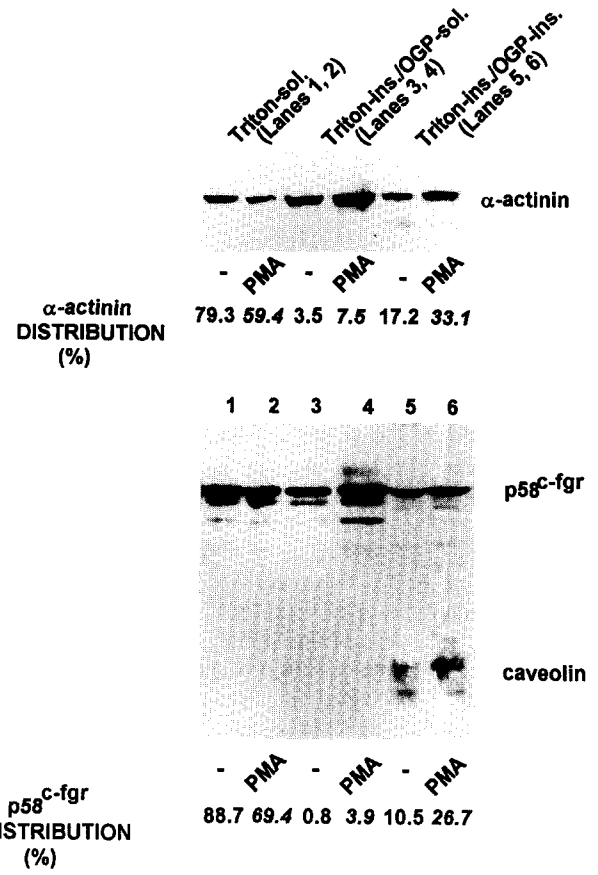

Fig. 5. PMNs express the marker of caveolar structures caveolin and this is not extracted from Triton-insoluble fractions with OGP. PMNs were stimulated with PMA and Triton-soluble and insoluble fractions prepared as described in Figs. 1 and 2 legends and section 2. 60 μg proteins of the three fractions were dissolved in heated (95°C) SDS-PAGE sample buffer and analysed by immunoblot assays with anti-α-actinin, anti-$p58^{c-fgr}$ and anti-caveolin Abs. Per cent of α-actinin and $p58^{c-fgr}$ distribution in the three fractions was calculated from the density of the bands in the autoradiogram (see Fig. 2 legend) and the amount of total proteins in the three fractions. Per cent of total proteins recovered in the three fractions was as follows. Triton-soluble: unstimulated PMNs, 72%, PMA-stimulated PMNs, 74%; Triton-insoluble/OGP-soluble: unstimulated PMNs, 3%, PMA-stimulated PMNs, 3%; Triton-insoluble/OGP-insoluble: unstimulated PMNs, 25%, PMA-stimulated PMNs, 23%. One representative experiment of three performed is reported.

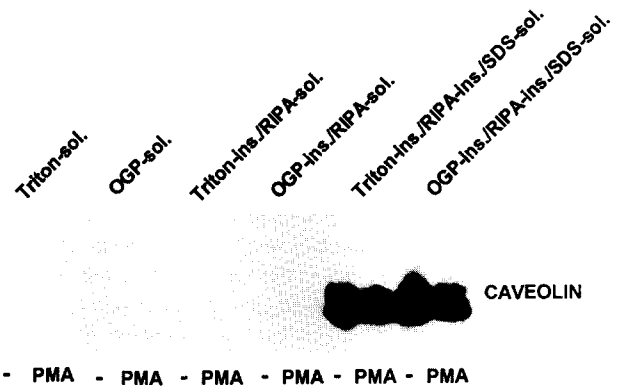

Fig. 6. In PMNs, caveolin is Triton-insoluble and can not be extracted either with OGP or RIPA buffer. Unstimulated or PMA-stimulated PMNs (see Fig. 2 legend) were lysed with buffers containing 1% Triton or 60 mM OGP for 30 min at 4°C. After centrifugation in a microfuge, the supernatants were removed (Triton-sol. and OGP-sol) and the pellets further extracted with RIPA buffer for 30 min at 4°C. After extraction with RIPA buffer, RIPA-soluble (Triton-ins./RIPA-sol. and OGP-ins/RIPA-sol.) and insoluble fractions were recovered by centrifugation in a microfuge. The final pellet was dissolved in heated (95°C) SDS-PAGE sample buffer (Triton-ins./RIPA-sol./SDS-sol and OGP-ins/RIPA-sol./SDS-sol.). 50 μg proteins of each fraction, dissolved in heated (95°C) SDS-PAGE sample buffer, were subjected to immunoblot assays with anti-caveolin Abs.

suggest that the OGP-insolubility reflects a tight association of caveolin-containing structures with a fraction of highly polymerised cytoskeleton. Proteins present in this multimolecular complex can be dissociated by detergent extraction to different extents. In fact, as reported in Figs. 2 and 3, RIPA buffer effectively solubilised $p58^{c-fgr}$ and $p53/56^{lyn}$ from the Triton-insoluble fraction; comparable results were obtained by analysing α-actinin (data not shown). Extraction of the Triton-insoluble/RIPA-insoluble fractions obtained in the experiments illustrated in Figs. 2 and 3 with SDS-PAGE sample buffer allowed us to calculate that a negligible fraction of the $p58^{c-fgr}$ and $p53/56^{lyn}$ protein (<5%), as well of α-actinin remained insoluble in RIPA buffer (data not shown). However, we found that caveolin remained insoluble also after extraction of the Triton X-100- or the OGP-insoluble fraction with RIPA (Fig. 6). These findings show that $p58^{c-fgr}$ and $p53/56^{lyn}$ present in the Triton-insoluble/RIPA-soluble fraction we analysed in the experiments illustrated in Figs. 2 and 3 are devoid of caveolin. They do not however exclude that the two kinases are associated with caveolin-containing structures and, in contrast with caveolin itself, are readily solubilised by RIPA buffer.

In conclusion, we demonstrated that PMN stimulation promotes the redistribution of two *src* family members to a Triton-insoluble fraction containing both cytoskeletal and caveolar proteins. Moreover, our findings provide the first evidence that $p58^{c-fgr}$ and $p53/56^{lyn}$ redistributed to a Triton-insoluble fraction display an enhanced autophosphorylating kinase activity thus substantiating the hypothesis that activation of *src* family members is closely linked to reorganisation of the cytoskeleton [28]. Redistribution of *src* family tyrosine kinases to a multimolecular complex of interconnected cytoskeletal structures, likely containing also caveolar components, as indicated by the presence of caveolin in these structures, may play a major role in regulation of selective PMN functions such as phagocytosis, secretion and regulation of gene transcription.

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