The CD45 tyrosine phosphatase regulates Campath-1H (CD52)-induced TCR-dependent signal transduction in human T cells

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Abstract

Campath-1H, a humanized mAb undergoing clinical trials for treatment of leukemia, transplantation and autoimmune diseases, produces substantial lymphocyte depletion in vivo. The antibody binds to CD52, a highly glycosylated molecule attached to the membrane by a glycosylphosphatidylinositol anchor. Cross-linked Campath-1H is known to activate T cells in vitro. We have investigated the molecular basis for these effects by comparing the protein tyrosine phosphorylation signals induced by Campath-1H and the CD3 mAb OKT3 in primary T cells, and in CD45⁺/c9059 TCR⁺/c9059, CD45⁻/c9059 TCR⁻ Jurkat subclones transfected with CD52. Our results show that Campath-1H triggers similar tyrosine phosphorylation events as OKT3 in both primary T cells and in the CD45⁺/c9059 TCR⁻ Jurkat sub-clone, albeit at quantitatively lower levels. However, no phospholipase Cγ₁ activation nor calcium signals were detected in response to CD52 ligation. The CD52-mediated induction of protein tyrosine phosphorylation was absolutely dependent upon the expression of both the TCR and the CD45 phosphotyrosine phosphatase at the cell surface. Cross-linking of Campath-1H was essential for signal transduction in all cells investigated. Fluorescence resonance energy transfer was used to demonstrate CD52 homo-association at the cell surface in Jurkat T cells in a TCR- and CD45-independent manner, and CD52–TCR association in CD45⁺/c9059 TCR⁺ cells. We propose a model to explain the activating effects of Campath-1H in which CD52 mAb cross-linking causes the trapping of TCR polypeptides within molecular complexes at the cell surface, thereby inducing signals via the TCR by a process which depends on the CD45-mediated regulation of the p56lck and p59fyn tyrosine kinases.

Introduction

T cell activation not only requires the engagement of the TCR, but also the participation of additional co-stimulatory receptors to induce optimal T cell proliferation (1). Several lymphocyte antigens with a diverse range of extracellular domains, but attached to the membrane with a glycosylphosphatidylinositol (GPI) anchor, have also been shown to transduce signals resulting in proliferation, IL-2 production and Ca²⁺ mobilization. These proteins include Thy-1 (2), Qa-2 (3), Ly-6 (4), human decay-accelerating factor (CD55) (5), CD59 (6) and CD73 (7). Antibodies to these molecules are mitogenic in vitro and may mimic the interaction of the natural ligand(s) in vivo. Campath-1H, a humanized antibody (8,9), is directed at CD52, a small heavily glycosylated protein expressed at high levels (5×10⁵ molecules/cell) on human lymphocytes and monocytes (10,11). CD52 is a very good target for complement-mediated lysis and antibody-dependent cellular cytotoxicity (12), and depletes T cells and B cells efficiently in vivo. Campath-1H has been used in clinical trials, many still in progress, to treat T and B cell malignancies, renal transplant rejection, rheumatoid arthritis, systemic vasculitis, and multiple sclerosis (13–17). The structure of CD52 has been extensively studied (18–20).
The molecule has a mol. wt of 21–28 kDa, as determined by SDS-PAGE and immunoblotting. The mature protein comprises a very short peptide of 12 amino acids with a single N-linked oligosaccharide. The protein is attached to the membrane via a GPI anchor (11) as determined by treatment with phosphoinositol-specific phospholipase C (PLC). The function and natural ligand(s) (if any) of CD52 remain unknown.

It has previously been shown that the combination of cross-linked Campath-1H with phorbol myristate acetate (PMA) is mitogenic in vitro (21), causing proliferation and cytokine production in resting CD4+ and CD8+ T cells. To understand the molecular basis for these effects we have studied intracellular signalling mediated by CD52 with particular reference to the regulatory role of the CD45 transmembrane phosphotyrosine phosphatase (22). The reasons for focusing on the role of CD45 are as follows: the physiological substrates for CD45 which have been identified so far are the Src-family protein tyrosine kinases p59
\(^\text{fyn}\) and p56
\(^\text{fyn}\) that associate with the TCR and CD4/8 antigens respectively (23–26). Interestingly, these kinases have also been found to co-precipitate with GPI-linked molecules (27–29). Furthermore, an intact TCR appears to be required for signalling through GPI-linked molecules (23,24). Since it is clear that CD45 controls the ability of the TCR to couple to intracellular signals by regulating the actions of p56
\(^\text{fyn}\) and p59
\(^\text{fyn}\) (22), it is possible that CD45 also regulates signalling through GPI-linked molecules. In fact, it has been reported that in the absence of CD45, T cell clones do not signal in response to antibodies to the GPI-linked molecule Thy-1 (25), and Thy-1 and CD45 have been reported to co-precipitate after chemical cross-linking (26).

