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ORIGINAL ARTICLE

Sebastian Virchow · Nikolaus Ansorge · Dieter Rosskopf
Herbert Rübben · Winfried Siffert

The G protein β3 subunit splice variant Gβ3-s causes enhanced chemotaxis of human neutrophils in response to interleukin-8

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Abstract A C825T polymorphism was recently described in GNB3, the gene encoding the Gβ3 subunit of heterotrimeric G proteins. The 825T allele is associated with the expression of a shorter splice variant (Gβ3-s) and enhanced signal transduction via pertussis toxin (PTX)-sensitive G proteins. Given the pivotal role of G protein βγ dimers in chemotaxis, we related the genotype at the GNB3 locus as a marker for Gβ3-s expression to chemotaxis of human neutrophils in response to stimulation with interleukin-8 (IL-8). IL-8, which activates a CXC receptor coupled to PTX-sensitive G proteins, induced at 10 nM an enhanced maximum chemotaxis of neutrophils from individuals with TC/TT genotype compared to CC genotype. Furthermore, migration of neutrophils from 825T allele carriers was 2.5-fold higher at 0.1 nM and 1 nM IL-8. At these concentrations of IL-8, no significant chemotaxis was observed in neutrophils from homozygous C825 allele carriers, indicating a genotype-dependent, different potency of IL-8 to chemoattract neutrophils. In contrast, IL-8-induced Ca^{2+} signals and O^{2-} generation were independent of genotype. The role of Gβ3-s in enhanced chemotaxis could be confirmed by determination of chemotaxis of COS-7 cells following transfection with either Gβ3-s or "wild-type" Gβ3. Upon stimulation of the transfected cells with the chemoattractant lysophosphatidic acid (LPA), we observed an enhanced chemotactic response of Gβ3-s-transfected compared to Gβ3-transfected COS-7 cells, confirming that Gβ3-s actually causes enhanced chemotaxis.

Key words Calcium · Leukocytes · Signal transduction

S. Virchow (✉) · N. Ansorge, D. Rosskopf · W. Siffert
Institut für Pharmakologie, Universitätsklinikum Essen,
Hufelandstrasse 55, D-45122 Essen, Germany
e-mail: sebastian.virchow@uni-essen.de, Fax: +49-201-7235968

H. Rübben
Urologische Klinik und Poliklinik, Universitätsklinikum Essen,
Hufelandstrasse 55, D-45122 Essen, Germany

Introduction

A C825T polymorphism was recently described in GNB3, the gene encoding the G protein β3 subunit (Gβ3) of heterotrimeric G proteins (Siffert et al. 1998). Cells from individuals carrying the 825T allele express a shorter Gβ3 splice variant, referred to as Gβ3-s, which is strongly associated with enhanced signaling through G protein-coupled receptors via pertussis toxin (PTX)-sensitive, Gi-type G proteins (Siffert et al. 1998). The majority of G protein-coupled receptors for chemoattractants, especially for chemokines, exert their actions via Gi-type G proteins (Taub 1996), and the βγ-dimers released from activated α subunits initiate signal transduction pathways finally resulting in chemotaxis (Arai et al. 1997; Neptune and Bourne 1997). Given this pivotal role of G protein βγ subunits in chemotaxis and the enhanced G protein reactivity in individuals carrying the G protein β3 subunit 825T allele, which is a genetic marker for the expression of Gβ3-s, we hypothesized that the chemotactic response of neutrophils from individuals with a 825T allele should be predictably enhanced compared to those from homozygous C825 allele carriers. To verify this notion, we quantified interleukin-8 (IL-8)-evoked neutrophil responses such as Ca^{2+} increases, O^{2-} generation and chemotactic activity and related them to genotype at the GNB3 locus. IL-8 is a member of the CXC chemokines, which mediate a variety of inflammatory and immune reactions (Baggiolini et al. 1994; Mukaida et al. 1998), and is a potent chemoattractant for human neutrophils (Loetscher et al. 1994; Baggiolini et al. 1997; Ludwig et al. 1997). The cellular actions of IL-8 are mediated via PTX-sensitive, Gi-type G proteins (Damaj et al. 1996). In another set of experiments, we quantified chemotaxis of COS-7 cells transfected with Gβ3-s in comparison with cells overexpressing "wild-type" Gβ3. In these experiments, cells were stimulated with lysophosphatidic acid (LPA), a potent chemoattractant for a variety of cells (Kundra et al. 1995; Lümmen et al. 1997; Moolenaar et al. 1997; Sakai et al. 1998). We report here that neutrophils from individuals carrying a Gβ3

825T allele display a significantly enhanced, IL-8-stimulated chemotaxis. This enhancement is due to the expression of Gβ3-s, as this phenotype could be fully reconstituted in COS-7 cells transfected with Gβ3-s but not "wild-type" Gβ3.

