Molecular Requirements for Sorting of the Chemokine Interleukin-8/CXCL8 to Endothelial Weibel-Palade Bodies*

Johanna Hol†1, Axel M. Küchler†5, Finn-Eirik Johansen†5, Bjørn Dalhus*, Guttorm Haraldsen†2, and Inger Øynebråten†‖

From the †Institute and ‡Division of Pathology, the †Institute of Clinical Biochemistry and Medical Microbiology, and the ‡Institute of Immunology, University of Oslo, Rikshospitalet University Hospital, Sognsvannsveien 20, 0027 Oslo, Norway

Sorting of proteins to Weibel-Palade bodies (WPB) of endothelial cells allows rapid regulated secretion of leukocyte-recruiting P-selectin and chemokines as well as procoagulant von Willebrand factor (VWF). Here we show by domain swap studies that the exposed aspartic acid in loop 2 (Ser44-Asp45-Gly46) of Willebrand factor (VWF). The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

The regulated secretion of proteins from vascular endothelial cells provides a mechanism for their rapid delivery in response to secretagogues (1–4). The best characterized organelle for such secretion is the cigar-shaped Weibel-Palade body (WPB) that contains a number of proteins important in hemostasis and inflammation. For example, release of WPB is most likely involved in the early events of acute inflammation, since P-selectin stored in this compartment (5) mediates the tethering and rolling of leukocytes (6) and even appears to be the dominant selectin involved in ischemia/reperfusion injury (7, 8). Moreover, we and others have previously shown that the chemokines interleukin-8 (IL-8)/CXCL8 (9, 10) and eotaxin-3/CCL26 (11) can also be stored in WPB and hence are prime candidates for converting the selectin-mediated rolling of leukocytes into integrin-mediated firm adhesion. In this respect, WPB can be considered a “Swiss army knife of leukocyte recruitment,” able to rapidly deliver selectins and chemokines to the surface of endothelial cells that constitutively express the integrin ligands intercellular adhesion molecule-1 and -2.

Targeting of proteins to compartments of regulated secretion is postulated to be an active process where proteins are segregated from the default route of exocytosis, the constitutive secretory pathway (12). The formation of WPB appears to be triggered by the expression of its main constituent, the 350-kDa hemostatic glycoprotein von Willebrand factor (VWF) (1, 13, 14). VWF entails a large propeptide (741 amino acids) that has been shown to be crucial for both the sorting of VWF (14, 15) and its multimerization (13, 14). Interaction of the propeptide and the mature part of VWF is moreover required for the formation of WPB in endothelial cells (16). Multimerization and organization of VWF and its propeptide into tubules takes place in the trans-Golgi network (TGN) and is followed by the formation of an extensive coat of AP-1/clathrin around the emerging organelles (17). Furthermore, it appears that other molecules can be targeted to WPB by interaction with VWF. For osteoprotegerin (18) and P-selectin (5, 19), this probably happens at the TGN level. IL-8 also binds to VWF under conditions mimicking those of the TGN, and subcellular fractionation analysis revealed that IL-8 is stored in a stoichiometric ratio to VWF, suggesting a direct molecular interaction (20).

Mechanisms of chemokine sorting to the regulated secretory pathway are currently not well understood, but recent data from blood platelets point to a role for an exposed loop between the second and third β-strands of the molecule, referred to as loop 2 or the 40s loop, in the sorting of PF4/CXCL4, RANTES/CCL5, and NAP-2/CXCL7 to platelet α-granules (21). Specifically, the sorting of PF4 appears to depend on loop 2, featuring the residues 46Leu-Lys-Asn-Gly58 (21), and loops with similar three-dimensional structures are also found in RANTES and NAP-2, suggesting that this motif may be of general significance for chemokine sorting in platelets (21). In this regard, it may be of relevance that GROα (growth-related oncogene α)/CXCL1 contains a loop 2 sequence identical to that of PF4 and is found together with MCP-1 (monocyte chemotactic protein-1)/CCL2 in a recently characterized endothelial compartment for regulated secretion that we have designated type II granules of regulated secretion (11, 22).

