Platelets achieve bleeding arrest at sites of vascular injury via secretion of secretory proteins from their storage granules, termed α-granules. We have recently analyzed granule targeting of platelet factor 4 (PF4), a secretory α-granule chemokine, and demonstrated that PF4 α-granule storage relied upon determinants within PF4 mature sequence. To define these determinants, PF4 mutants fused to the fluorescent reporter protein green fluorescent protein were generated by progressive deletions and site-directed mutagenesis. They were then transfected in AtT20 cells and assessed for granule targeting by colocalization with ACTH-containing granules, using laser scanning confocal microscopy. This strategy identified the amino acid 41–50 (LIATLKNGRK) sequence as most critical for PF4 granule targeting and/or storage; its deletion from PF4 induced a marked decrease in granule storage (from 81 ± 2% to 17 ± 3%, p ≤ 0.0001). Allo-scanning mutagenesis of LIATLKNGRK narrowed down the targeting motif to LKNG. A direct role for LKNG in α-granule targeting was confirmed in the thrombopoietin-induced megakaryocytic Dami cells, in which the LKNG-green fluorescent protein chimera exhibited an 82.5 ± 1.8% colocalization with the α-granule proteins von Willebrand factor and P-selectin. LKNG is poorly conserved within the chemokine family. However, three-dimensional alignments of the human α-granule chemokines Nap-2 (neutrophil-activating peptide) and RANTES (Regulated upon Activation Normal T Cell Expressed and Secreted) with PF4 revealed that LKNG, a surface-exposed hydrophilic turn/loop, matched Nap-2 (LKDG) and RANTES (TRKN) peptides with similar features. Moreover, Nap-2 and RANTES peptides exhibited the same α-granule targeting efficiency than LKNG. We therefore postulate that the three-dimensional and physicochemical characteristics of PF4 LKNG are of general relevance to α-granule targeting of chemokines and possibly of other α-granule proteins.

Proteins from the regulated secretory pathway are sorted within the late Golgi, the trans-Golgi network (1–4). Two models are proposed to explain the biogenesis of regulated storage granules. First, the sorting receptor model, for which the carboxypeptidase E (CPE) stands as a paradigm, as it is involved in Golgi sorting of the propiomelanocortin, insulin, or somatostatin (5, 6). However, the sorting receptor model may not be relevant to non hormonal proteins (7). Second, the granule maturation model, which implies that an immature granule generated from the Golgi, undergoes a maturation step consisting in the exclusion of non granular proteins, leading to mature granules (4). Which of these models, or possibly both, is actually at work in secretory cells is still a matter of debate.

Platelets, which are blood-circulating anucleated cells, fulfill hemostatic functions via regulated secretion of their storage granules at sites of vascular injury (8). All platelet secretory proteins are stored in the storage granule, termed α-granule (9). Defective storage of secretory proteins in the α-granule is found in the inherited hemorrhagic disease, the gray platelet syndrome (GPS), the molecular basis of which is unknown (10). So far, little is known regarding the mechanism by which megakaryocytic soluble proteins are sorted and retained within these storage granules. Sorting to α-granules of the membrane protein P-selectin has been shown to rely on its cytoplasmic domain (11, 12). This suggests involvement of soluble cytoplasmic partners, but this pathway appears unlikely for granular soluble proteins, because they are not in contact with the cytoplasm. This would be consistent with observations indicating that granule membrane proteins and their soluble variants do not follow the same routes (13). Among the well described soluble proteins of the α-granule and exclusively synthesized by platelets is platelet factor 4 (PF4), a 70-amino acid-long member of the CXC chemokine family (14). PF4 is an antiangiogenic factor that inhibits megakaryocyte and endothelial cell proliferation (15, 16). Importantly, the PF4 structure has been solved by x-ray crystallography, allowing structure-function predictions (17, 18). PF4 is released along with other α-granule proteins upon physiological and pathological stimulation (9).

