Monocyte chemoattractant protein 1 causes differential signalling mediated by proline-rich tyrosine kinase 2 in THP-1 cells

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Monocyte chemoattractant protein 1 (MCP-1) has a crucial role in atherogenesis and inflammation. However, MCP-1-mediated signalling pathways in monocytes have not been fully elucidated. In the present study we investigated the role of tyrosine kinases such as proline-rich tyrosine kinase 2 (Pyk2) in MCP-1-mediated signal transduction in the monocytic cell line THP-1. Pyk2 was tyrosine phosphorylated very quickly after stimulation with MCP-1. We found that Lyn, Shc and paxillin were also tyrosine phosphorylated by MCP-1. We examined the association of these molecules by immunoprecipitation and immunoblot analysis. The association of Pyk2 with Lyn was dependent on stimulation with MCP-1 and on tyrosine phosphorylation of Pyk2. Phosphorylation of p38 was also dependent on tyrosine phosphorylation of Pyk2. However, the association of Pyk2 with paxillin and Grb2 was not affected by stimulation with MCP-1. Phosphorylation of ERK (extracellular-signal-regulated protein kinase) was not affected by overexpression of kinase-negative Pyk2. Our results indicate that Pyk2 forms a complex with paxillin, Grb2 and Lyn in THP-1 cells. However, Pyk2 is not always involved in MCP-1-mediated signalling pathways.

Key words: chemokine receptor 2, ERK, G-protein-coupled receptor, p38.

INTRODUCTION

Chemokines (chemotactic cytokines) are small basic peptides that induce the directed migration and activation of leucocytes [1,2] and are thought to be important in controlling inflammation. There are two major families of chemokines, termed CC and CXC, which differ in the positions of the first two of four conserved cysteine residues. Monocyte chemoattractant protein 1 (MCP-1) is a member of the CC family and is a potent agonist for monocytes, T lymphocytes, natural killer cells and basophils. MCP-1 binds to and activates a seven-transmembrane domain receptor known as CC chemokine receptor 2 (CCR2) [3].

MCP-1 has been implicated as an important factor in mediating monocytic infiltration in early atherosclerosis as well as in a number of chronic inflammatory diseases [4]. In the initial stage of atherosclerosis, monocytes migrate into the subendothelial space along a concentration gradient of chemoattractants, where they are differentiated into macrophages and become foam cells after taking up modified lipoproteins. Recent studies have shown that both CCR2-deficient mice crossed with apolipoprotein E knock-out mice and MCP-1-deficient mice crossed with apolipoprotein B transgenic mice have a decreased formation of atherosclerotic lesions [5,6]. These results suggest that MCP-1-mediated chemotaxis has a crucial role in the initiation of atherosclerosis.

However, the signal transduction mechanism leading to MCP-1-induced chemotaxis has not been fully characterized. We have previously demonstrated that the $\beta\gamma$ subunit of heterotrimeric G protein, G_i, has a key role in MCP-1-induced chemotaxis [7]. In that study we suggested that the activation of extracellular-signal-regulated protein kinase (ERK) is not involved in chemotaxis by MCP-1. However, little is known about the role of tyrosine kinases other than mitogen-activated protein (MAP) kinase in chemotaxis.

Protein tyrosine kinases (PTKs) transduce key extracellular signals that trigger various biological events such as cytoskeletal rearrangement and mitogenesis. PTKs transmit signals to the nucleus by making a complex with other signalling molecules such as SH2–SH3 adaptor molecules. Among the PTKs, focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2) are structurally related tyrosine kinases, which exhibit approx. 48 % amino acid identity [8]. FAK is localized mainly in focal contacts, whereas Pyk2 exists mainly in the cytoplasm [9]. Pyk2 is abundantly expressed in haemopoietic cells and in the brain. We therefore chose to focus on the role of Pyk2 in chemokine-mediated signal transduction.

Recent studies have shown that Pyk2 is activated by stimuli that increase intracellular Ca^{2+} concentrations by various agonists such as growth factors and cytokines [10]. Pyk2 is also associated with other signalling molecules such as Src-family kinases and paxillin [11,12]. The activation of MAP kinase through G-protein-coupled receptors is mediated by Pyk2 [12,13]. However, the role of Pyk2 in chemokine-mediated signalling in monocytic cells is not fully understood. We therefore examined the role of Pyk2 in MCP-1-mediated signalling in the monocytic cell line THP-1.

