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CHIPS binds to the phosphorylated N-terminus of the C5a-receptor

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ABSTRACT

Replacement of the sulfate groups, present in vivo on the N-terminus of the C5a-receptor (C5aR), by phosphate groups is explored. Phosphorylated mimics of the C5a-receptor N-terminus are synthesized and their binding to Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS) is studied by ITC and NMR. The phosphorylated C5aR mimics showed comparable binding affinity and a similar binding mode towards CHIPS compared to their sulfated forms. The activities of the phosphorylated peptides in a biological assay, however, were significantly lower compared to their sulfated counterparts.

Post-translational modifications (PTMs) are widely used in nature to regulate the function of proteins.[1] PTMs can be structurally very different, ranging from, for example, glycosidation to methylation of arginine or lysine side-chains. On the other hand, seemingly very similar PTMs, like phosphorylation and sulfation, exist as well. These groups have roughly the same size, are both negatively charged and can both be present on the side-chain of tyrosine residues (Fig. 1). Despite their similarity, sulfated and phosphorylated tyrosine residues are believed to have very different biological functions and localization. Phosphorylation is mainly found intracellularly and is involved in signal transduction pathways, while sulfation is believed to be an irreversible extracellular enhancement mechanism for protein–protein interactions.[2]

In the literature several cases have been described in which sulfated tyrosine residues could be replaced by their phosphorylated counterparts without loss of biological activity.[3–6] Recent reports, however, indicate substantial differences between sulfation and phosphorylation. Hoffhines et al. developed an anti-sulfotyrosine monoclonal antibody (PSG2), which shows a 13-fold higher affinity for sulfotyrosine over phosphotyrosine.[7] In a peptide containing a sulfated tyrosine the affinity was even 10^3-fold higher compared to the same peptide with the tyrosine phosphorylated. The sulfated N-terminus of the CCR5-receptor plays a crucial role in HIV-1 infection by forming a binding site for the gp120:CD4 complex and in this way facilitating viral entry.[8] Lam et al. showed that a phosphorylated CCR5 peptide did not compete with native sulfated CCR5 peptide for binding to the CD4-activated gp120 complex.[9]

The extracellular N-terminus of the C5a-receptor (C5aR) contains in vivo two sulfated tyrosine residues at position 11 and 14 (Fig. 2). These two sulfated tyrosine residues are essential for activation of this receptor by its endogenous ligand C5a.[10] The activation of the C5aR triggers recruitment of specific white blood cells to potential sites of infection. This process can be inhibited by the Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS), which binds with high-affinity exclusively to the sulfated N-terminus of the C5aR.[11–13]

A truncated version of CHIPS missing 30 residues at the N-terminus and designated $CHIPS_{31-121}$ appeared to have the same inhibitory potency compared to native CHIPS.[14] Peptides representing the N-terminal portion of the C5aR are valuable models for studying the interactions with CHIPS. Recently, we reported a 380-fold increase in affinity of $CHIPS_{31-121}$ for C5aR peptide 7–28

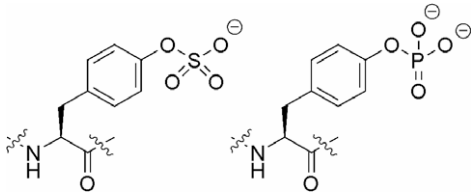

Figure 1. Structures of sulfo- and phosphotyrosine.

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(see Fig. 2) upon sulfation of tyrosine 11 and 14.[13] Here, we explore the effects on binding and potency of replacing the C5aR sulfate groups by phosphate moieties in order to gain more insight in the molecular interactions of CHIPS with the C5aR and in the biological significance of both PTMs.

Figure 2. Amino acid sequence of residues 7–28 of the human C5aR (numbering according to Swiss-Prot entry P21730). The positions of the sulfated or phosphorylated tyrosine residues 11 and 14 are indicated in black.

We successfully synthesized two C5aR peptides with a single phosphorylated tyrosine at either position 11 ($C5aR_{7-28}P^{11}$) or 14 ($C5aR_{7-28}P^{14}$) and one C5aR peptide with both positions 11 and 14 phosphorylated ($C5aR_{7-28}P_2$) using Fmoc SPPS. The phosphorylated tyrosine was introduced using the commercially available building block, Fmoc-Tyr(PO(OBzl)OH)-OH, in an adapted coupling scheme. This procedure included one additional equivalent of base during coupling of the phosphorylated building block and an additional ionic washing step (1.1 M D*i*PEA, 1 M TFA in NMP) after each Fmoc deprotection during the remaining coupling cycles to exchange the piperidine counterions for tertiary amines.[15,16]