To bring up more insight into the trafficking mechanisms of soluble megakaryocytic proteins to storage granules, we have chosen PF4 as a model and the widely used AtT20 cells as a model cell line (11, 19–22). We recently demonstrated that PF4 is efficiently routed to secretory granules when transfected into AtT20 cells (23). We demonstrated that the PF4 signal peptide is not sufficient to promote granule targeting. We thus hypothesized that a PF4 targeting determinant lay within the PF4 mature sequence itself (23). The present study confirms this hypothesis. Using green fluorescent protein (GFP)-tagged PF4

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Platelets, which are blood-circulating anucleated cells, fulfill hemostatic functions via regulated secretion of their storage granules at sites of vascular injury (8). All platelet secretory proteins are stored in the storage granule, termed α-granule (9). Defective storage of secretory proteins in the α-granule is found in the inherited hemorrhagic disease, the gray platelet syndrome (GPS), the molecular basis of which is unknown (10). So far, little is known regarding the mechanism by which megakaryocytic soluble proteins are sorted and retained within these storage granules. Sorting to α-granules of the membrane protein P-selectin has been shown to rely on its cytoplasmic domain (11, 12). This suggests involvement of soluble cytoplasmic partners, but this pathway appears unlikely for granular soluble proteins, because they are not in contact with the cytoplasm. This would be consistent with observations indicating that granule membrane proteins and their soluble variants do not follow the same routes (13). Among the well described soluble proteins of the α-granule and exclusively synthesized by platelets is platelet factor 4 (PF4), a 70-amino acid-long member of the CXC chemokine family (14). PF4 is an antiangiogenic factor that inhibits megakaryocyte and endothelial cell proliferation (15, 16). Importantly, the PF4 structure has been solved by x-ray crystallography, allowing structure-function predictions (17, 18). PF4 is released along with other α-granule proteins upon physiological and pathological stimulation (9).

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constructs and progressive deletions, as well as site-directed mutagenesis, we demonstrate that PF4 L45KNG48 is essential for granule targeting in the AtT20 pituitary mouse cell line. Moreover, in the megakaryocytic context of the human Dami cell line induced with thrombopoietin and phosphor 12-myristate 13-acetate (PMA) (24), LKNG was efficiently targeted to $\alpha$-granules. LKNG was not found in other $\alpha$-granule proteins, but three-dimensional alignments with the human $\alpha$-granule chemokines Nap-2 and RANTES showed peptides (LKDG and TRKN, respectively) exhibiting the same surface-exposed hydrophilic turn/loop features of LKNG. Moreover both sequences were efficiently targeted to $\alpha$-granules. This strongly suggests that LKNG three-dimensional features are critical for $\alpha$-granule targeting. Consequences in terms of granule targeting mechanisms in megakaryocytes are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco's modified Eagle's medium and RPMI 1640, both containing GlutaMAX™ I, 4500 mg/liter $\alpha$-glucose, 50,000 units/liter penicillin, and 50 mg/liter streptomycin were from Invitrogen (Cergy Pontoise, France); Fetalclone® II was from Perbio Science (Cergy Pontoise, France); thrombopoietin-mimetic peptide (TPOmp) was from Tebu (Le Perray en Yvelines, France); rabbit anti-human P-selectin was from Pharmingen, a mix of six mouse monoclonal human VWF antibodies was a kind gift from Dr. Baruch; expression vector pCMV/EGFP-N1 was from Clontech (Palo Alto, CA); expression vector pcDNA3 was from Invitrogen; the Expand™ high fidelity PCR system (Roche Applied Science, Meylan, France); the QuikChange™ XL site-directed mutagenesis kit was from Stratagene (La Jolla, CA); 4′,6-diamidino-2-phenylindole-containing Vectashield® was from Vector Laboratories (Burlingame, CA); Leica TCS 4D and Bio-Rad MRC 1024 confocal laser scanning microscopes were from Leica Microsystems (Rueil-Malmaison, France) and Bio-Rad (Marnes-la-Coquette, France), respectively.

