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Phosphorylation of JAK2 in thrombin-stimulated human platelets

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Abstract We show the presence of the tyrosine kinase JAK2 in human platelets and demonstrate that it undergoes phosphorylation on tyrosine residues on challenge with the G protein receptor stimulus, thrombin, or the tyrosine phosphatase inhibitor, peroxovanadate. Thrombin-induced phosphorylation of JAK2 is inhibited by two structurally distinct inhibitors of tyrosine kinases, staurosporine and the tyrphostin ST271. The protein kinase C (PKC) inhibitor, Ro 31-8220, and intracellular Ca^{2+} chelator, BAPTA-AM, also inhibit thrombin-induced phosphorylation of JAK2, while the phorbol ester, phorbol dibutyrate (PDBu), and Ca^{2+} ionophore, A23187, induce tyrosine phosphorylation of JAK2. These results suggest that tyrosine phosphorylation of JAK2 stimulated by thrombin may be mediated downstream of phosphoinositide metabolism.

Key words: JAK2; Tyrosine kinase; Human platelet; Tyrosine phosphorylation

1. Introduction

There is a marked increase in protein tyrosine phosphorylation in association with platelet activation by a wide variety of agonists which occurs in three distinct phases, namely rapid, intermediate and late-stage events [1–3]. The latter stages of tyrosine phosphorylation are associated with activation of the fibrinogen receptor and onset of platelet aggregation [1,2]. The significance of the early stage of tyrosine phosphorylation in platelet activation is uncertain.

The majority of agonists induce platelet stimulation through activation of phospholipase C which hydrolyses phosphoinositides generating diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). The former activates PKC and the latter releases calcium from intracellular stores. Stimuli which activate seven transmembrane receptors such as thrombin and thromboxanes induce activation of phospholipase $C\beta$ isoforms through a G protein-dependent pathway [4], while other stimuli, such as collagen and immune complexes, are thought to induce activation of phospholipase $C\gamma$ isoforms through a tyrosine kinase-dependent pathway [5,6].

Recently, a new family of tyrosine kinases, called the JAK family (Janus kinase or just another kinase), has been identified and implicated in signal transduction induced by cytokines and growth factors (for review see [7–9]). The family currently consists of four members, JAK1, JAK2, JAK3 and TYK2, which act in combination or in isolation depending on the stimulus [10–14]. For example, JAK2 plays an important role in mitogenesis induced by the haematopoietic factor, erythropoetin, in myeloid cells by virtue of its association to the membrane-proximal region of the erythropoietin receptor and subsequent activation [14]. The JAK family of kinases induce gene regulation through a miscellaneous family of proteins known as STATS (signal transducers and activators of transcription) [9,15]. The existence of this new family of tyrosine kinases in haemotopoietic cells prompted us to investigate their presence in platelets.

2. Materials and methods

2.1. Reagents and materials

Staurosporine, indomethacin, protein A-sepharose CL-4B, Tween 20, thrombin, A23187, phenylmethanesulphonyl difluoride (PMSF), PDBu and bovine serum albumin (BSA) fraction V were purchased from Sigma (Poole, Dorset, UK). Antisera against JAK1 and JAK2 and the anti-phosphotyrosine monoclonal antibody, 4G10, were from Upstate Biotechnology Inc., (TCS Biologicals Ltd., Buckinghamshire, UK). Affinity-purified anti-JAK2 and anti-TYK2 were obtained from Santa Cruz Biotechnology (NBS Biologicals, Hatfield, UK). Horseradish peroxidase-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG antibodies (NA931 and NA934), ECL reagents and Hyperfilm were from Amersham International (Cardiff, UK). BAPTA-AM was from Calbiochem (Calbiochem-Novabiochem, Nottingham, UK). Triton-X100 was purchased from BDH-Merck (Poole, Dorset, UK). ST271 was kindly donated by the Wellcome Foundation. Ro 31-8220 was a gift from Roche Products (Welwyn Garden City, Herts., UK). Polyvinylidene difluoride (PVDF) membranes were from Bio-Rad (Bio-Rad Laboratories Ltd., Hertfordshire, UK). All other reagents were of analytical grade.