MATERIALS AND METHODS

Reagents and materials

MCP-1 was obtained from Pepro Tech EC (London, U.K.). PMA was obtained from Sigma (St Louis, MO, U.S.A.). LIPOFECTAMINE and Opti-MEM were purchased from Life Technologies (Rockville, MD, U.S.A.). DMEM (Dulbecco's modified Eagle's medium) and RPMI 1640 medium were obtained from Nissui Pharmaceuticals Co. (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Intergen (Grand Cayman,

Abbreviations used: CCR, CC chemokine receptor; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated protein kinase; FAK, focal adhesion kinase; FCS, fetal calf serum; GFP, green fluorescent protein; HA, haemagglutinin; MAP, mitogen-activated protein; MCP-1, monocyte chemoattractant protein 1; PTK, protein tyrosine kinase; Pyk2, proline-rich tyrosine kinase 2.

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British West Indies). L-Glutamine and penicillin/streptomycin were obtained from Bio Whittaker (Walkersville, MD, U.S.A.). Anti-Pyk2 antibody (N19), anti-Hck antibody, anti-Lyn antibody and anti-ERK1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-phosphotyrosine antibody (4G10) and anti-Grb2 antibody were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-(phospho-ERK), anti-(phospho-p38), anti-ERK and p38 antibodies were purchased from New England Biolabs (Beverly, MA, U.S.A.). Anti-Shc antibody was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-Pyk2 phosphospecific antibodies pY402, pY580 and pY881 were obtained from Biosource International (Camarillo, CA, U.S.A.). Anti-haemagglutinin antibody (12CA5) and Protein G–agarose were obtained from Roche (Mannheim, Germany). Anti-paxillin antibody was kindly provided by Dr H. Sabe (Osaka Bioscience Institute, Osaka, Japan).

DNA constructs

The cDNA for Pyk2 was cloned in our laboratory. Total RNA from Jurkat cells was used to prepare cDNA by using reverse transcriptase in accordance with the manufacturer's instructions. The cDNA was amplified by PCR with eight degenerate oligonucleotide primers corresponding to amino acid sequences of human Pyk2. This cDNA was subcloned into the pEGFP vector (Clontech Laboratories, Palo Alto, CA, U.S.A.). A kinase-negative mutant of Pyk2 was constructed by replacing Lys-457 with Ala. The cDNA for CCR2B was cloned as previously described [14]. The cDNA encoding Lyn was a kind gift from Dr T. Yamamoto (University of Tokyo, Tokyo, Japan). The haemagglutinin (HA)-tagged ERK2 construct was provided by Dr H. R. Bourne (University of California, San Francisco, CA, U.S.A.). The Myc-tagged p38 construct was a gift from Dr E. Nishida (Kyoto University, Kyoto, Japan).

Cell culture and transfection

THP-1 cells were provided from K. Nishida (Daiichi Pharmaceuticals Co., Tokyo, Japan) and were cultured in RPMI 1640 medium supplemented with 10% (w/v) FCS, 100 i.u./ml penicillin and 100 μg/ml streptomycin at 37 °C in air/CO_2 (19:1). COS-7 cells were obtained from the American Type Tissue Culture Collection (Manassas, VA, U.S.A.) and were grown in DMEM supplemented with 10% (v/v) FCS, 100 i.u./ml penicillin and 100 μg/ml streptomycin at 37 °C in air/CO_2 (19:1). For transient transfection of COS-7 cells, cells were transfected with the same quantity of expression vectors by using LIPOFECTAMINE in accordance with the manufacturer's instructions.

Stimulation of cells

THP-1 cells were serum-starved overnight at 37 °C in FCS-free RPMI 1640 medium supplemented with 0.1% BSA. The cells were washed once with RPMI 1640 supplemented with 0.1% BSA, resuspended at 3×10^7/ml in the same medium, and cultured for 4 h at 37 °C. The cells were stimulated for the indicated durations with 25 nM MCP-1 or 1.6 μM PMA at 37 °C. After stimulation, cells were immediately lysed in lysis buffer [10 mM Hepes (pH 7.4)/1% (v/v) Triton X-100/25 mM NaCl/25 mM NaF/5 mM sodium pyrophosphate/2 mM Na_3VO_4/0.5 μg/ml aprotinin/0.5 μg/ml leupeptin/2 mM PMSF]. COS-7 cells were transfected as described above. The cells were starved at 37 °C in DMEM supplemented with 2% (v/v) dialysed FCS. The cells were washed once with serum-free DMEM and were stimulated with 25 nM of MCP-1 at 37 °C for the indicated times. After stimulation, cells were lysed with lysis buffer [10 mM Hepes (pH 7.4)/0.5% (v/v) Triton X-100/25 mM NaCl/25 mM NaF/5 mM sodium pyrophosphate/1 mM Na_3VO_4/0.5 μg/ml aprotinin/0.5 μg/ml leupeptin/1 mM PMSF].

