Leucine-based Receptor Sorting Motifs Are Dependent on the Spacing Relative to the Plasma Membrane*

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Many integral membrane proteins contain leucine-based motifs within their cytoplasmic domains that mediate internalization and intracellular sorting. Two types of leucine-based motifs have been identified. One type is dependent on phosphorylation, whereas the other type, which includes an acidic amino acid, is constitutively active. In this study, we have investigated how the spacing relative to the plasma membrane affects the function of both types of leucine-based motifs. For phosphorylation-dependent leucine-based motifs, a minimal spacing of 7 residues between the plasma membrane and the phospho-acceptor was required for phosphorylation and thereby activation of the motifs. For constitutively active leucine-based motifs, a minimal spacing of 6 residues between the plasma membrane and the acidic residue was required for optimal activity of the motifs. In addition, we found that the acidic residue of leucine-based motifs must be located amino-terminal to the dileucine sequence for proper function of the motifs and that residues surrounding the motifs affect the activity of the motifs. Thus, our observations suggest that the position, the exact sequence, and surrounding residues are major determinants of the function of leucine-based receptor sorting motifs.

At least two types of leucine-based receptor sorting motifs can be described. One type is constitutively active, and receptors with this type of Leu-based motifs are sorted from the trans-Golgi network to late endosomes/lysosomes, e.g., the cation-dependent mannose 6-phosphate receptor, Limp-II, and the invariant chain of the major histocompatibility complex class II (II1) (1–3). The other type of Leu-based motifs is activated following phosphorylation. Most receptors carrying this type of Leu-based motifs are expressed at the cell surface and become internalized after protein kinase activation, leading to phosphorylation and activation of the Leu-based motifs (4–6).

Among other receptors, the T cell receptor (TCR) is internalized following phosphorylation of protein kinase C (PKC). PKC-induced internalization of the TCR is mediated via the Leu-based motif 5YFDFKTL132 in the cytoplasmic tail of the TCR subunit CD3γ (5, 7) (amino acid numbering of human CD3γ according to Ref. 8). The Leu-based motif in CD3γ has been extensively characterized, and PKC-induced internalization of the TCR can be described as a two-step process. In the first step, recognition and phosphorylation of CD3γ Ser126 by PKC, basic amino acids surrounding Ser126 are important (7). The phosphorylation of CD3γ Ser126 most probably induces a conformational change of CD3γ, leading to the second step, recognition and binding of clathrin-coated vesicle adaptor proteins to CD3γ. In this step, CD3γ Asp127, Leu131, and Leu132 constitute the binding motif for adaptor proteins (9).

Several studies have demonstrated that in addition to binding of Leu-based receptor sorting motifs, adaptor proteins have the capacity to bind tyrosine-based motifs (10–16). Furthermore, it has been shown that the position of Tyr-based sorting motifs influences the function of this type of receptor sorting motif. Thus, the YYRF motif of the transferrin receptor requires a spacing of at least 7 residues relative to the plasma membrane (PM) to function as an internalization motif (17), and the function of the YXXI sorting motif of lamp1 is also strictly dependent on the position within the cytoplasmic tail of lamp1 (18). Theoretically, the position of Leu-based motifs within the cytoplasmic tail of receptors may influence their activity at the trans-Golgi network and the cell surface (5). However, this possibility has not yet been experimentally addressed.

In this study, the role of the position of the CD3γ Leu-based motif in receptor sorting was examined in the complete TCR and in chimeric CD4/CD3γ molecules. We found that a minimal spacing of 7 residues between the PM and Ser126 was required for phosphorylation and activation of the CD3γ Leu-based motif in the TCR. Furthermore, the phosphorylation-independent, constitutively active Leu-based motif in chimeric CD4/CD3γ molecules required a minimal spacing of 6 residues between the PM and the acidic residue for optimal activity. Finally, we found that the acidic residue of Leu-based motifs must be located amino-terminal to the dileucine sequence for proper function of the motifs and that residues surrounding the motifs affect the activity of the motifs.
**EXPERIMENTAL PROCEDURES**

