A sequence conserved between CD5 and CD6 binds an FERM domain and exerts a restraint on T-cell activation

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Summary
CD5 and CD6 are related surface receptors that limit and promote T-cell responses. Co-stimulatory effects of CD6 depend on binding a cell surface ligand, CD166, and recruitment of the intracellular adaptor proteins GADS and SLP-76 by C-terminal phosphotyrosines. We have continued to identify interactions of CD5 and CD6 to understand their roles in T-cell activation. In a screen to identify binding partners for peptides containing a cytoplasmic sequence, SDSDY conserved between CD5 and CD6, we identified ezrin radixin moesin (ERM) proteins, which link plasma membrane proteins to actin. Purified radixin FERM domain bound directly to CD5 and CD6 SDSDY peptides in a phosphorylation-dependent manner ($K_D = 0.5-2 \mu M$) at $37^\circ$. In human T-cell blasts, mutation of the CD6 SDSDY sequence enhanced CD69 expression in response to CD3 monoclonal antibody. In this proximal readout, interactions of the SDSDY sequence were dominant compared with the C-terminal tyrosines of CD6. In contrast, in a more downstream readout, interleukin-2 expression, in response to immobilized CD3 and CD6 monoclonal antibodies, the C-terminal tyrosines were dominant. The data suggest that varying functional effects of CD6 and potentially CD5 depend on interactions of different cytoplasmic regions with the cytoskeleton and alter depending on the stimuli.

Keywords: inhibitory/activating receptors; signal transduction; T cell.

Introduction
CD5 and CD6 are T-cell surface receptors that regulate responses to antigen. The two receptors are related, both containing three scavenger receptor cysteine-rich (SRCR) domains in their extracellular regions and cytoplasmic tails with signalling potential. They have roles in limiting and promoting T-cell responses in vitro and in vivo.\textsuperscript{1-5} Progress has been made in identifying molecular interactions that regulate activating effects of CD6. Co-stimulatory effects of CD6 depend on binding to a cell surface ligand, CD166, and on recruitment of the intracellular adaptor proteins SLP-76 and GADS.\textsuperscript{6,7} Characterization of genetically manipulated mice lacking CD5 or CD6 revealed that both these receptors could exert inhibitory effects on T-cell activation.\textsuperscript{2,5} However, challenge of CD5- or CD6-deficient mice resulted in attenuation of autoimmune disease, implicating these receptors in promoting responses.\textsuperscript{8,9} In a different mouse background in a model with the less severe disease, lack of CD6 exacerbated disease.\textsuperscript{5} At the molecular level and in the whole organism, there seems to be a general trend for CD6 that with reduced stimulus, inhibition prevails.\textsuperscript{2,3,6,10} CD5 and CD6 appear to have the potential to mediate activating and inhibitory effects.

Parallels between the behaviour of the two related receptors suggest mechanisms in common. There is a conserved sequence, SDSDY in the cytoplasmic tails of CD5 and CD6. In CD5, there is in vitro and in vivo evidence for activating and inhibitory functions of the SDSDY sequence.\textsuperscript{11,12} In genetically manipulated mice, knocked-in CD5 with a deletion in the SDSDY sequence had a similar effect in reducing autoimmune disease compared with CD5-deficient cells, indicating that the SDSDY sequence is critical for CD5-mediated regulation.\textsuperscript{11} In in vitro analyses, the effects of mutant CD5 were inhibitory or activating depending on the stimulus and assay.\textsuperscript{11} Mapping sites for serine phosphorylation of CD5 and CD6 showed that the SDSDY...
sequence can be phosphorylated and is a substrate for casein kinase II.\textsuperscript{13,14} Phosphorylated forms of both serines and tyrosine residues in the SDSDY sequence in native CD5 in activated cells have been identified in mass spectrometry screens.\textsuperscript{15}

We report interactions of ezrin, radixin and moesin (ERM) proteins with the SDSDY sequence of CD5 and CD6, identified using a proteomics approach. We measured a direct interaction between a FERM domain of an ERM protein and CD5 and CD6 peptides using surface plasmon resonance. The affinities of the interactions were dependent on phosphorylation of the SDSDY sequence. In cells expressing recombinant receptors, mutation of the SDSDY sequence to prevent phosphorylation altered responses. An inhibitory effect of the CD6 SDSDY sequence on proximal signalling in primary CD4\textsuperscript{+} T cells was observed whereas the activating effect of C-terminal phosphotyrosines, which bind GADS and SLP-76, dominated in a more downstream readout.