Immunoprecipitation and Western blot analysis

The cell lysates were subjected to immunoprecipitation for 2 h at 4 °C with primary antibodies for each experiment. Antibody–antigen complexes were collected by incubation for 45 min with Protein G–agarose at 4 °C. After the beads had been washed twice with lysis buffer, bound proteins were solubilized in SDS sample buffer [50 mM Tris/HCl (pH 6.8)/2% (w/v) SDS/6% (v/v) 2-mercaptoethanol/10% (v/v) glycerol]. The samples were separated on SDS/PAGE and then transferred to nitrocellulose membranes. The membranes were blocked and probed with primary antibody for 2 h at room temperature or 4 °C overnight. Immunoreactive bands were revealed with horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence (ECL®) system (Amersham Pharmacia Biotech).

RESULTS

MCP-1 tyrosine phosphorylates Pyk2 in THP-1 cells

Pyk2 tyrosine phosphorylation is associated with an increase in its kinase activity. To determine the effect of MCP-1 on tyrosine phosphorylation of Pyk2, we stimulated THP-1 cells, which have endogenous CCR2, with 25 nM MCP-1 for the indicated durations. Cell lysates were immunoprecipitated with anti-Pyk2 antibody and subjected to immunoblot analysis with anti-phosphotyrosine antibody (4G10) and anti-Pyk2 antibody. Pyk2 was quickly phosphorylated on tyrosine residues by MCP-1,

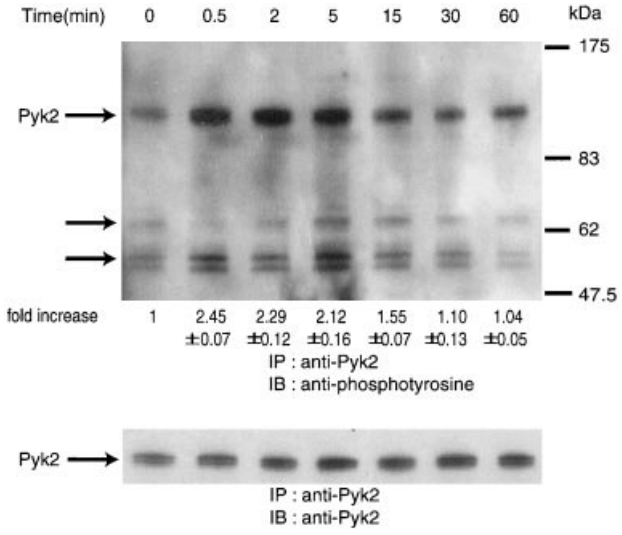

Figure 1 MCP-1 enhances tyrosine phosphorylation of Pyk2 in THP-1 cells

THP-1 cells (1.5×10^7) were serum-starved overnight and then stimulated with 25 nM MCP-1 for the indicated durations. The cell lysates were immunoprecipitated (IP) with anti-Pyk2 antibody. The immune complexes were resolved by SDS/PAGE [8% (w/v) gel] and subjected to serial immunoblotting (IB) with anti-phosphotyrosine antibody (4G10) and anti-Pyk2 antibodies. The bands were analysed with NIH Image; the fold increases in phosphorylation of Pyk2 are shown below the blot (means ± S.D. for three independent experiments).

