Chemokine receptor signals: role in migration, invasion and cancer metastasis
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Citation for published version (APA):

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Chapter 5

Fusion of docked vesicles, driven by synaptotagmin, is required for chemokine-induced T-cell migration.

To be submitted
Fusion of docked vesicles, driven by synaptotagmin, is required for chemokine-induced T-cell migration

I.S. Zeelenberg, Y.M. Wijnands, E. Roos

Lymphoid cells react very rapidly to chemokines. We hypothesized that this involves rapid fusion of docked vesicles with the plasma membrane. Calcium-triggered fusion of docked synaptic vesicles in neurons depends on oligomerization of synaptotagmins. The isolated synaptotagmin C2B domain blocks this oligomerization and therefore inhibits fusion. We found that T-lymphoma cells express synaptotagmin-3. Transfection of the C2B domain fully inhibited CXCR4-triggered migration, whereas a C2B mutant, that binds calcium but does not inhibit fusion, had no effect. Migration was enhanced in cells overexpressing full-length synaptotagmin-3, but inhibited when synaptotagmin-3 contained the same mutation. Finally, we found complexes of v- and t-SNAREs in the T cells, indicating that docked vesicles are present. These complexes were dissociated when fusion was induced by calcium influx, and also when cells were treated with the CXCR4-binding chemokine CXCL12. We propose that docked vesicle fusion regulated by the calcium sensor synaptotagmin is essential for chemokine-induced migration.

Introduction

The migration of leukocytes into tissues in vivo is directed by chemokines and their receptors. This occurs mainly in inflamed tissues, in which expression of certain chemokines is upregulated. However, also in the absence of inflammation, tissues contain chemokines that are probably involved in the normal recirculation of leukocytes. In particular, CXCL12 (SDF-1) is constitutively expressed in many organs. Recently it has been described to play a role in the metastasis of various malignancies.

Chemotaxis towards CXCL12 is dependent on the Rho GTPase Cdc42. The prototype effect of Cdc42 is actin polymerization and the formation of filopodia. Recently, however, Cdc42 was also implicated in fusion of vesicles with the membrane. In yeast, Cdc42 has a role in fusion of vesicles, or even at the docking stage before fusion, independent of its role in actin polymerization. Migrating cells display intense exocytosis at the leading edge, which is considered necessary to supply membrane for formation of membrane-rich structures such as filopodia and lamellipodia. These vesicles may also contain signaling molecules and adhesion molecules necessary for migration. Since responses to chemokines are very rapid, we hypothesized that the vesicles are docked at the membrane, so that they can fuse immediately after a stimulus.

Vesicle fusion in eukaryotic cells is mediated by proteins termed SNAREs (soluble N-ethyl maleimide-sensitive factor (NSF) attachment protein receptors). A v-SNARE on the vesicle membrane interacts with two t-SNAREs on the target membrane. They form a four-stranded coiled-coil core complex that promotes the association of the vesicle with the membrane. If fusion is blocked at this stage, the vesicles remain docked. In neurons, vesicles loaded with neurotransmitter are docked at the membrane and remain quiescent until an action potential arrives. This opens voltage-activated channels leading to the influx of Ca\(^{2+}\) that activates the calcium-sensor synaptotagmin, resulting in vesicle fusion. Synaptotagmin-1 and synaptotagmin-2 undergo Ca\(^{2+}\)-dependent oligomerization, but the structure of these oligomers is still unclear. It has been speculated that the oligomers form a ring-like structure and that Ca\(^{2+}\)-driven rearrangements dilate the neck of a fusion pore, resulting in secretion.

Synaptotagmins are transmembrane proteins with a short extracellular domain. The cytoplasmic part consists of two calcium binding domains, C2A and C2B. Thirteen synaptotagmins have been identified. The neuron-specific synaptotagmin-1 and -2 have low affinity for Ca\(^{2+}\). The other synaptotagmins are more ubiquitously expressed and can be activated by lower Ca\(^{2+}\) levels, in the micromolar range. Synaptotagmin homologues have been found in plants and therefore the synaptotagmins probably arose early in evolution, before nerve cells existed. This suggests that synaptotagmins have more general functions than the regulation of neurotransmitter release in the central nervous system. Indeed, synaptotagmins have recently
been implicated in regulated exocytosis\textsuperscript{21,22}, membrane repair\textsuperscript{23,24} and enzyme release by sperm cells\textsuperscript{25}.