**Material and methods**

**Peptides, pulldown and mass spectrometry**

Peptides representing human sequences and biotinylated at the N-terminus were obtained from Peptide Protein Research Ltd (Hampshire, UK): CD5 pSDpSDpY, PDNSpSDpSDpYDLHGAQRL; CD5 SDSDY, PDNSSDSDYDLHGAQRL; CD6 pSDpSDpY, GpSDpSDpYEHYDFSAQ; CD6 SDSDY: GSDSDYEHYDFSAQ; control peptide (CD5), HVDNEYSQPRNS; ICAM-2 (NP_001093259 FERM binding site (amino acids 251–275): HLRQQRMGTGYVRAAWRRLPQQAFRP.\textsuperscript{16}

Pulldowns and mass spectrometry have been described elsewhere.\textsuperscript{6} Briefly, peptides CD5 pSDpSDpY, CD6 pSDpSDpY or a control peptide derived from a region around a more proximal cytoplasmic tyrosine of CD5 were immobilized on magnetic beads and used to isolate interacting proteins from Jurkat T-cell lysates. Interacting proteins were processed, then digested on a filter, and the peptides were loaded onto the Ultimate 300 RSLCnano high-performance liquid chromatography ( Dionex, Sunnyvale, CA) -tandem mass spectrometry Q Exactive Orbitrap machine using a 25 cm by 75 \( \mu \)m inner diameter picotip analytical column (New Objective, Woburn, MA) in the central proteomics facilities at the Sir William Dunn School of Pathology, University of Oxford. Spectra analysis was conducted using the Mascot search engine.\textsuperscript{17}

**Soluble recombinant proteins and surface plasmon resonance**

Recombinant GST-tagged FERM domain of radixin constructed from mouse cDNA encoding amino acid residues 1–310, which are 100% identical between mouse and human in the pET49b(+) vector was kindly provided by Toshio Hakoshima (Nara Institute of Science and Technology, Japan).\textsuperscript{16} Radixin FERM was expressed in Rosetta (DE3) pLysS competent cells (Novagen, Madison, WI) and affinity purified using Glutathione Sepharose 4B (GE Healthcare, Chicago, IL). The GST tag was cleaved with HRV 3C protease (Novagen). Cleaved radixin FERM domains were then further purified using size exclusion chromatography.

For surface plasmon resonance analysis, CD5 pSDpSDpY, CD5 SDSDY, CD6 pSDpSDpY and CD6 SDSDY and ICAM-2 peptides were immobilized on streptavidin-coated CM5 chips in a BIAcore 3000 machine. Radixin FERM was injected over the immobilized peptides at 37\(^\circ\). Background signals of radixin FERM over a streptavidin-coated flow cell were subtracted and equilibrium binding data were fitted using a 1:1 Langmuir binding model using GraphPad Prism 7 software.

**Expression of recombinant CD6**

Chimeric CD6 (Genbank: HSU34623) containing rat CD6 domain 1 and cytoplasmic mutants were expressed as fusion proteins with enhanced green fluorescent protein (EGFP) using lentiviral vector (pHR-EGFP) details and detected with rat CD6 domain 1 (OX52) and human CD6 domain 3 (OX124) monoclonal antibodies (mAbs) as described elsewhere.\textsuperscript{6}

**Cellular assays**

Primary T-cell assays were conducted as described previously.\textsuperscript{6} Briefly, primary CD4\textsuperscript{+} T cells were isolated using RosetteSep\textsuperscript{TM} and transduced with lentivirus. Cells were stimulated for 18 hr at 37\(^\circ\) with platebound CD3 (UCHT1) at a range of concentrations and/or platebound rat CD6 domain 1 mAbs (OX52; 1–5 \( \mu \)g/ml) for CD69 expression and for interleukin-2 (IL-2) expression, with immobilized CD3 (2 \( \mu \)g/ml), rat CD6 domain 1 (OX52; 5 \( \mu \)g/ml) or a combination of CD3 and OX52 immobilized together at 5 \( \mu \)g/ml.\textsuperscript{6} CD69 and IL-2 expression were measured (percentage positive cells) by flow cytometry, gating on EGFP-positive cells. Experiments were performed once or twice for each donor with duplicate samples for CD69 and once with duplicate or single samples for IL-2. Data from donors for CD69 and IL-2, respectively: untransduced \( n = 11, n = 14 \); wild-type \( n = 11, n = 14 \); SDSDF \( n = 6, n = 10 \); ADADY \( n = 6, n = 10 \); ADADF \( n = 10, n = 14 \); Y629F Y662F \( n = 11, n = 13 \); ADADY Y629F Y662F \( n = 5, n = 8 \); ADADF Y629F Y662F \( n = 5, n = 6 \) were combined. Statistical analyses using analysis of variance with correction for multiple comparisons (Sidak) were carried out using GraphPad Prism 7 software.
**Results**

