Cell. Mol. Life Sci. 66 (2009) 1974 – 1984
1420-682X/09/111974-11
DOI 10.1007/s00018-009-9057-y

Cellular and Molecular Life Sciences

Research Article

Signalling processes involved in C-peptide-induced chemotaxis of CD4-positive lymphocytes

M. Aleksic[a, †], D. Walcher[a, †], K. Giehl[b], H. Bach[a], M. Grüb[a], R. Durst[a], V. Hombach[a] and N. Marx[a, *]

[a] Department of Internal Medicine II – Cardiology, University of Ulm, Robert Koch Str. 8, D-89081 (Germany), Fax: +49-731-500-45025, e-mail: nikolaus.marx@uniklinik-ulm.de
[b] Department of Pharmacology and Toxicology, University of Ulm (Germany)

Received 22 January 2009; received after revision 23 March 2009; accepted 23 March 2009
Online First 14 April 2009

Abstract. Previous data from our group demonstrated that C-peptide induces chemotaxis of CD4-positive lymphocytes *in-vitro*, mediated by activation of G-protein and PI 3-kinase γ, but additional signalling pathways involved in this process remained unexplored. In the present study we further analyze intracellular signalling pathways which lead to C-peptide-induced CD4-positive lymphocyte migration. We provide evidence that C-peptide-induced chemotaxis of CD4-positive lymphocytes is critically dependent on activation of Src-kinase and RhoA, Rac-1 and Cdc42 GTPases. Furthermore, C-peptide stimulates phosphorylation of PAK, LIMK and cofilin downstream of Rac-1 and Cdc42, leading to cofilin inactivation and actin filament stabilization. In addition, C-peptide induces ROCK kinase activity and MLC phosphorylation downstream of RhoA, thereby stimulating myosin mediated cell contraction. In contrast, C-peptide does not activate ERK1/2, p38 or Akt in CD4-positive lymphocytes. Our data support an active role of C-peptide in CD4-positive lymphocyte chemotaxis and elucidate molecular mechanisms in C-peptide-induced cell migration.

Keywords. C-peptide, CD4-positive lymphocytes, migration, signalling, atherosclerosis.

Introduction.

Patients with insulin resistance and type 2 diabetes mellitus exhibit an increased propensity to develop vascular disease with its sequelae of acute coronary syndrome and stroke. These patients typically show elevated levels of C-peptide, a cleavage product of proinsulin, released into the bloodstream in equimolar concentrations to insulin [1] . Originally, C-peptide has been considered to be biologically inactive, but over the last couple of years several studies suggest that C-peptide may have important physiological functions [2, 3]. A previous study from our group demonstrated colocalisation of C-peptide with intimal monocytes/macrophages and CD4-positive lymphocytes in early arteriosclerotic lesions of diabetic patients as well as chemotactic activity of C-peptide in these cells *in-vitro* [4, 5]. Moreover, we showed that C-peptide colocalizes with vascular smooth muscle cells in the arterial media in lesions of diabetic subjects and also demonstrated that C-peptide induces smooth muscle cell proliferation via activation of Src-kinase, PI-3 kinase and MAP-kinase [6].
These results suggest a potential causal role of C-peptide in early atherogenesis in patients with diabetes and insulin resistance [4, 5]. Although we showed that C-peptide-induced CD4-positive cell

† These two authors contributed equally to this work.
* Corresponding author.

migration is mediated by a yet unidentified G-protein-coupled receptor as well as by activation of phosphoinositide 3-kinase gamma (PI 3-kinase γ) [4], additional signalling pathways involved in this process remained unexplored.

The process of directed migration of leukocytes (chemotaxis) is governed by various chemokines which attract cells to sites of inflammation or developing arteriosclerotic lesions. Binding of chemokines to their respective receptors activates Src-kinases and PI 3-kinases with a subsequent production of phosphatidylinositol(3,4,5)-triphosphate [7], which can activate guanine nucleotide-exchange factors (GEFs) for Rho GTPases [8]. Downstream of these GEFs RhoA, Rac1, and Cdc42, members of the Rho family of small GTPases, play a critical role in coordinating cellular responses required for cell migration [9–11] . These proteins cycle between an active, GTP-bound and an inactive, GDP-bound conformation, where the activation process is controlled by GEFs [1, 8, 12]. In migrating cells, RhoA regulates the formation of stress fibers, focal adhesion complexes and contractile actin myosin filaments, while Rac1 and Cdc42 regulate actin polymerization to form lamelipodia and filopodia, respectively [13, 14]. Once present in its active form, RhoA activates Rho kinase (ROCK), a serine/threonine kinase which stimulates activation of myosin, either directly by phosphorylation of myosin light chain (MLC), or indirectly, by activation of myosin light chain kinase (MLCK) and inactivation of MLC phosphatase [15]. In contrast, activated Rac1 and Cdc42 induce phosphorylation of p21 activated kinase (PAK), thus stimulating its kinase activity [16, 17]. Activated PAK then phosphorylates and activates LIM domain-containing protein kinase (LIM kinase), which results in subsequent phosphorylation of cofilin at Ser3 [18, 19]. This phosphorylation inhibits the actin depolymerizing activity of cofilin, leading to actin filament accumulation at the leading edge of migrating cells.

The MAP kinases ERK1/2 (extracellular signal-regulated kinase 1 and 2) and p38 MAP kinases, as well as Akt kinases are additional downstream targets of the Rho GTPases Rac1 and Cdc42 [20–22]. ERK1/2 and p38 primarily regulate cell proliferation and apoptosis, respectively, but some studies demonstrate their involvement in chemokine-induced cell migration [23–25]. Moreover, Akt, a signalling molecule with anti-apoptotic functions, has also been shown to be involved in cell migration [26, 27].

To date, it is unknown whether such chemokine-activated pathways are involved in C-peptide-induced CD4-positive lymphocyte migration. Therefore, the present study examined the effect of C-peptide stimulation on the activity of signalling proteins that control cytoskeleton rearrangements in the process of CD4-positive lymphocyte migration.

Material and methods

Cells. Human CD4-positive lymphocytes were isolated from freshly drawn blood of healthy volunteers using gradient centrifugation with subsequent magnetic beads (Miltenyi Biotec, Gladbach, Germany) isolation, as previously described [28]. Similar isolation techniques were used to isolate $CD8^+$-, CD4/$CD45RA^+$- and CD4/$CD45RO^+$ cells. After isolation, lymphocytes were cultured in serum-free RPMI media (PAA, Pasching, Austria) for 16 h and then assayed as described below.

***In-vitro* cell migration assay.** CD4-positive lymphocyte chemotaxis was assayed under serum-free conditions in a 48-well microchemotaxis chamber (Neuroprobe, Gaithersburg, Germany) as previously described [4]. In some experiments, CD4-positive lymphocytes were incubated with 50 ng/mL *Clostridium difficile* toxin B (Sigma, St. Lois, MO, USA), 25 μg/mL *Clostridium botulinum* exoenzyme B (Calbiochem, La Jola, CA) or the Src-kinase inhibitor PP2 (Sigma) for 30 minutes before assays were performed.