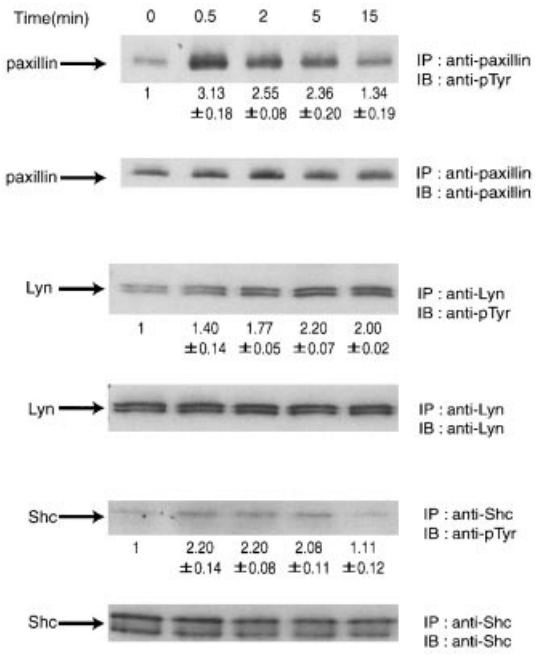

Figure 2 MCP-1 tyrosine phosphorylates paxillin, Lyn and Shc in THP-1 cells

THP-1 cells (1.5×10^7) were serum-starved overnight and then stimulated with 25 nM MCP-1 for the indicated durations. The cell lysates were immunoprecipitated (IP) with anti-paxillin, anti-Lyn and anti-Shc antibodies. The immune complexes were resolved by SDS/PAGE [8% (w/v) gel] and subjected to immunoblotting (IB) with 4G10, anti-paxillin, anti-Lyn and anti-Shc antibodies. The bands were analysed with NIH Image; the fold increases in phosphorylation of paxillin, Lyn and Shc are shown below the blot (means ± S.D. for three independent experiments).

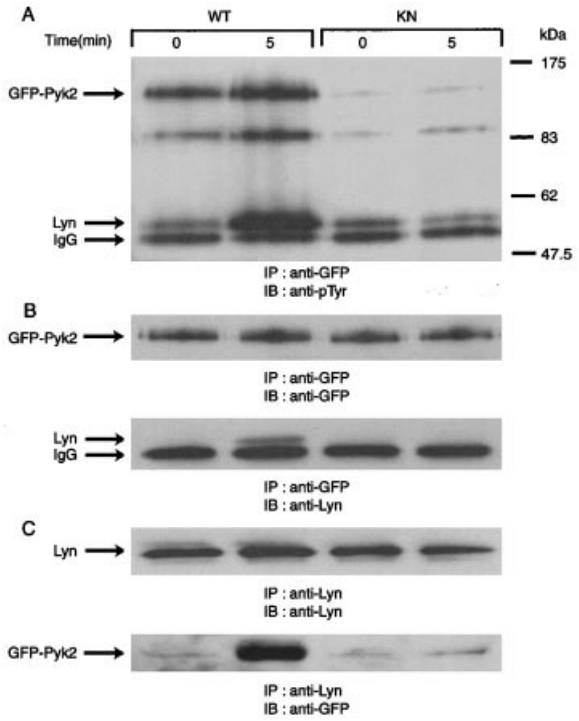

Figure 4 Increased association of Pyk2 with Lyn by MCP-1 is dependent on the kinase activity of Pyk2

COS-7 cells were transfected with 1 μg of enhanced GFP-Pyk2 [wild-type (WT) or kinase-negative mutant (KN)], 2 μg of pCMV1-FLAG/CCR2B and 1 μg of pME-Lyn per well in a six-well dish as described in the Materials and methods section. After incubation at 37 °C for 24 h, the cells were serum-starved overnight, then stimulated with 25 nM MCP-1 for the indicated durations. (**A**, **B**) The cell lysates were immunoprecipitated (IP) with anti-GFP antibody and immunoblotted (IB) with 4G10 (**A**), anti-GFP (**B**) and anti-Lyn (**B**) antibodies. (**C**) The cell lysates were immunoprecipitated with anti-Lyn antibody and immunoblotted with anti-Lyn and anti-GFP antibodies.

with a maximal response at 0.5–2 min (approx. 2.5-fold) (Figure 1). We found that additional proteins at about 70 and 55 kDa, which associated with Pyk2, were also tyrosine phosphorylated.

MCP-1 tyrosine phosphorylates paxillin, Lyn and Shc in THP-1 cells

As possible candidates for the 55–70 kDa protein tyrosine phosphorylated by MCP-1 in THP-1 cells, paxillin, Shc and Src-family kinases were examined to determine whether they were also phosphorylated by MCP-1. After immunoprecipitation with anti-paxillin, Shc or Src kinase antibody, the immunoprecipitates were subjected to immunoblot analysis with 4G10. We found that paxillin and Shc were also tyrosine phosphorylated by