CD5 and CD6 pSdpSDpY peptides isolated ERM proteins

To identify potential interacting binding partners for the CD5 and CD6 cytoplasmic SDSDY sequence, we conducted pulldown experiments using Jurkat cell lysates and CD5 or CD6 serine and tyrosine-phosphorylated pSdpSDpY peptides. Five proteins were repeatedly isolated with CD5 and CD6 pSdpSDpY peptides and were not identified in pulldowns with an unrelated control peptide (Table 1). There was no enrichment for SH2 or PTB domain-containing proteins by the phosphorylated peptides. Proteins isolated specifically by the pSdpSDpY peptides include the 4-1, (FERM) domain-containing ERM proteins and the Na\(^+\)/H\(^+\) exchange regulatory cofactor (NHE-RF) 1 and 2. ERM proteins have an established role in connecting plasma membrane proteins to the underlying actin cytoskeleton. NHE-RF is a well-established ERM protein binding partner. These pulldown results suggest that CD5 and CD6 pSdpSDpY sequences associate with ERM proteins or possibly NHE-RF but do not give an indication which of these proteins bind directly or if they are part of a larger protein complex.

The radixin FERM domain directly binds to CD5 and CD6 pSdpSDpY

It was plausible that the cytoplasmic regions of CD5 and CD6 bound directly to FERM domains of the ERM proteins in a manner comparable with several other transmembrane proteins. We tested for direct binding of CD5 and CD6 to FERM domains using surface plasmon resonance. We chose a peptide from the transmembrane protein ICAM-2 as a positive control. The finding that radixin FERM domain to test for direct binding to CD5 and CD6.

Recombinant monomeric radixin FERM was injected over CD5 or CD6 pSdpSDpY or non-phosphorylated SDSDY peptides or a peptide derived from a known FERM domain binding site in ICAM-2 at 37\(^\circ\). Representative traces for radixin FERM domain over CD5 and CD6 phosphorylated peptides are typical for low-affinity equilibrium binding by monomeric protein (Fig. 1, right panels). Dissociation constants were measured by equilibrium binding, which is not affected by mass transport, which limits analysis of rapid kinetics. Fitting equilibrium binding data for three experiments for binding of radixin FERM domain to the CD5 and CD6 phosphorylated pSdpSDpY-containing peptides gave a \(K_D\) = 1–2 \(\mu\)M for CD5 and \(K_D\) = 0.5–2 \(\mu\)M for CD6. The affinity of radixin FERM for the ICAM-2 peptide was measured as \(K_D\) \(\geq\) 0.2 \(\mu\)M, indicating that the radixin FERM domain was active (Fig. 1c). A higher \(K_D\) compared with previous measurements for binding of radixin FERM domain to a mouse ICAM-2 peptide may be explained by differences in temperature and peptide sequence. Binding of radixin FERM domain to CD5 and CD6 non-phosphorylated SDSDY peptides was weak and did not reach saturation (Fig. 1a, b). These data indicate that not only are the interactions between CD5 and CD6 and the ERM proteins direct but they are dependent on phosphorylation of the SDSDY sequence.

The CD6 SDSDY sequence inhibits proximal T-cell activation

We tested the importance of phosphorylation of the SDSDY sequence for CD6-mediated signalling in primary human CD4\(^+\) cells. T-cell blasts were transduced with CD6 containing rat CD6 domain 1; wild-type cytoplasmic region (CD6), a single tyrosine mutant (SDSDY), a double serine mutant (ADADY), a triple mutant (ADADF), the C-terminal mutant (Y629F Y662F) or with both regions mutated, (ADADY Y629F Y662F or ADADF Y629F Y662F) as EGFP fusion proteins. All recombinant CD6 proteins were expressed at similar levels measured by rat CD6 domain 1 mAb (OX52) staining (Fig. 2a, left panel and data not shown). The relative increase in CD6 expression was revealed with a human CD6 domain 3 mAb (OX124) (Fig. 2a, middle panel). Transduction of wild-type or mutant CD6 did not affect the expression of CD3 (Fig. 2a, right panel).