Immunoblotting. CD4-positive lymphocytes were left untreated or incubated at 37 °C with 10 nmol/L C-peptide (Sigma) or with 200 ng/mL SDF-1α (Upstate, Lake Placid, NY, USA) for times indicated. Cells were lysed in lysis buffer (50 mmol/L Hepes pH 7.4, 150 mmol/L NaCl, 1 % (w/v) NP40, 1 % (w/v) glycerol, 1 mmol/L $MgCl_2$, 1 mmol/L $MnCl_2$, 10 mmol/L NaF, 1 mM Na_3VO_4, 10 μg/μl aprotinin, 10 μg/μl leupeptin, 0.1 mmol/L PMSF). Aliquots of cell lysates were boiled in Laemmli buffer before running on SDS-PAGE. Immunoblotting was performed by running samples on SDS-PAGE with subsequent electrotransfer onto nitrocellulose membranes (Amersham Pharmacia Biotech, Amersham, England), blocking with 5 % skim milk in TBS buffer with 0.1 % Tween 20 for 1 h, and incubating with primary antibody (anti-RhoA was from Santa Cruz Biotechnology [Santa Cruz, CA, USA], anti-Rac1 and anti-Cdc42 were from Upstate [Lake Placid, NY, USA], anti-phospho-LIMK, anti-phospho-PAK, anti-phospho-MLC2, anti-phospho-cofilin, anti-phospho-ERK1/2, anti-phospho-Akt, anti-phospho-Src, anti-Src, anti-phospho-p38 and anti-p38 were from Cell Signalling [Beverly, MA, USA], anti-α-tubulin was from Sigma, anti-ERK1/2 was from Promega [Madison, WI, USA] and anti-AKT1 was from Pharmingen [San Diego, CA, USA])

and 1:2000 dilution of the secondary antibody (anti-goat, anti-rabbit or anti-mouse horseradish peroxidase [DAKO, Glostrup, Denmark]). Development was done by using enhanced chemiluminescence reagents (Pearce, Rockford, IL, USA) according to the manufacturer's specifications.

GTPase activity assays. For detection of GTP-bound Rac1, Cdc42 and RhoA, isolated CD4-positive lymphocytes were treated at 37 ° with 10 nmol/L C-peptide for indicated periods of time. Cells were lysed for Rac1/Ccd42-GTP pull-down experiments in 500 µl Rac1/Cdc42-RIPA buffer (50 mmol/L Tris/HCl, pH 7.2, 150 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 % (v/v) Triton X-100, 0.5 % (w/v) sodium deoxyholate, 10 µg/µl aprotinin, 10 µg/µl leupeptin, 0.1 mmol/L PMSF) or for RhoA-GTP pull-down assays in RhoA-RIPA buffer (50 mmol/L Tris/HCl, pH 7.2, 500 mmol/L NaCl, 10mmol/L $MgCl_2$, 1 % (v/v) Triton X-100, 0.5 % (w/v) sodium deoxyholate, 10 µg/µl aprotinin, 10 µg/µl leupeptin, 0.1 mmol/L PMSF). Cell lysates were cleared by centrifugation in a benchtop centrifuge at 15 800 x *g* for 15 minutes at +4 °C. Lysates were then incubated with 20–40 µg glutathione S-transferase (GST)-fusion proteins immobilized to Glutathione Sepharose 4B beads for 45 minutes at +4 °C. The active, GTP-bound form of Rac1 and Cdc42 was determined by using a GST-PAK fusion proteins and GTP-bound RhoA was detected by using recombinant GST-Rhotekin. GST-PAK and GST-Rhotekin were prepared as previously described [29]. Beads were washed four times with washing buffer (50 mmol/L Tris/HCl, pH 7.2, 150 mmol/L NaCl, 10 mmol/L $MgCL_2$, 1 % (v/v) Triton X-100, 10 µg/µl aprotinin, 10 µg/µl leupeptin, 0.1 mmol/L PMSF). Precipitated proteins were separated on 12 % SDS-polyacrylamide gels, transferred to membrane and detected by using appropriate antibodies.

***In-vitro* kinase assay of ROCK.** CD4-positive lymphocytes were left untreated or incubated at 37 °C with 10 nmol/L C-peptide for indicated periods of time. After washing with PBS cells were lysed in ROCK lysis buffer (50 mmol/L M Tris/HCl pH 7.4, 100 mmol/L NaCl, 10 % (v/v) glycerol, 0.5 % (v/v) Triton X-100, 10 mmol/L $MgCl_2$, 1 mmol/L Na_3VO_4, 10 mmol/L NaF, 10 µg/µl aprotinin, 10 µg/µl leupeptin, 0.1 mmol/L PMSF). Lysates were incubated with anti-ROCK2 antibodies (Santa Cruz, Santa Cruz, CA, USA) for 2 h at +4 °C, followed by incubation with protein-G agarose beads (Santa Cruz, Santa Cruz, CA, USA) for an additional 1 h. Beads were washed twice with ROCK lysis buffer and twice with ROCK kinase buffer (50 mmol/L Tris pH 7.4, 100 mmol/L NaCl, 10 % (v/v) glycerol, 0.05 % (v/v) Triton X-100, 2 mmol/L $MgCl_2$, 2 mmol/L $MnCl_2$, 1 mmol/L Na_3VO_4, 10 mmol/L NaF, 10 µg/µl aprotinin, 10 µg/µl leupeptin, 100 nmol/L PMSF). *In-vitro* kinase assays of precipitated ROCK were performed in 30 µL of ROCK kinase buffer with 1 µg of recombinant MYPT-1 substrate (Upstate, Lake Placid, NY, USA) and 5 µCi of radioactively labelled γ-ATP for 30 minutes at 30 °C. Phosphorylated substrate was separated on 8 % SDS-polyacrylamide gel, transferred to membrane and detected by autoradiography.

Short interference RNA transfection. Short interference RNA (siRNA) targeting Src and control siRNA were obtained from Upstate (Lake Placid, NY, USA). Freshly isolated CD4-positive lymphocytes were transfected using Amaxa nucleofector kit (Amaxa, Cologne, Germany) according to the manufacturer's protocol. After 36 h cells were treated with C-peptide, and immunoblotting for p-MLC as a read-out for migrating cells was performed.

Statistical analysis. Results of the experimental studies are reported as mean ± SD. Differences were analyzed by one-way ANOVA followed by the appropriate post hoc test. $P < 0.05$ was regarded as significant.

Results

C-peptide induces migration of different cell-types of T-cells. First we examined the chemotactic effect of C-peptide on different subtypes of T-cells. C-peptide significantly induced migration of freshly isolated human $CD4^+$- and $CD8^+$-cells as well as naïve (CD4/$CD45RA^+$) and memory T-cells (CD4/$CD45RO^+$) (Fig. 1A). Since CD4-positive lymphocytes represent the most important lymphocyte population in atherogenesis, further experiments were focussed on these cells.

C-peptide activates Src-kinase in CD4-positive lymphocytes. To examine the molecular mechanisms involved in C-peptide-induced CD4-positive lymphocyte migration, we employed an inhibitor of Src-kinase (PP2), to block a crucial upstream signalling molecule involved in cell migration. 30 minutes pretreatment of cells with PP2 significantly reduced C-peptide-induced CD4-positive T-cell migration (Fig. 1B), suggesting the involvement of Src-kinase in these signalling pathways. PP2 did not affect baseline migration or cell viability (data not shown). Given the inhibitory effect of PP2 on C-peptide–induced CD4-positive lymphocyte migration, we next investigated whether C-peptide activates Src-kinase in these cells. As shown in Figure 1C, C-peptide

induced Src phosphorylation in CD4-positive cells significantly within 3 minutes, suggesting that Src-kinase activation is involved in C-peptide–mediated T-cell migration.

C-peptide stimulates CD4-positive cell migration through activation of RhoA, Rac1, and Cdc42. Rho GTPases are important signalling molecules involved in leukocyte migration. To examine the role of Rho GTPases in C-peptide-induced migration of CD4-positive lymphocytes we first performed *in-vitro* chemotaxis assays employing *Clostridium difficile* toxin B, an inhibitor of Rho GTPases. As shown in Figure 2A, *Clostridium difficile* toxin B significantly reduced C-peptide-induced cell migration. In addition, treatment of human CD4-positive lymphocytes with *Clostridium botulinum* C3 transferase, a specific RhoA inhibitor, abolished the effect of C-peptide on cell migration, suggesting that RhoA activation is crucial for this process. Neither *Clostridium difficile* toxin B nor *Clostridium botulinum* C3 transferase affected baseline migration or cell viability (data not shown).

