

# Calcium Signaling through the $\beta_2$ -Cytoplasmic Domain of LFA-1 Requires Intracellular Elements of the T Cell Receptor Complex\*

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**The  $\beta_2$  integrin LFA-1 is an important cell-cell adhesion receptor of the immune system. Evidence suggests that the molecule also participates in signaling and co-stimulatory function. We show here that clustering of the intracellular domain of the  $\beta_2$  chain but not of the  $\alpha_L$ - or  $\beta_1$ -cytoplasmic domains, respectively, triggers intracellular  $Ca^{2+}$  mobilization in Jurkat cells. A  $\beta_2$ -specific NPXF motif, located in the C-terminal portion of the  $\beta_2$  tail, is required for  $Ca^{2+}$  signaling, and we show that this motif is important for the induction of allo-specific target cell lysis by cytotoxic T cells *in vitro*. Significantly, the  $Ca^{2+}$ -signaling capacity of the  $\beta_2$  integrin is abrogated in T cells that do not express the T cell receptor but may be reconstituted by co-expression of the T cell receptor- $\zeta$  chain. Our data suggest a specific function of the cytoplasmic domain of the  $\beta_2$  integrin chain in T cell signaling.**

Activation of a T cell by cognate antigen is a complex series of events of which specificity and efficiency are critical for the development and maintenance of adaptive vertebrate immunity. The final outcome of T cell activation, *i.e.* triggering of effector functions or induction of cytokine gene expression, is dependent on the precise orchestration of plasma membrane proximal events, both at the level of the involved receptors and the subsequent cytoplasmic signal transduction cascades.

The specificity of T cell activation relies on the interaction of an idiotypic TCR<sup>1</sup> with antigenic ligand on an antigen-presenting cell (APC). The activated TCR in turn couples to an intracellular signal transduction apparatus, which is predominantly based on specific tyrosine phosphorylation events (1, 2). After phosphorylation of so-called immunoreceptor tyrosine-based activation motifs (ITAMs), present in the intracellular elements of the TCR-associated  $\zeta$  chains or the CD3 complex (3, 4), a cross-talk of non-receptor tyrosine kinases of the Src and Syk/ZAP-70 families is initiated (5, 6) that relays signals between the TCR and distal functions (*i.e.* cytokine promoter

activation) (1, 7). Specific hematopoietic adaptor proteins play important roles in this information flow.

Signal transduction from the TCR, however, is not sufficient to fully activate T cells. It has become evident that so-called accessory or co-stimulatory receptors, *e.g.* CD28, expressed at the surface of the T cell, are important determinants of this process. Co-stimulatory interactions between other T cell surface proteins and their self-ligands on APC have been hypothesized to deliver a qualitatively different “signal 2” (as to distinguish it from “signal 1” triggered by the TCR) (8, 9) and to influence cell-cell tethering (10).

LFA-1 ( $\alpha_L\beta_2$ , CD11a/CD18) belongs to a family of heterodimeric cell surface proteins termed  $\beta_2$  integrins, which have primarily been shown to play important roles in T cell adhesion to both endothelial cells and APC (11, 12). Recently, LFA-1 has also been implicated in signal transduction (13–19). In one study it was shown that the interaction of LFA-1 with its APC ligand ICAM-1 was required for potentiation of  $Ca^{2+}$ -signaling by the TCR but was dispensable for T cell adhesion and spreading to major histocompatibility complex-antigen complexes embedded in lipid membranes (15). It was, therefore, suggested that the signaling function of LFA-1 might even be more important for T cell activation than its adhesive properties. On the other hand, Zuckerman *et al.* (16) demonstrated that although LFA-1 cooperated initially with the TCR to induce proliferation of naive T cells, this signal led to apoptotic cell death after prolonged incubation periods. Finally, recent studies indicate that after stimulation with specific antigen, both the TCR and LFA-1 undergo specific reorientation and reorganization processes at the plasma membrane, forming so-called supramolecular activation clusters (20). A different study documented similar structures, which were referred to as the immunological synapse (21, 22). Supramolecular activation clusters also include other co-stimulatory receptors as well as intracellular signaling molecules, such as protein kinase C $\theta$  (20).