To investigate the possible role of synaptotagmins in migration and invasion, induced by the chemokine CXCL12, we used a T-cell hybridoma as a model.\textsuperscript{26} Like the activated T cell from which it was generated, the T-cell hybridoma expresses CXCR4 and is highly migratory and invasive. Because of the autonomous growth capacity derived from the lymphoma fusion partner, these cells are easier to culture than freshly isolated T cells and thus more suitable to generate stable transfectants.

Desai et al.\textsuperscript{16} showed that the isolated C2B domain of synaptotagmin-1 inhibits oligomerization of all synaptotagmins. Consequently, Ca\textsuperscript{2+} -induced fusion of docked vesicles is blocked, as shown by adding recombinant C2B to permeabilized PC12 cells. A mutant of the C2B domain in which the two lysines 326 and 327 are replaced with alanines, does not block oligomerization, but still binds calcium. We generated stable transfectants expressing either the C2B domain or this mutant, termed C2B(KA). The C2B domain inhibited migration and invasion, whereas C2B(KA) had no effect. Moreover, overexpression of synaptotagmin-3 resulted in enhanced migration, suggesting that the expression level of synaptotagmin-3 is a limiting factor. In contrast, overexpression of a mutated synaptotagmin-3 (syt3(KA)), in which the same two lysines as in C2B(KA) were replaced with alanines (K482,483A), caused inhibition of migration. Furthermore, the presence of docked vesicles was strongly suggested by the co-immunoprecipitation of the v- and t-SNAREs VAMP-2 and SNAP-23. These SNAREs were dissociated upon Ca\textsuperscript{2+} influx and after CXCR4 activation by CXCL12. These results suggest a critical role for docked vesicles, and the regulation of their fusion by synaptotagmin-3, in chemokine-induced migration and invasion.

Materials and methods

Cell culture. The mouse T cell hybridoma TAM2D2 was generated by fusion of noninvasive BW5147 lymphoma cells with normal activated T lymphocytes.\textsuperscript{26} The cells were cultured in RPMI-1640 medium with L-glutamine (GIBCO BRL, Paisley, United Kingdom) supplemented with 12.5 mM NaHCO\textsubscript{3}, 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, 10% NCTC 135 (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA), 0.26 g/l L-glutamine, 0.05 mM 2-mercaptoethanol, 0.5 mM Na-pyruvate 1 mM oxaloacetic acid, 0.2 IU/ml bovine insulin, 100 IU/ml of penicillin, 100 µg/ml of streptomyacin (GIBCO BRL) and 10% fetal calf serum (FCS) (GIBCO/Invitrogen, Carlsbad, CA, USA).\textsuperscript{27,28} TAM2D2 transfectants were cultured in the same medium supplemented with 0.4 µg/ml puromycin (Sigma Chemical Co., St. Louis, Missouri, USA) or 0.3 mg/ml zeocin (Invitrogen, Carlsbad, CA).

Rat embryo fibroblasts (REFs) were cultured in DMEM medium (GIBCO BRL) supplemented with 100 IU/ml of penicillin, 100 µg/ml of streptomyacin (GIBCO BRL) and 10% newborn calf serum (GIBCO/Invitrogen). They were used for invasion assays between passages 5 and 15.

The virus-packaging cell line ΦNXE\textsuperscript{29} was cultured in DMEM medium (GIBCO BRL) supplemented with 100 IU/ml of penicillin, 100 µg/ml of streptomyacin (GIBCO BRL), 10% FCS (GIBCO/Invitrogen) and 0.584 g/l L-glutamine (GIBCO BRL).

Antibodies. The antibodies against synaptotagmin-3 and -7 were a generous gift of B.A. Wolf and R.A. Young.\textsuperscript{21} The synaptotagmin-1, VAMP-2 and SNAP-23 antibody were purchased from Synaptic Systems (Göttingen, Germany). The 2B11 anti-CXCR4 antibodies used for flow cytometry, were a generous gift of Dr. R. Förster (Hannover, Germany).