In order to gain insight into the molecular mechanisms of CD52 signal transduction, we have studied early events such as protein tyrosine phosphorylation and calcium signalling. Using purified human primary T cells and mutant Jurkat sub-clones lacking either CD45 or TCR expression, we show that CD3 and Campath-1H mAb induce protein tyrosine phosphorylation in a similar array of substrates, including TCR ζ, and that the expression of both CD45 and the TCR are essential for coupling CD52 to intracellular signals. An intimate relationship between CD52 and the TCR is supported by our observation that CD52 associates with the TCR on the surface of intact cells. Surprisingly, however, Campath-1H failed to induce detectable calcium signals in either primary T cells or in Jurkat cells, suggesting that CD52-mediated signals are not identical to those triggered by CD3 mAb. We discuss the ways in which CD52 mAb such as Campath-1H may utilize the molecular signalling machinery of the TCR to induce activation signals in T cells.

**Methods**

**Reagents**

The following antibodies were used: CD45.2 (anti-human pan-C5, mouse IgG1), YTH360.10.1 (anti-human CD52, rat IgG2c), Campath-1H (anti-human CD52, human IgG1), OKT3 (mouse-anti-human CD3, IgG2a; Orthoclone, Cilag, France), Fb2 (mouse anti-phosphotyrosine, kindly provided by Dr D. Cantrell, London, UK), CT11 (goat anti-TCR ζ chain, kind gift from Dr Cox Terhorst, Boston, MA), anti-TCR ζ, anti-fyn and anti-PLCy1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-lck (Santa Cruz and kindly provided by L. Samuelson, Washington, DC), 4G10 (mouse anti-phosphotyrosine), linker for activation of T cells (LAT) and Vav mAb (Upstate Biotechnology, Lake Placid, NY), horseradish peroxidase-conjugated goat anti-mouse (GAM) (Dako, Glostrup, Denmark), sheep anti-mouse Ig (SAM) (Sigma, St Louis, MO), ZAP-70 mAb (Signal Transduction Laboratories), goat anti-human F(ab)\(^2\) (GAH) (ICN, Basingstoke, UK), and the following mouse anti-human mAb: anti-CD16 and anti-HLA-DR (Becton Dickinson, San Jose, CA), anti-CD19, anti-CD33, anti-CD8 (Serotec, Oxford, UK), and anti-glycophorin (Sigma). The Gst–GRB-2 fusion protein was a kind gift of D. Cantrell (London, UK). F(ab’\(^2\)) fragments were prepared from mAb as previously described (27).

**Flow cytometric analysis**

Flow cytometry was performed by staining cells with primary antibody in 50 µl PBS/10 mM Na\(_2\) on ice for 30 min. Cells were then incubated with either rabbit anti-mouse Ig–FITC (Dako) or, in the case of Campath-1H, with mouse anti-human–FITC (Sigma). After washing samples, viable cells were analysed (10,000 events) on a FACScan flow cytometer using CellQuest software (Becton Dickinson).

**Cells**

Primary T cells were isolated from buffy coats obtained from the National Blood Transfusion Service (Addenbrooke’s Hospital, Cambridge, UK). Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation from buffy coats prepared from the blood of healthy donors. PBMC were depleted of adherent cells by incubation in plastic tissue culture flasks (Nunclon; Nunc, Roskilde, Denmark) for 60 min at 37°C. T cells were then isolated from non-adherent cells by immuno-magnetic negative selection. Cells were resuspended at 10\(^7\) cells/ml in HEPES-modified RPMI 1640 supplemented with antibiotics (penicillin and streptomycin), L-glutamine and 5% heat-inactivated FCS, and incubated for 20 min at 4°C with saturating concentrations of anti-CD16, anti-CD19, anti-CD33 and anti-glycophorin mAb. Cells were washed twice, resuspended at 10\(^8\) cells/ml in medium, and incubated with SAM IgG-coupled Dynabeads (4×10\(^6\) beads/10\(^7\) cells) for 30 min at 4°C. The cells were then resuspended in 1 ml medium, and incubated with a Dynal magnetic particle concentrator for 2 min in order to remove monocytes, NK cells, B cells, dendritic cells and erythrocytes. The supernatant containing non-magnetized cells was removed, the cells were pelleted by centrifugation and the selection process performed once more. Cells were routinely >95% CD3\(^+\) as determined by flow cytometric analysis.

Jurkat sub-clones used were E6 (CD45\(^{-}\)TCR\(^+\)), J45.01 (CD45\(^{-}\)TCR\(^+\)) and 31-13 (CD45\(^{-}\)TCR\(^-\), kindly provided by A. Alcover, Paris). Jurkat cells were cultured in RPMI 1640 supplemented with antibiotics, l-glutamine and 5% heat-inactivated FCS. For electroporation with CD52 cDNA, Jurkat cells were grown in 500 µl RPMI supplemented with 20 % FCS and electroporated at 250V, 960 µF, using 20 ng of a purified CD52-containing plasmid (kind gift of Dr Masahide F.}
Tone, University of Oxford, UK). Stable transfectants were isolated using 1 mg/ml G418 (Sigma). Clones appeared after 3 weeks and were selected according to their phenotype as determined by FACS analysis.