Materials and methods

Isolation of human neutrophils. Venous blood was obtained from healthy, male, non-smoking individuals after informed consent. Neutrophils were isolated through sedimentation of erythrocytes with dextran, followed by centrifugation through Ficoll-Paque gradient and hypotonic lysis of residual erythrocytes (Boyum 1968). Cells were resuspended into RPMI 1640 medium or HBSS, containing 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM D-glucose, buffered at pH 7.4 with 20 mM Hepes. All preparations consisted of at least 95% neutrophils as determined by staining with Diff-Quick (Dade) according to the manufacturer's instructions. In some experiments, cells were treated for 2 h with 100 ng/ml PTX prior to the experiments.

Cell culture and transfection. COS-7 cells were maintained in DMEM containing 10% (v/v) fetal calf serum and antibiotics. One day before transfection, cells were seeded at a density of 1.5×10^4 cells/cm^2 on 94-mm culture dishes. Plasmid DNA for transfection of COS-7 cells was prepared with the Qiagen plasmid kit. Cells were transfected by lipofection with DAC 30 (Eurogentec) according to the manufacturer's recommendation. Briefly, DAC 30 was diluted in HBS (150 mM NaCl buffered at pH 7.4 with 20 mM HEPES) to a concentration of 10 μg/ml and plasmid DNA to a concentration of 3.2 μg/ml (Gβ3 and Gβ3-s in pRK5 vector, Gγ5 in pcDNA3 vector) in a final volume of 5 ml. DAC 30 and DNA were incubated at room temperature (30 min) to form stable complexes. Immediately before adding the DAC 30/DNA complexes containing HBS to the cell culture dishes, the growth medium was replaced by an equal volume of serum- and antibiotic-free DMEM. After 4 h, transfection was stopped by replacing the medium by DMEM containing 10% FCS. Cells were harvested by trypsinization and used for experiments 48 h thereafter. The transfection efficacy was estimated at 40%–50% by control transfection with enhanced green fluorescent protein.

Western-blot analysis. Proteins of crude membranes from transfected COS-7 cells were separated by SDS-PAGE on 8%–20% polyacrylamide gels and then transferred onto nitrocellulose membranes. Western-blot analysis was performed with polyclonal rabbit anti-human $G\beta_{common}$ antibodies (Santa Cruz Biotechnology) and a peroxidase-conjugated goat anti-rabbit antibody (Sigma) according to the manufacturers' instructions. Immunoreactive bands were visualized with enhanced chemoluminescent reagent (Amersham).

Measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was determined using the fluorescent calcium indicator Fura-2 (Molecular Probes) in a Perkin-Elmer LS 5B spectrofluorometer at 37 °C as described elsewhere (Siffert et al. 1995). Briefly, neutrophils were loaded with 2.5 μM Fura-2/AM at 37 °C in RPMI 1640 containing 0.1% BSA. After 45-min incubation, cells were pelleted and resuspended in HBSS. Calibration of fluorescence data in terms of $[Ca^{2+}]_i$ was performed as described (Siffert et al. 1995). Data represent maximal increases (peak values) of $[Ca^{2+}]_i$ after stimulation. In some experiments, extracellular Ca^{2+} was depleted by addition of EGTA to a final concentration of 4 mM 30 s before stimulation of neutrophils to determine the amount of Ca^{2+} released from intracellular stores (Siffert et al. 1995).