RT-PCR. The brain of a BALB/c nude mouse and 10\textsuperscript{5} TAM2D2 cells were directly lysed in 1 ml RNAzol (Teltest Inc., Friendswood, TX, USA). Total RNA was extracted and an RT-PCR was performed using a one-step RT-PCR kit (Clontech, Palo Alto, CA, USA) and the primers (syt-1: 5'-GGAGGAGCCCAAGGAAGAGGAAGAA-3' (forward) and 5'-AGCAGGAGAGGAAGCAGATGCAC-3' (reverse), syt-3: 5'-CTGCCGGGTGAGAAGAAAAA3' (forward) and 5'-AGGGCCACCGAAGACTGAAACG-3' (reverse), syt-7: 5'-GAAATCTAAAACCCGACTGGAATGAG-3' (forward) and 5'-CGTTGGCGGCTAGCTTTGTTTT-3' (reverse)). Amplification of β-actin RNA was performed simultaneously using actin-specific primers. Amplified products were electrophoresed on a 1.5% agarose gel.

Generation and transduction of DNA constructs. The C2B domain (residues 248-421) was generated by PCR with the primers (5'-ATCGATCGATGGATGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTGAGGCTTATCGGGATTGTTT
AT-3' (forward) and 5'-GTACTACGTATTACTCTTGACAGGCGCAT-3' (reverse)). The C2B(K326,327A) mutant was generated by mutagenesis with primers (5'-CGCAGAAGGCTGGAACAGCATGATTAGAAGG AACAC-3' (forward) and 5'-GGTCTCCTCTAATGGAGGTCGCC-3' (reverse)) and mutated with the primers (Stratagene, Amsterdam, The Netherlands) using PCR with the primers (5'-CGGCAAGAGAAACAC-3' (forward) and S'-GTGTTCTTCCTGAAGAAGGCAGCGACAACGATTAAGAGGGGACCTCC (reverse)) and mutated with the primers (5'-GGCCCGCCTCCTGAAGAAGGCCGACCTCCATTAGAAGA-3' (forward) and 5'-GGTCTCTTAAATGGAGGTCGCCGCTTCCTTCAGTCTCCTGC-3' (reverse)) using the mutagenesis kit from Stratagene. The synaptotagmin-3 construct was cloned into the retroviral vector pLZRS-IRES-zeo. The other constructs were cloned into the retroviral vector pLZRS-IRES-puro-EGFP. The latter vector was based on the pLZRS-IRES-zeo vector, in which the zeocin resistance cDNA was replaced by a cDNA encoding a puromycin resistance-enhanced green fluorescence protein (EGFP) fusion protein.

Because it contains an internal ribosomal entry site (IRES), both the construct and the puromycin-resistance EGFP fusion protein are translated from one bicistronic mRNA. Therefore, EGFP levels are expected to correlate with levels of the proteins encoded by the inserted cDNAs. The vector plasmids were transfected by calcium-phosphate precipitation into the virus-packaging cell line ONXE. After 8 h, the medium was refreshed, and 48 h later the virus supernatant was collected and used to infect the TAM2D2 cells. Three days later, puromycin was added, and after a few days the selected cells were FACS-sorted to select bulk populations with high EGFP expression.

Migration assay. Migration assays were performed as described previously. Briefly, Transwells with 5 μm pores were treated for 2 h with 0.5% ovalbumin at room temperature. The lower chamber was filled with 250 μl RPMI1640 supplemented with 0.1% ovalbumin and 100 ng/ml CXCL12 (PeproTech Inc., Rocky Hill, NJ, USA). Cells were kept in fresh medium for 30 min at 37°C and washed with ice-cold serum-free medium. 103 cells were added to the upper chamber of the Transwells in RPMI1640 supplemented with 0.1% ovalbumin. After incubation for 2 h at 37°C and 5% CO2, the migrated cells in the lower chamber were counted.

Invasion assay. Invasion assays were performed as described previously. Briefly, confluent REF monolayers in 24-well plates and TAM2D2 transfectants were washed and the latter were added to the monolayers in serum-free medium. After incubation for 1 or 4 h at 37°C and 5% CO2, the monolayers were extensively washed and fixed with 2% paraformaldehyde. The invaded cells were counted using phase-contrast microscopy.