The affinity of CHIPS for these three phosphorylated C5aR peptides was measured by Isothermal Titration Calorimetry (ITC). The dissociation constants (K_d) reveal that the phosphate groups have a significant influence on the binding affinity with CHIPS. Introduction of a phosphate group at position 11 results in a 13-fold increase in binding affinity compared to the unphosphorylated peptide $C5aR_{7-28}$ (Table 1). Clearly, the phosphate group at position 14 is involved in even stronger interactions: This phosphate group increases the binding affinity 70-fold. Finally, a 100-fold higher affinity compared to its unphosphorylated version was determined for the doubly phosphorylated peptide $C5aR_{7-28}P_2$ (K_d = 29.4 nM). We observed a similar trend for sulfated C5aR peptides.[13,17]

In order to obtain more information about the mode of binding of CHIPS to phosphorylated C5aR mimics we recorded ^{15}N-HSQC spectra of ^{15}N-labelled CHIPS before and after addition of an excess of doubly phosphorylated receptor mimic $C5aR_{7-28}P_2$. Based upon previous assignments we could determine the chemical shifts changes of the backbone amides of CHIPS upon titration of $C5aR_{7-28}P_2$.[13] A comparison of the amide ^{1}H–^{15}N chemical shift perturbations upon binding of either peptide $C5aR_{7-28}S_2$ or $C5aR_{7-28}P_2$ is presented in Figure 3. The data for residues 31–36, 54–56, and 93 were omitted from the perturbation plot of the CHIPS:$C5aR_{7-28}P_2$ complex as these residues could not be assigned unambiguously on the bases of previous spectra.

The phosphorylated C5aR mimics were also evaluated in a calcium mobilization assay. Activation of the C5aR by C5a was monitored in this assay by measuring the fluorescence of a U937/C5aR cell line loaded with a calcium sensitive Fluo-3 AM probe. Inhibition of the activation by CHIPS can be blocked by C5aR mimics, restoring receptor activation by C5a.[13] At concentrations >1 μM the phosphorylated peptides $C5aR_{7-28}P^{14}$ and $C5aR_{7-28}P_2$ compete with the native C5aR N-terminus present on the surface of the U937/C5aR cells. Peptide $C5aR_{7-28}P^{11}$ competes with the native C5aR N-terminus at concentrations >10 μM (Fig. 4).

Table 1
Dissociation constants of CHIPS:$C5aR_{7-28}$ phosphopeptide complexes determined by ITC

C5aR mimic	K_d (nM)[a]
$C5aR_{7-28}$[b]	$(3.2 \pm 0.1) \times 10^3$
$C5aR_{7-28}P^{11}$	244 ± 35
$C5aR_{7-28}P^{14}$	47.5 ± 3.1
$C5aR_{7-28}P_2$	29.4 ± 4.3

[a] Values are averages ± S.E.M. of three independent experiments.
[b] Data published previously.[13]

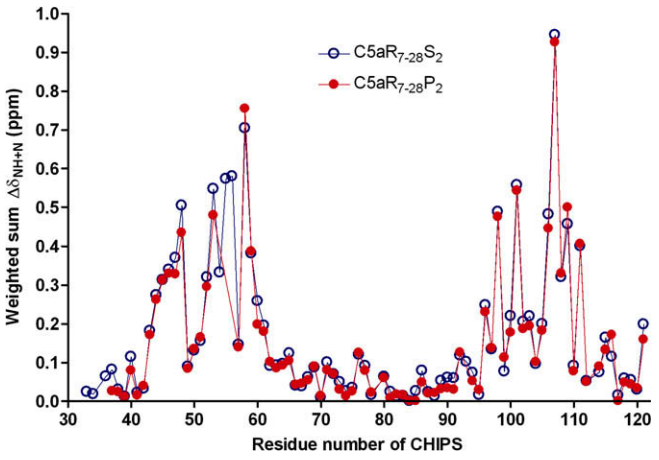

Figure 3. Weighted sum of CHIPS amide ^{15}N–^{1}H chemical shift changes ($\Delta\delta$ (ppm) = $[(\Delta\delta_{NH})^2 + (0.1\Delta\delta_N)^2]^{1/2}$) upon binding of $C5aR_{7-28}S_2$ (○) or $C5aR_{7-28}P_2$ (●). Chemical shift perturbation data were plotted versus residue number.