To further investigate the role of these small Rho GTPases upon C-peptide stimulation, we assessed RhoA, Rac1, and Cdc42 activity performing affinity precipitation experiments with GST-Rhotekin or GST-PAK, to which only the active, GTP-bound form of RhoA, or Rac1 and Cdc42, respectively, can bind. Stimulation of cells with C-peptide activated all GTPases, RhoA, Rac1 and Cdc42, in a time-dependent manner with a maximal effect after 3 to 5 minutes of stimulation, declining thereafter (Fig. 2B–D).

C-peptide induces ROCK activation as well as MLC phosphorylation in CD4-positive lymphocytes. Next, we investigated signalling pathways downstream of RhoA. RhoA activation is known to stimulate actin-myosin contractility through activation of ROCK and MLC [15, 30]. Treatment of cells with the specific ROCK inhibitor Y-27632 significantly reduced C-peptide-induced CD4-positive cell migration, indicating that ROCK activity is required for this process (Fig. 3A). Y-27632 did not affect baseline migration or cell viability (data not shown). In addition, stimulation of cells with C-peptide induced ROCK activity as assessed by an *in-vitro* kinase assay (Fig. 3B). Moreover, C-peptide treatment of CD4-positive lymphocytes resulted in phosphorylation of the downstream target MLC (Fig. 3C), suggesting that both, ROCK as well as MLC are involved in C-peptide-induced cell activation. These mechanisms may then lead to myosin contraction induced by activated MLC.

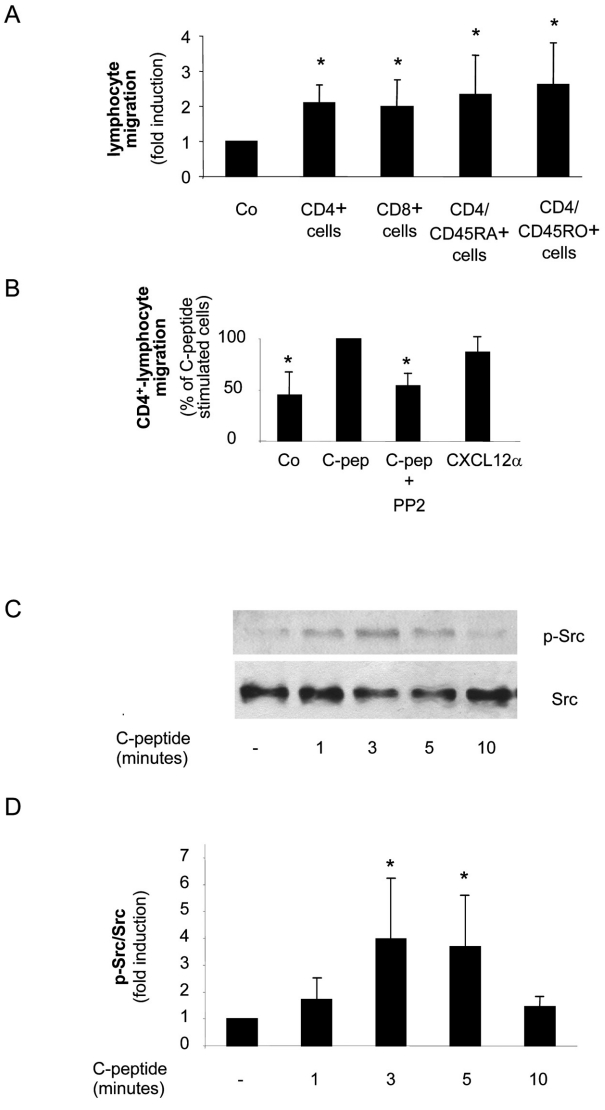

Figure 1. C-peptide induces cell migration in different subtypes of T-cells and activates Src-kinase in CD4-positive lymphocytes. (*A*) Freshly isolated human $CD4^+$- and $CD8^+$-cells as well as naïve ($CD4/CD45RA^+$) and memory-cells ($CD4/CD45RO^+$) were stimulated with C-peptide for three hours and cell migration was assessed by using a modified Boyden chamber. Data are expressed as fold induction compared with unstimulated cells. Bars represent mean±SD ($n = 7$; $*p < 0.05$). (*B*) C-peptide–induced CD4-positive-cell migration involves Src-kinase. CD4-positive lymphocytes were pretreated for 30 minutes with the Src-kinase inhibitor PP2 (1 μM) and then stimulated with C-peptide (10 nmol/L) for three hours and migration assays were performed. Bars represent mean ± SD. * $p < 0.05$ compared with C-peptide-stimulated cells; $n = 5$. CXCL12α served as a positive control. (*C*) CD4-positive lymphocytes were stimulated with C-peptide at 10nmol/L for times indicated before phosphorylation of Src was determined by Western blot analysis (upper panel). Equal loading of intact protein was confirmed by staining for non-phosphorylated Src (lower panel). (*D*) Densitometric analysis of five independent experiments. Data are expressed as p-Src normalized to Src. Bars represent mean ± SD. *$p < 0.05$ compared with unstimulated cells; $n = 5$.

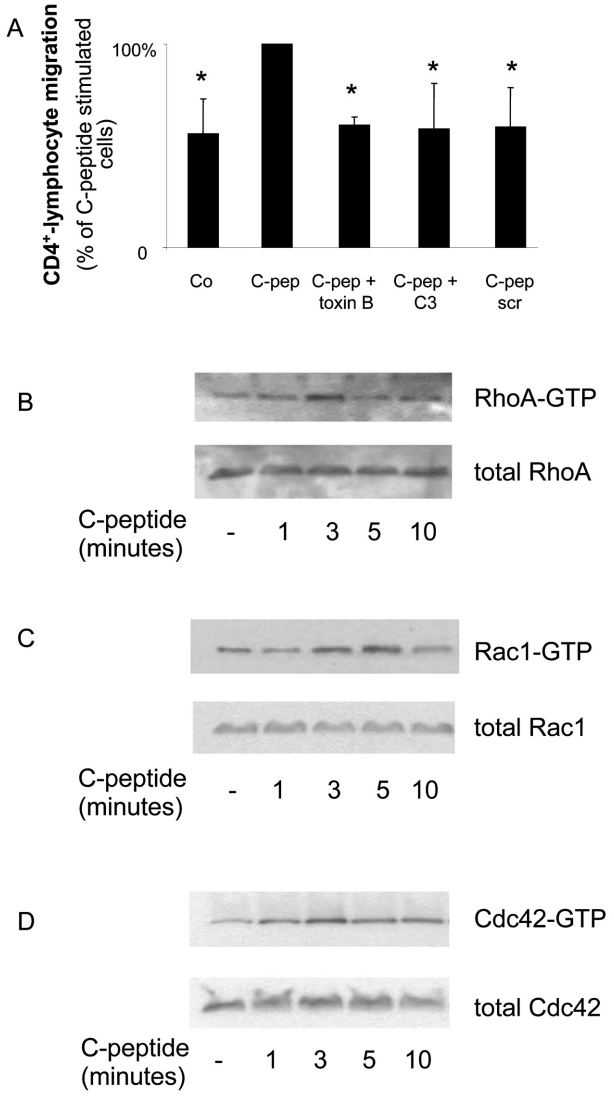

Figure 2. Activation of Rho GTPases is required for C-peptide-induced CD4-positive lymphocyte migration. (*A*) Human CD4-positive lymphocytes were pretreated with 50 ng/mL *Clostridium difficile* toxin B (toxin B) or 25 μg/mL *Clostridium botulinum* C3 transferase (C3) for three hours before stimulation with 10 nmol/L C-peptide (C-pep) for migration experiments. Stimulation of cells with scrambled C-peptide (C-pep scr, 10 nmol/L) served as a negative control. Data are expressed as percent of C-peptide-stimulated cells. Bars represent mean ± SD (n = 13 for toxin B; n = 5 for C3 transferase); * $P < 0.01$ vs. C-peptide-stimulated cells. B–D) C-peptide activates RhoA, Rac1, and Cdc42. CD4-positive lymphocytes were stimulated with 10 nmol/L C-peptide for times indicated. Cells were then lysed and lysates were subjected to affinity precipitation assays to determine RhoA, Rac1, and Cdc42 activity in the presence of GST-Rhotekin (*B*) or GST-PAK (*C* and *D*). Equal loading of protein was confirmed by immunoblotting of aliquots of the cell lysates used in the assays against total RhoA, Rac1, or Cdc42. Three independent experiments yielded similar results.