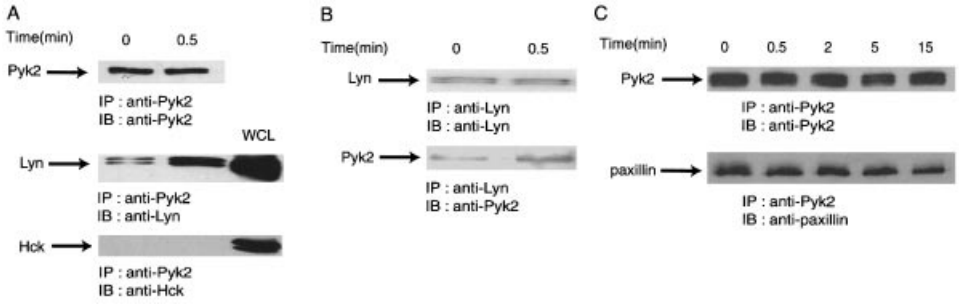

Figure 3 MCP-1 increases association of Pyk2 with Lyn in THP-1 cells

THP-1 cells (1.5×10^7) were serum-starved overnight and then stimulated with 25 nM MCP-1 for the indicated durations. (**A**) The cell lysates were immunoprecipitated (IP) with anti-Pyk2 antibody. The immune complexes were resolved by SDS/PAGE [8% (w/v) gel] and subjected to serial immunoblotting (IB) with anti-Pyk2, -Lyn and -Hck antibodies, with THP-1 whole cell lysates (WCL) as a control. (**B**) The cell lysates were immunoprecipitated with anti-Lyn antibody. The immune complexes were resolved by SDS/PAGE [8% (w/v) gel] and subjected to serial immunoblotting with anti-Lyn and anti-Pyk2 antibodies. (**C**) The cell lysates were immunoprecipitated with anti-Pyk2 antibody. The immune complexes were resolved by SDS/PAGE [8% (w/v) gel] and subjected to serial immunoblotting with anti-Pyk2 and -paxillin antibodies.

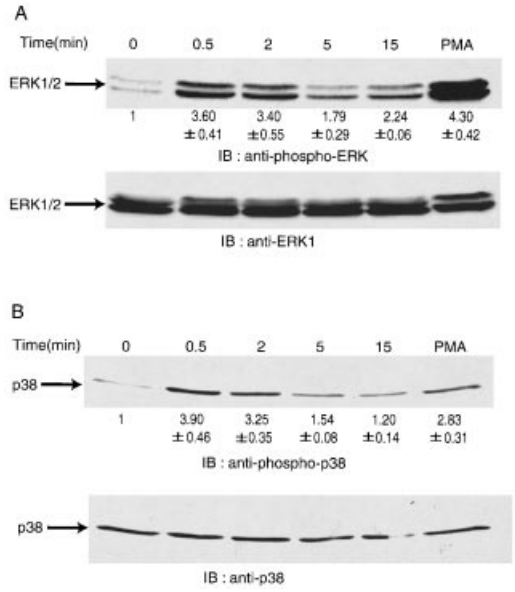

Figure 5 MCP-1 phosphorylates ERK and p38 in THP-1 cells

THP-1 cells (1.5×10^7) were serum-starved overnight and then stimulated with 25 nM MCP-1 for the indicated durations or with 1.6 μM PMA for 15 min. The cell lysates were resolved by SDS/PAGE [10% (w/v) gel] and subjected to serial immunoblotting (IB) with anti-(phospho-ERK), anti-ERK1, anti-(phospho-p38) and anti-p38 antibodies. The fold increases in ERK and p38 activity are shown (means ± S.D. for three independent experiments).

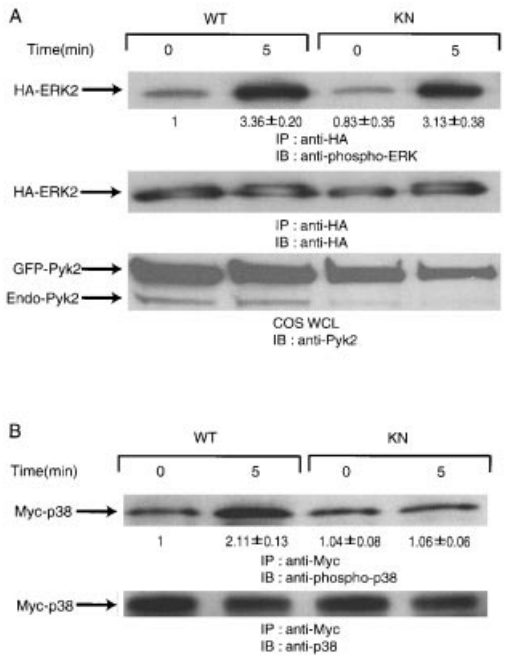

Figure 6 Effect of a kinase-negative mutant of Pyk2 on activation of ERK and p38 by MCP-1