**Recombinant DNA Constructs**—PF4-EGFP was reconstituted as described previously (23). Progressive deletions of the PF4 mature sequence from the C terminus fused to the GFP fluorescent reporter, PF4-(1–60)-GFP, PF4-(1–50)-GFP, PF4-(1–40)-GFP, PF4-(1–30)-GFP, PF4-(1–20)-GFP, PF4-(1–13)-GFP, PF4-(L41-K50)-GFP and PF4-(LKNG)-GFP chimerae were all achieved using SOEing PCR (25). PCR fragments corresponding to deletions within PF4 mature sequence, fused to GFP, were generated using the following strategy; all shared the same 5′-most sequence (23 nucleotides) of the GFP linker (lin22) (black box) and are complementary to the last 3′ 21 nucleotides immediately preceding the corresponding PF4 deletion (here from amino acids 60–70). Primers C (here $C_w$) are complementary to primers B. Illustrated here as an example is PF4-(1–60)-GFP, deleted from the last 10 amino acids of PF4. Fragments $AB_w$ and $CD_w$ were amplified in two separate steps (1 and 2), before full-length PF4-(1–60)-GFP construct was generated by PCR (step 3) using both fragments as templates and primers A and D, strategy for SP-(LIAILKNGRK)-GFP, SP-(LKNG), SP-(LKDG), SP-(TRKN), and SP-(AAAA) fused to GFP. Mature PF4 was deleted from PF4-(56–60)-GFP by PpuMI/BamHI-mediated double digestion. Oligonucleotides encompassing LIAILKNGRK, LKNG, LKDG, TRKN, AAAA, complementary to each other and with PpuMI and BamHI cohesive ends, respectively, were inserted by ligation between PF4 signal peptide ($SP$) and GFP linker (lin22).
fragments, the 5′-sequences of which correspond to the C termini of the PF4 deleted fragments PF42–60, PF42–50, and PF42–40. PF42–30, PF42–20, PF42–15, PF42–10, and PF42–50 (Fig. 1B).

Products AB and CD were mixed in equal molarity and the final fusions, PF4(1–60) through 13-GFP, PF4L/IAITLKNGKR-GFP, or PF4L/(L41-K50)-GFP and PF4V(LKNG)-GFP, were generated by SOE-PCR with primers forward A and reverse D (Fig. 1B and supplemental Table I).

Final fusion products were subcloned into the pcdNA3 expression vector using HindIII and XbaI sites introduced within primers A and D (italic).

SP-(L41-LTAKNGKR)-GFP (or SP-(L41-K50)-GFP), SP-(LKNG)-GFP (PF4 peptides), SP-(LKDGL)-GFP (the human Np2-tetrapeptide), SP-(TRKN)-GFP (the human RANTES tetrapeptide), and SP-(AAGA)-GFP chimeras were prepared in three steps (Fig. 1C and supplemental Table I: 1) PF4-GFP plasmid was double digested by PpuMI in 5′ and BamHI in 3′, to delete the PF4 mature sequence and to preserve the signal peptide, and the reporter GFP; 2) two overlapping complementary oligonucleotides corresponding to the six short peptides and containing overhanged sequences for PpuMI in 5′ and BamHI in 3′, respectively, were synthesized and annealed each other. Supplemental Table I displays the oligonucleotides sequences and names for each construct. 3) ligations of PpuMI/BamHI double digested PF4-GFP plasmid with the annealed oligonucleotides were performed to generate the chimeras.

Site-directed Mutagenesis—Ala-scanning mutagenesis substituting an alanine for each residue from Leu-41 to Lys-50 (except for A43 substituted for G) was achieved using the Stratagene mutagenesis kit and according to the manufacturer’s instructions. Briefly, substitution mutations were generated by annealing the SP-(L41-K50)-GFP construct with two synthetic oligonucleotide primers containing the desired Ala-scan codon, and complementary to each other, followed by PCR. DpnI endonuclease digestion eliminated the methylated non-mutated parental DNA. XL10-Gold ultracompetent cells were transformed with the synthesized circular nicked mutated DNA, and colonies were screened by sequencing.