The aim of this study was to elucidate the molecular properties of IL-8 that enable its targeting to endothelial WPB. Chemokines have a highly similar tertiary structure, consisting of a typical Greek key structural motif with three consecutive β-strands forming a β-sheet stabilized by one or two disulfide bridges and a C-terminal α-helix inclined at a 45° angle to the...
β-sheet (Fig. 2, A and B) (23). This similarity in protein fold and the existence of endothelial cell-derived chemokines that are not sorted to WPB (11) make them ideal candidates for domain swap studies. By means of alanine mutations or chimerization with IP-10 (interferon-γ-induced protein-10), a chemokine not targeted to WPB (11), we demonstrated that loop 2 of IL-8, consisting of the residues Ser43–Asp45–Gly46, is essential for sorting of the chemokine to WPB. Moreover, a chimera containing loop 2 and the α-helix from IL-8 on an IP-10 backbone sorted to WPB with the same efficiency as IL-8. The loop 2 area of IL-8 maintains a neutral net charge, and we propose that WPB targeting of IL-8 can occur when the overall charge is neutral or negative, whereas a positive charge is not tolerated. The fingerprint of the loop 2 region differs among chemokines and may confer specificity for sorting.

**EXPERIMENTAL PROCEDURES**

Reagents—Recombinant human epidermal growth factor and basic fibroblast growth factor were obtained from R&D Systems (Abingdon, UK). MCDB 131 and Opti-MEM I medium, fetal calf serum, gentamicin, fungizone, and L-glutamine were purchased from Invitrogen, and trypsin-EDTA was from Bio-Whittaker (Walkersville, MD). Restriction enzymes were purchased from Invitrogen, and trypsin-EDTA was from Bio-Whittaker (Walkersville, MD). Restriction enzymes were from New England Biolabs (Hitchin, UK). Unless otherwise noted, all other reagents were from Sigma.

Cell Culture—Umbilical cords were obtained with the mothers’ permission from the Department of Gynecology and Obstetrics, Rikshospitalet, according to a protocol approved by the Regional Committee for Medical Research Ethics (Health Region South, Norway, Approval 5-05152). Human umbilical vein endothelial cells (HUVECs) were isolated as described by Jaffe et al. (24) and cultured in MCDB 131 containing 7.5% fetal calf serum, 10 ng/ml EGF, 1 ng/ml basic fibroblast growth factor, 2 mM L-glutamine, 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, and 250 ng/ml fungizone (complete HUVEC medium). The cells were maintained at 37 °C in a humid 95% air, 5% CO2 atmosphere and split at a ratio of 1:3. Cultures were used at passage levels between 1 and 6.

Plasmid Construction—Full-length human IL-8 and IP-10 were amplified by PCR from plasmids kindly provided by J. Oppenheim (NCI-Fredrick) and A. D. Luster (Massachusetts General Hospital, Boston, MA), respectively. The restriction sites Nhel and KpnI were added before subcloning into the expression vector pcDNA3.1 (+) (Invitrogen). Next, the 3' fusion with the HA tag was made by insertion of HA tag-encoding DNA oligomers containing KpnI and XhoI sites. Mutagenesis was performed by QuickChange PCR (Stratagene, La Jolla, CA) or by splice overlap extension and subcloning into an Nhel- and XbaI-digested pcDNA3.1 (+). Details of all plasmids will be provided upon request. Plasmid DNA was isolated using the Plus SV Minipreps kit from Promega (Madison, WI) or the QIAfilter Plasmid Maxi Kit from Qiagen (Madison, WI). The entire open reading frame of all plasmids was verified by DNA sequencing (Seqlab, Göttingen, Germany). Note that IL-8 exists in at least two forms, with the 72-amino acid form representing N-terminally truncated IL-8 (25). To avoid deviation from the generally accepted nomenclature, the numbering of residues in this paper (Fig. 1) is based on the 72-residue variant.

**RESULTS**

To identify motifs of IL-8 important for targeting of the protein to WPB, we designed mutants of IL-8 as well as chimeras of WPB targeting of IL-8 can occur when the overall charge is neutral or negative, whereas a positive charge is not tolerated. The fingerprint of the loop 2 region differs among chemokines and may confer specificity for sorting.

**Molecular Requirements for Sorting of IL-8 to WPB**

Transfection—HUVECs, grown to 70–80% confluence, were trypsinized by 0.05% trypsin-EDTA and washed once in complete HUVEC medium before resuspension at 2 × 10^6 cells/ml in Opti-MEM I containing 2.5% fetal calf serum. The cell suspension (400 µl) was mixed with 25 µg of DNA, transferred to a 4-mm disposable cuvette (model 640, Bridge Bioscience Inc., Portsmouth, NH), and electroporated with a BTX ECM830 ElectroSquarePorator (BTX Instrument Division, Harvard Apparatus Inc., Holiston, MA) in LV mode (single pulse of 160 V, 70 ms). The cells were seeded in gelatin-coated 8-well LabTek™ chamber slides (Nalge Nunc International, Rochester, NY) at a density of 3 × 10^4 cells/well and cultured for 24–27 h before fixation in 4% paraformaldehyde for 10 min. One well with cells transfected with HA-tagged wild type (WT) IL-8 was included on every chamber slide to provide an internal control for normalization of the results.