**Cells and Antibodies**—JGN cells, a TCR cell-surface negative variant of the human T cell line Jurkat that does not synthesize CD3ε (19), were cultured in RPMI 1640 medium supplemented with 2 × 10^4 units/liter penicillin (Leo Pharmaceutical Products, Ballerup, Denmark), 50 mg/liter streptomycin (Merck, Darmstadt, Germany), and 10% (v/v) FCS (Life Technologies, Inc., Paisley, United Kingdom) at 37 °C in 5% CO₂. The mouse mAb UCHT1 directed against human CD3e was obtained purified and phycoerythrin (PE)-conjugated from Dakopatts A/S, and the phorbol ester PDB. At the indicated times, cells were transferred to ice-cold phosphate-buffered saline containing 2% FCS and 0.1% NaN₃ and washed twice. The cells were stained directly with PE-conjugated mAb UCHT1 and analyzed in a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Mean fluorescence intensity (MFI) was recorded and used in the calculation of percent anti-CD3ε binding: \((\text{MFI of phorbol ester-treated cells})/\text{MFI of untreated cells} \times 100\%\). For each construct, at least three different clones were analyzed.

**Function of Leu-based Receptor Sorting Motifs**

**Transfection, and TCR Down-regulation**—All CD3ε mutagenesis and chimeric CD4/CD3ε molecules were constructed as described previously (5, 9, 20) by polymerase chain reaction using Vent DNA polymerase containing 3’ → 5’ proofreading exonuclease activity (New England Biolabs Inc., Beverly, MA) and the plasmids pJ6T3y-2 (8) and pCD-L3T4 25 (21) as templates. The primers used for each mutant are listed in Table I. The CD3ε polymerase chain reaction products were digested with XhoI and EcoRI and cloned into the 6.2-kilobase XhoI/EcoRI fragment of the expression vector pMH-Neo-CD3ε-WT. The CD4/CD3ε polymerase chain reaction products were digested with XhoI and EcoRI and cloned into the expression vector pMH-Neo-CD3ε (22). Mutations were confirmed by DNA sequencing. Transfections were performed using the Bio-Rad Gene Pulser at a setting of 270 V and 960 microfarads with 40 µg of plasmid/2 × 10⁷ cells. After 3–4 weeks of selection, G418-resistant clones were expanded and maintained in medium without G418. Approximately 30% of the clones expressed the transfected molecules. Clones expressing comparable levels of TCR were selected if possible. The expression level of the chimeric CD4/CD3ε molecules varied considerably between the different constructs, and clones expressing the highest level of each construct were selected for further studies.

**For TCR down-regulation, cells were adjusted to 2 × 10⁵ cells/ml of medium (RPMI 1640 medium and 10% FCS) and incubated at 37 °C with various concentrations of the phorbol ester PDB. At the indicated times, cells were transferred to ice-cold phosphate-buffered saline containing 2% FCS and 0.1% NaN₃ and washed twice. The cells were stained directly with PE-conjugated mAb UCHT1 and analyzed in a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Mean fluorescence intensity (MFI) was recorded and used in the calculation of percent anti-CD3ε binding: \((\text{MFI of phorbol ester-treated cells})/\text{MFI of untreated cells} \times 100\%\). For each construct, at least three different clones were analyzed.

The phosphorylation and internalization of the TCR and Chimeric CD4/CD3ε Molecules—Phosphorylation assays were performed as described previously (5, 7). The phosphorylated CD3ε chain with a molecular mass of 26–30 kDa was coprecipitated with CD3e (20 KDa) using anti-CD3ε mAb UCHT1 and subsequently analyzed by SDS-polyacrylamide gel electrophoresis. For each construct, at least two different clones were analyzed.