All these observations are consistent with the view that LFA-1 may not deliver a second signal but rather could aid in amplifying the TCR-dependent signal 1. However, its mode of action in this function is obscure. Here we use a chimeric receptor approach to investigate the formal requirements of LFA-1 cytoplasmic domain elements in T cell signal transduction.

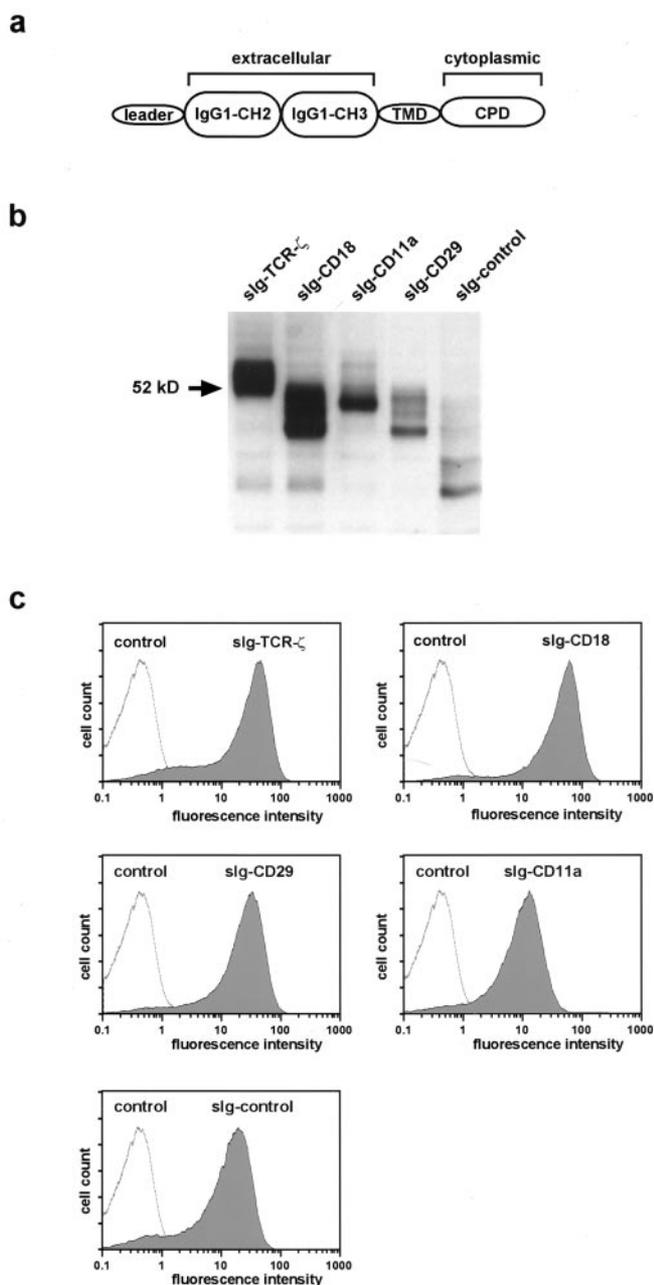
## EXPERIMENTAL PROCEDURES

**Cell Lines and Antibodies**—TAG-Jurkat cells and Jurkat J.RT3-T3.5 T cells deficient in surface expression of the TCR were maintained in RPMI 1640 supplemented with 10% fetal calf serum and 10  $\mu$ g/ml gentamicin sulfate. The following antibodies were used in this study: antigen affinity-purified goat anti-human IgG, Fc- $\gamma$  fragment-specific polyclonal antibody (Jackson ImmunoResearch, West Grove, PA),