Cell activation and immunoblotting

YTH 360.10.1 and Campath-1H were used to ligate CD52 at concentrations at 5–50 µg/ml with 2–10×10^6 cells in 100 µl. The best signal:noise ratio was obtained using 2×10^6 cells per assay and 5 µg/ml Campath-1H or 10 µg/ml YTH 360.10.1. The concentration of cross-linking antibody was kept constant at 100 µg/ml. Cells in 100 µl serum-free RPMI were incubated with antibody for 1 min followed by addition of cross-linking antibody for 5 min (unless otherwise stated), washed in medium and then lysed in lysis buffer (1% NP-40, 3 ml of 0.1 M formic acid/1 M ammonium formate and sub-

For labeling of antibodies and F(ab’)-cross-linking antibody for 5 min (unless otherwise stated), or F(ab’)/H11032 fragment was removed by washing the cells twice in PBS. Cells were either used immediately for measurements or were fixed in 1% formaldehyde. Results obtained with live and fixed cells were similar. Antibodies were centrifuged at 100,000 g for 30 min before use to avoid possible aggregation.

Flow cytometric energy transfer measurements were carried out using a modified Becton Dickinson FACStar Plus flow cytometer to determine FRET efficiency between F- and R-conjugated mAb and F(ab’)-fragments on a cell-by-cell basis. A detailed description of the method has been given elsewhere (31,32). In brief, fluorescence was excited with the 488 and 514 nm lines of an argon ion laser, and fluorescence emission was detected at 540 ± 20 and >580 nm for the 488 nm excitation, and >580 nm for the 514 nm excitation. Correction factors for the spectral overlap between the fluor-

Inositol phosphate (InsP) assay

InsP analysis was carried out according to the method of Bernidge et al. (29). Briefly, cells were labelled overnight with 1 µCi/ml myo-[3H]inositol (Amersham Pharmacia Biotech). Cells (2×10^6) cells were incubated in a final volume of 200 µl for 10 min with OKT3 (5 µg/ml), Campath-1H (20 µg/ml) and then GAH (100 µg/ml) or left untreated. Incubations were performed in triplicate. The reactions were terminated by addition of 750 µl chloroform: methanol and left on ice. Then 200 µl chloroform and water were added sequentially, mixed and spun for 1 min in a microfuge, and 800 µl of supernatant was removed and applied to a 750 µl Dowex column. After washing, total InsP was eluted with 3 ml of 0.1 M formic acid/1 M ammonium formate and sub-

subjected to liquid scintillation counting.

Fluorescence resonance energy transfer (FRET)

For labeling of antibodies and F(ab’)-fragments with fluorescent probes, purified aliquots of purified mAb and F(ab’)-fragments were conjugated with 6-(fluorescein-5-carboxamido)-hexanoic acid succinimidyl ester and 6-(tetramethylrhodamine-5-(and -6)-carboxamido)-hexanoic acid succinimidyl ester (Molecular Probes, Eugene, OR) as described (30). The use of a capital F or R in front of the name of an antibody or F(ab’)-fragment designates fluoresceinated and rhodaminated antibodies respectively. The dye:protein ratios varied between 2 and 4, and were separately determined for each labeled aliquot by spectrophotometric measurements. The fluorescently tagged antibodies and F(ab’)-fragments retained their binding capacity according to competition assays with identical, but unlabeled antibodies.

For labeling of cell surface antigens, cells were washed twice and resuspended in PBS. About 10^6 cells in 50 µl of PBS were incubated with fluorescently tagged antibodies or F(ab’)-fragments at saturating concentration for 30 min on ice in the dark. The applied antibody concentration (100–200 µg/ml) saturated all available binding sites and produced at least partly monomeric binding of the antibodies that reduced the possibility of cross-linking. The excess mAb or F(ab’)-fragment was removed by washing the cells twice in PBS. Cells were either used immediately for measurements or were fixed in 1% formaldehyde. Results obtained with live and fixed cells were similar. Antibodies were centrifuged at 100,000 g for 30 min before use to avoid possible aggregation.

Flow cytometric energy transfer measurements were carried out using a modified Becton Dickinson FACStar Plus flow cytometer to determine FRET efficiency between F- and R-conjugated mAb and F(ab’)-fragments on a cell-by-cell basis. A detailed description of the method has been given elsewhere (31,32). In brief, fluorescence was excited with the 488 and 514 nm lines of an argon ion laser, and fluorescence emission was detected at 540 ± 20 and >580 nm for the 488 nm excitation, and >580 nm for the 514 nm excitation. Correction factors for the spectral overlap between the fluor-

Results

CD52-induced signal transduction in primary human T cells

In light of previous results implicating p56^ck and p59^yn in the signals mediated by GPI-linked molecules, we expected that CD52 mAb also would induce increases in protein tyrosine phosphorylation in T cells. This was demonstrated by prepar-
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Fig. 1. CD52-induced protein tyrosine phosphorylation in primary human T cells (A) Purified human T cells were incubated without (C) or with CD52 mAb (YTH 360.10.1) for 2 min and anti-Ig added for the times indicated. Lysates were prepared, separated by SDS-PAGE and immunoblotted with phosphotyrosine mAb 4G10. The migration of protein molecular mass standards are shown on the left. (B) Lysates of human T cells unstimulated (lane 1) or with CD52 mAb (Campath-1H) and anti-Ig (lanes 2 and 3) were subjected to solid-phase immunoprecipitation with phosphotyrosine mAb Fb2, followed by SDS–PAGE separation and immunoblotting with 4G10. Lanes 2 and 3 show the results using two separate T cell preparations.