Table 1. CD5 and CD6 pSdpSDpY peptides isolated ERM proteins from Jurkat T-cell lysates

| Protein       | % sequence coverage | Unique peptides |
|---------------|---------------------|----------------|
| Ezrin         | 42–46               | 24             |
| Moezin        | 49–45               | 25–35          |
| Radixin       | 25–31               | 7–13           |
| NHE-RF        | 25–50               | 7–13           |
| NHE-RF        | 18–33               | 6–8            |

1 Peptides only found in the target protein, each number represents one independent experiment.
2 Peptides found in three experiments.
3 Peptides found in one experiment.
4 Protein sequence covered by the identified peptides.
Cells were stimulated with platebound CD3 mAb. A consistent pattern was observed, and data from several donors (n = 5 to n = 11) are collated in Fig. 2(b). Differences among CD6 and mutants were most clearly detected at intermediate concentrations of CD3 mAb. Mutation of the residues, which could be phosphorylated in the SDSDY sequence, showed a trend toward increased responses reaching statistical significance in response to CD3 mAb (shown for 0.25 μg/ml) when all three were mutated (Fig. 2b, ADADF). These data indicated that the phosphorylation of the SDSDY sequence regulates CD6 signalling in a manner that differs from the C-terminal tyrosine motifs (Fig. 2b). The effect of the SDSDY sequence was diminished and became insignificant when the cells were stimulated with CD3 and CD6 mAbs (data not shown).

The CD6 SDSDY sequence attenuates proximal T-cell activation

We assessed the relative effects of the two regions of the cytoplasmic region of CD6 on proximal T-cell receptor-mediated signalling by combining mutations of the SDSDY and the C-terminus (ADADFY629F Y662F and ADADFY629F Y662F). In the proximal signalling readout of CD69 expression, responses by ADADFY629F Y662F in which just the two serines in the SDSDY sequence were mutated and ADADFY629F Y662F matched those of the SDSDY mutants, not the C-terminal mutant (Y629F Y662F). The responses of ADADFY629F Y662F and ADAFY629F Y662F were significantly (shown for CD3 mAb 0.25 μg/ml) raised above that of Y629F Y662F (Fig. 2b). The SDSDY region appeared dominant, and this was observed in the absence of ligation of the extracellular region of CD6 in these assays.

Interactions of the CD6 C terminus compared with SDSDY are dominant in a downstream readout of T-cell activation

The analysis of the effects of mutating CD6 on CD69 expression indicated that the SDSDY sequence can exert a restraint on proximal events in T-cell activation. To assess the relative effects of the SDSDY sequence and the C-terminus, we combined mutations in these regions (ADADFY629F Y662F). The responses of ADADFY629F Y662F and ADADFY629F Y662F were significantly raised above that of Y629F Y662F (Fig. 2b). The SDSDY region appeared dominant, and this was observed in the absence of ligation of the extracellular region of CD6 in these assays.
terminus of CD6 on a downstream measure of activation, we analysed IL-2 production in a single cell assay. Figure 3 shows combined data of the means from donors (n = 6 to n = 14). As we have shown previously, expression of CD6 increased the percentage of IL-2-positive cells in response to CD3 mAb and more markedly in the presence of the rat CD6 domain 1 mAb (Fig. 3a). Stimulation with the combination of CD3 and CD6 mAbs revealed a reduced percentage of IL-2-positive cells expressing the C-terminal mutant, Y629F Y662F of CD6 compared with transduced wild-type CD6 (Fig. 3b). The percentage of IL-2-positive cells remained reduced in cells expressing ADADY Y629F Y662F or ADADF Y629F Y662F (Fig. 3). The C-terminal mutations were dominant in a downstream readout when the T-cell receptor and the extracellular region of CD6 were ligated.