Chemotaxis assay. Chemotaxis of human neutrophils was determined in a 48-well microchemotaxis chamber as described in detail elsewhere (Allport et al. 1996; Lümmen et al. 1997) with minor modifications: chemoattractants were diluted in RPMI 1640, and the neutrophil suspension was adjusted to a density of 0.5×10^6 cells/ml. Fifty microliters of the cell suspension were applied to the upper wells of the microchemotaxis chamber separated from the lower wells by a polyvinylpyrolidone-free polycarbonate filter membrane with a pore size of 3 μm coated with collagen type I (100 μg/ml for 2 h). Migration of neutrophils was allowed for 1 h in a humidified atmosphere of 5% CO_2 at 37 °C. Migrated cells were quantified on the lower side of the filter by counting three random areas of each well under a microscope with a magnification of 400-fold (high power field), and mean ± SD for each well was calculated. Migration of COS-7 cells was determined with a cell suspension adjusted to a density of 1×10^6 cells/ml in DMEM buffered at pH 7.4 with 20 mM Hepes, and migration was allowed through a collagen type I-coated filter membrane with a pore size of 8 μm for 6 h. Migrated COS-7 cells were quantified on the lower side of the filter by counting three random areas of each well under a microscope with a magnification of 200-fold (high power field). As the number of migrated COS-7 cells differed between experiments, a migration index was calculated which represents the number of cells migrated in response to stimulation with chemoattractant divided by the number of cells migrated towards control medium without agonist (Arai et al. 1997; Neptune and Bourne 1997). To determine random migration (chemokinesis), chemoattractants were also added to the cells in the upper wells of the microchemotaxis chamber in some experiments.

Measurement of O^{2-} generation. O^{2-} generation was measured with the lucigenin assay in a luminometer (Bhakdi and Martin 1991). Briefly, neutrophils resuspended in HBSS at a density of 1×10^6 cells/ml were transferred to luminometer cuvettes. During continuous stirring lucigenin was added to a final concentration of 0.1 mg/ml and equilibration was allowed for 2 min. Finally, cells were stimulated with receptor agonist or PMA in a total volume of 400 μl, and responses were recorded continuously. Stimulation with 100 nM PMA served as positive control for all cells and represent maximal, G protein-independent O^{2-} generation. The chemoluminescence peak in response to 100 nM PMA was defined as 100% value.

DNA genotyping. DNA extraction from blood and genotyping was performed as described (Siffert et al. 1998).

Reverse transcriptase-polymerase chain reaction. RNA was prepared with the RNeasy Kit (Qiagen) according to the manufacturer's instructions. The reverse transcription was performed with Superscript (Gibco/BRL) using oligo-dT-primers followed by PCR with the Gβ3-specific primers 5′-CGGGAGCTTTCTGCTCACAC-3′ (sense) and 5′-GGCCAGAGAGGATGCCCAC-3′ (antisense). PCR reactions began with denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C (1 min), annealing at 61 °C (1 min), extension at 72 °C (1 min) and a final extension at 72 °C (7 min). PCR products were size-fractionated on 2.5% (w/v) agarose gels containing ethidium bromide and visualized under UV illumination. RT-PCR products encoding Gβ3-s with a size of 400 bp are 123 bp smaller than those encoding wild-type Gβ3 with a size of 523 bp. Neutrophils from each of the three homozygous C825 and 825T allele carriers as well as heterozygous 825T allele carriers were investigated. Results were confirmed using samples from independent reverse transcription reactions.

Data presentation and analysis. For final analysis, CC genotypes were compared to combined TC/TT genotypes, since the 825T allele is associated with alternative splicing (Siffert et al. 1998). All experiments were performed in a blinded fashion, i.e. investigators were unaware of the individuals' genotypes. Data are means ± SEM from independent experiments as indicated in the figure legends. Concentration response curves were calculated using iterative, non-linear regression analysis (Prism2, GraphPAD Software; San Diego, Calif., USA). Statistical analysis of COS-7 cell migration was done by paired Student's *t*-test, all other comparisons with unpaired *t*-tests. Differences were regarded significant at $P < 0.05$.