To determine the internalization rates of TCR and chimeric CD4/CD3ε molecules, cells were incubated in RPMI 1640 medium and 10% FCS at a cell density of 2 × 10⁵ cells/ml at 37 or 4 °C with PE-conjugated anti-CD3ε or anti-CD4 mAb, respectively. At the times indicated, aliquots of the cell suspension were washed in ice-cold RPMI 1640 medium and 10% FCS, divided in two equal parts, and subsequently treated with 300 µl of 0.5 M NaCl and 0.5 M acetic acid (pH 2.2) for 10 s or left untreated. The fluorescence of the cells was measured in the FACSCalibur. The percentage of internalized mAb to cell-surface bound mAb was subsequently calculated using the following equation:

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\text{Internalization} = \frac{\text{MFI of phorbol ester-treated cells} - \text{MFI of untreated cells}}{\text{MFI of untreated cells}} \times 100\%
\]

**TABLE I**

| Construct | CD3ε/CD4 | CD3ε/CD3 | CD3ε/CD4 | CD3ε/CD3 |
|-----------|----------|----------|----------|----------|
| CD3ε/CD4  |         |          |          |          |
| CD3ε/CD3  |         |          |          |          |

**Primers used to make the CD3ε mutations and the chimeric CD4/CD3ε molecules**

| CD3ε/CD4 | CAA GGA CCC TCG AGG GAT GTA TCA |
|----------|----------------------------------|
| CD3ε/CD3 | TGA GTC GAA TCC GTC TCA ATT CCT CCA AGG CTG |
| CD3ε/CD4Xb | CGA CTG CGC ACC GTC TGG TCC ATC CTT GTC TTG |
| CD3ε/CD3Xb | GGA CAG GAT GGA CAA GAC GGT GTT GTG CAG TCG AGA GCT |
| CD3ε/CD4X2b | CGA CTG CGC ACC GTC TGG TCC ATC CTT GTC TTG |
| CD3ε/CD3X2b | GGA CAG GAT GGA CAA GAC GGT GTT GTG CAG TCG AGA GCT |
| CD3ε/CD4X3b | CGA CTG CGC ACC GTC TGG TCC ATC CTT GTC TTG |
| CD3ε/CD3X3b | GGA CAG GAT GGA CAA GAC GGT GTT GTG CAG TCG AGA GCT |
| CD3ε/CD4X4b | CGA CTG CGC ACC GTC TGG TCC ATC CTT GTC TTG |
| CD3ε/CD3X4b | GGA CAG GAT GGA CAA GAC GGT GTT GTG CAG TCG AGA GCT |
| CD3ε/CD4X5b | CGA CTG CGC ACC GTC TGG TCC ATC CTT GTC TTG |
| CD3ε/CD3X5b | GGA CAG GAT GGA CAA GAC GGT GTT GTG CAG TCG AGA GCT |
| CD3ε/CD4X6b | CGA CTG CGC ACC GTC TGG TCC ATC CTT GTC TTG |
| CD3ε/CD3X6b | GGA CAG GAT GGA CAA GAC GGT GTT GTG CAG TCG AGA GCT |
| CD3ε/CD4X7b | CGA CTG CGC ACC GTC TGG TCC ATC CTT GTC TTG |
| CD3ε/CD3X7b | GGA CAG GAT GGA CAA GAC GGT GTT GTG CAG TCG AGA GCT |
| CD3ε/CD4X8b | CGA CTG CGC ACC GTC TGG TCC ATC CTT GTC TTG |
| CD3ε/CD3X8b | GGA CAG GAT GGA CAA GAC GGT GTT GTG CAG TCG AGA GCT |
**Function of Leu-based Receptor Sorting Motifs**