Internalization assay. Cells (0.5 x 106) were washed and incubated for 30 min in RPMI1640 with 0.1% ovalbumin at 37°C. CXCL12 (100 ng/ml) was added to the cells at various time points during this period. Then, cells were washed again stained with the 2B11 anti-CXCR4 supernatant and analyzed by flow cytometry as described above.

Immunoprecipitation. Cells (5 x 106) were resuspended in 250 μl RPMI medium and added to 750 μl warm RPMI medium. Subsequently the cells were incubated for 5 min. at 37°C and ionomycin (Calbiochem, Darmstadt, Germany) or CXCL12 (PeproTech Inc.) was added to the cells for 30 sec. or 1 min. during this period. Then, cells were immediately washed with ice-cold PBS supplemented with 1 mM EGTA and lysed in 1 ml lysis buffer (1% Triton X-100, 0.15 M NaCl, 20 mM Heps, pH 7.4) for 30 min. After centrifugation for 5 min. at 1200 rpm, the supernatant was incubated at 4°C with anti-VAMP2 and subsequently with protein-G Sepharose beads (Amersham, Pharmacia Biotech., Uppsala, Sweden). Precipitated complexes were washed three times in lysis buffer and eluted with 1 M ethanolamine pH11. The eluted proteins were mixed with sample

Flow cytometry. GFP and CXCR4 levels were assessed by flow cytometry. GFP levels were measured directly. To assess CXCR4 levels, cells were incubated for 45 min with 20 μl of 1:5 diluted 2B11 hybridoma supernatant, and next for 45 min with phycoerythrin (PE)-conjugated anti-rat antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). The analyses were performed on a Becton Dickinson FACScan using CellQuest software.

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buffer and boiled for 5 min. Complexes were analyzed by immunoblotting.

**Immunoblotting.** SDS-PAGE-separated cell lysates were blotted to nitrocellulose, which was then blocked with 1% BSA and 3% nonfat dried milk. The membranes were incubated for 1 h with the appropriate antibody at room temperature, followed by incubation with a secondary antibody coupled to horseradish peroxidase (Amersham Life Sciences, Little Chalfont, U.K.). Stained proteins were visualized by enhanced chemiluminescence (ECL kit, Amersham).

**Results**

**Expression of synaptotagmins in TAM2D2 cells.** The two synaptotagmin (syt) family members that have been implicated in regulated exocytosis in non-neuronal cells, are syt-3 and syt-7. Therefore they are the main candidates for being involved in chemotaxis. We tested their expression in TAM2D2 T cell hybridoma cells by RT-PCR. As shown in Fig. 1, we found that TAM2D2 cells express syt-3, but not syt-7. We confirmed the expression of syt-3 in TAM2D2 cells by Western blotting (Fig. 5).

![Fig. 1. Expression of synaptotagmins in TAM2D2 cells. (A) RT-PCR of syt-1 (1), syt-3 (3) and syt-7 (7). As controls, the expression of these synaptotagmins in brain is shown, and that of actin (A) in TAM2D2 cells and brain are shown, as well as a negative water control (w). (B) Western blot showing the expression of syt-3 and not syt-7 in TAM2D2 cells](image)

**Blocked synaptotagmin oligomerization impairs chemotaxis.** To demonstrate a role for regulated exocytosis in chemokine-induced migration, we expressed the isolated C2B domain of synaptotagmin-1 in the TAM2D2 cells. This C2B domain inhibits oligomerization of all synaptotagmin isoforms tested, and thus blocks fusion of docked vesicles and regulated exocytosis. We used a retroviral vector with an IRES downstream of the C2B cDNA, followed by a cDNA encoding a fusion protein of the puromycin-resistance protein and EGFP. Both C2B and the EGFP are expressed from one bicistronic mRNA and the expression levels are likely to correlate. To obtain cells with high or medium C2B expression, transfectants with high (C2B(h)) or medium (C2B(m)) EGFP levels were FACSorted (Fig. 2A). Of the control TAM2D2 cells, almost 36% migrated towards CXCL12. In contrast, the C2B-expressing cells with high EGFP expression did not migrate at all (Fig. 2B). An intermediate effect was seen with the C2B(m) cells. Clearly, the C2B domain has a major impact on the chemotactic process.