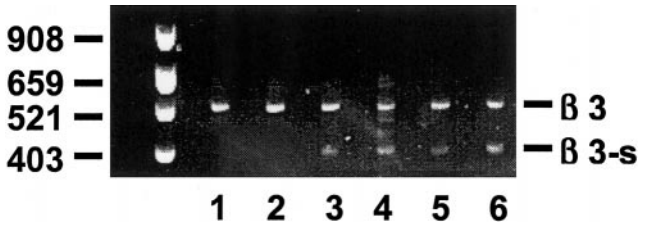

Fig. 1 Expression of Gβ3- and Gβ3-s-specific PCR products in human neutrophils. Displayed is a representative result from RT-PCR (*lanes 1–2* CC genotype, *lanes 3–4* TC genotype, *lanes 5–6* TT genotype; size standard: *Alu*I-digested pBR 322 DNA)

Results

Expression of Gβ3 and Gβ3-s in human neutrophils

The expression of Gβ3- and Gβ3-s-specific mRNA was investigated by RT-PCR using Gβ3-specific primers. Only one specific RT-PCR product with the predicted size of 523 bp was found in neutrophils from individuals homozygous for the 825C allele. An additional, smaller band of approximately 400 bp was exclusively detected in RNA preparations from neutrophils of heterozygous or homozygous 825T allele carriers, suggesting the presence of an alternatively spliced, shorter variant of Gβ3, i.e. Gβ3-s (Fig. 1).

IL-8-stimulated neutrophil chemotaxis

IL-8-induced chemotactic responses of neutrophils were completely PTX-sensitive regardless of genotype and not due to enhanced chemokinesis which was excluded by adding IL-8 to the cells in the upper wells of the microchemotaxis chamber while the chemokine was absent in the lower wells. Under these conditions, there was no migration above basal (data not shown). We observed a significantly enhanced IL-8-stimulated chemotaxis of human neutrophils from individuals carrying a 825T allele compared to homozygous C825 allele carriers in a concentration range of 0.1–10 nM (Fig. 2). Stimulation with IL-8 at concentrations of 0.1 nM and 1 nM led to an approximately 2.5-fold higher chemotactic response of neutrophils from 825T allele carriers compared to homozygous C825 allele carriers – indicating a higher potency of IL-8 in cells from 825T allele carriers. Maximum and enhanced chemotactic responses of neutrophils with TC/TT genotype were observed at 10 nM IL-8 and averaged 110.1 ± 8.5 vs. 80.3 ± 11.7 cells per high power field (CC genotype; $P < 0.05$). At 100 nM, neutrophil chemotaxis was slightly reduced compared to the maximum response at 10 nM IL-8 with no significant difference between genotypes (Fig. 2). We found no difference or further enhancement of neutrophil chemotaxis from individuals with TT compared to TC genotype (data not shown), suggesting that one T allele suffices to induce a dominant phenotype. No attempt was made to further enrich the

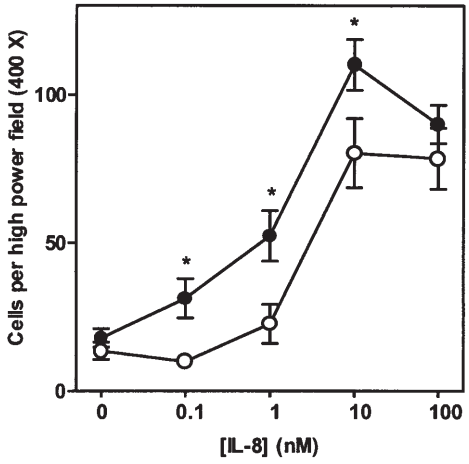

Fig. 2 IL-8-stimulated chemotaxis of human neutrophils. Data represent means ± SEM of migrated neutrophils per high power field. Experiments were performed in quadruplicate for each individual and concentration [*open circles* CC genotype ($n = 13$), *filled circles* TC ($n = 14$) and TT ($n = 5$) genotypes]. $*P < 0.05$