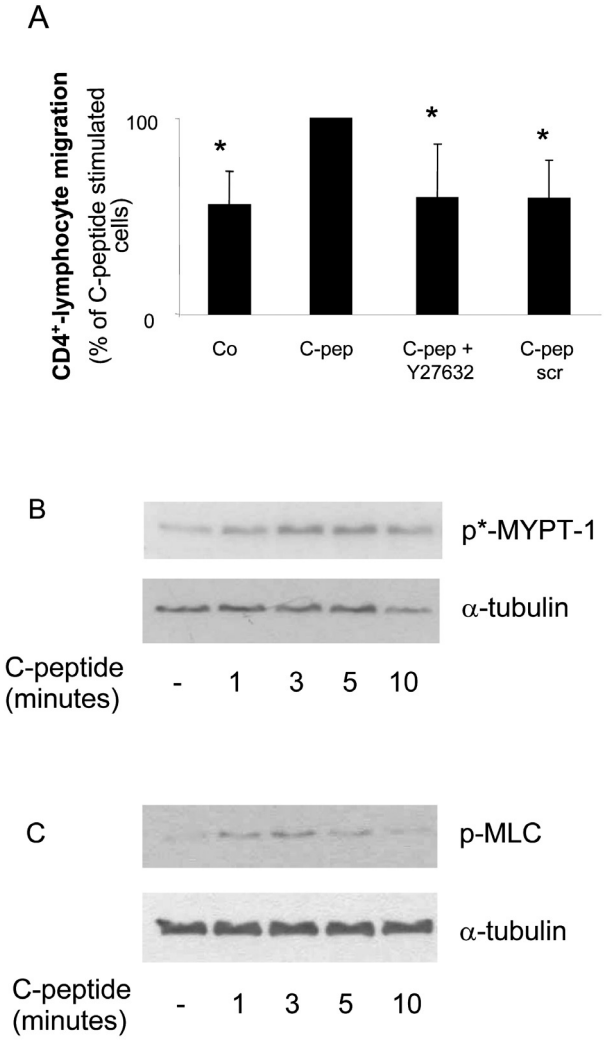

Figure 3. C-peptide stimulates ROCK kinase activity and MLC phosphorylation in CD4-positive cells. (*A*) C-peptide induced CD4-positive migration is dependent on ROCK activation. Freshly isolated human CD4-positive lymphocytes were pretreated with the ROCK inhibitor Y-27632 (1 μM) for three hours before migration experiments using 10 nmol/L C-peptide were performed. Data are expressed as percent of C-peptide-stimulated cells. Bars represent mean ± SEM (n = 6); * $P < 0.01$ vs. C-peptide-stimulated cells. (*B*) C-peptide activates ROCK kinase activity. CD4-positive lymphocytes were stimulated with 10 nmol/L C-peptide for times indicated. Cells were then lysed and the lysates were subjected to affinity precipitation assays to determine ROCK activity using MYPT-1 as a substrate. Equal loading of intact protein was confirmed by staining for α-tubulin in cell lysates. (*C*) C-peptide leads to phosphorylation of MLC. Isolated CD4-positive lymphocytes were stimulated with 10 nmol/L C-peptide for times indicated. Total lysates were analyzed by immunoblotting employing antibodies against phospho-MLC. Equal loading of intact protein was confirmed by staining for α-tubulin. Similar results were obtained in three independent experiments.

C-peptide induces activation of PAK and LIMK leading to cofilin phosphorylation in CD4-positive lymphocytes. Since C-peptide stimulation of CD4-positive cells also activates Rac1 and Cdc42, we then investigated the effect of C-peptide on downstream targets of these GTPases. During chemokine-induced cell migration, activation of Rac1 and Cdc42 has been shown to induce threonine phosphorylation of PAK, a process critical for the maintenance of full PAK

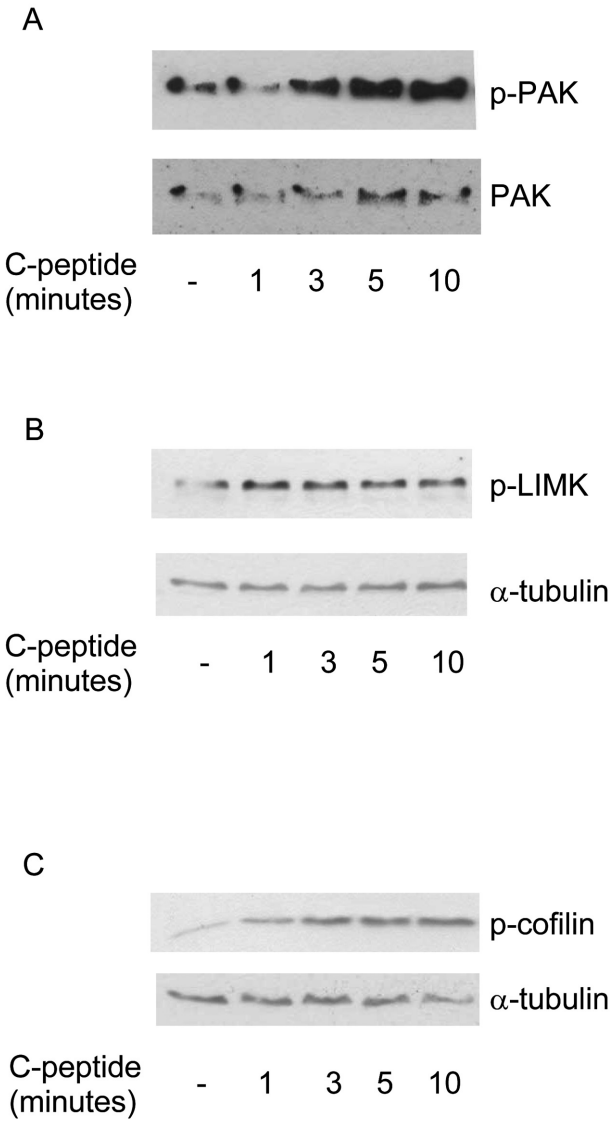

Figure 4. C-peptide induces phosphorylation of PAK, LIMK, and cofilin in CD4-positive lymphocytes. Human CD4-positive lymphocytes were stimulated with 10 nmol/L C-peptide for times indicated, lysed, and aliquots of total lysates were analyzed by immunoblotting using antibodies against phospho-PAK (*A*), phospho-LIMK (*B*), and phospho-cofilin (*C*). Equal loading of intact protein was confirmed by staining for PAK or α-tubulin, respectively. Three independent experiments yielded similar results.

activity [16]. Activated PAK then phosphorylates and activates LIMK, with subsequent phosphorylation and inactivation of cofilin [31]. Stimulation of cells with C-peptide induced PAK phosphorylation within three minutes and this process was prolonged up to 10 minutes after stimulation (Fig. 4A). Treatment of human CD4-positive lymphocytes with C-peptide also led to a time-dependent threonine phosphorylation of LIMK (Fig. 4A) as well as to serine phosphorylation of cofilin (Fig. 4C), thus rendering this mediator of actin filament depolymerization inactive.