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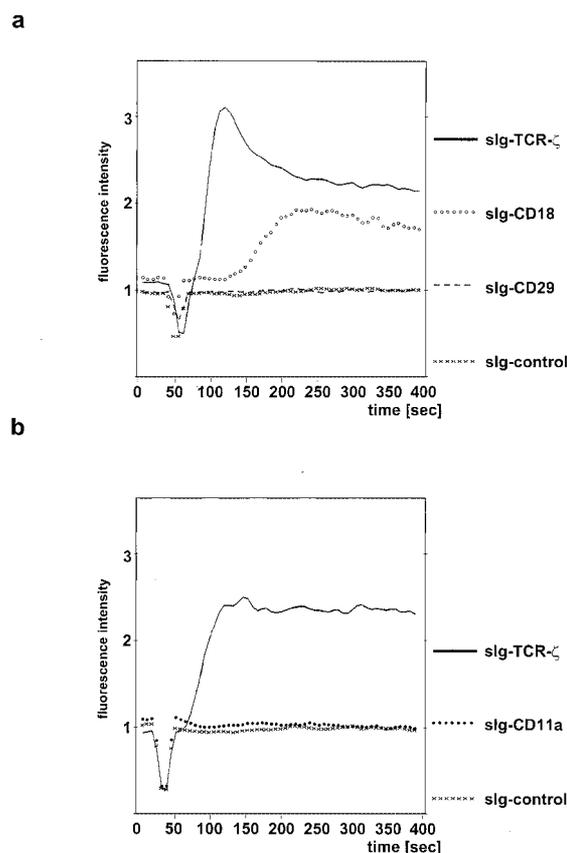
<sup>1</sup> The abbreviations used are: TCR, T cell receptor; LFA-1, lymphocyte function-associated antigen-1; APC, antigen-presenting cell; ICAM-1, intercellular adhesion molecule-1; CTL, cytotoxic T lymphocyte; LAT, linker in T cell activation.



**FIG. 1. Construction and characterization of transmembrane sIg chimeras.** *a*, schematic diagram of the sIg fusion proteins. Two extracellular immunoglobulin domains were genetically fused to the cytoplasmic portion (*CPD*) of either the TCR- $\zeta$  chain, CD18, CD29, or CD11a or lacked any intracellular tail (*sIg-control*). Expression of these sIg chimeras was achieved by infecting TAG-Jurkat T with recombinant vaccinia viruses. Expression levels were verified using anti-human IgG reagents either by SDS-polyacrylamide gel electrophoresis of cell lysates followed by Western blot analysis (*b*) or by measurement of cell surface expression using flow cytometry (*c*). *TMD*, transmembrane domain.

monoclonal anti-CD4 antibody MT-151 (kindly provided by Peter Rieber, University of Dresden), anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY), and anti-LFA-1 antibody MEM-95 (kindly provided by Vaclav Horejsi, University of Prague, Czech Republic). All secondary anti-IgG reagents were purchased from Jackson ImmunoResearch.

**DNA Constructs and Mutagenesis**—The cytoplasmic portions of the transmembrane chimeras were amplified using PCR techniques and cloned into a sIg expression plasmid p5C7 using the *MluI* and *NotI* restriction sites (23). For construction of recombinant vaccinia viruses, the coding segments were subcloned into the pTKG vector (4). The intracellular domains of the respective transmembrane fusion proteins



**FIG. 2. Clustering of the sIg-CD18 chimera induces intracellular calcium mobilization.** TAG-Jurkat T cells were infected with recombinant vaccinia virus expressing sIg fusion proteins, loaded with the calcium-sensitive fluorescent indicator Fluo-3, and analyzed by flow cytometry. Clustering of the CD18 chimera with an anti-human IgG-Fc-specific, affinity-purified antibody resulted in a significant increase of intracellular free calcium concentration. In contrast, both sIg-CD29 (*a*) and sIg-CD11a (*b*) failed to induce a calcium signal.

comprised the following amino acid sequences: residues 725–769 (ALI...AES\*) of CD18, residues 1114–1170 (VGF...GKD\*) of CD11a, residues 753–798 (LLM...EGK\*) of CD29 and residues 183–220 (RSR...YRS\*) of CD28. The sIg-TCR- $\zeta$  construct (23) and the CD4-TCR- $\zeta$  construct (4, 24) were described before.