![Fig. 2. The synaptotagmin-1 C2B domain inhibits migration of TAM2D2 cells. (A) FACS analysis of EGFP expression of the C2B transfectants. Filled histograms represent the fluorescence in the transfectants, open histograms the fluorescence of untransfected control cells. The median of the filled histograms is shown in the upper right corner. (B) Migration towards 100 ng/ml CXCL12 of C2B transfectants. Results are given as the mean +/- SEM of the migration index, which is set to 1 for the control cells in each individual experiment. Shown are averages of 14 experiments.](image)

The C2B domain binds calcium, so it might influence migration by acting as a calcium sink. To rule out this possibility, we expressed a mutant C2B (K326,327A) which does not inhibit oligomerization and fusion, but still binds calcium. This mutant will be referred to as C2B (KA). We selected by FACS sorting a population with similar levels of C2B(KA) as in the cells expressing the non-mutated C2B, as judged by EGFP levels (Fig. 2A). CXCL12-induced migration of these cells was similar to
the TAM2D2 cells, showing that inhibition by C2B was not due to calcium binding (Fig.2B).

**Blocking oligomerization of synaptotagmin impairs invasion.** The T-cell hybridoma cells rapidly invade into monolayers of rat embryo fibroblasts. We have shown previously that this depends completely on CXCL12 bound to the surface of the fibroblasts and therefore on CXCR4 signaling. One of the effects of the signal is the rapid activation of the integrin LFA-1 which is required for this invasion. As shown in Fig. 3, invasion was completely blocked by C2B(h). In contrast, C2B(KA), expressed at the same high level, had only a minor effect. This inhibition was comparable to that seen in C2B(m) cells that express low levels of C2B.

**Fig. 3. Invasion of C2B transfectants into REF monolayers.** Results are given as the mean +/- SEM of the invasion index, which is set to 1 for the control cells in each individual experiment. Shown are averages of 8 experiments.

**Blocking oligomerization of synaptotagmin has no effect on internalization.** To rule out the possibility that the C2B domain affects endocytosis and recycling of the receptor, we determined whether CXCL12-induced internalization of CXCR4 was affected. The extent of internalization was comparable for all cells and, importantly, similar for both C2B transfectant populations, of which migration was blocked, and C2B(KA) transfectants, of which the migration was not affected (Fig.4).

**Fig. 4. Internalization of CXCR4 by C2B transfectants.** FACS analysis of CXCR4 expression of TAM2D2 and C2B-transfectants after 30 min incubation with CXCL12. Black histograms represent the CXCR4 levels before, and white histograms after 30 min incubation. One representative experiment is shown out of three with similar results.

**Synaptotagmin-3 overexpression promotes migration.** Both synaptotagmin-3 (syt-3) and synaptotagmin-7 (syt-7) have been implicated in regulated exocytosis in non-neuronal cells, and are therefore the main candidates for being involved in chemotaxis. As described above, TAM2D2 cells express syt-3, but not syt-7. To investigate its putative involvement, we overexpressed syt-3. A mutated synaptotagmin-3 (syt3(KA)), in which the same two lysines as in C2B(KA) were replaced with alanines (K482,483A) was also expressed. This syt-3(KA) mutant cannot oligomerize and is expected to prevent oligomerization by competing with endogenous syt-3. Protein levels of endogenous syt-3, overexpressed syt-3 and syt3(KA) are shown in Fig. 5A. As shown

**Fig. 5. Effect of syt-3 on migration.** (A) Expression of syt-3 in the transduced and non-transduced cells, as detected by Western blotting with antibodies against syt-3. (B) Migration of C2B transfectants towards 100 ng/ml CXCL12. Results are given as the mean +/- SEM of the migration index, which is set to 1 for the control cells in each individual experiment. Shown are averages of 10 experiments.
in Fig.5B, syt-3 overexpression led to 75% more migration, suggesting that the syt-3 levels in the cells are limiting and determine the extent of migration. In contrast, expression of the mutant syt-3(KA) led to reduced migration, probably because it competes with the endogenous syt-3 and thus inhibits fusion. However, inhibition was not complete, probably because the expression levels were not sufficient.