2.2. Platelet isolation and stimulation

Human platelets were isolated from aspirin-free donors on the day of the experiment as described previously [16] and, following two washings, resuspended at a concentration of $4–8 \times 10^8$ cells/ml in a modified Tyrode buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, 5 mM glucose, pH 7.3) containing indomethacin (10 μM) and EGTA (1 mM). All experiments were performed at 37°C. Platelet suspensions (0.4 ml) were prewarmed for 5 min before addition of compounds (1–4 μl) and incubated for the times indicated before addition of the stimulus. Peroxovanadate was generated by adding orthovanadate (400 μM) 60 s before H_2O_2 (2 mM).

2.3. Immunoblotting

Samples of platelets (4×10^8/ml) were heated for 5 min at 100°C in Laemmli buffer. Proteins were separated by 10% SDS-PAGE and transferred to PVDF blotting membranes by semi-dry transfer (120 min, 15 V). Non-specific binding was blocked with Tris-buffered saline-Tween (TBS-T: 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 10% BSA (w/v). Membranes were incubated with the primary antibody dissolved in the same buffer (1 μg/ml anti-phosphotyrosine 4G10, 1:500 dilution anti-JAK1 serum, 1:1000 dilution anti-JAK2 serum and 0.1 μg/ml anti-TYK2 antibody) for 60 min at room temperature. Membranes were washed five times in TBS-T before detection of bound antibody with horseradish peroxidase-conjugated

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Abbreviations: BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acetoxymethylester; DG, diacylglycerol; PKC, protein kinase C; IP_3, inositol 1,4,5-trisphosphate; PDBu, phorbol dibutyrate; TBS-T, Tris buffered saline-Tween; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PMSF, phenylmethanesulphonyl difluoride.

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sheep anti-mouse IgG (NA 931) or donkey anti-rabbit IgG (NA 934), depending on the nature of the first antibody. After washing, the blots were treated with ECL reagents and exposed to Hyperfilm. Membranes were stripped of bound antibody by washing in TBS-T containing 2% SDS for 40 min at 80°C. After verifying the stripping through the use of the secondary antibody, blots were re-probed with a different primary antibody as appropriate.

2.4. Immunoprecipitation

Immunoprecipitation of proteins carried out under non-denaturing conditions was performed as follows: platelets (8×10^8/ml) were lysed in 1% Triton X-100, 150 mM NaCl, 10 mM Tris, 0.5 mM PMSF, 5 mM EDTA, 1 mM Na_3VO_4 for 30 min at 4°C. Samples were centrifuged 5 min at $13,000 \times g$ and supernatant was pre-cleared with 40 μl of protein A-Sepharose CL 4B. After adding the antibody (1:300 dilution for anti-JAK2 serum, 1 μg/ml for anti-JAK2 and anti-TYK2 antibodies) and 40 μl of protein A-Sepharose CL 4B, samples were left to mix gently overnight and then centrifuged at $13,000 \times g$ and the pellet washed twice with extraction buffer and twice with TBS-T. Immunoprecipitated protein was solubilized in Laemmli buffer, boiled for 10 min at 100°C, separated by 10% SDS-PAGE and analysed by immunoblotting as detailed before.

Immunoprecipitation in denaturing conditions was carried out with the use of 2% SDS in the extraction buffer. The samples were then diluted 1:20 in TBS-T containing 2 mg/ml BSA, 1 mM PMSF and 1 mM EDTA; subsequent steps were the same as for immunoprecipitation under the non-denaturing conditions described above, except that a 1:20,000 dilution for anti-JAK2 serum was used.

3. Results

3.1. Presence of JAK2 and tyrosine phosphorylation

Western blotting revealed the presence of JAK2 (~125 kDa) and TYK2 (~135 kDa) in human platelets (Fig. 1A). Both proteins were weakly phosphorylated on tyrosine residues under basal conditions. However, only JAK2 underwent an increase in phosphorylation on tyrosine residues in platelets challenged with thrombin or the tyrosine phosphatase inhibitor, peroxovanadate, in the absence of aggregation (Fig. 1B). Stripping of blots and reprobing with anti-JAK2 confirmed that the increase in tyrosine phosphorylation was due to incorporation of phosphate rather than recovery of more protein (Fig. 1B). Tyrosine phosphorylation of JAK2 induced by either stimulus was slow in onset relative to several platelet responses, including secretion and shape change, with significant increases being detected only 30–60 s after stimulation; phosphorylation was maintained for at least 10 min (Fig. 2). Thrombin-induced a similar increase in tyrosine phosphorylation of JAK2 under conditions which enabled platelet aggregation to take place, i.e. absence of EGTA (not shown). The increase in tyrosine phosphorylation of JAK2 induced by thrombin was inhibited completely by staurosporine (10 μM), a non-specific inhibitor of both serine/threonine and tyrosine kinases [5], and by the tyrphostin ST271 (300 μM) [17], a more selective inhibitor of tyrosine kinases (Fig. 3A).