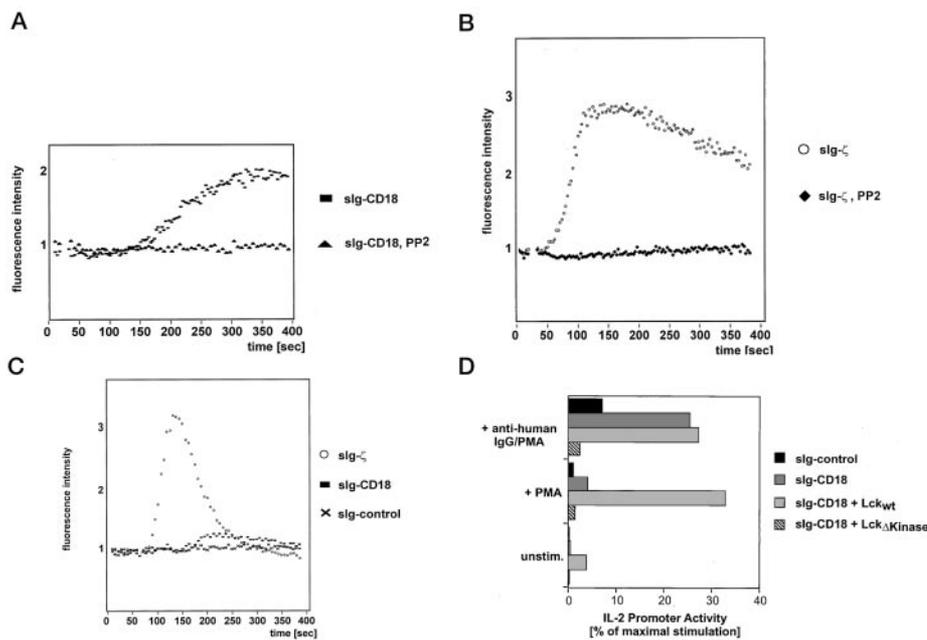
Deletion mutants of the CD18 cytoplasmic tail comprised sIg-CD18–762\* residues 753–762 (ALI...TVM\*) or sIg-CD18–747\* residues 753–747 (ALI...SQW\*). Point mutants of diverse chimeras were generated using polymerase chain reaction-based strategies and resulted in substitution of phenylalanine 766 of CD18 for either alanine or tyrosine or of tyrosine 795 of CD29 for phenylalanine.

**Transfection of Jurkat T cells**—TAG-Jurkat and J.RT3-T3.5 T cells were transfected with constructs coding for the chimeric proteins by infection with recombinant vaccinia virus as described before (24). Alternatively, Jurkat J.RT3-T3.5 T cells were transiently transfected using the Easyject Plus electroporation system (Eurogentec, Seraing, Belgium) as described previously (23).

**Western Blot Analysis**—To verify protein levels of the respective sIg chimeras, transfected TAG-Jurkat cells were lysed by adding SDS to a final concentration of 1%. After adding 3 $\times$  loading buffer, samples were boiled for 5 min, separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Immunodetection was performed using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence detection (PerkinElmer Life Sciences). Alternatively, analysis of protein tyrosine phosphorylation was performed by incubating transfected J.RT3-T3.5 T cells with anti-human IgG antibody at 2  $\mu$ g of antibody/6  $\times$  10<sup>6</sup> cells for 5 min in Hanks' buffered saline solution at 37  $^{\circ}$ C before lysis with radioimmune precipitation buffer containing 1 mM Na<sub>3</sub>VO<sub>4</sub>. Tyrosine-phosphorylated proteins were visualized by Western blot analysis using 4G10 monoclonal antibody.