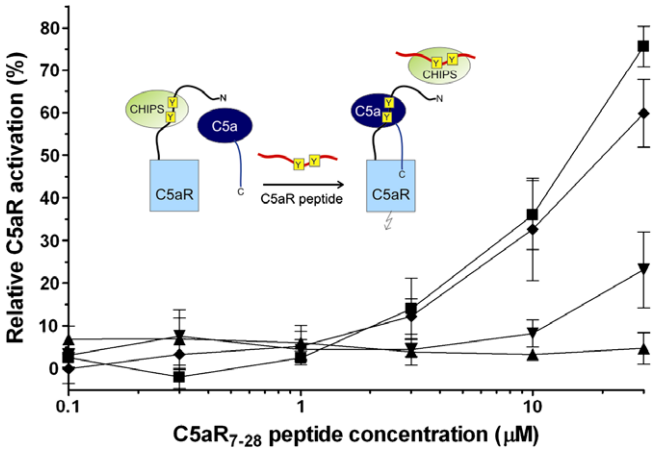

Figure 4. Calcium mobilization experiments: U937/C5aR cells were activated by 4 nM C5a in the presence of 10 nM $CHIPS_{31-121}$ and increasing concentrations of $C5aR_{7-28}$ (▲), $C5aR_{7-28}P^{11}$ (▼), $C5aR_{7-28}P^{14}$ (♦) or $C5aR_{7-28}P_2$ (■).

In conclusion, both the mono phosphorylated and the double phosphorylated C5aR mimics, $C5aR_{7-28}P^{11}$, $C5aR_{7-28}P^{14}$, and $C5aR_{7-28}P_2$, were successfully synthesized by applying Fmoc SPPS. ITC experiments with these peptides and $CHIPS_{31-121}$ revealed that the presence of phosphate groups results in significantly higher affinities compared to unphosphorylated peptides. The K_ds vary between 244 nM for $C5aR_{7-28}P^{11}$ and 29.4 nM for doubly phosphorylated $C5aR_{7-28}P_2$. The latter peptide binds 100-fold times stronger to $CHIPS_{31-121}$ than unphosphorylated peptide $C5aR_{7-28}$ (K_d = 3.2 μM).[13]

Comparison of the phosphorylated peptides described here with the previously measured sulfated peptides reveals that phosphorylation of the C5aR N-terminus results in similar binding affinities as sulfation.[13,17] Simultaneous modification of both tyrosine 11 and 14 leads to the highest affinity for CHIPS. The difference in contribution of tyrosine 11 and 14 to the overall affinity shows

a similar trend. In case of sulfation we concluded previously that tyrosine 14 of the C5aR contributes most to the affinity with CHIPS. This can be rationalized by numerous interactions between modified tyrosine 14 and the CHIPS 52–59 binding loop.[13] The difference between the two functionalized tyrosines is, however, larger for the sulfated form: $C5aR_{7-28}P^{11}$ binds stronger to CHIPS than $C5aR_{7-28}S^{11}$.[17]

Similarity in structure between phosphorylated versus sulfated C5aR peptides in complex with $CHIPS_{31-121}$ was supported by NMR spectroscopy. We observed in ^{15}N-HSQC spectra an almost identical pattern of chemical shift perturbations of the backbone amide signals of $CHIPS_{31-121}$ upon titration with either $C5aR_{7-28}S_2$ or $C5aR_{7-28}P_2$. This is highly indicative for the presence of similar structural features in both cases.

Surprisingly, the phosphorylated peptides were less active in the calcium mobilization assay as compared to sulfated peptides. Approximately 30-fold higher concentrations of $C5aR_{7-28}P_2$ are needed to compete with the native sulfated C5aR N-terminus.[13] This observation is in apparent contradiction with the results we obtained from ITC and NMR experiments. Several factors can account for this difference in behavior: The presence of doubly charged cations (Mg^{2+}, Ca^{2+}) and high concentrations of Human Serum Albumin (HSA) in the growth medium of the U937/C5aR cells, the presence of fast acting phosphatases or a higher affinity of other membrane bound proteins for phosphorylated peptides might interfere with the ability of the phosphorylated C5aR mimics to compete with the native receptor.

In summary, sulfated and phosphorylated peptide mimics comprising residues 7–28 of the C5aR have comparable affinity for the Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS). This makes the phosphorylated C5aR peptide mimic an attractive alternative compared to its sulfated version in in vitro studies with respect to simplicity of its chemical synthesis and improved stability. Replacement of sulfated C5aR mimics by phosphorylated mimics in in vivo experiments is less evident as the potency of the phosphorylated peptides was significantly less compared to the sulfated peptides in a biological assay. Apparently other extracellular constituents or mechanisms present in such assays or in vivo interfere with the activity of sulfated or phosphorylated constructs. This is subject to further studies.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.04.028.

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