The function of Leu-based motifs on the surface of chimeric molecules can be assessed by monitoring their internalization rates following PKC activation. Motifs such as SDKQTLL and ADKQTLL, which promote receptor down-regulation, are highly conserved in lymphocytes and are thought to mediate the internalization of the TCR complex following PKC activation. To test the function of Leu-based motifs in a chimeric context, we created a series of constructs in which the spacing between the DKQTLL motif and the PM was varied. These constructs were transfected into JGN cells, and transfectants expressing the wild-type chimeric molecules were isolated. JGN-CD4/3-tS126 and JGN-d2 cells expressed the chimeras with high intensity, while JGN-CD4/3-d6 cells expressed the chimeras with high intensity at the cell surface. PKC-mediated TCR down-regulation was abolished in JGN-CD4/3-tS126 and JGN-d2 cells as in JGN-WT cells following PKC activation. In contrast, phosphorylation of CD3γ was abolished in JGN-CD4/3-d6 cells (Fig. 2C). These data indicate that the first step in PKC-mediated TCR internalization, namely recognition and phosphorylation of the Leu-based motif by PKC, is critically dependent on a spacing of at least 7 residues between Ser126 and the PM. Optimal Activity of Phosphorylation-independent Leu-based Motifs Is Dependent on a Spacing of at Least 6 Residues between the Acidic Amino Acid and the PM—We have previously shown that the SDRKQTLT motif functions as a constitutively active receptor sorting motif in chimeric CD4/CD3γ molecules independently of phosphorylation and that the DXXXL sequence constitutes a binding motif for adaptor proteins (9). Thus, chimeric CD4/CD3γ molecules expressing the wild-type SDRKQTLT motif or the ADKQTLT motif have high spontaneous internalization rates and are quickly degraded in the lysosomes. To determine the role of the spacing between the Leu-based motif and the PM for adaptor binding and receptor sorting, we took advantage of these observations. Constructs were produced in which the spacing between the DKQTLT motif and the PM was stepwise reduced by successive deletions of 1, 2, 4, 5, and 6 residues, respectively (Fig. 3A). These and the CD4/3-3S126 and CD4/3-SA constructs were separately transfected into JGN cells, and transfectants expressing the chimeric molecules were isolated. JGN-CD4/3-3S126 and JGN-CD4/3-d6 cells expressed the chimeras with high intensity at the PM; JGN-CD4/3-SA, JGN-CD4/3-d1, and JGN-CD4/3-d4 cells expressed the chimeras with low intensity; and JGN-CD4/3-d2 and JGN-CD4/3-d5 cells expressed the chimeras with intermediate intensity (Fig. 3B). To determine the activity of the Leu-based motif in the chimeras, the spontaneous internalization rates of the chimeras were measured. Three groups of the chimeras could be distinguished according to their internalization rates. Thus, the CD4/3-3S126 and CD4/3-d6 chimeras had low internalization rates; the CD4/3-SA, CD4/3-d1, and CD4/3-d4 chimeras had high internalization rates; and the CD4/3-d2 and CD4/3-d5 chimeras had intermediate internalization rates (Fig. 3C). Thus, the internalization rates of the chimeras inversely paralleled the cell-surface expression levels of the chimeras. Furthermore, pulse-chase metabolic labeling

**RESULTS**

Function of Leu-based Motifs Is Preserved When the Spacing Relative to the PM Is Increased—To investigate whether an increase of the spacing between the PM and the Leu-based motif of CD3γ influenced the function of the motif, the CD3γ-QDGx2 and CD3γ-QDGx3 constructs were made. In CD3γ-QDGx2 and CD3γ-QDGx3, the Leu-based motif was moved down from the PM by inserting 3 and 6 residues between the motif and the PM, respectively (Fig. 1A). These constructs and wild-type CD3γ were separately transfected into the CD3γ-negative Jurkat variant JGN (19), and TCR-positive transfectants were isolated. Both JGN-QDGx2 and JGN-QDGx3 cells down-regulated the TCR as efficiently as JGN-WT cells following PKC activation (Fig. 1, B and D). This demonstrated that the function of the CD3γ Leu-based motif was not affected by increasing the spacing between the Leu-based motif and the PM with at least 6 residues.

It has been suggested that a membrane-proximal versus a membrane-distal position of Leu-based motifs may influence their activity (5). To test the function of Leu-based motifs in a membrane-distal position, the CD3γ-dWT, CD3γ-dSα, and CD3γ-dDA constructs were made using the previously described CD3γ-LLAA as template (5). In CD3γ-LLAA, Leu124 and Leu127 are mutated to alanines, and PKC-mediated TCR down-regulation is abolished in JGN-LLAA cells, although Ser126 phosphorylation is intact (Fig. 1, A, C, and D). The CD3γ-dWT construct coded for the CD3γ-LLAA chain with the wild-type Leu-based motif at the C-terminal end. The CD3γ-dSα and CD3γ-dDA constructs coded for the CD3γ-LLAA chain with a mutated Leu-based motif corresponding to a Ser126 → Ala and an Asp127 → Ala mutation at the C-terminal end, respectively (Fig. 1A). These different types of Leu-based motifs were chosen as we have recently shown that they all function as sorting motifs in chimeric CD4/CD3γ molecules (9). Thus, the SDRQTLT and ADKQTLT motifs are constitutively active, and the SAKQTLT motif is activated by phosphorylation in chimeric CD4/CD3γ molecules. All of the transfectants expressed the TCR at comparable levels, indicating that the CD3γ-dWT and CD3γ-dSα constructs were separately transfected into JGN cells, and transfectants expressing the chimeric molecules were isolated. JGN-CD4/3-3S126 and JGN-CD4/3-d6 cells expressed the chimeras with high intensity at the PM; JGN-CD4/3-SA, JGN-CD4/3-d1, and JGN-CD4/3-d4 cells expressed the chimeras with low intensity; and JGN-CD4/3-d2 and JGN-CD4/3-d5 cells expressed the chimeras with intermediate intensity (Fig. 1F). Thus, when positioned membrane-distal, the SAKQTLT version of Leu-based motifs functioned as efficiently in PKC-mediated TCR down-regulation as the membrane-proximal wild-type Leu-based motif.