Fig. 2. Campath-1H and OKT3 induce tyrosine phosphorylation in similar proteins in human primary T cells. (A) Lysates of human T cells unstimulated (lane 1) or stimulated with cross-linked Campath-1H (lane 2) or OKT3 (lane 3) were separated by SDS–PAGE and then immunoblotted with 4G10. (B) Lysates of human T cells unstimulated (lane 1) or stimulated with cross-linked Campath-1H (lane 2) or OKT3 (lane 3) were immunoprecipitated with CT11 TCR ζ antibody (right-hand panel), (C) Grb2-fusion protein precipitates of human T cells unstimulated (lane 1) or stimulated with cross-linked Campath-1H (lane 2) or OKT3 (lane 3) were separated by SDS–PAGE and immunoblotted with 4G10.

T cell activation through GPI-linked molecules is thought to be dependent on the expression of a functional TCR (23,24). In order to investigate whether CD52-mediated signalling is related to TCR-induced tyrosine phosphorylation events, we compared CD52-induced phosphorylation with that caused by ligation of the TCR. Figure 2(A) shows that cross-linked Campath-1H (Fig. 2A, lane 2) and cross-linked CD3 mAb OKT3 (Fig. 2A, lane 3) induced increases in tyrosine phosphorylation in an apparently similar array of proteins. However, in this as in other experiments, the phosphorylation signals induced by Campath-1H were significantly lower than those triggered upon TCR ligation.

Many of the proteins which become tyrosine phosphorylated upon TCR ligation have been previously identified, and include the TCR ζ chain, the ZAP-70 tyrosine kinase, the proto-oncogenes p95Vav and p120Cbl, and the adaptor proteins SLP-76 and pp 36 (LAT) (34,35). It is thought that the
phosphorylation of TCR ζ by a process requiring CD45-activated p56fyn (28,36), plays a key role in initiating a signalling cascade which couples the TCR to intracellular signals. Figure 2(B, left panel) shows that both Campath-1H (Fig. 2B, lane 2) and OKT3 (Fig. 2B, lane 3) triggered increases in the p21 TCR ζ phosphorylation, although the level of phosphorylation induced by Campath-1H was much lower than that induced by OKT3, despite the presence of comparable amounts of the non-phosphorylated TCR ζ polypeptide in the immunoprecipitates (cf. Fig. 2B, right panel). Immunoblotting with a ζ chain-specific antibody also revealed that the amount of ζ chain polypeptide phosphorylated by OKT3 was small in relation to the total TCR ζ precipitated (cf. the small zeta-p signal at 21 kDa with the major zeta-0 signal at 16 kDa) and in fact was non-detectable upon CD52 ligation. These results confirm that the 21 kDa tyrosine phosphorylated protein illustrated in Figs 1(B) and 2(A) was TCR ζ.

The adaptor protein Grb-2 is known to bind to multimeric molecular complexes in T cells containing proteins which become tyrosine phosphorylated upon TCR ligation. These include p120cbl, p95vav, SLP-76 and LAT (37,38). To investigate the tyrosine phosphorylation of these proteins in response to Campath-1H, immobilized Grb-2 fusion protein precipitates from activated cells were immunoblotted with phosphotyrosine mAb. Figure 2(C) shows that the profile of phosphorylated proteins appeared very similar irrespective of whether the cells were activated with Campath-1H (Fig. 2C, lane 2) or OKT3 (Fig. 2C, lane 3). There was a marked increase in phosphorylation in both cases in the pp36 protein recently cloned and re-named LAT (37), as well as in proteins provisionally identified by their electrophoretic mobility as SLP-76, p95vav and p120cbl. Taken together these results suggest that CD52 ligation induces increases in tyrosine phosphorylation in many or even all of the same proteins which become phosphorylated upon TCR stimulation, suggesting that CD52 may utilize the molecular signalling machinery of the TCR to stimulate intracellular signalling pathways.

The role of CD45 and the TCR in CD52-mediated signalling

To obtain a fuller understanding of the role of the TCR in CD52-mediated signal transduction and, in particular, to elucidate the possible role of CD45 in this process, we used the Jurkat E6 (CD45–TCR+, A12), J45.01 (CD45–TCR+, B8) and 31-13 (CD45–TCR+, C2) sub-clones. Initial analysis revealed that CD52 expression on Jurkat cells was ~10-fold less than its expression on primary T cells (data not shown). Therefore we transfected each sub-clone with CD52 cDNA and selected stable transfectants expressing CD52 at levels comparable with those found in primary human T cells, as illustrated in Fig. 3. The low CD52 expression detected on the J45.01 sub-clone (B8) was similar to that earlier described for these cells, a level insufficient to promote TCR-mediated signals (38). The expression of p56fyn and p59fyn in the selected transfectants was similar, as established by immunoblotting, although the CD45–TCR– C2 sub-clone expressed approximately twice as much p59fyn as the A12 and B8 sub-clones (data not shown).