Docked vesicles in TAM2D2 cells. Vesicle fusion is mediated by SNAREs. In docked vesicles the v- and t-SNAREs are associated at the membrane, but fusion is blocked. The SNAREs are dissociated upon an increase in Ca\(^{2+}\) concentration. The v-SNARE VAMP-2 and the t-SNARE SNAP-23 are expressed in the TAM2D2 cells. To demonstrate association, we immunoprecipitated VAMP-2 and tested for associated SNAP-23 by Western blotting with SNAP-23 antibodies. As shown in Fig. 6, we did detect SNAP-23, showing that VAMP2-SNAP-23 complexes are present in the cell. This strongly suggests the presence of docked vesicles. When the intracellular calcium concentration is raised using ionomycin, the complex is dissociated after 30 sec. Strikingly, the same is seen within 1 min after treatment of the cells with the chemokine CXCL12. This indicates that fusion of docked vesicles is induced by CXCL12.

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Fig. 6. Interaction of the v-SNARE VAMP-2 and the t-SNARE SNAP-23, detected by immuno-precipitation of VAMP-2 and Western blotting with SNAP-23 antibodies. The TAM2D2 cells were treated with either ionomycin (iono) or CXCL12 for the indicated time period. A control immuno-precipitation without VAMP-2 antibodies is shown to demonstrate the specificity of the interaction.

Discussion
Here we provide evidence supporting our hypothesis that rapid fusion of docked vesicles, regulated by synaptotagmin-3 (syt-3), is required for chemokine-induced migration and invasion of T cells. This is based on the inhibition of migration by a C2B domain of syt-1, expressed in these cells, but not by a mutant that does not affect fusion. Furthermore, overexpression of full-length syt-3 enhanced migration, whereas a fusion-incompetent syt-3 mutant reduced migration. Finally, we found complexes of v- and t-SNAREs in the T cells, indicating that docked vesicles are present. These complexes were dissociated when fusion was induced by calcium influx, and also when cells were treated with the chemokine CXCL12.

Synaptotagmins are integral membrane proteins that serve as Ca\(^{2+}\)-sensors for vesicle fusion. They block fusion in the absence of calcium but promote rapid fusion upon binding of calcium. The cytoplasmic part contains two C2 domains, C2A and C2B, which bind Ca\(^{2+}\). The Ca\(^{2+}\)-induced conformational change enables the C2B domain to interact with C2B domains of other synaptotagmin molecules. This results in oligomerization, which is required for fusion of the docked vesicles. The isolated C2B domain binds to the C2B domains of endogenous intact synaptotagmins and thus impairs oligomerization in a dominant-negative fashion. In permeabilized PC12 cells, recombinant proteins consisting of only the C2B domain thus prevented neurotransmitter release.\textsuperscript{18} Desai et al.\textsuperscript{18} showed that the C2B domain of syt-1 inhibits oligomerization of all synaptotagmins tested. We therefore expressed this C2B domain, expecting that it would inhibit oligomerization of the endogenous syt-3 or any other synaptotagmin, which might be expressed in the T-cell hybridoma cells. The complete inhibition of migration by this C2B strongly suggests that synaptotagmin oligomerization, and therefore fusion of docked vesicles, is required for chemokine-induced migration.

Chemokines induce a rapid rise in intracellular Ca\(^{2+}\) concentration. This is mainly due to the activation of phospholipase C (PLC).\textsuperscript{30} PLC generates inositol trisphosphate (IP\(_3\)) from phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)).\textsuperscript{34} This IP\(_3\) releases Ca\(^{2+}\) from the endoplasmic reticulum. However, such large global Ca\(^{2+}\) responses are not required for chemokine-induced chemotaxis.\textsuperscript{35-37} Indeed, chemotaxis can be induced by very low concentrations of chemokines that are unlikely to trigger substantial changes in global calcium concentrations. It is therefore more likely that chemotaxis is induced by a transient very local increase in Ca\(^{2+}\) concentration, perhaps due to the opening of an ion channel rather than release from the endoplasmic reticulum. In this respect, it is of interest that the affinity of syt-3...
for Ca\(^{2+}\) is much higher than that of the neuronal-specific syt-1 and syt-2, so that it will be activated by much lower Ca\(^{2+}\) concentrations (in the micromolar range).\(^9\)