C-peptide does not activate ERK1/2, p38 and Akt in CD4-positive lymphocytes. Given the involvement of the MAP Kinases ERK1/2 and p38 in cell migration downstream of Rac1 and Cdc42, we next focused on this signalling pathway. By western blot analyses we demonstrated that treatment of human CD4-positive lymphocytes with C-peptide does not lead to ERK1/2 or p38 phosphorylation (Fig. 5A and 5B). Moreover, C-peptide stimulation of CD4-positive lymphocytes did not result in phosphorylation of Akt, another Rac1/Cdc42 downstream molecule implicated in cell migration (Fig. 5C). These data suggest that ERK1/2, p38, as well as Akt are not involved in C-peptide-induced CD4-positive cell migration.

Transfection with Src siRNA inhibits C-peptide–induced phosphorylation of MLC. Because Src-kinase is the most upstream signalling molecule involved in C-peptide-induced migration, we performed Src siRNA transfection and assessed MLC phosphorylation as a read-out for cell migration after C-peptide treatment. C-peptide stimulation of mock transfected cells significantly increased phosphorylation of MLC whereas transfection of cells with Src siRNA abolished this induction (Fig. 6), underscoring the importance of Src in C-peptide induced CD4-positive lymphocyte migration.

Discussion

For decades C-peptide was considered to be physiologically inert but recent data have demonstrated that C-peptide is a bioactive molecule with various cellular effects [3, 32]. Specific binding of C-peptide to cell membranes at physiological concentrations has been shown [33] but the respective C-peptide receptor remains to be identified. *In-vitro* studies have shown that binding of C-peptide to its receptor activates various signalling pathways in many different cell lines [32]. It has been reported that C-peptide stimulation activates Na^+/K^+-ATPase [34–36], ERK1/2 and JNK kinases [6, 34, 37, 38], p38 [39, 40], PI3-kinase and Akt [6, 38, 41], Src kinase [6] and protein kinase C [38, 41]. C-peptide treatment may also lead to increase in nitric oxide production [40, 42], and intracellular Ca^{2+} concentration [38, 43].

Most of the clinical studies on the physiological function of C-peptide describe its beneficial effects in type 1 diabetic patients. Conventional therapy of these patients involves insulin treatment, but they still develop several complications such as neuropathy,

nephropathy and retinopathy. Additional treatment of such patients with C-peptide improves these microvascular complications [3, 44, 45]. C-peptide is also able to promote disaggregation of insulin oligomers and in that way facilitate the effect of insulin on glucose uptake [46].

Much less is known about effect of C-peptide in type 2 diabetic patients. Previous studies from our group suggest that, in type 2 diabetic patients, C-peptide may have a deleterious effect by promoting development of atherosclerosis [4–6, 47]. These patients usually develop endothelial dysfunction with increased endothelial permeability and C-peptide may enter the vessel wall and build deposits there, as demonstrated in [4]. These C-peptide deposits may contribute to atherogenesis by stimulating monocyte and CD4-positive lymphocyte migration into the vessel wall [4, 5], thus promoting inflammation, a key event in developing atherosclerosis [48]. Interestingly, C-peptide treatment of other cell-types such as neutrophils and B-cells did not show any migratory effect (data not shown). C-peptide may also stimulate smooth muscle cell proliferation in atherosclerotic lesion [6], leading to further atherosclerotic complications. The extent of C-peptide–induced $CD4^+$ cell migration is similar to the effect of the established T-cell chemokine CXCL12. Moreover, combined stimulation of cells with both C-peptide as well as CXCL12 does not lead to a further increase in chemotactic activity, suggesting that these mediators may share similar intracellular signalling pathways. C-peptide's chemotactic effect is mediated by a yet unidentified G-protein-coupled receptor as well as by activation of PI3-kinase γ [4], but additional signalling pathways involved in this process remained unexplored. The present study sheds more light on such pathways by demonstrating that C-peptide stimulation of CD4-positive lymphocytes activates Src-kinase and leads to downstream activation of PI-3 kinase γ. Previous work has shown that Src-kinase is involved in, e. g. IP-10 (interferon-gamma-inducible protein-10) mediated chemotactic response of lymphocytes [7]. A specific inhibitor of Src-kinase, PP2, as well as transfection of Src siRNA, abolished C-peptide induced T-cell migration in our experiments, suggesting that C-peptide also signals through this pathway.

Various chemokines, such as CXCL12α (SDF-1α) and CCL5 (RANTES) stimulate lymphocyte migration through activation of small Rho GTPases. As such, CXCL12α, the best studied CD4-positive lymphocyte chemokine, induces Rac1, Cdc42 and RhoA activation shortly after cell stimulation [49]. Our data, showing a reduction of C-peptide-induced chemotaxis by inhibitors of Rho GTPases as well as the time-dependent activation of RhoA, Rac1, and Cdc42 by C-peptide

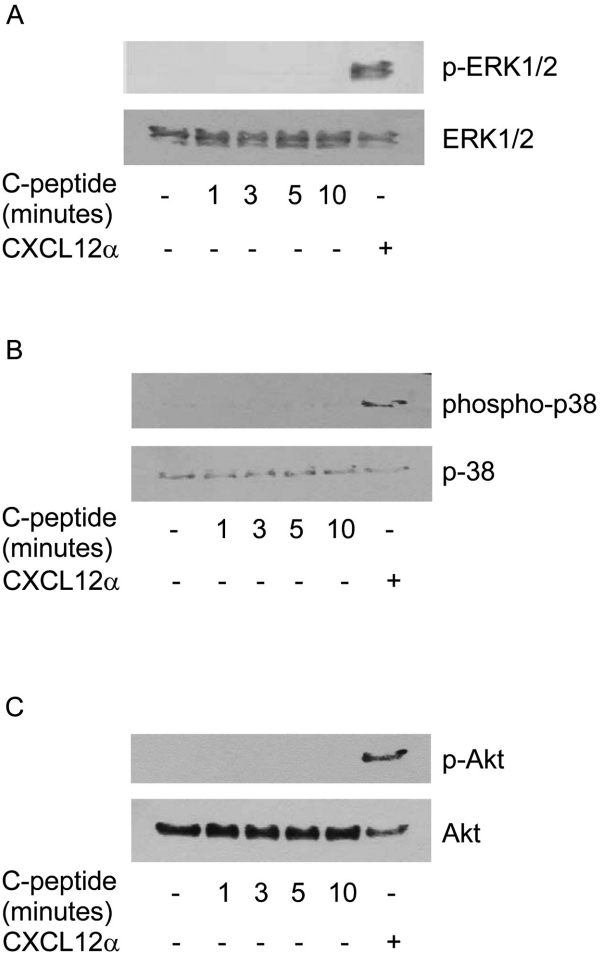

Figure 5. C-peptide does not activate ERK1/2, Akt and p38 in human CD4-positive lymphocytes. (*A–C*) Freshly isolated human CD4-positive lymphocytes were incubated with 10 nmol/L C-peptide or 200 ng/mL CXCL12α for times indicated and phosphorylation of ERK1/2, p38, and Akt was determined by Western blot analysis using specific anti-phospho-ERK1/2, phospho-p38, and phospho-Akt antibodies. For loading control, membranes were reprobed with antibodies against the non-phosphorylated forms of proteins. Blots represent one of three similar experiments.

suggest that similar mechanisms are involved in C-peptide mediated CD4-positive lymphocyte migration. PAKs are important effectors downstream of Rac1 and Cdc42, leading to actin filament stabilization. Once activated, Rac1 and Cdc42 bind to PAKs, relieving their inhibitory constraints, and thus enabling their phosphorylation at threonine residues, leading to PAKs full kinase activity [16]. After downstream activation of LIMK, the final effector molecule in this cascade is cofilin [31, 50, 51]. Cofilin stimulates actin dynamics in two ways: 1) by inducing F-actin fragmentation (severing) to create new free barbed ends for actin polymerization catalyzed by Arp2/3 and, 2) by inducing actin depolymerization from pointed ends of actin filament, thus creating new elements for *de novo* actin polymerization [52, 53]. As