3.2. Relationship of phosphorylation of JAK2 to phosphoinositide metabolism

Since signal transduction by thrombin involves synergistic effects mediated through the mobilization of Ca^{2+} and activation of PKC, we examined the role of these second messenger pathways in the regulation of JAK2 phosphorylation. The PKC inhibitor Ro 31-8220 (10 μM) and intracellular Ca^{2+} chelator

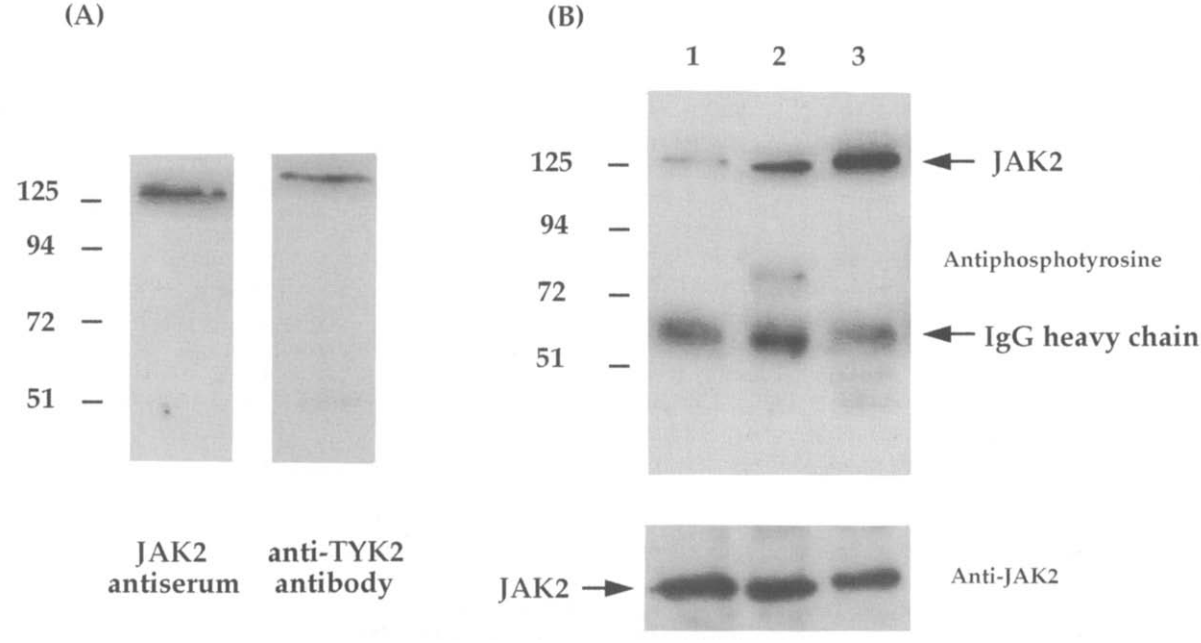


Fig. 1. (A) Presence of JAK2 and TYK2 in human platelets. Whole cell lysates were immunoblotted as described in section 2. The results with JAK2 were obtained with the antiserum from Upstate Biotechnology Inc. (UBI) which was raised against residues 758–776 of murine JAK2; similar results were obtained with a second JAK2 antibody from Santa Cruz Biotechnology raised against a peptide corresponding to amino acids 1110–1129 of murine JAK2 (not shown) (B) Upper panel: JAK2 undergoes tyrosine phosphorylation on activation of platelets with thrombin (1 unit/ml) (lane 2) and peroxovanadate (400 μM sodium vanadate/4 mM H_2O_2) (lane 3). Lane 1 shows weak basal tyrosine phosphorylation. Platelets (8×10^8/ml) were stimulated for 3 min at 37°C under stirring. Samples were lysed in non-denaturing conditions and JAK2 was immunoprecipitated with anti-JAK2 antibody from Santa Cruz Biotechnology, separated and immunoblotted with antiphosphotyrosine as detailed above. The 80 kDa band observed in thrombin-stimulated platelets was observed in some but not all studies, and could be revealed through the use of the secondary antibody alone. Lower panel: Western blot of anti-JAK2 for the same region of the membrane, following removal of the antiphosphotyrosine antibody (see section 2). In all cases, similar results were obtained in at least three other experiments.