Immunofluorescent Staining and Microscopy—To evaluate sorting, the fixed HUVECs were incubated with mouse monoclonal primary antibody against HA or IP-10 for 20 h at 4 °C, washed in phosphate-buffered saline containing 0.1% saponin, incubated with biotinylated horse anti-mouse IgG (H + L) and rabbit-anti-human VWF for 90 min at room temperature, washed, incubated with streptavidin-Cy2 and pig anti-rabbit IgG-TRITC for 60 min, washed, dipped in distilled H2O, dried, and mounted in polyvinyl alcohol. All antibodies were diluted in phosphate-buffered saline containing 1.25% bovine serum albumin and 0.1% saponin. Details of antibodies, manufacturers, and concentrations used are given in Table 2. Images were obtained using a Leica TSC XP confocal microscope (Leica Microsystems, Heidelberg, Germany) equipped with an argon (488 nm) and two helium/neon (543 and 633 nm) lasers. PL Apo ×40/1.25 – 0.75 and N Plan apochromat ×100/1.4 oil objectives were used.

**Enumeration of Sorting Index**—Sorting of chimeras and mutants to WPB is indicated by a sorting index (SI), defined as the ratio between the number of double positive HA/VWF granules and the total number of either HA-containing or VWF-containing granules. Control experiments showed that mean SI calculated from the two reference points (total number of HA- or VWF-containing granules) were similar but that the range of results obtained by the former method varied more (data not shown). 10 cells were counted manually for each construct per experiment, and the counts from individual cells were normalized by division by the mean of WT IL-8 counts on the same chamber slide. All constructs were tested in at least three experiments with cells from different donors.

**Statistics**—Histograms of data indicated an approximately normal distribution. SPSS 16.0 was used to calculate mean and 95% confidence interval as well as to perform one-way variance analysis (analysis of variance) with post hoc Bonferroni testing (α-value of 0.05), providing two-tailed p values for differences in SI between each construct and WT IL-8 (values shown in Table 1).
Molecular Requirements for Sorting of IL-8 to WPB

IL-8 and IP-10. In HUVECs, IP-10 is released via constitutive secretion (11), apparently lacking the properties required to direct it to compartments of regulated secretion (26, 27). Moreover, the activity of such IL-8/IP-10 hybrids has been examined with regard to neutrophil activation and compared with that of WT IL-8, demonstrating that several of them retain a structure sufficient for receptor activation (28). On these grounds, IP-10 was considered a good candidate for chimera formation. An alignment of the two chemokines is shown in Fig. 1.

Sorting to WPB of the chimeras and mutants was examined in transiently transfected HUVECs incubated for 24–27 h before fixation and immunostaining. Subsequently, the ratio of double-positive HA/VWF granules was enumerated by fluorescent confocal microscopy. The ability of a given construct to sort to WPB is presented by an SI, defined as the percentage of colocalization relative to that of the WT control (see “Experimental Procedures”). Representative images of wild type IL-8, IP-10, mutants, and chimeras are shown in Fig. 2C. A summary of the results for all constructs examined is presented in Table 1.

The Presence of IL-8 in Endothelial WPB Depends on Amino Acids within Stretch 23–51 of IL-8—In the first series of experiments (Fig. 3), we examined a chimera (Ch1) in which the N-terminal part of IL-8, including the first amino acid of β-strand 1 was substituted with that of IP-10 (IP-10-(1–21)-IL-8-(23–72)), and observed an SI of 81% (Fig. 3 and Table 1). Likewise, a chimera (Ch2) in which loop 3 and the C-terminal α-helix of IL-8 were substituted with that of IP-10 (IL-8-(1–51)-IP-10-(52–74)) showed an SI of 78% (Figs. 3 and Table 1). On the other hand, the complementary chimera (Ch3) (IP-10-(1–51)-IL-8-(52–72)) demonstrated an SI of only 29% (Fig. 3 and Table 1). Based on these results, we wanted to examine the importance of residues 23–51 of IL-8, which constitute most of β-strand 1 as well as loop 1, β-strand 2, loop 2, and β-strand 3, and designed chimera 4 (IP-10-(1–21)-IL-8-(23–51)-IP-10-(52–74)) that showed an SI of 76% (Figs. 2C and 3). These data (Fig. 3 and Table 1) indicate that amino acids within the sequence 23–51 of IL-8 comprise the most important residues for sorting to WPB. Furthermore, the C-terminal α-helix together with the preceding loop 3 of IL-8 (Ch3) was not enough to induce sorting.