Phosphorylation of the Leu-based Motif Is Critically Dependent on a Spacing of at Least 7 Residues between Ser126 and the PM—To investigate whether a reduction of the spacing between the PM and the Leu-based motif of CD3γ influenced the function of the motif, the CD3γ-d1, CD3γ-d2, CD3γ-d3, and CD3γ-d6 constructs were made. In these constructs, successive deletions of 1, 2, 3, and 6 residues stepwise reduced the spacing between the Leu-based motif and the PM (Fig. 2A). The constructs were separately transfected into JGN cells, and TCR-positive transfectants were isolated. The ability of the transfectants to down-regulate the TCR following PKC activation was tested. As shown in Fig. 2B, JGN-d1 and JGN-d2 cells down-regulated the TCR as efficiently as JGN-WT cells. However, PKC-mediated TCR down-regulation was abolished in JGN-d3 and JGN-d6 cells (Fig. 2, B and D). To determine whether phosphorylation of Ser126 was affected by reducing the spacing relative to the PM, phosphorylation assays were performed. CD3γ was as efficiently phosphorylated in JGN-d1 and JGN-d2 cells as in JGN-WT cells following PKC activation. In contrast, phosphorylation of CD3γ was abolished in JGN-d3 and JGN-d6 cells (Fig. 2C). These data indicate that the first step in PKC-mediated TCR internalization, namely recognition and phosphorylation of the Leu-based motif by PKC, is critically dependent on a spacing of at least 7 residues between Ser126 and the PM.
Function of Leu-based Receptor Sorting Motifs

**A**

| Cell line   | CD3γ cytoplasmic tail sequence | TCR down-regulation |
|-------------|-------------------------------|---------------------|
| JGN-WT      | QDGVRQSRAS DKOTLLFNDQ LYQPLKRD | +                   |
| JGN-QDGx2   | QDGVRQSRAS DKOTLLFNDQLYQPLKRD  | +                   |
| JGN-QDGx3   | QDGVRQSRAS DKOTLLFNDQLYQPLKD   | +                   |
| JGN-ILAA    | QDGVRQSRAS DKOTLLFNDQLYQPLKD   | +                   |
| JGN-doWT    | QDGVRQSRAS DKOTLLFNDQLYQPLKD   | +                   |
| JGN-doSA    | QDGVRQSRAS DKOTLLFNDQLYQPLKD   | +                   |
| JGN-doDA    | QDGVRQSRAS DKOTLLFNDQLYQPLKD   | +                   |

**B**

![Graph showing TCR down-regulation](image)

**C**

![Graph showing TCR down-regulation](image)

**D**

![FACS histograms](image)

**E**

![Graph showing internalization rates](image)

**F**

![Graph showing internalization rates](image)

