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Hypophosphorylated and inactive Pyk2 associates with paxillin at the microtubule organizing center in hematopoietic cells

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ABSTRACT

Pyk2 is a non-receptor tyrosine kinase that regulates cellular adhesion. We generated antibodies to a peptide corresponding to the N-terminus (NT) of Pyk2 and another to a portion of the C-terminal (CT) domain. Only the CT antiserum recovered paxillin-associated Pyk2. These antibodies recognized overlapping but biochemically distinct molecular species of Pyk2 since the CT antiserum recovered Pyk2 after NT antibody immunodepletion. Furthermore, the CT antibody could not immunoblot NT antibody-captured Pyk2. Phosphorylation partially accounts for the differential binding of these antibodies as dephosphorylation of Pyk2 recovered with the NT antibodies allows for recognition by the CT antibody. Additionally, Pyk2 recovered with the NT antibody displays increased serine/threonine phosphorylation. We suggest that the NT epitope is inaccessible to the antibody because Pyk2 is in a closed confirmation in association with paxillin. Upon induction of serine and/or threonine phosphorylation of Pyk2, it opens to a confirmation that allows for antibody binding to the NT epitope but at the same time no longer binds paxillin or the CT antiserum. These antibodies also display differential staining of Pyk2 in both T cells and macrophages. Pyk2 recognized by the CT antibody, but not the NT antibody, colocalized with paxillin at the microtubule-organizing center (MTOC). The MTOC-bound Pyk2 was not tyrosine phosphorylated upon T cell activation. We hypothesize that a reservoir of primarily inactive Pyk2 associates with paxillin at the MTOC, which may allow for rapid delivery of Pyk2 to specific sites of adhesion.

1. Introduction

Pyk2 is a non-receptor tyrosine kinase expressed in numerous cell types but which is highly expressed in hematopoietic and neuronal cells. It is closely related to focal adhesion kinase (FAK); they share approximately 45% amino acid identity and 65% similarity [1–5]. These proteins have similar domain structure with an N-terminal divergent FERM (Protein 4.1, Ezrin, Radixin, Moesin) domain, a centrally located kinase domain, two proline-rich regions in the C-terminus, and a FAT (focal adhesion targeting) domain within the extreme C-terminus [6–8]. These proteins also have four conserved tyrosine residues that can become phosphorylated upon activation. Tyrosine 402 of Pyk2 is located in the linker between the FERM and kinase domains and serves as an autophosphorylation site [9–12]. Two tyrosine residues, Y579/Y580 in Pyk2, are located within the activation loop of the catalytic domain and function to enhance catalytic activity upon phosphorylation [11,12]. The fourth tyrosine residue, Y881 in Pyk2, is located in the C-terminus and has been implicated in recruiting the adaptor protein Grb2 upon phosphorylation [10]. The current model for activation of Pyk2 is that upon stimulation it first becomes autophosphorylated, providing a docking site for Src family kinases (SFK) [6,9,10]. The recruited SFK then phosphorylates additional tyrosine residues within Pyk2 thus enhancing catalytic activity and providing new docking sites for SH2 domain-containing proteins [7,12].

It is not entirely clear how Pyk2 kinase activity is regulated, however structural studies have revealed that FAK undergoes autoinhibition by binding of the FERM domain to the kinase domain [13,14]. The mechanism(s) that relieve the autoinhibition are not known but may include protein binding, phosphorylation or sumoylation [15]. Given the high degree of sequence similarity between FAK and Pyk2, it is likely that Pyk2 would be similarly regulated, however strong support for this molecular inhibition is currently lacking. Further studies are required to understand the contribution, if any, of the FERM domain to Pyk2 regulation.

The regions of highest homology between FAK and Pyk2 exist within the kinase domain (~60%) [1–4] and the FAT domain (~61%) [4,16]. High homology within the FAT domain led to speculation that Pyk2 would bind the cytoskeletal adapter protein paxillin as it had been shown that this region was responsible for FAK binding to

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paxillin [17–19]. Indeed, earlier work demonstrated that paxillin coimmunoprecipitated with Pyk2 and that the association was constitutive [20–23]. The interaction was later confirmed to occur specifically within the FAT domain of Pyk2 as fusion proteins that were lacking part of the FAT domain failed to bind paxillin [24].

While the association between Pyk2 and paxillin is known to occur, it is not known if this association is regulated and where the association occurs within non-adherent cells. We generated antibodies to two distinct regions of Pyk2 and found that they recover overlapping, but biochemically distinct, Pyk2 populations in both T cells and macrophages. One population of Pyk2 associated with paxillin while the other displayed hyperphosphorylation on serine and/or threonine residues suggesting that Pyk2 is found in multiple conformational states that depend on serine and/or threonine phosphorylation of the molecule. We further examined the colocalization of Pyk2 and paxillin and found that the population of Pyk2 that strongly associates with paxillin does so at the microtubule-organizing center (MTOC) of hematopoietic cells. These studies suggest that a reservoir of inactive Pyk2, in association with paxillin, localizes at the MTOC in resting and activated T cells.

2. Materials and methods

2.1. Cell culture

The murine $CD8^+$ cytotoxic T lymphocyte (CTL) clones AB.1 and Cl 11 have been described previously [25,26]. Clones were stimulated weekly with irradiated allogeneic C57BL/6 splenocytes and recombinant IL-2 and were used for experiments 4–6 days after stimulation. Clones were maintained in RPMI supplemented with 10% FCS, L-glutamine, penicillin/streptomycin, non-essential amino acids, sodium pyruvate and β-mercaptoethanol. The L1210 and $L1210^{Kb/Dd}$ cell lines were a gift from Dr. K.P. Kane (University of Alberta, Edmonton, AB) and were cultured in DMEM with 8% defined bovine calf serum [27]. The RAW 264.7 macrophage cell line was purchased from Sigma-Aldrich (Mississauga, ON) and was maintained in RPMI supplemented with 10% FCS and penicillin/streptomycin. NIH 3T3 cells were obtained from Dr. J.C. Stone (University of Alberta, Edmonton, AB) and were maintained in DMEM supplemented with 8% FCS.