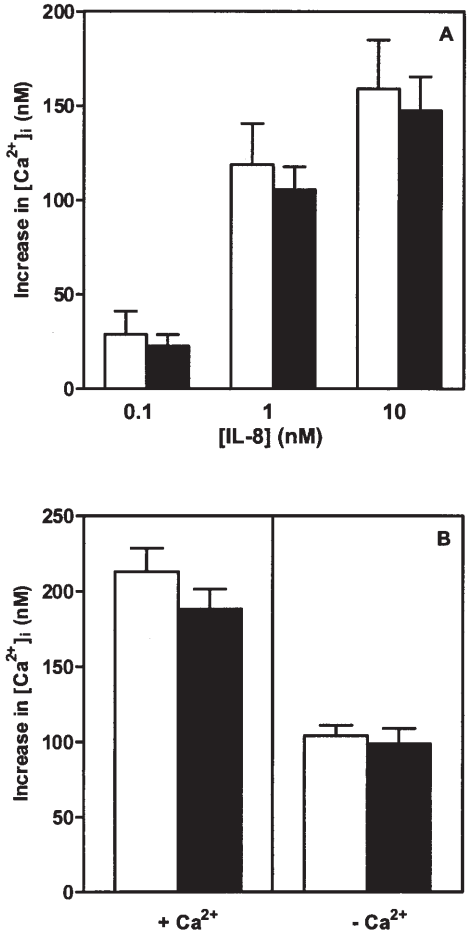

Fig. 3 A,B IL-8-stimulated $[Ca^{2+}]_i$ rise. **A** Concentration-dependent increase in $[Ca^{2+}]_i$. Values represent peak increases in $[Ca^{2+}]_i$ [*open bars* CC genotype ($n = 8$), *filled bars* TC genotype ($n = 14$)]. **B** Increase in $[Ca^{2+}]_i$ after addition of 10 nM IL-8 in the presence or absence of 1 mM Ca^{2+}. Values represent peak increases in $[Ca^{2+}]_i$ [*open bars* CC genotype ($n = 14$), *filled bars* TC genotype ($n = 7$)]

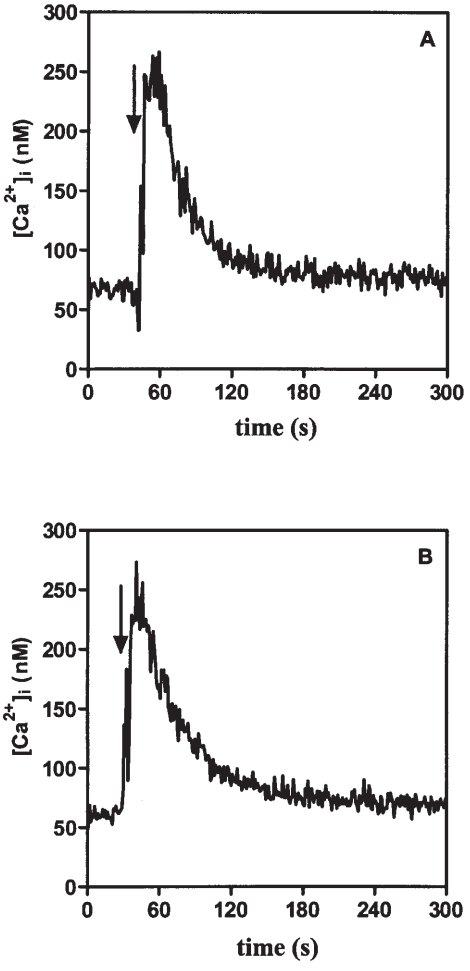

Fig. 4 A,B Time courses of changes in $[Ca^{2+}]_i$ upon addition of 10 nM IL-8 to human neutrophils. **A** CC genotype; **B** TC genotype. *Traces* are representative of at least 20 independent experiments. *Arrows* indicate addition of IL-8

sample with cells from individuals homozygous for the 825T allele as the TT genotype is relatively rare (approximately 10%) in Caucasian populations (Siffert et al. 1998).

IL-8-stimulated $[Ca^{2+}]_i$ mobilization

To determine whether the genotype-dependent difference found in IL-8-stimulated chemotaxis is related to other signalling pathways, IL-8-evoked changes in $[Ca^{2+}]_i$ were quantified. The IL-8-stimulated increase in $[Ca^{2+}]_i$ was completely PTX-sensitive (not shown). Baseline $[Ca^{2+}]_i$ was 110.8 ± 10.8 nM in neutrophils with CC genotype and 99.2 ± 7.5 nM in neutrophils with TC/TT genotypes, respectively ($P > 0.2$). Stimulation with 0.1 nM, 1 nM and 10 nM IL-8 induced peak increases in $[Ca^{2+}]_i$ that averaged 28.8 ± 12.4 nM (CC) vs. 22.7 ± 6.0 nM (TC/TT), 118.8 ± 21.7 nM (CC) vs. 105.5 ± 12.2 nM (TC/TT), and 159.1 ± 52.8 nM (CC) vs. 147.3 ± 18.1 nM (TC/TT), respectively (Fig. 3A). In addition, we determined the contribution of Ca^{2+} released from intracellular compartments to the total increase in $[Ca^{2+}]_i$ in nominally Ca^{2+}-free medium. Under these conditions, 10 nM IL-8 increased $[Ca^{2+}]_i$ by 104.1 ± 6.9 nM (CC) and 98.6 ± 10.2 nM (TC/TT), respectively (Fig. 3B). The time courses of the IL-8-evoked Ca^{2+} transients were similar in neutrophils from individuals with CC and TC genotypes (Figs. 4A,B).