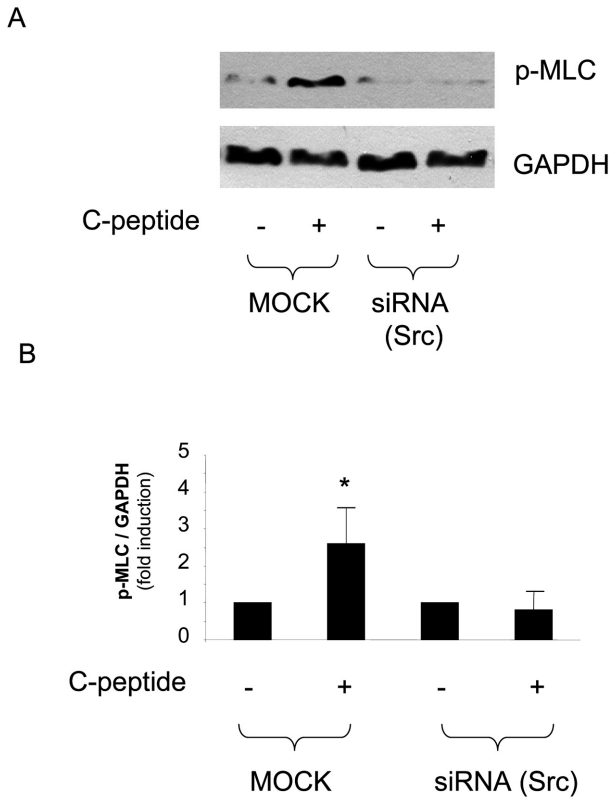

Figure 6. Transfection with Src siRNA inhibits C-peptide–induced phosphorylation of MLC. (*A*) Mock- or Src siRNA-transfected CD4-positive lymphocytes were stimulated with C-peptide (10 nmol/L) for three minutes before Western blot analyses for p-MLC were performed. (*B*) Densitometric analysis of eight independent experiments. Data are expressed as p-MLC normalized to GAPDH. Bars represent mean±SD. $^*P < 0.05$ compared with unstimulated cells, respectively; n = 8.

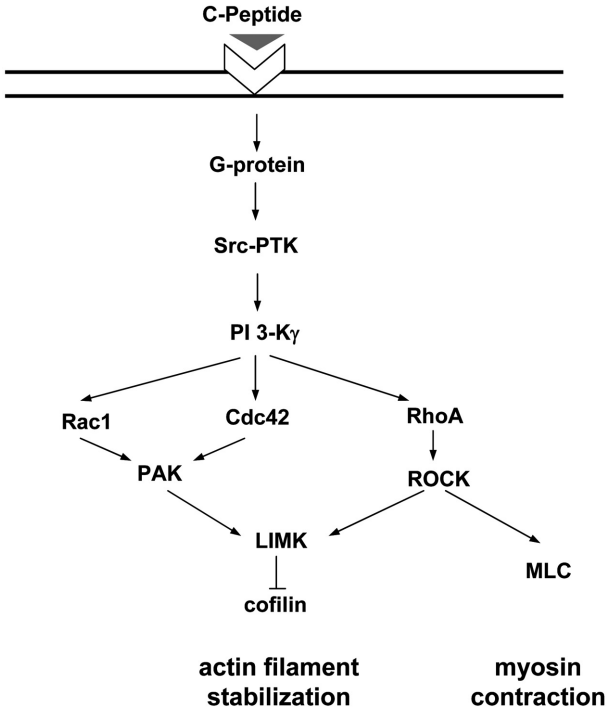

Figure 7. C-peptide-induced signalling pathways in CD4-positive lymphocytes. C-peptide binds to a yet unidentified G-protein-coupled receptor and stimulates Src Kinase with subsequent activation of PI 3-kinase γ. Activation of PI3-kinase leads to an activation of members of Rho GTPase family. Rac1 and Cdc42 act via PAKs to stimulate LIMK, which phosphorylates and inhibits cofilin, a process that allows increased accumulation of polymerized actin at the leading edge of cells. In addition, RhoA stimulates MLC phosphorylation via ROCK activation, a process needed for cell body contraction and for migration.

suggested for CXCL12α [49], chemokine stimulated activity of LIMK and subsequent cofilin inactivation probably increase the rate of cofilin recycling, a critical process for chemokine induced lymphocyte migration. We show that similar pathways seem to be involved in C-peptide-mediated CD4-positive cell migration: C-peptide stimulates phosphorylation of LIMK on threonine residues as well as phosphorylation of cofilin on serine 3 in CD4-positive lymphocytes.

Activation of ROCK and its effector MLC is required for chemokine-induced cell motility downstream of RhoA [15]. ROCK phosphorylates MLC either directly or via activation of MLCK. Active myosin forms contractile force for detachment of migrating leukocytes [54]. Our results indicate that C-peptide-induced migration is mediated via similar pathways, as treatment with C-peptide increases *in-vitro* kinase activity of ROCK and stimulates MLC phosphorylation. A recent study by Lindahl et al. demonstrated that in HEK-293 cells C-peptide becomes internalized and that it can bind to various cytoplasmic proteins, including MLCK [55]. It is possible that this association with MLCK is responsible for the effect of C-peptide on MLC phosphorylation in CD4-positive lymphocytes, but this possibility remains to be tested. Data on the importance of activation of ERK, Akt and p38 kinases for chemokine-induced CD4-positive cell migration are controversial. Some studies have shown that ERK1/2 activation is crucial for CXCL12α-induced CD4-positive lymphocyte migration [24], while others suggest that this MAP kinase pathway may not be involved [56]. Moreover, CXCR3 ligand-activated T-cell chemotaxis is also considered to be independent of ERK and Akt activation [57]. Previous studies employing C-peptide suggest that the effect of C-peptide on Akt, p38 and ERK1/2 activation is cell-type dependent [6, 39, 41, 58]. Our results on phosphorylation of these kinases show that ERK1/2, Akt and p38 are not involved in C-peptide-induced CD4-positive lymphocyte migration. Since our previous studies demonstrated activation of PI 3-kinase γ by C-peptide, the lack of Akt activation, a typical effector of PI 3-kinases, suggests that additional

mechanisms, e. g. phosphorylation by PDK1 [21], may be required to achieve Akt activity in CD4-positive lymphocytes.

Together with other studies describing signalling pathways activated by C-peptide in various cell types (reviewed in [32]) our study describes a unique set of signalling proteins that is activated by C-peptide in CD4-positive lymphocytes. In other cell types C-peptide activates different pathways leading to different functional outcome. These diverse effects of C-peptide might be the consequence of the existence of more than one C-peptide receptor or different mechanism of action in various cells. Recently demonstrated internalization of C-peptide and its localization in the cell nucleus of HEK-293 cells [55] is a good demonstration of its versatile roles in various cells. Much of this controversy will be cleared upon identification of the receptor for C-peptide.

Our results further elucidate the effect of C-peptide on CD4-positive T-cell chemotaxis by describing activated signalling pathways leading to cytoskeleton rearrangements. We demonstrate that C-peptide-induced migration of CD4-positive lymphocytes is mediated by activation of Src-kinase, an upstream signalling-molecule of PI-3 kinase, and the small Rho GTPases RhoA, Rac1, and Cdc42. Subsequently, RhoA and ROCK activation leads to MLC phosphorylation, while activation of Rac1 and Cdc42 results in cofilin inactivation via phosphorylation of PAK and LIMK. Together, these mechanisms mediate cell migration by inducing myosin contraction at the uropod of the cell and actin filament stabilization at the leading edge (Fig. 7). These data underscore the role of C-peptide as a biologically active molecule with potential implications for the activation of CD4-positive lymphocytes during early lesion development in diabetic subjects.