2.2. Antibodies and reagents

The source and purification of antibodies from hybridomas producing 145-2C11 (anti-CD3ε) and PY72.10.5 (anti-phosphotyrosine) have been described previously [22]. The polyclonal anti-Pyk2 antibodies F298 and F245 were generated in our laboratory by injecting New Zealand White rabbits with a peptide corresponding to amino acids 2–12 (F298) or 720–826 fused to GST (F245), and have been described previously [28] and have been used in additional studies [28,29]. Monoclonal antibodies specific for Pyk2, paxillin and phosphoserine/threonine were purchased from BD Biosciences (Mississauga, ON). Anti-Pyk2 (C-19 and N-19), and anti-goat IgG-HRP (horseradish peroxidise) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-tubulin (mouse monoclonal) and anti-rabbit-Alexa Fluor 488 were purchased from Molecular Probes (Eugene, OR). Anti-α-tubulin (rabbit polyclonal) was purchased from Abcam Inc (Cambridge, MA). Goat anti-hamster IgG, anti-mouse IgG-HRP, anti-mouse-rhodamine, anti-rabbit-Texas red and streptavidin-DTAF were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Protein A-HRP was purchased from Pierce (Rockford, IL). Protein A Sepharose was obtained from Amersham Biosciences (Piscataway, NJ). CIAP was acquired from Invitrogen (Carlsbad, CA). Ionomycin was purchased from Calbiochem (San Diego, CA). Colchicine and propyl gallate were purchased from Sigma (St. Louis, MI). Protease inhibitors were obtained from Roche (Indianapolis, IN). The pC1-Neo vector was obtained from Promega (Madison, Wisconsin) and Effectene from Qiagen (Mississauga, ON).

2.3. CTL stimulation with anti-CD3

Anti-CD3 was immobilized by incubating 60 mm non-tissue culture-treated plates with 10 μg/ml 145-2C11 overnight at 4 °C. The plates were washed twice with Dulbecco's phosphate buffered saline (PBS), blocked with 2% BSA in PBS for 30–60 min at 37 °C, and then washed three times with PBS prior to use for stimulation of CTL. Cloned CTL were harvested and washed three times with PBS (Life Technologies) then resuspended at 2×10^7 cells/ml PBS or RPMI and were added to BSA- or 145-2C11-coated dishes and incubated at 37 °C for 20 min time. Cells were lysed in the dishes by incubation at 4 °C in lysis buffer (1% NP-40, 10 mM Tris, 5 mM EDTA, 150 mM NaCl, 1 mM orthovanadate) for 20 min. Lysates were removed from the dishes and post-nuclear lysates were used for immunoprecipitation. A sample of post-nuclear lysate containing 2.7×10^5 cell equivalents was saved for further analysis.

2.4. Cell stimulation with ionomycin

Cells were harvested, washed and resuspended at 1×10^7 cells/ml PBS and chilled on ice. Ionomycin or DMSO (carrier control) was added and the cells were incubated at 37 °C for 10 min. After 10 min, the cells were pelleted and lysed in 1% NP-40 lysis buffer for 20 min on ice. Post-nuclear lysates were then ready to use for immunoprecipitation. A sample of post-nuclear lysate containing 4×10^5 cell equivalents was saved for further analysis.

2.5. Immunoprecipitation

Post-nuclear 1% NP-40 cell lysates containing 1×10^7 cell equivalents were incubated with anti-Pyk2 antisera (F298 or F245) or other indicated antibody for 15 min on ice. Protein A Sepharose was then added (30 μl of a 50% slurry) and samples were rotated at 4 °C for approximately 2 h. Beads were pelleted and washed three times with lysis buffer and resuspended in Laemmli reducing sample buffer and boiled for 3 min. For sequential Pyk2 immunoprecipitates, post-nuclear lysates were subjected to immunoprecipitation with the first Pyk2 antiserum (either F298 or F245) for 12 h at 4 °C at which time, the beads were pelleted and saved as the first immunoprecipitate and the supernatant was transferred to a new tube. Beads from the first immunoprecipitation were washed three times with lysis buffer containing 0.1% SDS, resuspended in Laemmli reducing sample buffer, and boiled for 3 min. The supernatant from the first immunoprecipitation was then subjected to a second round of immunoprecpitation with the second Pyk2 antiserum (either F298 or F245) for 12 h at 4 °C. Beads were washed and resuspended as described above, and the procedure repeated as indicated. For alkaline phosphatase treatment, Pyk2 immunoprecipitates were prepared by incubation with lysate for 2 h then washed three times with lysis buffer. Beads were resuspended in 70 μl of water and 10 μl of 10× reaction buffer (provided with enzyme) was added along with 20 μl of CIAP (20 units) or dilution buffer. Reactions were allowed to proceed at 37 °C for 3 h with occasional agitation. After 3 h, beads were pelleted, washed twice with lysis buffer, resuspended in Laemmli reducing sample buffer, and boiled.

2.6. Western blotting

Total cell lysates or immunoprecipitates were loaded onto SDS-PAGE gels. The gels were then transferred to polyvinylidene difluoride (PVDF) membranes. Immunoblotting was performed using the appropriate primary and HRP-coupled secondary antibodies and was visualized by enhanced chemiluminescence (ECL) (PerkinElmer