Sequence—All constructs were sequenced in both directions prior to transfection (Genome Express, Meylan, France).

Cells—Dami cells were grown onto sterile glass coverslips, before immunofluorescence processing. Dami cells were seeded 5–7 days prior to immunofluorescence processing. Dami cells were grown onto sterile glass coverslips, before immunofluorescence processing. Dami cells were seeded 5–7 days prior to immunofluorescence experiments to induce α-granules upon addition of PMA (1 μM) and the TP0mp (10 nM) as described previously (24). Cells were washed three times with 0.5 mM CaCl2 PBS. Between each of the following steps, cells were also washed three times at room temperature. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, then permeabilized for 40 min at room temperature with permeabilization buffer containing 20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl2, 1% Triton X-100, pH 7.0. Next, cells were incubated in the presence of 5% BSA (w/v) for 30 min at 37°C.

For AtT20 cells, primary mouse anti-ACTH IgGs (1:50, v/v), diluted in 2.5% BSA (w/v), were incubated for 30 min at 37°C, followed by Alexa Fluor® 594 goat anti-mouse IgGs (1:200, v/v), diluted in 2.5% BSA (w/v) for 30 min at 37°C.

For Dami cells, two sequential primary antibodies and their corresponding secondary antibodies, all incubated for 30 min at 37°C, were used: mouse anti-VWF IgGs (1:100, v/v), diluted in 2.5% BSA (w/v), followed by Alexa Fluor® 594 goat anti-mouse IgGs (1:200, v/v) diluted in 2.5% BSA (w/v), and rabbit anti-P-selectin IgGs (1:150, v/v), diluted in 2.5% BSA (w/v), followed by Alexa Fluor® 633 goat anti-rabbit IgGs (1:200, v/v) diluted in 2.5% BSA (w/v), and rabbit anti-P-selectin IgGs (1:150, v/v), diluted in 2.5% BSA (w/v), followed by Alexa Fluor® 633 goat anti-rabbit IgGs (1:200, v/v) diluted in 2.5% BSA (w/v). Then, glass coverslips were mounted with 4,6-diamidino-2-phenylindole-containing Vectashield® onto microscope slides for fluorescence analyses using a confocal laser scanning microscope.

Statistical Analysis—To quantitatively assess granule targeting efficiency, all granules were counted in an average of 15 cells. Percent of colocalization was calculated for each cell as the following ratio: number of yellow granules × 100/whole granules. Results are expressed as means ± S.E.; p values were calculated using the Student’s t test.

RESULTS

Progressive Deletions of PF4 Uncover a 10-Amino Acid Sequence Necessary for Targeting—To look for a putative granular storage sequence within PF4, we generated progressive deletions starting from the PF4 C-terminal end, to preserve its signal peptide, which is required for endoplasmic reticulum entry (23). After transfection in AtT20 cells, analysis by confocal scanning microscopy in Fig. 2 shows that like PF4-GFP (Fig. 2, a–c), PF4(1–60)-GFP and PF4(1–50)-GFP were efficiently stored in ACTH-containing granules (Fig. 2, d–i), as evidenced by numerous yellow granules. Table I recapitulates the statistical analyses of the number of yellow granules per cell, relative to the total number of GFP-containing granules. These data demonstrate that deletion of the last 20 C-terminal amino acids does not affect PF4 targeting. By contrast, PF4(1–40)-GFP, PF4(1–30)-GFP, PF4(1–20)-GFP and PF4(1–13)-GFP exhibited poor granule storage, as shown by the low number of yellow granules (Fig. 2, j–u), and the low targeting efficiency (about 10% versus 80 ± 2% for wild type PF4, p ≤ 0.0001; Table I). Thus the first 40 residues of the PF4 sequence do not support granule targeting, confirming our previous observation that the PF4 signal peptide was not sufficient per se to support granule targeting (23). In contrast, the striking decrease in targeting efficiency when comparing PF4(1–50) and PF4(1–40) mutants (83 ± 2% versus 10.6 ± 0.4%, p ≤ 0.0001) suggests that the L41ATLKNGKR sequence is involved in granule targeting.