Loop 2 (Ser44-Asp45-Gly46) of IL-8 Is Necessary for the Localization of IL-8 to WPB—The three loops of IL-8 consist mainly of non-hydrophobic amino acids and are exposed on the surface of the globular structure (Fig. 2, A and B). Since protein-interacting sites typically are found in such exposed loops, we wanted to investigate their involvement in sorting by exchanging loop residues with alanine (Fig. 4). To test a potential role of residues in loop 1 (Ser29-Gly32-Phe33) in sorting, the two constructs 29AAA33 and 36AAA37 were designed (Fig. 4). The disulfide bridge-forming Cys43 was not altered due to its role in maintenance of the IL-8 structure, nor was Ala35. None of the constructs showed any significant reduction in SI (Fig. 4 and Table 1), suggesting that the charged or polar amino acids of loop 1 are not important for WPB localization. Alanine mutations of the residues in loop 3 (Ser43-Pro-Glu35) resulted in a weak but significant reduction in sorting (Fig. 4 and Table 1).

In contrast, replacement of loop 2 (43Leu-Ser-Asp-Gly46) with alanines (43AAA46) led to a significant reduction in sorting efficiency (Fig. 4 and Table 1). On these grounds, loop 2 was dissected further by performing individual alanine mutations of the loop residues as well as the preceding lysine and leucine (Fig. 4; K42A, L43A, S44A, D45A, and G46A). K42A, L43A, S44A, and G46A sorted efficiently to WPB, whereas mutation of Asp45 induced a strong reduction in SI to 26% (Fig. 4 and Table 1). The D45A mutant appears to be correctly folded, because it was secreted from our cultures at levels similar to those of WT (supplemental Fig. S1). To examine if hydrophobicity of the residue was sufficient for sorting or whether a negative charge was required, the residue was exchanged for the positive amino acid lysine. This mutation similarly resulted in an SI of 23% (Figs. 2C and 4 and Table 1). In this context, it is interesting to note that replacement of Asp45 with the positively charged arginine (D45R) retained binding to both IL-8 receptors with an affinity similar to that of WT IL-8 (29). Taken together, it would appear that D45A and D45K are correctly folded and functionally intact, and our data therefore indicate that the negative charge of Asp45 is of importance.

Replacement of loop 2 of IL-8 with the corresponding region of IP-10 (Fig. 4, 44KKKG46) abrogated sorting to WPB (Fig. 4 and Table 2). However, the grafting of loop 2 of IL-8 into IP-10 (Fig. 4, IP-10-44SDG46) did not induce sorting of IP-10 (Fig. 4 and Table 1). Moreover, loop 2 fused to the signal peptide of IL-8 did not colocalize with VWF (data not shown). The Ser44-Asp45-Gly46 motif of IL-8 is hence necessary for sorting to endothelial WPB, whereas the linear sequence on its own appears not to be sufficient.

The C-terminal α-Helix and Loop 2 of IL-8 Are Together Sufficient for Sorting to WPB—To shed further light on the criteria for sorting to WPB, chimeras containing loop 2 and varying regions of IL-8 were designed. First, we examined a chimera containing loop 2 and the flanking β-strands 2 and 3 of IL-8 (Fig. 5, Ch5), and observed a SI of only 30% (Fig. 5 and Table 1). Similarly, chimera 6, containing loop 2 and the N-terminal amino acids 1–28 of IL-8, showed an SI of 20% (Fig. 5 and Table 1). Hence, neither the adjacent β-strands of loop 2 nor the N-terminal part of IL-8 appeared to assist in sorting of the molecule to WPB. On the other hand, chimera 7, incorporating loop 3 and the C-terminal α-helix in addition to loop 2 of IL-8, efficiently increased the SI to 70% (Fig. 5 and Table 1), indicat-
Molecular Requirements for Sorting of IL-8 to WPB