In the A12 CD45+ Jurkat sub-clone, both Campath-1H and OKT3 (Fig. 4A, lanes 2 and 3) induced a similar profile of protein tyrosine phosphorylation as in primary T cells, although as with primary T cells the phosphorylation signals induced by CD52 mAb were lower than those triggered by OKT3. It should also be noted that Jurkat cells were stimulated with OKT3 without further addition of a cross-linking reagent, whereas increases in tyrosine phosphorylation in response to CD52 stimulation were only observed following cross-linking of CD52 mAb (data not shown). Furthermore, the induction of TCR ζ chain phosphorylation was weak in Jurkat cells in response to OKT3 and was not detectable upon CD52 ligation. Interestingly, in the B8 and C2 sub-clones no Campath-1H- or OKT3-induced tyrosine phosphorylation was observed (Fig. 4A, lanes 5 and 6, and data not shown), showing that CD52 signal transduction is dependent upon CD45 and TCR expression. In addition, CD45+ cells had less background protein phosphorylation than CD45+ cells (Fig. 4A, cf. lanes 1 and 4).

CD52 signal transduction was further characterized in the Jurkat sub-clones by precipitating tyrosine phosphorylated proteins using the Fb2 mAb and then identifying the proteins by immunoblotting. Figure 4(B) shows that ligation of Campath-1H induced increased tyrosine phosphorylation of ZAP-70, Vav and LAT. The induction of LAT phosphorylation was confirmed by precipitating LAT with the immobilized Grb-2 fusion protein followed by immunoblotting with phosphotyrosine mAb (data not shown). In contrast, no increase in phosphorylation of PLCγ1 was detectable, although a clear PLCγ1 phosphorylation signal was observed in response to OKT3.

Campath-1H fails to trigger Ca2+ or InsP in human T cells

Mobilization of intracellular Ca2+ is an important event preceding cell proliferation in response to TCR ligation. As cross-linked Campath-1H plus PMA induces proliferation of human T cells in vitro (21), we investigated Ca2+ signalling in primary T cells and in the CD52-transfected Jurkat sub-clones. Whereas cross-linked OKT3 triggered a transient Ca2+ signal in primary T cells, in which ~50% of the cells responded, cross-linked Campath-1H failed to do so (Fig. 5). Similarly, in the CD45–TCR– A12 Jurkat sub-clone, as also illustrated in Fig. 5, no detectable calcium signal was induced in response to the cross-linking of Campath-1H, although the OKT3-stimulated calcium signal was readily detectable. As expected, no OKT3 or Campath-1H-stimulated calcium signals were detectable in the B8 or C2 sub-clones (data not shown). Cross-linked Campath-1H was tested at mAb concentrations in the range 1–100 µg/ml, but in no case was a calcium signal detected in either primary T cells or Jurkat cells (data not shown). Since the assays illustrated in figures were carried out over short periods of only 6 min, we considered that Campath-1H might be inducing a much slower and more gradual rise in intracellular Ca2+. However, monitoring of cells over a 30 min period following CD52 ligation did not lead to any detectable changes in calcium levels (data not shown). We also considered that Campath-1H might be inducing a negative signal which counteracted its possible ability to mobilise intracellular calcium. To test this hypothesis A12 cells were preincubated with cross-linked Campath-1H and then stimulated with OKT3 to determine whether the OKT3-triggered Ca2+ signal would be reduced. However, no inhibi-
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Fig. 3. Phenotypic characteristics of CD52-transfected Jurkat subclones. Jurkat sub-clones were stained for CD45, CD3 and CD52, and their relative cell-surface expression was compared to that expressed in primary T cells by flow cytometry. The control panel indicates staining with SAM–FITC mAb alone.

The failure of Campath-1H to induce a calcium signal is consistent with the lack of inducible PLCγ1 tyrosine phosphorylation following CD52 cross-linking (Fig. 4B). To assess whether Campath-1H could stimulate activation of PLCγ1, we measured InsP3 production in the A12 CD45⁺ Jurkat subclone in response to either TCR or CD52 ligation. As Table 1 illustrates, a 2- to 3-fold increase in InsP3 was generated in response to CD3 ligation, as expected, but no detectable InsP3 production was observed in response to cross-linked Campath-1H.

In summary, therefore, CD52 ligation induces a profile of tyrosine phosphorylated proteins which appears qualitatively similar but quantitatively lower to that induced upon TCR ligation in both primary and Jurkat T cells, and these signalling events are dependent upon both CD45 and TCR expression. However, CD52 signal transduction is not identical to the signals triggered by a CD3 mAb because neither PLCγ1 activation nor calcium signals were detected in response to CD52 ligation in contrast to the large PLCγ1 activation and calcium signals measured in response to TCR ligation.