LIATLKNGKR Supports Granule Targeting Directly—Deletion of L41ATLKNGKR from PF4 (PF4L/(L41-K50)-GFP) induced poor colocalization of PF4-GFP with ACTH granules (17 ± 3%, p = 0.0001; Table I and Fig. 3A, panels a–c), confirming the role the LIATLKNGKR sequence in PF4 granule targeting. To test whether LIATLKNGKR was directly involved in granule targeting, LIATLKNGKR was inserted between SP, the PF4 signal peptide (to allow its entry into the secretory pathway), and GFP (SP-(LIATLKNGKR)-GFP) (see “Experimental Procedures” for details). After transient or stable expression in AtT20 cells, SP-(LIATLKNGKR)-GFP was efficiently targeted to ACTH-containing storage granules (Fig. 3A, panels d–f, 81 ± 2% targeting efficiency; Table I). Altogether these results indicate that the PF4 sequence L41ATLKNGKR is critically and directly involved in PF4 targeting to storage granules.

Ala Scanning of SP-(LIATLKNGKR)-GFP Discloses a 4-Amino Acid Sequence Essential for Targeting—To define within the L41ATLKNGKR sequence which amino acid residues are relevant to targeting, Ala-scanning site-directed mutagenesis of SP-(LIATLKNGKR)-GFP was performed (Fig. 3B). Fig. 3B, panels e–h, and Table I clearly show that L45A, K46A, N47A, and G48A mutations lead to a loss in targeting efficiency to storage granules: from 81% for SP-(LIATLKNGKR)-GFP down to 38, 35, 32, and 33% (p < 0.001), respectively. In contrast, L41A, I42A, A43G, T44A, R49A, and K50A mutations did not affect the targeting efficiency of SP-(LIATLKNGKR)-GFP (Table I and Fig. 3B, panels a–d and i–j).

The L45KNGKR Sequence Is Necessary and Sufficient for Granular Targeting—Confirming involvement of the L45KNGKR sequence in PF4 granule targeting, deletion of L45KNGKR from the PF4 sequence (PF4Δ(LKNG)-GFP) induced
suggest that L45KNG48 is directly involved in and critical for efficient granule storage (70% between the PF4 signal peptide and GFP (SP-(LKNG)-GFP) led with a direct granule targeting ability, insertion of LKNG to PF4 targeting to storage granules. A significant decrease in the granule storage efficiency (27 ± 8%, p ≤ 0.0001; Table I and Fig. 3C, panels a–c). Consistent with a direct granule targeting ability, insertion of LKNG between the PF4 signal peptide and GFP (SP-(LKNG)-GFP) led to efficient granule storage (70 ± 7%, targeting efficiency; Table I and Fig. 3C, panels d–f). Thus, these results strongly suggest that L45KNG48 is directly involved in and critical for PF4 targeting to storage granules.

Fig. 2. Progressive deletion mutants of PF4. PF4-GFP (a–c), PF4-(1–60)-GFP (d–f), PF4-(1–50)-GFP (g–i), PF4-(1–40)-GFP (j–l), PF4-(1–30)-GFP (m–o), PF4-(1–20)-GFP (p–r) and PF4-(1–13)-GFP (s–u) constructs were transfected in AtT20 cells. Cells were fixed, permeabilized, and incubated with a monoclonal anti-ACTH antibody, which was detected with Alexa® Fluor 594-conjugated goat anti-mouse IgGs and analyzed by confocal laser scanning microscopy. a, d, g, j, m, and s, anti-ACTH (red); b, e, h, k, n, q, and t, GFP fluorescence (green); c, a and b merged; f, d and e merged; i, g and h merged; j, f and k merged; o, m and n merged; r, p and q merged; u, s and t merged. Total magnification: ×1800.