COS-7 cells were transfected with 1 μg of enhanced GFP-Pyk2 [wild-type (WT) or kinase-negative mutant (KN)], 2 μg of pCMV1-FLAG/CCR2B and 1 μg of pCMV1-HA/ERK2 or pcDL-SRα-myc/p38 per well of a six-well dish as described in the Materials and methods section. After incubation at 37 °C for 24 h, the cells were serum-starved overnight and then stimulated with 25 nM MCP-1 for the indicated durations. (**A**) The cell lysates were immunoprecipitated (IP) with anti-HA antibody (12CA5). The immune complexes were resolved by SDS/PAGE [10% (w/v) gel] and subjected to serial immunoblotting (IB) with anti-(phospho-ERK) antibody and 12CA5. The cell lysates were also subjected to immunoblotting with anti-Pyk2 antibody. Abbreviations: Endo-Pyk2, endogenous Pyk2 of COS-7 cells; WCL, whole cell lysates. (**B**) The cell lysates were immunoprecipitated with anti-Myc antibody. The immune complexes were resolved by SDS/PAGE [10% (w/v) gel] and subjected to serial immunoblotting with anti-(phospho-p38) and anti-p38 antibodies. The fold increases in ERK and p38 activity are shown (means ± S.D. for three independent experiments).

MCP-1; this phosphorylation peaked at 0.5 min and increased by approx. 3-fold and 2-fold respectively (Figure 2). The phosphorylation of the p46 isoform of Shc is very weak at the basal level, and MCP-1 slightly increased its phosphorylation, as shown in Figure 2. The results indicate that the p52 isoform might have a major role in signalling in THP-1 cells.

Association of Pyk2 with Lyn is enhanced by MCP-1

Among the Src-family kinases that we studied, Lyn was the only member that showed increased phosphorylation and increased association with Pyk2 in response to MCP-1 in THP-1 cells (Figures 2, 3A and 3B). However, the phosphorylation of Lyn peaked later than that of the other two molecules. Although Hck was expressed in THP-1 cells, we did not find any increase in association of Hck with Pyk2. The immunoprecipitates with anti-Pyk2 antibody were also subjected to immunoblot analysis with anti-paxillin antibody. We found that the association of Pyk2 with paxillin was not changed (Figure 3C). These results indicate that MCP-1 increased the phosphorylation of Lyn and paxillin associated with Pyk2, despite the fact that the association of Pyk2 with paxillin was not changed. It is therefore conceivable that the 70 and 55 kDa tyrosine-phosphorylated proteins that associates with Pyk2 were paxillin and Lyn respectively.

Lyn is downstream of Pyk2 in MCP-1-mediated signalling

To determine whether the activation of Lyn is dependent on the kinase activity of Pyk2, we transiently transfected COS-7 cells with cDNA encoding CCR2B, green fluorescent protein (GFP)-tagged Pyk2 (wild-type or kinase-negative mutant) and Lyn. After stimulation of the cells with MCP-1, cell lysates were immunoprecipitated with anti-GFP or anti-Lyn antibody, followed by immunoblot analysis with 4G10, anti-GFP and anti-Lyn antibodies. MCP-1 treatment increased the phosphorylation of Pyk2 in the cells transfected with wild-type Pyk2, but not in those with kinase-negative Pyk2 (Figure 4A). The phosphorylation of Lyn associated with Pyk2 was also enhanced by MCP-1. Immunoprecipitates with anti-GFP antibody showed an increased association of Pyk2 and Lyn when the cells were transfected with wild-type Pyk2, but not with kinase-negative mutant Pyk2 (Figure 4B). This result was confirmed in the experiment with immunoprecipitates with anti-Lyn antibody. Immunoprecipitates with anti-Lyn antibody showed an increased association of Lyn with Pyk2 when the cells were transfected with wild-type Pyk2, but not with kinase-negative mutant Pyk2 (Figure 4C). These results indicate that Pyk2 is upstream of Lyn in MCP-1-mediated signalling.

Pyk2-dependent and Pyk2-independent activation of MAP kinase by MCP-1

We have already shown that ERK is activated by MCP-1 in the pre-B cell line expressing CCR2B [7]. We therefore examined whether MCP-1 can activate ERK as well as p38 in THP-1 cells expressing endogenous CCR2B. As shown in Figure 5, ERK and p38 were rapidly phosphorylated on treatment with MCP-1 in THP-1 cells. Phosphorylation of these MAP kinases peaked at