**Discussion**

Regulation of T-cell activation by non-catalytic tyrosine-phosphorylated receptors is generally mediated by phosphorylation-dependent interactions of tyrosine residues and direct recruitment of SH2 domain-containing proteins, which lead to activating or inhibitory signalling. Identification of physiologically relevant specific SH2 domain binding partners for the C-terminal region of these receptors using highly multivalent immobilized peptides has been successful. In a reciprocal manner, a complementary approach based on preservation of protein complexes associated with intracellular proteins also identified the interaction between SLP-76 and CD6. This latter approach identified CD5 together with SH2 domain-containing proteins in protein complexes. However, using the multivalent CD5 and CD6 peptides, we did not isolate a specific SH2 domain binding partner for the SDSDY sequence in CD5 or CD6. Identification of ERM proteins as binding partners for a phosphorylated SDSDY sequence in common between CD5 and CD6 suggests a phosphorylation-dependent mechanism that regulates the cytoskeleton and is relevant for regulating the activating and inhibitory effects by these receptors.

Binding of the cytoplasmic sequences in CD5 and CD6 to ERM proteins was novel in that it was dependent on phosphorylation. Dependence on phosphorylation suggested that negative charge was important for the interaction. This contrasts with basic residues in an ICAM-2 peptide being important for the affinity of the interaction. Transmembrane protein cytoplasmic regions containing less basic and more acidic residues have a reduced affinity generally by as much as ~50-fold for the radixin FERM compared with ICAM-2 in measurements made at 25°C. Compared with ICAM-2, CD5 and CD6 bound to the radixin FERM domain with only a fivefold to 10-fold lower affinity at 37°C. The affinity (K_D = 0.5 μM at 37°C) of the interaction between the phosphorylated CD6 SDSDY peptide and the radixin FERM domain is comparable with the phosphorylated C-terminal tyrosine interaction with the SH2 domain of SLP-76. These hierarchies indicate that interactions between CD5 and CD6 and ERM proteins are potentially physiologically significant in terms of ERM binding partners for type I transmembrane proteins.

The presence of NHE-RF adaptor proteins which bind to a different region of the PTB-like FERM subdomain among proteins isolated by the CD5 and CD6 peptides is consistent with the same or overlapping binding site for ICAM-2 and the phosphorylated SDSDY sequence. On the other hand, it is conceivable that the highly negatively
charged pSDpSDpY binds to the same positively charged surface as phospholipids such as IP₃, leading to ERM protein activation and subsequent binding to other transmembrane proteins.¹⁸

Detection of phosphorylation of a cluster of serines including the two in the SDSDY sequence in the absence of stimulation is consistent with the functional effects of mutating these serines being observed in the absence of a strong stimulus (Fig. 2B¹³). Phosphorylation of the tyrosine in SDSDY and possibly the tyrosine (Y489) three residues downstream (SDSDYEHY₁₄₉) may contribute to the electrostatic interaction with ERM proteins. Mutation of Y489 resulted in increased phosphorylation of CD6, indicating an inhibitory function and, similarly to the SDSDY sequence, was subservient to the effect of a C-terminal mutation in IL-2 production.¹⁰ Inhibitory effects of mutating the SDSDY sequence in recombinant CD6 were more apparent where the stimulus was limited to a CD3 mAb in the absence of CD6 ligation. In genetically manipulated mice, inhibitory effects of CD6 deficiency were observed in the presence of CD3 mAbs and obscured by the addition of co-stimulation with a CD28 mAb.³

Tipping the balance from inhibitory to co-stimulatory effects of CD6 seems to depend on the stimulus including engagement of other receptors. Increasing the number of antigen-presenting cells obscured the inhibitory effects of transduced CD6 in a murine T-cell hybridoma line.¹⁰ The effect appeared to be dependent on the antigen-presenting cells, not antigen dose.¹⁰ A strong stimulus with substantial up-regulation of CD166 on antigen-presenting cells will recruit more CD6 to cell-contact sites, resulting in activating effects dependent on tyrosine phosphorylation and recruitment of GADS/SLP-76 complexes.⁶,²⁵ The dominant effect of the C-terminal interactions compared with the SDSDY sequence on downstream events in T-cell activation supports the conclusion that recruitment of a dominantly activating and inhibitory effects of CD6 and by association, CD5. In preliminary experiments, mutation of the

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SDS DY sequence in CD5 in primary cells resulted in enhanced or reduced CD69 expression depending on the donor (JB and MHB, unpublished observations). In Jurkat cells, mutation of the SDS DY sequence in CD6 revealed that it had an activating effect on CD69 expression (JB and MHB, unpublished observations) leading to speculation that interactions of the SDS DY sequence contribute to the balance between inhibitory and activating functional effects by affecting transport or segregation of receptors rather than a direct interaction with a signalling enzyme.21,32,33

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Disclosure

None declared.

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