**Intracellular Calcium Measurement**—Calcium mobilization analysis was performed as described before (23). Briefly, TAG-Jurkat and J.RT3-

**FIG. 3. Characterization of sIg-CD18-mediated signal transduction in TAg-Jurkat cells.** Calcium mobilization by clustered sIg chimeras was measured as described above but was specifically tested in the presence of Src kinase inhibitor PP2 (*a* and *b*) or in the absence of extracellular calcium (*c*). *d*, interleukin-2 (IL-2) promoter-dependent luciferase induction by sIg-CD18 chimera was measured as described under "Experimental Procedures." Luciferase activity of individual samples was normalized against the maximal induction obtained by simultaneous stimulation of cells with phorbol ester phorbol 12-myristate 13-acetate (PMA) and calcium ionophore. *wt*, wild type.



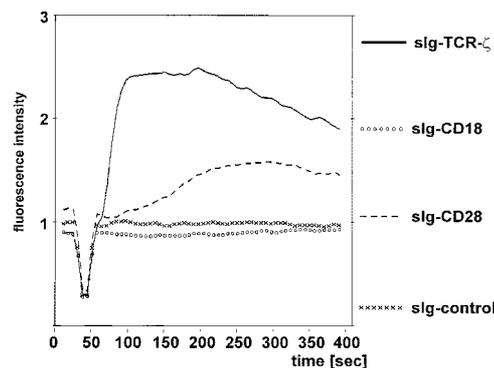
T3.5 T cells were transfected with chimeric transmembrane constructs by infection with recombinant vaccinia virus. After 5 h cells were washed, resuspended in Hanks' buffered saline solution, and loaded with the fluorescent calcium probe Fluo-3 (Molecular Probes, Inc., Eugene, OR) at 25 °C for 30 min. Fluorescence was measured by flow cytometry for 30 s before anti-human IgG antibody was added for stimulation of cells. The calcium influx was analyzed for a further 370 s, and mean values were calculated using the kinetic analysis software Multitime 3.0 (Phoenix Flow Systems, San Diego, CA). Src-kinase inhibitor PP2 (Calbiochem) was added at 10  $\mu$ M to Hanks' buffered saline solution where indicated. Calcium-free media consisted of 10 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, and 2  $\mu$ M EGTA, respectively.

**Cell-mediated Lympholysis**—CTL clone 234 was infected with recombinant vaccinia viruses and incubated for 4 h. Cells were harvested and subsequently incubated with target cells (BW-LCL, HLA-A24<sup>+</sup> or DS-LCL, HLA-A24<sup>-</sup>). Cell-mediated lysis was quantified with the help of a standard 4-h chromium-51 release assay as described (25). Spontaneous release was determined by incubating target cells alone in complete medium. Total release was determined by directly counting an aliquot of labeled cells. The percent cytotoxicity was calculated according to the formula % lysis = (experimental cpm - spontaneous cpm) / (total cpm - spontaneous cpm)  $\times$  100. Duplicate measurements of three to six step titrations of effector cells were used for all experiments. To evaluate the influence of LFA-1 on cytotoxicity, CD18 specific monoclonal antibody was incubated with clone 234 at room temperature for 30 min before the addition of target cells. The anti-CD18 monoclonal antibody MEM-95 (ascites) was used at a dilution of 1:100. After preincubation with the antibody, the standard 4-h chromium release assay was performed.