CD52–TCR and CD52–CD52 association at the T cell surface

To investigate further the possible relationship between CD52 and the TCR at the T cell surface, we employed the FRET technique to measure possible associations of CD52 with the TCR and other cell surface receptors. FRET analysis has the advantage that it avoids the use of detergent and enables interactions to be detected on intact cells when molecules are juxtaposed within distances of 10 nm or less. In the present work fluorescein (F) and rhodamine (R) were used as the fluorescent probes, and the relevant mAb were directly conjugated to one or other of the probes. R-CD52 and F-CD52 were used to study CD52 homo-association. Previous experience using this technique suggests that energy transfer values of >10% indicate that a significant degree of association is taking place (27,30). Figure 6(A) shows that substantial energy transfer occurred between CD52 molecules on the surface of the CD52⁺-transfected Jurkat T cells, demonstrating homo-association of CD52. This homo-association was independent of the expression of CD45 or the TCR, because similar levels of energy transfer between R-CD52 and F-CD52 were found on all three Jurkat subclones (Table 2). The homo-association was not the result of
aggregation caused by Campath-1H IgG itself since R-F(ab') and F-F(ab') fragments derived from Campath-1H also showed similar energy transfer values (Table 2). Interestingly, a significant association of CD52 with the TCR also occurred on the TCR+CD45+ sub-clone (Fig. 6A), but the energy transfer efficiency significantly decreased from 25 ± 8% to 7 ± 3% in the cells lacking CD45 expression (Table 2). No significant levels of CD4–CD52, CD4–CD45 or CD52–CD45 association were observed in the three Jurkat sub-clones investigated (Table 2), so confirming the selectivity of the CD3–CD52 and CD52–CD52 associations. A low degree of association was, however, detected between the TCR and CD45.

We considered that if significant CD3–CD52 association was occurring on the surface of TCR+CD45+ cells, then unlabeled Campath-1H might perturb the binding of F-CD3 mAb to the TCR. Figure 6(B) shows that this in fact occurred and in the presence of the CD52 mAb the binding of F-CD3 to the TCR was substantially reduced (overall by 37%) on the A12 TCR+CD45+ sub-clone. Two distinct populations of cells differing in their ability to bind CD3 mAbs were observed when Campath-1H was present, presumably reflecting differences in the degree of interaction between CD52 and the TCR on different cells. For example, some cells might contain a higher level of large CD3–CD52 complexes in which Campath-1H would only mask the binding of F-CD3 to those TCR closest to CD52, whereas masking by Campath-1H would be expected to be more effective for smaller CD3–CD52 aggregates. When we repeated the experiment illustrated in Fig. 6(B), only now using the B8 TCR+CD45+ sub-clone, Campath-1H binding reduced F-CD3 binding overall by only 22%, consistent with the lower CD3–CD52 association detected on this sub-clone by FRET analysis (cf. Table 2). Why CD3–CD52 association is lower on cells lacking CD45 expression is currently unknown. No competitive effects were noted between other mAb pairs tested, including CD52 and CD4, and CD52 and CD45, on either A12 or B8 sub-clones (data not shown), consistent with the apparent lack of association between these antigens as revealed by FRET energy transfer values (Table 2).

**Discussion**

The findings in this paper represent the first molecular analysis of the signalling functions of the CD52 antigen. Ligation of CD52 in either primary T cells or in CD52-transfected Jurkat T cells results in the induction of a panoply of tyrosine phosphorylation events similar to those observed upon TCR stimulation, including TCR ζ phosphorylation (Figs 1–4). However, CD52 ligation did not trigger detectable increases in the calcium signalling pathway (Fig. 5). The signalling functions of CD52 mAb are dependent upon the expression of both the TCR and CD45 at the cell surface (Fig. 4), where CD52 molecules are found in homo-associated clusters as well as in interaction with the TCR (Fig. 6).

Our results are consistent with previous reports that the signalling functions of the GPI-linked molecules Thy-1 (23, 39), Ly-6A/E (24, 40), CD55 (41) and CD59 (42) are dependent on TCR expression at the cell surface, and in particular on the transducing actions of the TCR ζ chain (39, 40). It is well established that TCR-mediated signalling events are dependent on the expression of the CD45 transmembrane phosphotyrosine phosphatase, as demonstrated by the uncoupling of the TCR from intracellular signals in mutant cells lacking CD45 expression (43–45), as well as the defects in thymic development and signalling which occur in CD45− mice (28, 46). Lack of CD45 expression correlates with hyper-phosphorylation and dysfunction of its p56ck and p59lyn.
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Fig. 5. Campath-1H fails to trigger calcium signals in primary T cells and Jurkat A12 sub-clone. Calcium assays were carried out in Indo-1-loaded cells. Cells were stimulated with 5 µg/ml OKT3 for 1 min followed by 50 µg/ml SAM (primary T cells), or with 20 µg/ml Campath-1H for 1 min followed by 100 µg/ml GAH, or with 5 µg/ml OKT3 alone (Jurkat A12 cells). Control experiments were performed with cross-linkers only (SAM or GAH as appropriate, labelled XL).

Table 1. InsP analysis in Jurkat sub-clone A12 either TCR or CD52 ligation, including TCR ζ, ZAP-70, Vav and LAT, although the phosphorylation events triggered by optimal concentrations of Campath-1H were normally considerably lower than those induced by the OKT3 mAb (Figs 1–4). Interestingly, the signalling functions of Thy-1 have also previously been reported to be CD45 dependent (25) and some CD55-induced signals are also CD45 dependent (D. R. Alexander, unpublished), so it may therefore prove to be generally the case that GPI-linked molecule signal transduction depends on the TCR and its CD45-regulated tyrosine kinases.