In contrast to the potential harmful effects of elevated levels of endogenous C-peptide in patients with insulin resistance and early type 2 diabetes, clinical studies in type 1 diabetic subjects suggest beneficial effects of C-peptide administration on microvascular complications such as diabetic neuropathy [44, 45, 59]. Still, the potential pro-atherogenic action of C-peptide described in our experiments is not necessarily in contrast to such clinical benefits of C-peptide treatment in patients with type 1 diabetes. The situation of C-peptide in type 1 and type 2 diabetic patients can be compared with the clinical presentation of hypo- and hyperthyroidism. L-thyroxine treatment in patients with hypothyroidism is beneficial without any doubt, but elevated levels of L-thyroxine in those with hyperthyroidism can cause serious clinical manifestations. Similar mechanisms may apply for C-peptide: supplementation of C-peptide in type 1 diabetic patients may be beneficial while an increase in C-peptide levels in patients with insulin resistance and type 2 diabetes may be harmful.

Acknowledgements. This work was supported by grants of the Deutsche Forschungsgemeinschaft (SFB 451, project B9 and B11; MA2047/4–1, WA2145 1–1) to Prof. Dr. Nikolaus Marx and Dr. Daniel Walcher as well as the Else-Kröner-Fresenius-Stiftung to Dr. Daniel Walcher. Mr. Milos Aleksic was funded by the Graduate College GRK 1041.

1 Tager, H. S., Emdin, S. O., Clark, J. L. and Steiner, D. F. (1973). Studies on the conversion of proinsulin to insulin. II. Evidence for a chymotrypsin-like cleavage in the connecting peptide region of insulin precursors in the rat. J. Biol. Chem. 248, 3476–82.
2 Brandenburg, D. (2008). History and diagnostic significance of C-peptide. Exp. Diabetes Res. 2008, 576862.
3 Wahren, J., Ekberg, K. and Jornvall, H. (2007). C-peptide is a bioactive peptide. Diabetologia 50, 503–9.
4 Walcher, D., Aleksic, M., Jerg, V., Hombach, V., Zieske, A., Homma, S., Strong, J. and Marx, N. (2004). C-peptide induces chemotaxis of human CD4-positive cells: involvement of pertussis toxin-sensitive G-proteins and phosphoinositide 3-kinase. Diabetes 53, 1664–70.
5 Marx, N., Walcher, D., Raichle, C., Aleksic, M., Bach, H., Grub, M., Hombach, V., Libby, P., Zieske, A., Homma, S. and Strong, J. (2004). C-peptide colocalizes with macrophages in early arteriosclerotic lesions of diabetic subjects and induces monocyte chemotaxis in vitro. Arterioscler. Thromb. Vasc. Biol. 24, 540–5.
6 Walcher, D., Babiak, C., Poletek, P., Rosenkranz, S., Bach, H., Betz, S., Durst, R., Grub, M., Hombach, V., Strong, J. and Marx, N. (2006). C-Peptide induces vascular smooth muscle cell proliferation: involvement of SRC-kinase, phosphatidylinositol 3-kinase, and extracellular signal-regulated kinase 1/2. Circ. Res. 99, 1181–7.
7 Kukhtina, N. B., Arefieva, T.I. and Krasnikova, T. L. (2005). Intracellular signal cascade in CD4+ T-lymphocyte migration stimulated by interferon-gamma-inducible protein-10. Biochemistry (Mosc) 70, 652–6.
8 Zheng, Y. and Quilliam, L. A. (2003). Activation of the Ras superfamily of small GTPases. Workshop on exchange factors. EMBO Rep. 4, 463–8.
9 Heasman, S. J. and Ridley, A. J. (2008). Mammalian Rho GTPases: new insights into their functions from in vivo studies. Nat. Rev. Mol. Cell Biol. 9, 690–701.
10 Burridge, K. and Wennerberg, K. (2004). Rho and Rac take center stage. Cell 116, 167–79.
11 Ridley, A. J. (2001). Rho GTPases and cell migration. J. Cell Sci. 114, 2713–22.
12 Raftopoulou, M. and Hall, A. (2004). Cell migration: Rho GTPases lead the way. Dev. Biol. 265, 23–32.
13 Sanchez-Madrid, F. and del Pozo, M. A. (1999). Leukocyte polarization in cell migration and immune interactions. Embo J. 18, 501–11.
14 Takai, Y., Sasaki, T. and Matozaki, T. (2001). Small GTP-binding proteins. Physiol. Rev. 81, 153–208.
15 Riento, K. and Ridley, A. J. (2003). Rocks: multifunctional kinases in cell behaviour. Nat. Rev. Mol. Cell Biol. 4, 446–56.
16 Bokoch, G. M. (2003). Biology of the p21-activated kinases. Annu. Rev. Biochem. 72, 743–81.
17 Chong, C., Tan, L., Lim, L. and Manser, E. (2001). The mechanism of PAK activation. Autophosphorylation events in both regulatory and kinase domains control activity. J. Biol. Chem. 276, 17347–53.
18 Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O. and Caroni, P. (1998). Regulation