Table 1

| Construct (figures providing details of chimeric structure) | Meana 95% CI | p valueb | Cells countedc |
|-----------------------------------------------------------|-------------|----------|----------------|
| WT IL-8                                                   | 100         | 98–102   | NA2           |
| Ch1 (Fig. 3)                                              | 81          | 73–89    | 0.003         | 40             |
| Ch2 (Fig. 3)                                              | 78          | 66–89    | <0.001        | 40             |
| Ch3 (Fig. 3)                                              | 24          | 16–32    | <0.001        | 40             |
| Ch4 (Fig. 3)                                              | 76          | 66–86    | <0.001        | 42             |
| 35AAAA45                                                | 100         | 90–109   | NS3           |
| 54AAAA45                                                | 94          | 86–103   | NS3           |
| 52AAAA45                                                | 41          | 32–51    | <0.001        | 53             |
| 35AAAA45                                                | 78          | 64–91    | 0.001         | 30             |
| K23A                                                    | 102         | 92–112   | NS3           |
| L43A                                                    | 96          | 87–105   | NS3           |
| S44A                                                    | 107         | 100–114  | NS3           |
| D45A                                                    | 26          | 19–33    | <0.001        | 51             |
| G46A                                                    | 98          | 89–107   | NS3           |
| D45K                                                    | 23          | 17–28    | <0.001        | 40             |
| IL-844KKKG46                                            | 8           | 6–10     | 0.001         | 60             |
| IP-1044SD-G46                                            | 21          | 17–26    | 0.001         | 38             |
| Ch5 (Fig. 5)                                             | 30          | 22–38    | <0.001        | 41             |
| Ch6 (Fig. 5)                                             | 20          | 15–25    | <0.001        | 30             |
| Ch7 (Fig. 5)                                             | 70          | 63–77    | <0.001        | 72             |
| Ch8 (Fig. 5)                                             | 61          | 52–70    | <0.001        | 42             |
| Ch9 (Fig. 5)                                             | 100         | 94–107   | NS4           |
| R20A/R60A/K65A/K67A/R68A                                 | 100         | 92–109   | NS3           |
| C7S/C9S/C34S/C50S                                        | 100         | 91–110   | NS2           |
| F17A                                                    | 97          | 88–106   | NS3           |
| 18AAAA45                                                | 93          | 79–107   | NS3           |
| 17AAAAAA45                                              | 50          | 39–62    | <0.001        | 30             |

a After normalization to internal control (WT IL-8).

b Difference from WT IL-8, adjusted by post hoc Bonferroni test; one-way analysis of variance between groups gave a significance level of <0.001.

c Total of HUVECs counted divided between at least three different donors.

d Not applicable.

e NS, not significant.

f Difference from IP-1044SD-G46, Student’s t test.

**FIGURE 2.** Structural properties and distribution of IL-8 and IP-10 constructs and IL-8/IP-10 chimeras in HUVECs. A and B, IL-8 shares the characteristic secondary structure of most chemokines, with an N-terminal flexible region culminating in an N-loop region and a short 310-helix, followed by three anti-parallel β-strands and a C-terminal α-helix. Loop 2 is located between strands β-2 and β-3. IL-8 may form homodimers, in which case two β-strands align to form an extended six-stranded β-sheet. Loop 2 of each monomer is exposed on opposite sides of the dimer. All central hydrophobic amino acid side chains making up the protein core connecting the β-sheet and α-helices of the two peptide chains are shown in ball-and-stick representations. The viewpoint in B is rotated 90° relative to A, C, transfected HUVECs were immunostained for a construct-derived HA tag (monoclonal antibody HA-7 or HA.11; green) and for VWF (red) as a marker of WPB. WT IL-8 colocalized with VWF, as demonstrated by the presence of yellow WPB (top). Mutation of Asp34 in loop 2 of IL-8 to K (D45K) arrested WPB localization. As previously shown, IP-10 does not colocalize with VWF. However, residues 23–51 of IL-8 (Ch4), loop 2 of IL-8 in combination with loop 3 of IL-8 (Ch8), or the residues of loop 2 of IL-8 plus the C-terminal α-helix of IL-8 (Ch9) grafted to IP-10 restored sorting to WPB. The corner insets show high magnification of framed areas. Scale bar, 10 μm.

**FIGURE 3.** Amino acids 23–51 of IL-8 encompass motifs involved in targeting to WPB. Chimeras 1–4 were designed to highlight areas of IL-8 important for sorting. Gray or white shading indicates IL-8- or IP-10-derived sequences, respectively. The diagram shows mean values and 95% confidence intervals of sorting indexes from pooled results of a minimum of three experiments, each with cells from different donors. The p values indicate the level of significance for the SI of the indicated construct compared with SI of WT IL-8. *, p < 0.001; †, p = 0.001.
Molecular Requirements for Sorting of IL-8 to WPB

tertiary structure of the chemokine, through the formation of stabilizing disulfide bridges (32). To prevent formation of disulfide bridges, which is reported to result in a molten globular tertiary structure of the molecule (32), the cysteines were replaced by serine residues (Fig. 6). However, the mutations did not induce any reduction in targeting to WPB (Fig. 6 and Table 1).