A surprising exception to the generalization that CD52 and CD3 mAb appear to trigger similar signals was the finding that the CD52 mAb failed to phosphorylate or activate PLCγ1 in Jurkat cells (Table 1) nor induce detectable calcium signals in either primary T cells or in Jurkat cells (Fig. 5). Since calcium assays were carried out by FACS analysis, it is unlikely that there was a small population of CD52-signalling responsive cells present, since these should have been detected using this technique. However, we cannot exclude the possibility that calcium transients were induced in single cells, possibly over a range of times longer than the 30 min employed in the present work. The finding that the mitogenic

tyrosine kinase substrates which are involved in the initiating events of TCR coupling to intracellular signalling pathways (28, 47–49), and evidence that p56{lck} is in its hyper-phosphorylated dysfunctional conformation has previously been reported for the CD45−J45.01 Jurkat sub-clone used in the present work (50). Our finding that CD52-induced signalling is dependent on the expression of CD45 is therefore consistent with a model in which CD52 transduces signals to the cell interior by utilizing the signalling functions of the TCR polypeptides in conjunction with the actions of CD45-activated p56{lck} and p59{fyn}. Such a model is supported by the similar profile of tyrosine phosphorylated substrates induced upon either TCR or CD52 ligation, including TCR ζ, ZAP-70, Vav and LAT, although the phosphorylation events triggered by optimal concentrations of Campath-1H were normally considerably lower than those induced by the OKT3 mAb (Figs 1–4). Interestingly, the signalling functions of Thy-1 have also previously been reported to be CD45 dependent (25) and some CD55-induced signals are also CD45 dependent (D. R. Alexander, unpublished), so it may therefore prove to be generally the case that GPI-linked molecule signal transduction depends on the TCR and its CD45-regulated tyrosine kinases.

A surprising exception to the generalization that CD52 and CD3 mAb appear to trigger similar signals was the finding that the CD52 mAb failed to phosphorylate or activate PLCγ1 in Jurkat cells (Table 1) nor induce detectable calcium signals in either primary T cells or in Jurkat cells (Fig. 5). Since calcium assays were carried out by FACS analysis, it is unlikely that there was a small population of CD52-responsive cells present, since these should have been detected using this technique. However, we cannot exclude the possibility that calcium transients were induced in single cells, possibly over a range of times longer than the 30 min employed in the present work. The finding that the mitogenic

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1 (c.p.m.)</th>
<th>Experiment 2 (c.p.m.)</th>
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</thead>
<tbody>
<tr>
<td>No antibody</td>
<td>7517 ± 1492</td>
<td>7399 ± 766</td>
</tr>
<tr>
<td>+ OKT3</td>
<td>16347 ± 1171</td>
<td>22603 ± 1157</td>
</tr>
<tr>
<td>+ Campath-1H + XL</td>
<td>7625 ± 395</td>
<td>7878 ± 446</td>
</tr>
</tbody>
</table>

Jurkat A12 cells were stimulated with the mAb shown as described in Methods. Values refer to total InsP c.p.m. ± SD.
Fig. 6. CD52–CD52 homo-association and CD52–TCR association in Jurkat T cells. (A) FRET analysis was carried out using the A12 Jurkat sub-clone to measure the energy transfer between F-CD52 and R-CD52, F-CD52 and R-CD45, and F-CD3 and R-CD52 as indicated. Further details are in Methods. (B) Jurkat A-12 cells were stained with F-CD3 alone (thick black line) or with F-CD3 in the presence of 100 μg/ml unlabelled Campath-1H (thin black line), as indicated. The dashed line refers to unstained cells.

Table 2. Energy transfer values (%)

<table>
<thead>
<tr>
<th>Antibody pairs</th>
<th>A12</th>
<th>B8</th>
<th>C2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 + CD52</td>
<td>25 ± 8</td>
<td>7 ± 3</td>
<td>ND</td>
</tr>
<tr>
<td>CD3 + CD45</td>
<td>9 ± 3</td>
<td>10 ± 3</td>
<td>ND</td>
</tr>
<tr>
<td>CD4 + CD52</td>
<td>−0.4 ± 1</td>
<td>0 ± 1</td>
<td>ND</td>
</tr>
<tr>
<td>CD4 + CD45</td>
<td>4 ± 2</td>
<td>−2 ± 2</td>
<td>ND</td>
</tr>
<tr>
<td>CD45 + CD52</td>
<td>3 ± 1</td>
<td>3 ± 3</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>CD52 + CD45</td>
<td>0 ± 1</td>
<td>ND</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>CD52 + CD52</td>
<td>21 ± 4</td>
<td>16 ± 4</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>CD52 + CD52 F(ab')</td>
<td>22 ± 5</td>
<td>19 ± 5</td>
<td>18 ± 4</td>
</tr>
</tbody>
</table>

Mean values of energy transfer histograms containing data derived from 10,000 cells are presented. SD values were calculated from four independent measurements. Antibodies used were IgG except for CD52 Campath-1H F(ab') fragments used as indicated. Antibodies written first were fluorescein labeled and were used as energy transfer donors for rhodaminated antibodies written second.