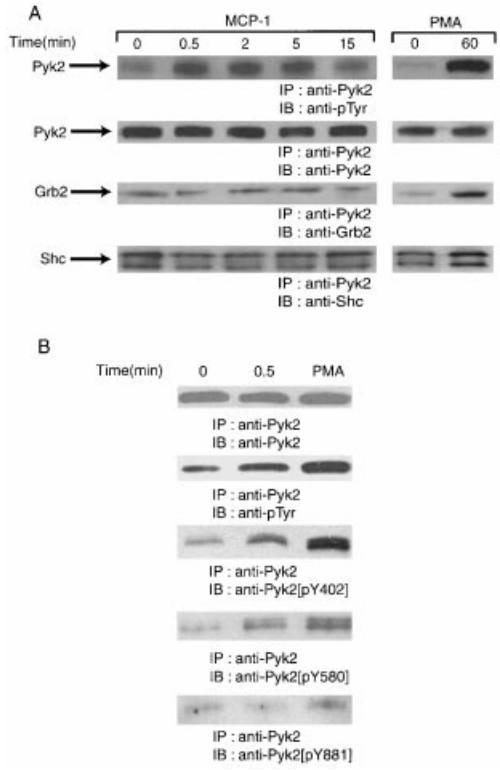

Figure 7 MCP-1 induces phosphorylation of Tyr-402 and Tyr-580, but not Tyr-881, of Pyk2 in THP-1 cells

THP-1 cells (1.5×10^7) were serum-starved overnight and then stimulated with 25 nM MCP-1 for the indicated durations or with 1.6 μM PMA for 60 min. (**A**) The immunoprecipitates (IP) with anti-Pyk2 antibody were immunoblotted (IB) with anti-Pyk2, anti-Shc and anti-Grb2 antibodies. (**B**) The cell lysates were immunoprecipitated with anti-Pyk2 antibody. The immune complexes were resolved by SDS/PAGE [6% (w/v) gel] and subjected to serial immunoblotting with anti-Pyk2, 4G10 and anti-Pyk2 phosphospecific antibodies (pY402, pY580 and pY881).

0.5–2 min by approx. 4-fold. We next examined whether Pyk2 is also involved in activation by these MAP kinases. COS-7 cells were transiently transfected with cDNA encoding CCR2B, GFP-tagged Pyk2 (wild-type or kinase-negative mutant) and HA-tagged ERK2 or Myc-tagged p38. Cell lysates were immunoprecipitated with 12CA5 or anti-Myc antibody and subjected to immunoblot analysis with the antibodies described in the Figure legends. The phosphorylation of ERK and p38 by MCP-1 peaked at 5 min in COS-7 cells (results not shown). Surprisingly, MCP-1 caused the phosphorylation of ERK even if the cells were transfected with kinase-negative mutant Pyk2 (Figure 6A). This was not because the expression of GFP-Pyk2 was insufficient, because GFP-tagged Pyk2 was expressed much more highly than endogenous Pyk2 in COS-7 cells. In contrast, the activation of p38 by MCP-1 was inhibited by overexpression of kinase-negative mutant Pyk2 (Figure 6B).

Activation of Pyk2 does not lead to increased association with Grb2

We immunoblotted Pyk2 immunoprecipitates with anti-Grb2 antibody because ERK activation is generally mediated through the Grb2–SOS–Ras pathway. However, there was no apparent increase in association between Pyk2 and Grb2 after stimulation with MCP-1 (Figure 7A); neither did the association between Pyk2 and Shc increase after stimulation with MCP-1. In contrast, PMA increased the association of Pyk2 with Grb2 and Shc in THP-1 cells. We also used anti-Pyk2 phospho-specific antibodies to confirm our observation. We found that stimulation with MCP-1 resulted in an increased tyrosine phosphorylation of Tyr-402 and Tyr-580, but not of Tyr-881, of Pyk2 (Figure 7B). This is consistent with the fact that MCP-1 did not enhance the association of Pyk2 with Grb2, because Tyr-881 is considered to be the Grb2-binding site. In contrast, PMA induced the phosphorylation of all three tyrosine residues, 402, 580 and 881, indicating that the association of Pyk2 with Grb2 would be mediated by the phosphorylation of Tyr-881 of Pyk2.

DISCUSSION

Here we have demonstrated that MCP-1 tyrosine phosphorylates Pyk2, Lyn, paxillin and Shc and increases the association of Pyk2 with Lyn, but not with paxillin, Grb2 or Shc, in THP-1 cells. The association of Pyk2 with Lyn is dependent on the kinase activity of Pyk2. We also have found that MCP-1 caused Pyk2-dependent activation of p38 and of ERK in THP-1 cells. These results indicate that MCP-1 induces the phosphorylation of a signalling complex of tyrosine kinases to transmit signals through downstream effectors. However, MCP-1 seems to transmit a Pyk2-dependent and Pyk2-independent signal to downstream effectors.