| Construct | Number of cells | Percentage of colocalization with ACTH-containing granules |
|-----------|-----------------|----------------------------------------------------------|
| PF4-GFP   | 10              | 80 ± 2                                                   |
| PF4-(1–60)-GFP | 10             | 80 ± 2                                                   |
| PF4-(1–50)-GFP | 10             | 83 ± 2                                                   |
| PF4-(1–40)-GFP | 9              | 10.6 ± 0.4<sup>a</sup>                                   |
| PF4-(1–30)-GFP | 13             | 10.2 ± 0.2<sup>a</sup>                                   |
| PF4-(1–20)-GFP | 9              | 10.0 ± 0.0<sup>a</sup>                                   |
| PF4-(1–13)-GFP | 7              | 10 ± 2<sup>a</sup>                                       |
| SP-(L41-I42A-K50)-GFP | 12             | 81 ± 2                                                   |
| PF4 Δ(LKNG)-GFP | 10             | 17 ± 2                                                   |
| SP-(L41A-K50)-GFP | 10             | 79 ± 3                                                   |
| SP-(L41A-K50)-GFP | 8              | 74 ± 4                                                   |
| SP-(L41A-K50)-GFP | 9              | 75 ± 5                                                   |
| SP-(L41A-K50)-GFP | 8              | 84 ± 2                                                   |
| SP-(L41A-K50)-GFP | 13             | 38 ± 6                                                   |
| SP-(L41A-K50)-GFP | 10             | 35 ± 10<sup>a</sup>                                     |
| SP-(L41A-K50)-GFP | 10             | 32 ± 8<sup>a</sup>                                       |
| SP-(L41A-K50)-GFP | 12             | 33 ± 10<sup>a</sup>                                     |
| SP-(L41A-K50)-GFP | 9              | 69 ± 6                                                   |
| SP-(L41A-K50)-GFP | 10             | 82 ± 2                                                   |
| SP-(L41A-K50)-GFP | 13             | 70 ± 7                                                   |
| PF4 Δ(LKNG)-GFP | 13             | 27 ± 8<sup>a</sup>                                       |

<sup>a</sup> p value ≤ 0.0001, Student’s t test.

<sup>b</sup> p value ≤ 0.001, Student’s t test.

L<sup>41</sup>LATLKN GRK<sup>30</sup>, or L<sup>45</sup>KNG<sup>48</sup> deletion, as well as its Ala substitutions induce a less drastic loss in targeting efficiency than progressive deletions (about 17–38% versus 10%). This suggests that other determinants within PF4 may have some granule targeting ability but to a lesser extent than LKNG.

LKNG Is an α-Granelle Leader Sequence in the Human Megakaryocytic Cell Line Dami—To confirm these results in a megakaryocytic context, granule targeting experiments were conducted using the megakaryocytic cell line Dami, which exhibits full α-granule maturation after induction with PMA and TPO (24). When transfected into PMA/TPO-treated Dami cells, PF4-GFP colocalized with both VWF and P-selectin as demonstrated by the high number of white granules (Fig. 4, a–c) while SP-GFP (PF4 signal peptide alone fused to GFP) exhibited very low targeting to α-granules (Fig. 4, d–f); 10.0 ± 0.0%, p ≤ 0.0001 in Table II), confirming our previous results (23). SP-(LKNG)-GFP was efficiently sorted to α-granules (Fig. 4, k–o, 82.5 ± 1.8% in Table II), while PF4Δ(LKNG)-GFP was only poorly targeted to α-granules (Fig. 4, p–t, 33 ± 12%).

Thus altogether our results demonstrate that the PF4 tetrapeptide LKNG sequence supports efficient targeting to α-granules.