CD45 regulates Campath-1H (CD52) T cell signal transduction

Effects of cross-linked Campath-1H are inhibited by cyclosporin A (21) certainly points to a role for Ca²⁺-activated calcineurin in the proliferative signalling cascade triggered by CD52 ligation. Our results contrast with those reported for CD59 mAb which induce calcium transients in U937 cells (6), human neutrophils (51) and in Jurkat T cells (42), and with Thy-1 mAb which also trigger calcium signals (23). On the other hand, a CD55 mAb did not induce calcium signals in Jurkat T cells (41). The failure of CD52 mAb in the present work to trigger a significant mobilization of intracellular Ca²⁺ may reflect the quantitatively lower levels of protein tyrosine phosphorylation, particularly LAT phosphorylation (53). Further work will be necessary to determine why Campath-1H does not induce PLCγ1 activation and recruitment to the plasma membrane, subsequent PLCγ1 activation with the consequent production of inositol trisphosphate, the generation of sufficient phosphatidylinositol bisphosphate substrate, and the regulation of ion channels (52, as well as the regulation of these events by LAT phosphorylation (53). Further work will be necessary to determine why Campath-1H does not induce PLCγ1 phosphorylation, InsP₃ production and a consequent calcium signal. The most likely explanation is that the stimulation of LAT phosphorylation is too low to allow efficient recruitment of PLCγ1 to the plasma membrane, thereby preventing its effective phosphorylation and activation.

Our finding that CD52 homo-associates, and at the same time associates with the TCR (Fig. 6A and B, Table 2), suggests a model of CD52 signal transduction which may explain how CD52 ligation utilizes the TCR to induce protein tyrosine phosphorylation signals, yet without triggering an observable calcium signal. GPI-anchored proteins are localized to the outer leaflet of the plasma membrane lipid bilayer and therefore require one or more transmembrane proteins to transmit their signals to the cell interior. It appears that the polypeptides of the TCR fulfill this role. Current evidence suggests that GPI-linked molecules are preferentially localized with glycosphingolipids in distinct microdomains in the cell membrane (53,54) and that membrane compartmentation is required for efficient T cell activation (54). GPI-linked molecules are not exclusively localized in such microdomains, but appear to be in dynamic equilibrium with other regions of the membrane (55). The extent to which transmembrane glycoproteins such as CD45 and the TCR polypeptides are or are not excluded from such microdomains has not been completely resolved. For example, when light-density vesicles enriched in GPI-linked molecules were prepared in the absence of detergent from T lymphocyte plasma membranes, they were found to contain only a small percentage of total CD45 (56), whereas Triton-insoluble GPI molecule-rich fractions prepared from various leukemic cell lines did contain significant levels of CD45 in one study (57), but not in another study (58). Investigations of intact cells in the absence of detergent will be required to resolve this issue (55). Our own finding that CD52 associates with the TCR (Fig. 6A and B, Table 2) is consistent with the presence of the TCR within GPI-rich lipid microdomains.

Irrespective of the precise location of the TCR and CD45
in respect to lipid microdomains, we propose that the cross-linking of CD52 mAb causes the ‘trapping’ of TCR polypeptides causing their dimerization or oligomerization, thereby mimicking the effects of a CD3 mAb. This is consistent with the observation that CD52 mAb cross-linking is essential for its mitogenic effects (21) and for inducing intracellular signals, as shown in the present work, as well as previous studies which have reported that the cross-linking of other GPI-linked molecules, such as Qa-2 (3) and CD59 (51), are required for signal transduction. Our model thus provides a simple explanation for the observed similarities between OKT3- and Campath-1H-triggered phosphorylation events (Figs 1–4), and explains why extensive cross-linking of GPI-linked molecules is necessary to induce intracellular signals. The unique way in which GPI-linked molecules are anchored to the outer leaflet of the plasma membrane may result in their effectiveness in trapping other cell-surface glycoproteins upon extensive cross-linking. In fact, it has been shown that ligation of certain molecules, such as Qa-2, induces T cell activation only when they are anchored by a GPI anchor and not when expressed as a transmembrane domain-containing protein (59). According to our model, therefore, the cross-linking antibody might cause trapping of TCR polypeptides within a molecular complex in a way which does not occur when non-mitogenic mAb to glycoproteins anchored to the plasma membrane via transmembrane domains, such as CD45, are utilized. Interestingly, OKT3 induces tyrosine phosphorylation and calcium signals in Jurkat T cells without the requirement for cross-linking, whereas mAb cross-linking is important for its mitogenic effects in primary T cells. This suggests that TCR dimerization is sufficient for signal transduction in Jurkat T cells, whereas TCR oligomerization is required for effective signal transduction in primary cells. In both cases it is noteworthy that the signals triggered by Campath-1H are considerably lower than those induced by OKT3 (Figs 2A and B, and 4), suggesting that the association of TCR polypeptides promoted as an indirect ‘trapping’ consequence of CD52 cross-linking might not be as effective as that induced by direct OKT3 mAb binding. This could also explain the inability of Campath-1H to trigger detectable calcium transients, since partial TCR polypeptide association might not be sufficient to promote the full gamut of signals, detailed above, which are essential for the mobilization of intracellular Ca2+. The situation may therefore be analogous to the incomplete signals induced by altered peptide ligands in T cell clones in which calcium signals are reduced or even absent (60).

In summary, our results show that Campath-1H mAb cross-linking mediates a panoply of intracellular signals which are similar to those induced upon TCR stimulation, and which are dependent upon expression of both the TCR and CD45. Our findings have important therapeutic implications, suggesting that the construction of humanized mAb mutated in the Fc region to prevent mAb cross-linking in situ may be important in the prevention of undesirable side effects.

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References

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