**Fig. 1.** Function of Leu-based motifs is preserved when the spacing between the motifs and the PM is increased. A, schematic representation of the amino acid sequences in the cytoplasmic tails of the CD3γ chains expressed in the indicated cell lines and a summation of the results from the TCR down-regulation analyses. B and C, TCR down-regulation of cells incubated with different concentrations of the PKC activator PDB for 1 h. TCR down-regulation was determined by staining the cells with PE-conjugated anti-CD3 ε mAb followed by flow cytometry comparing MFI of PDB-treated cells with MFI of untreated cells. D, FACS histograms of untreated cells (white areas) and cells treated with PDB (110 nM) for 1 h (black areas). The cell line and the percent anti-CD3 binding following PDB treatment are given in the upper left corner of each histogram. The ordinate gives the relative cell number, and the abscissa gives the fluorescence intensity in a logarithmic scale in arbitrary units. MFI of the cell lines stained with irrelevant monoclonal antibodies varied between 2 and 6 arbitrary units (data not shown). E and F, internalization rates of the TCR in cells preincubated for 60 min at 37 °C in normal medium or in medium containing 12 nM PDB, respectively. The internalization rates were determined as described under "Experimental Procedures."
experiments demonstrated that highly expressed chimeras with low internalization rates were stable for at least 4 h, whereas weakly expressed chimeras with high internalization rates were degraded during the 4-h chase period (Fig. 3D).

Taken together, these data demonstrated that a spacing of at least 6 residues relative to the PM is required for optimal activity of phosphorylation-independent Leu-based motifs. The results obtained with the CD4/3-d2 chimera indicated that parameters other than the spacing relative to the PM might also influence the activity of Leu-based motifs.

A Basic Amino Acid at Position -1 Reduces the Activity of Phosphorylation-independent Leu-based Motifs—We (9) and others (23, 24) have previously demonstrated that an acidic amino acid (Asp or Glu) positioned 4 or 5 residues N-terminal to the dileucine sequence is required for optimal activity of Leu-based motifs. In this study, the CD4/3-d2 chimera had a reduced internalization rate as compared with the CD4/3-SA, CD4/3-d1, and CD4/3-d4 chimeras (Fig. 3). In contrast to the CD4/3-SA, CD4/3-d1, and CD4/3-d4 chimeras, a basic amino acid (Arg) immediately preceded the DKQTL motif of the CD4/3-d2 chimera. Thus, the possibility existed that the reduced internalization rate of the CD4/3-d2 chimera was not due to the altered spacing between the DKQTL motif and the PM, but was caused by the Arg located immediately N-terminal to the DKQTL motif. To test this hypothesis, the CD4/3-SR construct, in which Arg was substituted for Ser126, was made (Fig. 4A). FACS analyses demonstrated that the CD4/3-SR chimera was expressed at a higher level at the cell surface as compared with the CD4/3-SA chimera (Fig. 4B). In agreement with this, the CD4/3-SR chimera had a reduced internalization rate and was more stable as compared with the CD4/3-SA chimera in pulse-chase metabolic labeling experiments (Fig. 4, C and D). These data indicate that the presence of a basic amino acid immediately N-terminal to the acidic amino acid in Leu-based motifs reduces the activity of the motifs.

Functional Leu-based motifs are found both in type I (e.g. CD3γ) and type II (e.g. Ii) integral membrane proteins, indicating that adaptor proteins have the ability to bind Leu-based motifs regardless of their orientation relative to the PM. Thus, in type I integral membrane proteins, the acidic amino acid of the Leu-based motif is located membrane-proximal relative to the dileucine sequence, whereas in type II integral membrane proteins, the acidic amino acid of the Leu-based motif is located membrane-distal relative to the dileucine sequence. To test whether a laterally reversed Leu-based motif consisting of an acidic amino acid located membrane-distal relative to the dileucine sequence had the ability to function as a receptor sorting motif in type I integral membrane proteins, the CD4/3-rev construct was made (Fig. 4A). This construct was made using the previously described CD4/3-SDAA as template (9). In