IL-8-evoked O^{2-} generation

O^{2-} generation was concentration-dependent after IL-8-stimulation of neutrophils with CC or TC genotype and completely blocked by PTX (data not shown). No genotype-dependent difference in O^{2-} generation was found in the range from 0.01 nM to 30 nM IL-8. Stimulation with 30 nM IL-8 was most effective and evoked a peak rise in O^{2-} generation of 8.1 ± 2.7% in relation to that evoked by 100 nM PMA (CC genotype), this value being 9.1 ± 0.6% in TC genotype (Fig. 5). Calculated EC_{50} values were 2.8 nM (CC) and 4.9 nM (TC; $P > 0.2$).

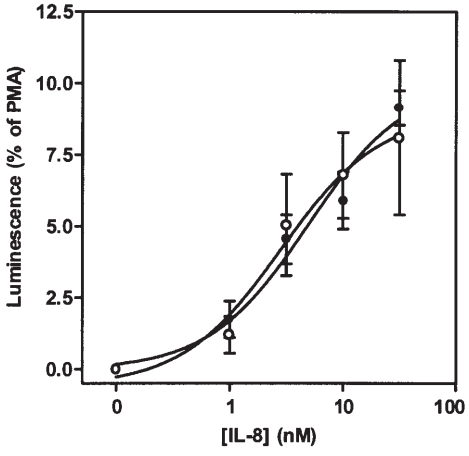

Fig. 5 O^{2-} generation in human neutrophils in response to IL-8-stimulation. O^{2-} generation was determined with the lucigenin assay and continuously monitored. Peak increases after IL-8 stimulation are related to maximum chemoluminescence determined in response to 100 nM PMA and are expressed as percent values. Data represent means ± SEM from independent experiments with CC genotype (*open circles*, $n = 6$) and TC genotype (*filled circles*, $n = 6$)

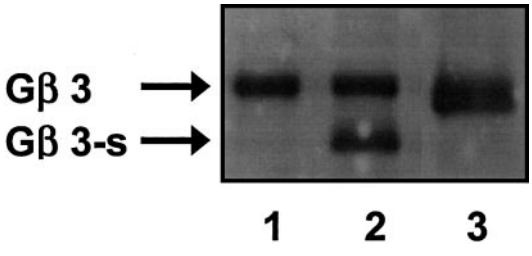

Fig. 6 Western-blot analysis of COS-7 cells overexpressing Gβ3 or Gβ3-s. Proteins of crude membranes of wild-type COS-7 cells (*lane 1*), COS-7 cells transfected with Gβ3-s (*lane 2*) and Gβ3 (*lane 3*) were separated on 8%–20% polyacrylamide gels and then transferred onto nitrocellulose membranes. Western-blot analysis was performed with polyclonal rabbit anti-human $G\beta_{common}$ antibodies and a peroxidase-conjugated goat anti-rabbit antibody

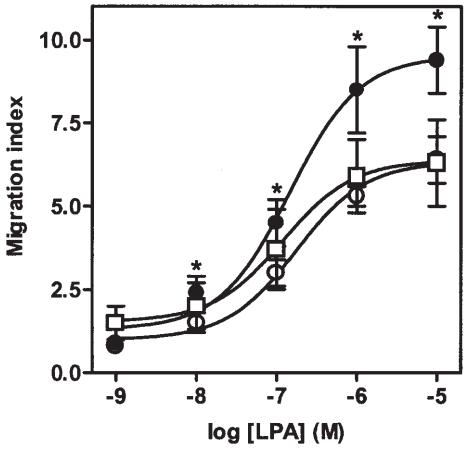

Fig. 7 Enhanced chemotactic response of COS-7 cells overexpressing Gβ3-s. COS-7 cells were cotransfected with either Gβ3 and Gγ5 (*open circles*), Gβ3-s and Gγ5 (*filled circles*) or plasmids alone (*open squares*). Thereafter, the concentration-dependent chemotactic response following stimulation with LPA was quantified in a 48-well microchemotaxis chamber. Shown are the results (means ± SEM) from six independent experiments. * $P < 0.05$