The most interesting finding in our study is that Pyk2 seems to act as a platform signalling molecule in MCP-1-mediated signalling in THP-1 cells and that an association with Grb2, paxillin and Lyn might have different biological implications. A Src-family member, Lyn, can bind to Pyk2 in THP-1 cells; the association between the two molecules is dependent on the kinase activity of Pyk2 in COS-7 cells, suggesting that Pyk2 is upstream of Lyn in MCP-1-mediated signalling. This is inconsistent with the results of Qian et al. [15], who showed that Pyk2 was not activated by a T-cell receptor in Fyn-deficient cells. However, other studies have shown that the Src family members are upstream of Pyk2 [12]. Previous studies have shown that Pyk2 associates with Src family members through Tyr-402, and we have shown that Tyr-402 is tyrosine phosphorylated by MCP-1. It is therefore conceivable that Pyk2 is upstream of Src-family members in MCP-1-induced signalling in THP-1 cells. Although other Src-family members such as Fyn and Lck have been shown to associate with Pyk2, this is the first report that demonstrates the association of Pyk2 with Lyn in G-protein-coupled receptor ('GPCR') signalling. These results indicate that the association of Pyk2 with Src-family members can be different in different cell types and might be dependent on each upstream signal. It is also possible that the activation of different Src-family members results in differential biological functions.

In contrast, the association of Pyk2 with paxillin and Grb2 is constitutive and is not dependent on the kinase activity of Pyk2. These results are consistent with the result that Tyr-881 of Pyk2, a putative Grb2-binding site, was not tyrosine phosphorylated by MCP-1 and that the activation of ERK by MCP-1 was not inhibited by overexpression of a kinase-negative mutant of Pyk2. These findings were supported by the result with PMA. PMA increased the association of Pyk2 with Grb2 and induced the phosphorylation of Tyr-881 of Pyk2. These results suggest that another signalling molecule is responsible for the Grb2–Ras pathway that leads to ERK activation in MCP-1 signalling. However, this is inconsistent with the results of Dikic et al. [12], who showed that Pyk2 is involved in ERK activation mediated by muscarinic receptors and lysophosphatidic acid receptors. They have shown that both G_q-coupled and G_i-coupled receptors can activate ERK via Pyk2. The reason for this difference is not clear. However, a recent paper [16] has shown that ERK

activation through the T cell receptor is independent of Pyk2 but that the activation of p38 and JNK (c-Jun N-terminal kinase) in response to the same stimulus is mediated by Pyk2. We confirmed their results in COS-7 cells expressing CCR2B. These data suggest that there is differential regulation of MAP kinase through Pyk2 and that this regulation might be dependent on the signal or cell types.

We have shown previously that MCP-1-dependent chemotaxis is dependent on the $\beta\gamma$ subunit of G_i and is not dependent on ERK [7]. Needham and Rozengurt [17] have shown that $G\alpha_{12}$ and $G\alpha_{13}$ can mediate the tyrosine phosphorylation of FAK and paxillin by thrombin. Although we have shown previously that CCR2 can couple with $G_q\alpha$ and $G\alpha_{16}$ [14], in the present study we found that the activation of Pyk2 by MCP-1 is sensitive to pertussis toxin (results not shown). The activation of Pyk2 by MCP-1 is therefore mediated through pertussis-toxin-sensitive $G_i\alpha$.

We have found that Pyk2 associates constitutively with paxillin in THP-1 cells. This is consistent with the previous report by Felsch et al. [18]. Because paxillin is associated with integrins [19], the association of Pyk2 with paxillin might be involved in the outside-in signalling of integrins. However, the role of paxillin in MCP-1-mediated signalling is not yet clear.

Pyk2 has been shown to have two splice-variant forms, one of which is preferentially expressed in haemopoietic cells: Pyk2-H [20]. We have made two antibodies that specifically recognize Pyk2 and Pyk2-H and have found that both isoforms of Pyk2 were tyrosine phosphorylated by MCP-1 in THP-1 cells (M. Yamasaki and H. Arai, unpublished work), suggesting that in THP-1 cells there is no specific role for Pyk2-H.

In summary, our studies indicate that MCP-1 can phosphorylate multiple signalling molecules, such as Pyk2, Shc, MAP kinase, Lyn and paxillin, in THP-1 cells. In this signalling cascade Pyk2 seems to be responsible for complex formation with Lyn, paxillin and Grb2. However, we found that the activation of p38, but not that of ERK, is dependent on the kinase activity of Pyk2, suggesting that association with Pyk2 does not always imply that the signalling is mediated by Pyk2. Guinamard et al. [21] have reported recently that B cells deficient in Pyk2 show a defect in migration. Taken together with our results that ERK is not involved in MCP-1-mediated chemotaxis, Pyk2 and p38 might have a role in MCP-1-mediated chemotaxis. The role of Pyk2 and MAP kinase in monocytes needs to be addressed further.

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