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**Fig. 2.** Phosphorylation of the Leu-based motif is critically dependent on a spacing of at least 7 residues between Ser126 and the PM. A, schematic representation of the amino acid sequences in the cytoplasmic tails of the CD3γ chains expressed in the indicated cell lines and a summation of the results from the CD3γ phosphorylation and TCR down-regulation analyses. B, TCR down-regulation of cells incubated with different concentrations of PDB for 1 h. TCR down-regulation was determined by staining the cells with PE-conjugated anti-CD3ε mAb followed by flow cytometry comparing MFI of PDB-treated cells with MFI of untreated cells. C, phosphorylation analyses of CD3γ from JGN-WT (lane 1), JGN-d1 (lane 2), JGN-d2 (lane 3), JGN-d3 (lane 4), and JGN-d6 (lane 5) cells treated with PDB (225 nM) for 10 min. D, FACS histograms of untreated cells (white areas) and cells treated with PDB (110 nM) for 1 h (black areas). The cell line and the percent anti-CD3 binding following PDB treatment are given in the upper left corner of each histogram. The ordinate gives the relative cell number, and the abscissa gives the fluorescence intensity in a logarithmic scale in arbitrary units. MFI of the cell lines stained with irrelevant monoclonal antibodies varied between 2 and 6 arbitrary units (data not shown).
CD4/3-SDAA, Ser<sup>126</sup> and Asp<sup>127</sup> were mutated to alanines, which resulted in an inactive Leu-based motif (Fig. 4A). As for the CD4/3-SDAA chimera, the CD4/3-rev chimera was highly expressed at the cell surface, had a low internalization rate, and was stable for at least 4 h (Fig. 4, B–D). Thus, the laterally reversed Leu-based motif in CD4/3-rev did not function as an active receptor sorting motif. This indicated that although adaptor proteins have the ability to bind Leu-based motifs of both type I and II integral membrane proteins, the acidic amino acid of Leu-based motifs must be located N-terminal to the dileucine sequence for proper function of the motifs.

**DISCUSSION**

This is the first study that defines the critical minimal spacing between Leu-based motifs and the PM required for proper function of Leu-based motifs. For phosphorylation-dependent Leu-based motifs, a minimal spacing of 7 residues between the PM and the phospho-acceptor was required for phosphorylation and thereby activation of the motifs. For constitutively active Leu-based motifs, a minimal spacing of 6 residues between the PM and the acidic amino acid was required for optimal activity of the motifs.
In contrast to defining a critical minimal spacing, an upper limit of the spacing between the Leu-based motifs and the PM was not found. Thus, increasing the spacing between the CD3γ- and CD3α-motifs did not affect PKC-mediated TCR down-regulation. Even when located at the C-terminal end of CD3γ, the SAKQTLL version of the motif functioned as efficiently as the membrane-proximal wild-type motif. These results are supported by the presence of phosphorylation-dependent Leu-based motifs in gp130 (6) and the cation-independent mannose 6-phosphate receptor (25), which are located 139 and 155 residues from the PM, respectively (Table II). Taken together, these observations indicate that phosphorylation-dependent Leu-based motifs can be found several residues from the PM even in a membrane-distal position.

Reducing the spacing between the Leu-based motif and the PM to 6 residues completely abolished the first step in PKC-mediated TCR internalization, namely phosphorylation of the serine corresponding to Ser126. This observation is in good agreement with previous studies of the TCR demonstrating agreement with previous studies of the TCR demonstrating

![Image](https://via.placeholder.com/120)
of the motif. To avoid the dependence of phosphorylation, we took advantage of the DKQTLL sequence functions as an active phosphorylation-independent Leu-based motif in chimeric receptors like Tac/CD3γ and CD4/CD3γ (9, 28). The data obtained with the chimeric CD4/CD3γ molecules demonstrated that a spacing of at least 6 residues relative to the PM was required for optimal activity of the phosphorylation-independent Leu-based motif. A spacing of 5 residues allowed suboptimal activity of the motifs, whereas a spacing of 4 residues did not allow activity of the motif. These data concur with the observation that lysosomal sorting is abolished in mutated Limp-II molecules in which the Leu-based motif is spaced only 4 residues from the PM (2) and are furthermore supported by the identification of a Leu-based motif in the β2-adrenergic receptor located only 5 residues from the PM (Table II) (29). Constitutively active Leu-based motifs are also found in Limp-II, CD44, and the cation-dependent mannose type I and type II integral membrane proteins (Table II). This might share common mechanisms.

Furthermore, residues surrounding the Leu-based motif probably affect adaptor binding, as might be suggested from the observation that the presence of a basic amino acid immediately N-terminal to the Leu-based motif reduces the activity of the motif. Thus, our observations suggest that the position, the sequence, and residues surrounding Leu-based receptor sorting motifs are major determinants of their function.

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