of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature 393, 805–9.
19 Edwards, D. C., Sanders, L. C., Bokoch, G. M. and Gill, G. N. (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat. Cell Biol. 1, 253–9.
20 Arai, A., Jin, A., Yan, W., Mizuchi, D., Yamamoto, K., Nanki, T. and Miura, O. (2005). SDF-1 synergistically enhances IL-3-induced activation of the Raf-1/MEK/Erk signaling pathway through activation of Rac and its effector Pak kinases to promote hematopoiesis and chemotaxis. Cell. Signal. 17, 497–506.
21 Cantrell, D. (2002). Protein kinase B (Akt) regulation and function in T lymphocytes. Semin. Immunol. 14, 19–26.
22 Zhang, S., Han, J., Sells, M. A., Chernoff, J., Knaus, U. G., Ulevitch, R. J. and Bokoch, G. M. (1995). Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J. Biol. Chem. 270, 23934–6.
23 Rousseau, S., Houle, F., Landry, J. and Huot, J. (1997). p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. Oncogene 15, 2169–77.
24 Vlahakis, S. R., Villasis-Keever, A., Gomez, T., Vanegas, M., Vlahakis, N. and Paya, C. V. (2002). G protein-coupled chemokine receptors induce both survival and apoptotic signaling pathways. J. Immunol. 169, 5546–54.
25 Wilflingseder, D., Mullauer, B., Schramek, H., Banki, Z., Pruenster, M., Dierich, M. P. and Stoiber, H. (2004). HIV-1-induced migration of monocyte-derived dendritic cells is associated with differential activation of MAPK pathways. J. Immunol. 173, 7497–505.
26 Fujita, T., Azuma, Y., Fukuyama, R., Hattori, Y., Yoshida, C., Koida, M., Ogita, K. and Komori, T. (2004). Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. J. Cell Biol. 166, 85–95.
27 Steinle, J. J., Meininger, C. J., Forough, R., Wu, G., Wu, M. H. and Granger, H. J. (2002). Eph B4 receptor signaling mediates endothelial cell migration and proliferation via the phosphatidylinositol 3-kinase pathway. J. Biol. Chem. 277, 43830–5.
28 Marx, N., Kehrle, B., Kohlhammer, K., Grub, M., Koenig, W., Hombach, V., Libby, P. and Plutzky, J. (2002). PPAR activators as antiinflammatory mediators in human T lymphocytes: implications for atherosclerosis and transplantation-associated arteriosclerosis. Circ. Res. 90, 703–10.
29 Stahle, M., Veit, C., Bachfischer, U., Schierling, K., Skripczynski, B., Hall, A., Gierschik, P. and Giehl, K. (2003). Mechanisms in LPA-induced tumor cell migration: critical role of phosphorylated ERK. J. Cell Sci. 116, 3835–46.
30 Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y. and Kaibuchi, K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J. Biol. Chem. 271, 20246–9.
31 Bamburg, J. R. (1999). Proteins of the ADF/cofilin family: essential regulators of actin dynamics. Annu. Rev. Cell Dev. Biol. 15, 185–230.
32 Hills, C. E. and Brunskill, N. J. (2008). Intracellular signalling by C-peptide. Exp. Diabetes Res. 2008, 635158.
33 Rigler, R., Pramanik, A., Jonasson, P., Kratz, G., Jansson, O. T., Nygren, P., Stahl, S., Ekberg, K., Johansson, B., Uhlen, S., Uhlen, M., Jornvall, H. and Wahren, J. (1999). Specific binding of proinsulin C-peptide to human cell membranes. Proc. Natl. Acad. Sci. U S A 96, 13318–23.
34 Zhong, Z., Davidescu, A., Ehren, I., Ekberg, K., Jornvall, H., Wahren, J. and Chibalin, A. V. (2005). C-peptide stimulates ERK1/2 and JNK MAP kinases via activation of protein kinase C in human renal tubular cells. Diabetologia 48, 187–97.
35 Tsimaratos, M., Roger, F., Chabardes, D., Mordasini, D., Hasler, U., Doucet, A., Martin, P. Y. and Feraille, E. (2003). C-peptide stimulates Na+,K+-ATPase activity via PKC alpha in rat medullary thick ascending limb. Diabetologia 46, 124–31.
36 Ohtomo, Y., Bergman, T., Johansson, B. L., Jornvall, H. and Wahren, J. (1998). Differential effects of proinsulin C-peptide fragments on Na+, K+-ATPase activity of renal tubule segments. Diabetologia 41, 287–91.
37 Grunberger, G., Qiang, X., Li, Z., Mathews, S. T., Sbrissa, D., Shisheva, A. and Sima, A. A. (2001). Molecular basis for the insulinomimetic effects of C-peptide. Diabetologia 44, 1247–57.
38 Al-Rasheed, N. M., Meakin, F., Royal, E. L., Lewington, A. J., Brown, J., Willars, G. B. and Brunskill, N. J. (2004). Potent activation of multiple signalling pathways by C-peptide in opossum kidney proximal tubular cells. Diabetologia 47, 987–97.
39 Kitamura, T., Kimura, K., Jung, B. D., Makondo, K., Sakane, N., Yoshida, T. and Saito, M. (2002). Proinsulin C-peptide activates cAMP response element-binding proteins through the p38 mitogen-activated protein kinase pathway in mouse lung capillary endothelial cells. Biochem. J. 366, 737–44.
40 Kitamura, T., Kimura, K., Makondo, K., Furuya, D. T., Suzuki, M., Yoshida, T. and Saito, M. (2003). Proinsulin C-peptide increases nitric oxide production by enhancing mitogen-activated protein-kinase-dependent transcription of endothelial nitric oxide synthase in aortic endothelial cells of Wistar rats. Diabetologia 46, 1698–705.
41 Kitamura, T., Kimura, K., Jung, B. D., Makondo, K., Okamoto, S., Canas, X., Sakane, N., Yoshida, T. and Saito, M. (2001). Proinsulin C-peptide rapidly stimulates mitogen-activated protein kinases in Swiss 3T3 fibroblasts: requirement of protein kinase C, phosphoinositide 3-kinase and pertussis toxin-sensitive G-protein. Biochem. J. 355, 123–9.
42 Scalia, R., Coyle, K. M., Levine, B. J., Booth, G. and Lefer, A. M. (2000). C-peptide inhibits leukocyte-endothelium interaction in the microcirculation during acute endothelial dysfunction. Faseb J. 14, 2357–64.
43 Shafqat, J., Juntti-Berggren, L., Zhong, Z., Ekberg, K., Kohler, M., Berggren, P. O., Johansson, J., Wahren, J. and Jornvall, H. (2002). Proinsulin C-peptide and its analogues induce intracellular Ca2+ increases in human renal tubular cells. Cell. Mol. Life Sci. 59, 1185–9.
44 Ekberg, K., Brismar, T., Johansson, B. L., Lindstrom, P., Juntti-Berggren, L., Norrby, A., Berne, C., Arnqvist, H. J., Bolinder, J. and Wahren, J. (2007). C-Peptide replacement therapy and sensory nerve function in type 1 diabetic neuropathy. Diabetes Care 30, 71–6.
45 Ido, Y., Vindigni, A., Chang, K., Stramm, L., Chance, R., Heath, W. F., DiMarchi, R. D., Di Cera, E. and Williamson, J. R. (1997). Prevention of vascular and neural dysfunction in diabetic rats by C-peptide. Science 277, 563–6.
46 Shafqat, J., Melles, E., Sigmundsson, K., Johansson, B. L., Ekberg, K., Alvelius, G., Henriksson, M., Johansson, J., Wahren, J. and Jornvall, H. (2006). Proinsulin C-peptide elicits disaggregation of insulin resulting in enhanced physiological insulin effects. Cell. Mol. Life Sci. 63, 1805–11.
47 Marx, N. and Walcher, D. (2008). C-Peptide and atherogenesis: C-Peptide as a mediator of lesion development in patients with type 2 diabetes mellitus? Exp. Diabetes Res. 2008, 385108.
48 Libby, P. (2002). Inflammation in atherosclerosis. Nature 420, 868–74.
49 Nishita, M., Aizawa, H. and Mizuno, K. (2002). Stromal cell-derived factor 1alpha activates LIM kinase 1 and induces cofilin phosphorylation for T-cell chemotaxis. Mol. Cell. Biol. 22, 774–83.
50 Ono, S. (2003). Regulation of actin filament dynamics by actin depolymerizing factor/cofilin and actin-interacting protein 1: new blades for twisted filaments. Biochemistry 42, 13363–70.
51 Pollard, T. D. and Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell 112, 453–65.
52 Carlier, M. F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G. X., Hong, Y., Chua, N. H. and Pantaloni, D. (1997). Actin depolymerizing factor (ADF/cofilin) enhances the rate

of filament turnover: implication in actin-based motility. J. Cell Biol. 136, 1307–22.
53 McGough, A., Pope, B., Chiu, W. and Weeds, A. (1997). Cofilin changes the twist of F-actin: implications for actin filament dynamics and cellular function. J. Cell Biol. 138, 771–81.
54 Alblas, J., Ulfman, L., Hordijk, P. and Koenderman, L. (2001). Activation of Rhoa and ROCK are essential for detachment of migrating leukocytes. Mol. Biol. Cell 12, 2137–45.
55 Lindahl, E., Nyman, U., Melles, E., Sigmundsson, K., Stahlberg, M., Wahren, J., Obrink, B., Shafqat, J., Joseph, B. and Jornvall, H. (2007). Cellular internalization of proinsulin C-peptide. Cell. Mol. Life Sci. 64, 479–86.
56 Cherla, R. P. and Ganju, R. K. (2001). Stromal cell-derived factor 1 alpha-induced chemotaxis in T cells is mediated by nitric oxide signaling pathways. J. Immunol. 166, 3067–74.
57 Smit, M. J., Verdijk, P., van der Raaij-Helmer, E. M., Navis, M., Hensbergen, P. J., Leurs, R. and Tensen, C. P. (2003). CXCR3-mediated chemotaxis of human T cells is regulated by a Gi- and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK nor Akt/PI-3 kinase. Blood 102, 1959–65.
58 Grunberger, G., Weston, W. M., Patwardan, R. and Rappaport, E. B. (1999). Rosiglitazone once or twice daily improves glycemic control in patients with type II diabetes (T2D). Diabetes 49, 0439.
59 Cotter, M. A., Ekberg, K., Wahren, J. and Cameron, N. E. (2003). Effects of proinsulin C-peptide in experimental diabetic neuropathy: vascular actions and modulation by nitric oxide synthase inhibition. Diabetes 52, 1812–7.