LKNG Is a Three-dimensional Motif for α-Granule Targeting—Next, we wondered whether LKNG was shared by other α-granule proteins. LKNG was not found in other α-granule proteins such as VWF, thrombospondin, platelet-derived growth factor, or transforming growth factor-β. Moreover, LKNG did not exhibit high conservation when PF4 was compared with other members of the chemokine family; Fig. 5A shows sequence alignments between the three human chemokines, PF4, Nap-2 (another CXC chemokine), and RANTES (a CC chemokine), in the region encompassing LKNG. While the LKNG-matching Nap-2 peptide LDGD shares three identical residues out of four (overall 54% identity between PF4 and Nap-2), RANTES peptide TRKN shows no identity (overall 18% identity). However three-dimensional alignments confirmed that the three chemokines, including the poorly homologous RANTES, are structurally homologous (data not shown).
FIG. 3. A, the decapeptide SP-(L41-K50), L^{141}ATLKNGRK^{50}, allows GFP granule storage in AtT20 cells. L^{141}ATLKNGRK^{50} was cloned between SP and GFP or deleted from PF4-GFP (PF4Δ(L41-K50)-GFP) and transfected into AtT20 cells. Panels a and d, anti-ACTH (red); panels b and e, GFP fluorescence; panels c and f, merge sections. Total magnification: \times 1800. B, alanine substitutions of L^{141}ATLKNGRK^{50}. Merged sections of GFP (green) and ACTH (red) are shown. Each amino acid of L^{141}ATLKNGRK^{50} was substituted for an alanine within the SP-(L41-LKNGRK)^-GFP construct (except for A, which was substituted for a G) and then transfected in AtT20 cells. Note the majority of yellow granules in panels a–d and i and j; whereas there is absence of yellow granules from panels e to h, i.e., absence of colocalization. Total magnification: \times 1800. C, the tetrapeptide LKNG supports efficient granule storage in AtT20 cells. SP-(LKNG)-GFP and PF4Δ(LKNG)-GFP constructs were transfected in AtT20 cells. Panels a and d, anti-ACTH (red); panels b and e, GFP fluorescence (green); panels c and f, merge sections. Total magnification: \times 1800.

TABLE II

| Construct           | Number of cells | Percentage of colocalization with VWF and P-selectin-containing granules |
|---------------------|-----------------|-------------------------------------------------------------------------|
| PF4-GFP             | 10              | 86.9 ± 1.4                                                              |
| SP-GFP              | 10              | 10.0 ± 0.0^a                                                             |
| SP-(LKNG)-GFP       | 10              | 82.5 ± 1.8                                                              |
| PF4Δ(LKNG)-GFP      | 10              | 33 ± 12^a                                                               |
| SP-(LKDG)-GFP       | 10              | 83 ± 2                                                                 |
| SP-(TRKN)-GFP       | 10              | 81.8 ± 1.8                                                              |
| SP-(AAAA)-GFP       | 10              | 11 ± 1^a                                                                |

^a p ≤ 0.0001, Student’s t test. ^b p ≤ 0.001, Student’s t test.

LKNG forms a \beta\text{ turn} between two anti-parallel \beta strands (18, 26, 27) (Fig. 5B a), a structure also found for the LKDG (Fig. 5B, panel b) and TRKN (Fig. 5B, panel c) peptides in Nap-2 and RANTES, respectively. Most importantly, when SP-(LKDG)-GFP and SP-(TRKN)-GFP were expressed in Dami cells, they exhibited granule targeting efficiencies comparable with LKNG (83 and 81.8\%, respectively, in Fig. 5C, panels a–j, and Table II), while a control SP-(AAAA)-GFP construct exhibited low granule targeting (11 ± 1\%; Table II and Fig. 5C, panels k–o). Thus, altogether our results show that LKNG and its homologs within divergent \alpha-granule chemokines are three-dimensional granule targeting motifs fully efficient in \alpha-granule storage.