Given the likely importance of small increases in \([\text{Ca}^{2+}]\), it was conceivable that the inhibition by C2B was due to Ca\(^{2+}\)-binding rather than impaired oligomerization. To exclude that possibility, we expressed a C2B mutant in which two lysines, which are essential for oligomerization\(^3\), were replaced by alanines. This mutant has been shown to bind Ca\(^{2+}\) similarly as intact C2B.\(^18\) The mutant, expressed at similar levels, as judged by co-expressed EGFP, did not inhibit migration. This shows that inhibition is not due to buffering of Ca\(^{2+}\) by the overexpressed isolated C2B domain. However, the invasive capacity of the cells transfected with the mutant C2B(KA) was somewhat lower than that of the untransfected cells, comparable to the C2B(m) cells that express lower levels of C2B. This suggests that calcium binding by the mutant does have an effect on invasion. However, the inhibition of invasion is much larger in the C2B(h) cells that express C2B at similar high levels as the C2B(KA) mutant in the C2B(KA) transfectant population. This difference indicates that the main effect of the C2B domain on invasion is due to inhibition of synaptotagmin oligomerization. To exclude the possibility that the C2B domain influences migration by an effect on receptor recycling, we tested whether CXCL12-induced internalization was altered, but this did not differ between C2B and C2B(KA) transfectants.

The role of synaptotagmins in exocytosis implies that docked vesicles are present in the T-cell hybridoma. This is very difficult to demonstrate since their number is likely to be small, compared to neurons. We show here that the t-SNARE SNAP-23 is co-precipitated with the v-SNARE VAMP-2. Such complexes are formed very transiently during exocytosis, so the presence of a sizable amount indicates that some vesicles are docked, i.e. tethered to membrane by the complex, but that their fusion is blocked. Indeed, the complexes are rapidly dissociated upon a rise in \([\text{Ca}^{2+}]\), induced by ionomycin. Such dissociation occurs after fusion\(^16\), so the most likely interpretation of this observation is that the docked vesicles have fused with the membrane. Strikingly, this dissociation also occurred upon treatment of the cells with the chemokine CXCL12. Thus, treatment with high concentrations of chemokine, when most receptors will be occupied, is apparently sufficient to induce fusion of most docked vesicles. Obviously, a cell moving in a chemokine gradient is exposed to lower concentrations and fusion will be limited and more localized. It remains to be demonstrated that dissociation is inhibited in cells expressing the C2B domain.

Synaptotagmins are best known for their role in regulation of neurotransmitter release. However, synaptotagmin homologues have been found in plants\(^4\) and therefore synaptotagmins arose early in evolution, before nerve cells existed. Furthermore, several synaptotagmins are expressed in many different non-neuronal cell types, also indicating a more general function. Indeed, syt-3 and -7 have been implicated in regulated insulin secretion in pancreatic cells.\(^21\) This exocytosis also depends on Cdc42 and G\(i\) protein signaling, similarly as chemokine-induced chemotaxis.\(^4\) This suggests that the possible roles of syt-3 and syt-7 imply a more general function. Indeed, syt-3 and syt-7 are expressed in non-neuronal cells,\(^21\) syt-3 and syt-7 were the main candidates for being involved in chemotaxis. Since syt-7 is not expressed in T-cell hybridoma, a role for syt-3 seemed likely. To show this, we expressed full-length syt-3 in which the two lysines on the C2B interaction surface were mutated. We expected this mutant to be incorporated into SNARE complexes on docked vesicles and to impair oligomerization and thus inhibit fusion. This mutant did inhibit migration, supporting a role for syt-3. As a control, we also overexpressed the intact full-length syt-3. Remarkably, this enhanced migration, indicating that the level of syt-3 is a limiting factor for chemotaxis. Taken together, these two results provide substantial evidence for the notion that syt-3 is required for chemokine-induced migration of T cells.

It has long been realized that vesicle trafficking and exocytosis are necessary for the migration of cells.\(^1\) This exocytosis at the leading edge was suggested to have two major roles.\(^1\) Firstly, it provides the cell with extra membrane necessary for membrane-rich structures such as filopodia. In this context, it is noteworthy that expression of synaptotagmins in fibroblasts has been reported to induce
Acknowledgements

We are grateful to Dr. B.A. Wolf and Dr. R.A. Young (Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine) for the antibodies against sys-3 and sys-7. We thank Dr. R. Förster (Institute of Immunology, Hannover Medical School, Hannover, Germany) for the anti-CXCR4 supernatant, 2B11.

References