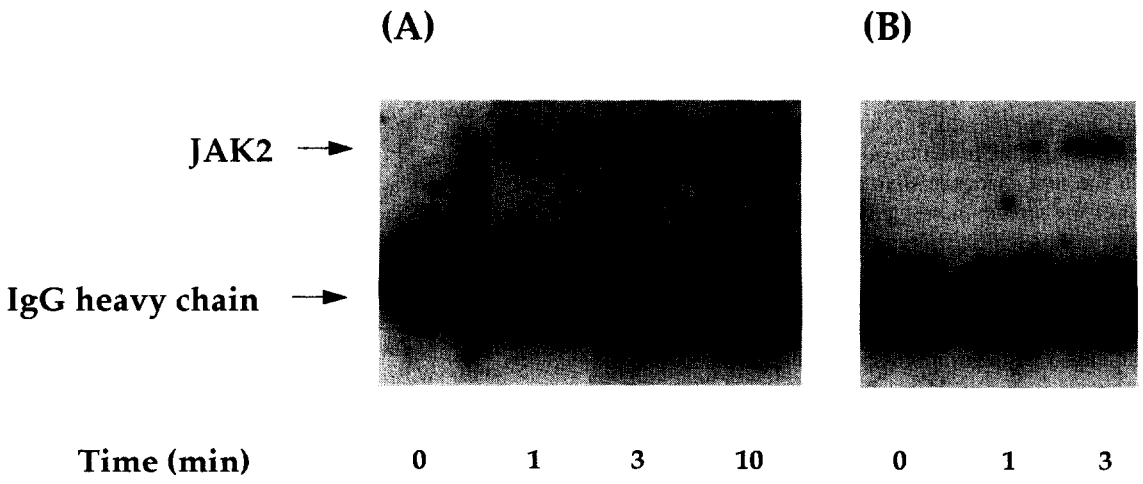
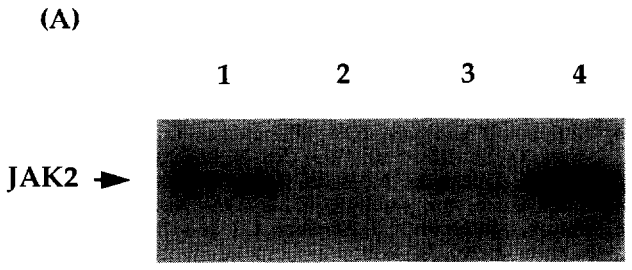
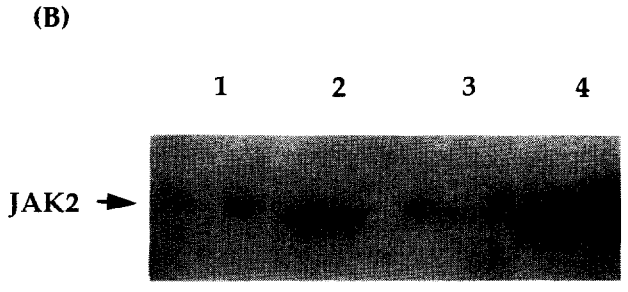
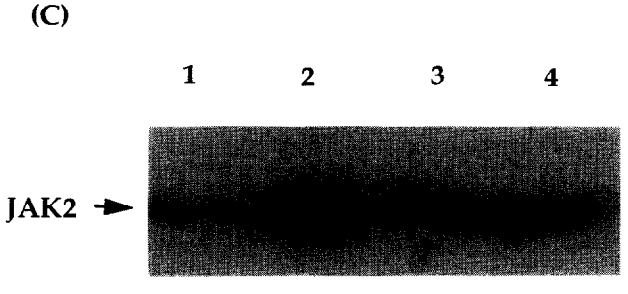


Fig. 2. Time-course for tyrosine phosphorylation of JAK2 induced by thrombin (1 unit/ml) (A) or peroxovanadate (400 μM sodium vanadate/4 mM H_2O_2) (B). Platelets (8×10^8/ml) were challenged with thrombin or peroxovanadate at 37°C for the time indicated. Cells were lysed and protein immunoprecipitated in denaturing conditions with anti-JAK2 serum from UBI. The immunoprecipitates were submitted to SDS-PAGE and protein was transfered to membrane and probed with antiphosphotyrosine antibody (see section 2). Two other experiments gave similar results.