LPA-induced chemotaxis
of COS-7 cells transfected with Gβ3-sγ5 or Gβ3γ5

The expression of Gβ3 and Gβ3-s in transfected COS-7 cells could be demonstrated by Western-blot analysis with anti-human $G\beta_{common}$ antibodies (Fig. 6). LPA-stimulated chemotaxis was not increased in cells overexpressing Gβ3γ5 compared to vector-transfected cells (Fig. 7). However, we observed a significantly enhanced chemotactic response to LPA stimulation in cells transfected with Gβ3-sγ5 compared to those transfected with Gβ3γ5 (Fig. 7) following stimulation with 0.01 μM, 0.1 μM, 1 μM and 10 μM LPA ($P < 0.05$) in six independent transfection experiments. This difference was most pronounced at 1 μM and 10 μM LPA. LPA-stimulated migration was chemotactic rather than chemokinetic as confirmed by experiments in which LPA was added to cells in the upper and not present in the lower wells of the microchemotaxis chamber. The chemotactic response to LPA was completely PTX-sensitive in all transfected COS-7 cells.

Discussion

The 825T allele of GNB3 appears to support alternative splicing of the gene most likely by increasing the use of a cryptic splice acceptor site in exon 9 of the gene (Siffert et al. 1998). This notion stays in line with the observations reported here which show that neutrophils from homozygous C825 allele carriers express only one Gβ3-specific RT-PCR product of the predicted size, whereas 825T allele carriers display a second specific, shorter RT-PCR product corresponding to Gβ3-s (Siffert et al. 1998). Since the occurrence of this splice variant is associated with enhanced activation of heterotrimeric, PTX-sensitive G proteins, we quantified IL-8-stimulated neutrophil responses that are mediated by PTX-sensitive G proteins, and related them to genotype at the GNB3 locus. We found a significantly, almost 2.5-fold higher chemotactic response of neutrophils from homo- and heterozygous 825T allele carriers at low concentrations of IL-8 that was still enhanced at 10 nM IL-8. This enhanced chemotaxis coincided with the expression of Gβ3-s-specific RT-PCR products. Although IL-8-mediated $[Ca^{2+}]_i$ rises and O^{2-} generation were also fully blocked by PTX, suggesting the involvement of Gi-type G proteins, neither the $[Ca^{2+}]_i$ rises nor O^{2-} generation were dependent on genotype. However, chemotaxis does not solely depend on a $[Ca^{2+}]_i$ rise and, vice versa, a variety of G protein-coupled receptors mediate a rise in $[Ca^{2+}]_i$ without evoking a chemotactic response (Arai et al. 1997; Lümmen et al. 1997; Neptune and Bourne 1997). At present our findings can be best explained by the notion that IL-8-induced neutrophil chemotaxis involves G protein βγ subunits comprising Gβ3 and Gβ3-s, whereas Ca^{2+} mobilization and O^{2-} generation may be mediated through other βγ dimers, e.g. involving Gβ1, Gβ2 or Gβ4. This argumentation can be further supported by similar results of an enhanced chemotaxis of human neutrophils with TC/TT genotype compared to the CC genotype following stimulation with the tripeptide fMLP (Virchow et al. 1998). Thus, genotyping at the GNB3 locus could represent a powerful tool to investigate the contribution of Gβ3-containing βγ subunits to G protein-mediated cell responses. The causal relationship between the expression of Gβ3-s and enhanced chemotactic response could be demonstrated in experiments in which we cotransfected COS-7 cells with Gβ3 or Gβ3-s together with the γ5 subunit, the latter forming functional βγ dimers with both Gβ3 and Gβ3-s (Siffert et al. 1998). Thus, our results of an enhanced neutrophil chemotaxis in 825T allele carriers are unlikely fortuitous.

Future studies will have to focus on the mechanism by which Gβ3-s increases the chemotactic response. One key step involves actin polymerization resulting in cytoskeletal remodeling in which the G protein β subunit plays a decisive role. Evidence for this comes from experiments on *Dictyostelium discoideum* in which null mutations of the Gβ subunit strongly impair chemotaxis as well as phagocytosis (Wu et al. 1995; Peracino et al. 1998).

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