An N-terminal stretch of residues, 17FHPKFI22, forms a 310 α-helix flanking a hydrophobic pocket adjacent to loop 2 of IL-8 (29). This pocket is important for IL-8-specific receptor binding (29), and Phe17 and Ile22 contribute to the hydrophobic core of IL-8 (Fig. 7A). Simultaneous replacement of the entire stretch of residues with alanines reduced the SI to 50%, whereas mutation of Phe17 alone or the residues 18–21 did not significantly alter sorting to WPB (Fig. 6 and Table 1). Hence, the reduced sorting appears to be caused by loss of Ile22, the side chain of which is deeply embedded in the hydrophobic core of IL-8 (Fig. 7A) and probably involved in stabilizing the structure of the chemokine.

DISCUSSION

This study demonstrated that loop 2 of IL-8 is essential for localization of the protein to WPB, since mutation of the loop residues to alanine or replacement with loop 2 of the constitutively secreted chemokine IP-10 both abrogated sorting to WPB. Alone, loop 2 of IL-8 could not direct sorting to WPB when fused to the HA tag or when introduced in IP-10; however, when loop 2 was grafted to IP-10 together with the α-helix of IL-8, targeting to WPB was completely restored.

Loop 2 is exposed on the surface of IL-8 and forms a 3:5 type I β-hairpin turn with a G1 β-bulge between β-strands 2 and 3, consisting of the residues Ser-Asp-Gly in positions 44–46 (33) (Fig. 7B). This combination of amino acids typically constitutes a very stable β-turn (34), and in IL-8, Ser44 stabilizes the loop by hydrogen-bonding to Phe21, whereas Leu43 forms hydrogen bonds to Gly46 and Arg47, involving atoms in the protein backbone (33, 35). Our finding that Leu43, Ser44, or Gly46 could be replaced by alanine without a reduction in sorting indicates that these residues are not crucial for targeting to WPB. In contrast, Asp45 is critically important, since mutation of Asp45 to alanine or lysine abolishes sorting. The negatively charged side chain of Asp45 forms a salt bridge with the positively charged side chain of Arg47 and in this manner conveys a neutral charge to the loop area (Fig. 7B). Accordingly, mutation of Asp45 to alanine or lysine will remove the negative component of the region, and the net charge becomes positive. Moreover, chimera 9 with the negatively charged Glu47 of IP-10 following loop 2 rather than the positively charged Arg47 of IL-8 sorted as efficiently as WT IL-8, suggesting that a negatively charged loop area can also mediate efficient sorting. Thus, we propose that sorting to

FIGURE 4. Single or multiple mutations and domain swaps with IP-10 illustrate the importance of the loop regions of IL-8 in sorting to WPB. Gray or white shading indicates IL-8- or IP-10-derived sequences, respectively. Multiple alanine mutations of loop 2 and loop 3 but not loop 1 significantly reduced sorting to WPB. Mutation of Asp45 (D45A or D45K) and replacement of loop 2 by the corresponding area of IP-10 respectively. Multiple alanine mutations of loop 2 and loop 3 but not loop 1 significantly reduced sorting to WPB (Fig. 6 and Table 1). Thus, we propose that sorting to WPB was completely restored.

DISCUSSION

This study demonstrated that loop 2 of IL-8 is essential for localization of the protein to WPB, since mutation of the loop residues to alanine or replacement with loop 2 of the constitutively secreted chemokine IP-10 both abrogated sorting to WPB. Alone, loop 2 of IL-8 could not direct sorting to WPB when fused to the HA tag or when introduced in IP-10; however, when loop 2 was grafted to IP-10 together with the α-helix of IL-8, targeting to WPB was completely restored.

Loop 2 is exposed on the surface of IL-8 and forms a 3:5 type I β-hairpin turn with a G1 β-bulge between β-strands 2 and 3, consisting of the residues Ser-Asp-Gly in positions 44–46 (33) (Fig. 7B). This combination of amino acids typically constitutes a very stable β-turn (34), and in IL-8, Ser44 stabilizes the loop by hydrogen-bonding to Phe21, whereas Leu43 forms hydrogen bonds to Gly46 and Arg47, involving atoms in the protein backbone (33, 35). Our finding that Leu43, Ser44, or Gly46 could be replaced by alanine without a reduction in sorting indicates that these residues are not crucial for targeting to WPB. In contrast, Asp45 is critically important, since mutation of Asp45 to alanine or lysine abolishes sorting. The negatively charged side chain of Asp45 forms a salt bridge with the positively charged side chain of Arg47 and in this manner conveys a neutral charge to the loop area (Fig. 7B). Accordingly, mutation of Asp45 to alanine or lysine will remove the negative component of the region, and the net charge becomes positive. Moreover, chimera 9 with the negatively charged Glu47 of IP-10 following loop 2 rather than the positively charged Arg47 of IL-8 sorted as efficiently as WT IL-8, suggesting that a negatively charged loop area can also mediate efficient sorting. Thus, we propose that sorting to