DISCUSSION

In this study, we have striven to define the sequence features of the megakaryocytic chemokine PF4, which are involved in granule targeting. We have found that the PF4 L^{145}KNG^{45} sequence is directly involved in targeting and/or storage of PF4 within granules. Finally, the granule targeting effect of LKNG is independent from the cellular context, since it is as efficient in the mouse pituitary AtT20 cell line or in the megakaryocytic human Dami cell line. This suggests that granule targeting may not be cell type-restricted but instead may be a rather unspecialized function.

LKNG was not found in other \alpha-granule proteins, suggesting that its effect is not dependent on its primary sequence. This
conclusion is consistent with the fact that equivalent granule targeting efficacy was obtained with the LKDG or TRKN peptides, which share some (LKDG) or no (TRKN) homology with LKNG. This suggests that LKNG may act through its three-dimensional turn conformation, which is shared by the other chemokine peptides within their respective parent molecule. It is also interesting to note that they all exhibit a high hydrophilicity (a minimum of three hydrophilic amino acids out of four), including ionic residues (K for LKNG, KD for LKDG, and RK for TRKN). The fact that LKNG is exposed at the surface of PF4, and located at the H2O/molecule interface (17) (Fig. 6), is consistent with its hydrophilicity. In addition, LKNG seems to fold spontaneously into a turn/loop in solution (28, 29). LKDG and TRKN turn/loops are likely to assume the same surface and water interaction, given their similar exposure at the surface of the molecule and hydrophilicity. We thus postulate that PF4 LKNG is a paradigm for a three-dimensional hydrophilic motif, possibly spontaneously folded into a turn/loop and directly involved in granule targeting. Whether this motif is engaged with a “sorting receptor” (5) or acts through another mechanism such as pH-dependent aggregation (30) remains to be determined. To this respect we note that in other chemokines such as Nap-2 or IL8, K42 or L41K42 have been suspected to be involved with either their receptor or glycosaminoglycans (26, 31), although these studies did not test direct interactions. Further studies are required to test the possibility that CXC receptors or glycosaminoglycans are involved in granule targeting.

Interestingly, neither LKNG, nor its chemokine analogs, or the LIATLKNGRK decapeptide resemble any of the structural sorting signals that have been described so far (32–37). For instance, LIATLKNGRK is different from the 13-amino acid amphipathic “hydrophobic acid” loop of proopiomelanocortin, the ACTH precursor that is recognized by the sorting receptor.
CPE (carboxypeptidase E) (5, 34). Although LIATLKNGRK is characterized by the presence of several ionizable groups, it is not hydrophobic acid. Thus LKNG, or its analogs, do not match the requirements for interaction with CPE. This is consistent with the fact that CPE may not be involved in sorting PF4 or any α-granule proteins since VWF is correctly routed to platelet α-granules in CPE−/− mice (7).

As expected, neither LIATLKNGRK nor LKNG share any primary homology with the cytoplasmic domain of the membrane protein P-selectin, which supports its targeting to the α-granule membrane (11, 12). This is consistent with the assumption that the soluble megakaryocytic protein PF4 follows a distinct sorting pathway than P-selectin and with the observation that post-trans-Golgi network trafficking of granule membrane proteins and their soluble variants is different (13).

The fact that LKNG alone supports granule targeting could be used to target ectopic proteins to platelet α-granules; LKNG-tagged proteins introduced into megakaryocyte progenitors would be targeted and stored into platelet α-granules and delivered upon platelet secretion at sites of vessel injury. This would allow high local concentrations and rapid delivery at specific sites while avoiding requirements for high blood levels, sometimes difficult to achieve (such as in the case of recombinant coagulation factors) or deleterious (seric immune response, toxicity . . . ). Such an approach might therefore be used for therapeutic strategies in congenital coagulation defects (hemophilia), in cancer therapy, or chronic inflammatory diseases.

Finally, our work may have a potential outcome for the future understanding of the human bleeding disorder GPS. Indeed, GPS effects (hemophilia), in cancer therapy, or chronic inflammatory response, toxicity . . . ). Such an approach might therefore be used for therapeutic strategies in congenital coagulation defects (hemophilia), in cancer therapy, or chronic inflammatory diseases.

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