BAPTA-AM (40 μM) were used to investigate whether thrombin induces phosphorylation of JAK2 through an increase in Ca^{2+} and/or activation of PKC [6]. Neither Ro 31-8220 or BAPTA-AM altered the degree of tyrosine phosphorylation of JAK2 under basal conditions (results not shown). However, both agents markedly inhibited tyrosine phosphorylation of JAK2 induced by thrombin (Fig. 3B). Consistent with this both PDBu (1 μM) and A23187 (2 μM) induced tyrosine phosphorylation of JAK2 and, when given in combination, produced an effect greater than that produced by either stimulus alone (Fig. 3C). These data suggest that phosphorylation of JAK2 by thrombin may occur downstream of second messenger formation.

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Fig. 3. (A) The effect of tyrosine kinase inhibitors on tyrosine phosphorylation of JAK2 stimulated by thrombin (1 unit/ml): lane 1, basal; lane 2, 10 μM staurosporine + 1 unit/ml thrombin; lane 3, 300 μM ST271 + 1 unit/ml thrombin; lane 4, 1 unit/ml thrombin. 400 μl aliquots of platelets (8×10^8/ml) were incubated with staurosporine (1 min) or ST271 (5 min) at 37°C before addition of stimuli. After 3 min, platelets were lysed and JAK2 was immunoprecipitated with anti-JAK2 antibody from Santa Cruz Biotechnology in non-denaturing conditions and immunoblotted with antiphosphotyrosine antibody as detailed above. (B) Effect of protein kinase C inhibition and intracellular calcium chelation on tyrosine phosphorylation of JAK2 stimulated by thrombin (1 unit/ml): lane 1, basal; lane 2, 10 μM Ro 31-8220 + 1 unit/ml thrombin; lane 3, 40 μM BAPTA-AM + 1 unit/ml thrombin; lane 4, 1 unit/ml thrombin. Platelets were treated with 10 μM Ro 31-8220 for 1 min or 40 μM BAPTA-AM for 15 min, and then exposed to 1 unit/ml thrombin for 3 min. Immunoprecipitation (with anti-JAK2 serum from UBI) and immunoblotting were carried out as described in part A. (C) Effect of ionophore A23187 and PDBu on tyrosine phosphorylation of JAK2. Washed platelets were incubated for 5 min with the following additions: lane 1, solvent; lane 2, 1 μM PDBu and 2 μM A23187; lane 3, 2 μM A23187; lane 4, 1 μM PDBu. JAK2 was immunoprecipitated from the lysed cells and immunoblotted with antiphosphotyrosine antibody as described in part A. In all cases, similar results were obtained in at least two other experiments.

4. Discussion

A number of studies have recently demonstrated the importance of the JAK family of tyrosine kinases in signal transduction triggered by cytokines and growth factors. In the present study we demonstrate the presence of JAK2 in human platelets and provide the first evidence that JAK2 is phosphorylated downstream of the second messengers DG/PKC and IP_3/Ca^{2+}. The molecular events which underlie JAK2 phosphorylation in platelets are not known, although it has been shown that JAK2 undergoes autophosphorylation in other cells [7–9].

The relatively slow onset in phosphorylation of JAK2 in platelets challenged with thrombin suggests that this kinase is unlikely to participate in the early events associated with platelet activation, such as secretion, shape change and the binding of fibrinogen to its receptor, the integrin $\alpha_{IIb}\beta_3$ (also known as glycoprotein IIb-IIIa). However, JAK2 phosphorylation may play a role in late events, such as cytoskeletal rearrangement or clot retraction, since these are associated with later stages of tyrosine phosphorylation [2]. Further studies are ongoing to establish the role of this protein kinase in platelet activation.

In conclusion, the present study has shown that JAK2 is phosphorylated on tyrosine residues on activation of the phosphoinositide pathway. The demonstration that G protein-coupled receptors are able to induce tyrosine phosphorylation of JAK2 in platelets has important implications for the regulation of gene expression in other cell types.

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