TABLE 2

Antibodies and conjugates used for immunostaining

| Epitope     | Specification (clone) | Source (product number)          | Working concentration |
|-------------|-----------------------|----------------------------------|-----------------------|
| HA          | Mouse IgG, (HA-7)     | Sigma (H9658)                    | 0.3 µg ml⁻¹           |
| HA          | Mouse IgG, (HA-11)    | Bio Site, Täby, Sweden (MMS-101P) | 1 µg ml⁻¹            |
| IP-10       | Mouse IgG, (33036.211)| R&D Systems Europe Ltd., Abingdon, UK (MAB266) | 1 µg ml⁻¹            |
| VWF         | Rabbit polyclonal     | DAKO Denmark (A0082)            | 1:1400 (v/v)          |
| Mouse IgG (H + L) | Horse polyclonal, biotin-conjugated | Vector Laboratories, Burlingame, CA (BA-2000) | 7.5 µg ml⁻¹          |
| Rabbit Ig   | Pig polyclonal, TRITC-conjugated | DAKO Denmark (R0156)            | 18 µg ml⁻¹           |
| Biotin      | Streptavidin, Cy2-conjugated | Amersham Biosciences UK Ltd., Buckinghamshire, UK (PA42001) | 1 µg ml⁻¹           |
WPB can take place when loop 2 is neutral or negatively charged, whereas a positive charge in this region is not tolerated. Interestingly, IL-8 is the only CXC chemokine fulfilling this criterion and also the only known CXC chemokine that is sorted to WPB. The loop 2 sequences of other CXC chemokines follow one of two trends; either the loop contains Lys-Asx-Gly, with Asx representing either Asp or Asn (including the CXCR2 agonists GRO/H9251/H9252/CXCL2, GRO/H9251/H9252/CXCL2, GRO/H9251/H9252/CXCL3, PF4, and IP-10). Blue, positive potential; red, negative potential. The molecules have been superimposed to maximize protein backbone overlap in the region around loop 2. Dashed boxes indicate the close-up regions. Coordinates have been retrieved from the Protein Data Bank, codes 3IL8, 1MSG, 1F9Q (chain C) and 1LV9 (NMR model number 1), respectively. The electrostatic potential was calculated using the APBS (adaptive Poisson-Boltzmann solver) software package (51) and projected onto the solvent-accessible surface as calculated by PyMol (Delano Scientific, LLC, Palo Alto, CA).

Although indispensable, loop 2 of IL-8 was not sufficient to induce sorting to WPB when grafted to IP-10 or fused to the HA tag, and the efficient sorting of chimera 9 containing loop 2 and the α-helix of IL-8 grafted to IP-10 (SI 100%), suggests that the α-helix is also important. Whereas the α-helices of IL-8 and IP-10 are both amphiphilic with hydrophobic residues facing the β-sheets and hydrophilic residues...
Molecular Requirements for Sorting of IL-8 to WPB

on the solvent-exposed surface (33, 35, 37), they do have notable differences. For example, both helices interact with GAGs, but the main GAG-binding sites are located in different positions (30, 38, 39). Nevertheless, replacement of all known GAG-interacting residues of IL-8 (Arg60, Lys65, Lys67, and Arg69 from the α-helix as well as Arg20) with alanine did not affect sorting, suggesting that the positively charged residues of the α-helix involved in GAG binding are not important for targeting to WPB. It cannot be excluded that the remaining surface-exposed residues of the α-helix contribute to sorting by interaction with specific receptor molecules. However, we find it more likely that the α-helix is of structural importance, because chimera 2 (IL-8-(1–51)-IP-10-(52–74)) sorted fairly efficiently (SI 78%), and we would expect that the differences in surface-exposed residues between IL-8 and IP-10 would cause a more dramatic reduction in SI if they were involved in specific interactions.

If structural features of the α-helix indeed facilitate sorting of IL-8, this could be explained by the numerous interactions between helix residues and amino acids distributed across the whole molecule. One example is the orientation of the α-helix relative to the β-sheet, which is stabilized by a number of hydrophobic interactions (33, 35). For instance, Trp57 has been reported to stabilize the interactions between the N-terminal end of the α-helix and the region around the 310-helix by stacking of the aromatic ring with the cyclic side chain of Pro16 and simultaneously being in close contact with Thr13, Phe17, and Leu51 (33). Although a proline residue is found in a similar position of IP-10, there are no aromatic residues in the α-helix to provide a strong interaction between the C-terminal helix and the loop around the 310-helix (37).

Our data also suggest a role for Ile22 (the first residue of β-strand 1), the side chain of which is deeply buried within the molecule and involved in interactions with residues in both the α-helix and the β-sheet (Fig. 7A) (33). Loss of the hydrophobic side chain of Ile22 is likely to perturb the IL-8 structure. Thus, Ile22 may be important for sorting of IL-8 to WPB by maintenance of structure-preserving interactions with the C-terminal α-helix. At dimerization, the hydrophobic network around Ile22 combine with the corresponding area of the opposing monomer to form a larger hydrophobic network (Fig. 2B). Although currently unknown, it is indeed possible that IL-8 is dimerized in the TGN and WPB, given that other proteins destined for sorting to regulated secretory granules are typically found in high concentrations in these compartments (40). It is worth noting that loop 2 of IL-8 is situated away from the dimerization interface and that mutations in this region are unlikely to directly influence dimerization of IL-8 (41) (Fig. 2A).

The CXC chemokines PF4 and NAP-2 are stored in α-granules of platelets (42), and like IL-8, PF-4 is targeted to its regulated secretory granule by loop 2 (21). The loop 2 sequences of PF4 (Leu-Lys-Asn-Gly) and NAP-2 (Leu-Lys-Asp-Gly) differ from that of IL-8 in that they contain positively charged residues, thereby exhibiting a signal with a fingerprint different from the neutral patch of IL-8. Accordingly, sorting of chemokines to the WPB and the α-granule, which both contain VWF, appears to result from different signatures of loop 2, suggesting that different mechanisms and/or receptors are involved in their targeting to the respective granules. This is further supported by the finding that GROα with an identical loop 2 region to PF4 appears to colocalize with PF4 in α-granules but targets the VWF negative type 2 granule when expressed in endothelial cells. In this manner, the fingerprint of loop 2 may provide specificity for sorting. VWF has been proposed to act as a scaffold protein able to co-translocate other proteins to WPB (5, 18, 19, 43). Whether VWF directly affects sorting of IL-8 is unknown, but IL-8 interacts with VWF in a biacore assay at Ca2+-concentrations and a pH that mimics those of the Golgi/TGN, indicating that VWF could be important for targeting of IL-8 to WPB (20).

Interestingly, the propeptide of VWF (pro-VWF) contains a stretch of residues 616Ser-Asp-Gly-Arg-Glu620 identical to the loop 2 sequence of IL-8. Pro-VWF has been demonstrated to be important for sorting of VWF (15, 44), but the region of pro-VWF enabling targeting to WPB has hitherto not been identified. Using tools for predicting secondary structure elements in proteins by sequence analysis (45–48), the region involving the first ~1250 residues of VWF was predicted to fold as a structurally ordered, single domain (45, 46). Particularly, the 616Ser-Asp-Gly-Arg-Glu620 region was predicted with high confidence to form a surface-exposed loop flanked by two α-helices (47, 48). Ser-Asp-Gly-Arg sequences are found in similar positions in canine and murine pro-VWF, suggesting that the motif is well conserved between species. Furthermore, a Ser-Asp-Gly loop connecting two β-strands forms the major ubiquitin-binding site of Vps23, a subunit of yeast ESCRT-1 complexes (49).

Sorting motifs have not been identified for WPB-resident proteins other than P-selectin and CD63 (5), and no universal sorting signal has been identified for the entry of proteins into organelles for regulated secretion (12, 50). Instead, such signals appear to differ, depending on the secretory compartment, the protein, and the type of secretory cell (50). Such heterogeneity in the requirements for sorting enables selective sorting of proteins, including chemokines, to different secretory compartments. It is therefore interesting to note that in endothelial cells, IL-8 with a neutrally charged patch in loop 2 sorts to WPB (9, 10), whereas GROα with a loop 2 sequence similar to PF4 sorts to the type 2 granule of endothelial cells (11), and IP-10 with a loop 2 signature that differs from both IL-8 and GROα is constitutively secreted (11). Differential sorting might enable different post-translational modifications of the chemokines as well as highly controlled selective release.

In conclusion, this study shows that loop 2 of IL-8 (Ser44-Asp45-Glu46) is crucial for sorting of the chemokine to WPB. We propose that an overall neutral or negative charge of this region is essential and that an overall positive charge is not tolerated. We further speculate that the loop 2 region has a general role in sorting of chemokines to compartments for regulated secretion.