**Interleukin-2 Promoter Reporter Assays**—Procedures for transient transfection of TAg-Jurkat cells and measurement of luciferase activity were described before (23). Briefly, 10  $\mu$ g of pIL2-GL2 reporter plasmid and 25  $\mu$ g of respective sIg constructs were cotransfected by electroporation. After 20 h, respective samples were stimulated for 8 h as indicated with calcium ionophore A23187 (0.5  $\mu$ g/ml) or phorbol 12-myristate 13-acetate (50 ng/ml) or by the addition of cross-linking anti-human Ig antibody (5  $\mu$ g/ml) or were left untreated. This was followed by the addition of reporter lysis buffer (Promega) and scintillation counting.

## RESULTS

Integrin adhesion receptors do not bind constitutively to their ligands. A large body of data indicates that the propensity of integrin molecules to interact with cell surface receptors or extracellular matrix ligands is regulated by intracellular signaling events that in turn are triggered by "activating" receptor/ligand interactions (integrin avidity regulation, "inside-out" signal transduction) (11, 12, 26–29). One example is the aforementioned TCR, the activation of which triggers enhanced



**FIG. 4. Aggregation of sIg-CD18 fails to induce calcium mobilization in T cells lacking TCR cell surface expression.** The TCR-negative mutant Jurkat cell line J.RT3-T3.5 was infected with recombinant vaccinia virus expressing sIg chimeras and analyzed as described before. Cross-linking of the CD18 fusion protein did not result in a detectable rise of intracellular calcium concentration in this cell type. Conversely, calcium signaling mediated by sIg-TCR- $\zeta$  or sIg-CD28 appears to be at least partially independent of TCR surface expression.

binding of LFA-1 to its ligand ICAM-1 on an APC (30). However, this property of the molecule complicates the study of LFA-1-dependent signaling functions because the integrin-mediated signal will be perturbed by the activation stimulus required for the engagement of LFA-1 with its ligand. We hypothesized that clustering of chimeric single-chain receptors bearing isolated integrin cytoplasmic domains may be sufficient to induce signaling events that would, under physiological conditions, emanate from these elements in the context of the much more complex T cell activation scenario. First, fusion proteins have been employed very successfully in delineating signal transduction events triggered by the complex T cell or B cell antigen receptors (4, 5, 31, 32). Second, strong evidence supports the notion that integrin-dependent signal transduction normally follows receptor aggregation or clustering. This not only holds true for T cells, as evidenced by the observed distribution of LFA-1 in supramolecular activation clusters (20), but also is valid for non-hematopoietic cells adhering to extracellular matrix components in which integrins signal through the formation of higher order protein complexes (33, 34).

The structure of the single chain receptors used in this study is shown in Fig. 1*a*, and the principal design of these fusion proteins was described earlier (4, 5, 23). The cytoplasmic domains of CD18 ( $\beta_2$  chain), CD11a ( $\alpha_L$  chain), or the  $\beta$ -cytoplasmic domain of the related  $\beta_1$  integrin CD29 were genetically fused to the transmembrane domain of the CD7 antigen and extended extracellularly by the CH2 and CH3 domains of human immunoglobulin G<sub>1</sub>. Secondary reagents directed against the constant immunoglobulin domains. Furthermore, the endoplasmic reticulum import signal sequence of CD5 (35) was used to mediate transport of the tripartite fusion proteins to the cell surface of TAG-Jurkat cells (Figs. 1, *b* and *c*).

We first investigated whether the chimeras induced signaling events in leukemic TAG-Jurkat T cells (36). We chose intracellular calcium mobilization, a generally accepted and important parameter of receptor proximal signaling events in T cells. Moreover, LFA-1 has been implicated in this function (13, 15). To this end, the fusion proteins were expressed in TAG-Jurkat cells by recombinant vaccinia viruses as described earlier (5). Intracellular calcium mobilization was monitored by flow cytometry using the fluorescent calcium chelator Fluo-3 (37). Fig. 2 shows that base-line calcium levels are similar for all chimeric constructs used. However, after clustering with antibody, specific induction of calcium mobilization was observed.

A strong and persistent  $\text{Ca}^{2+}$ -mobilization was induced by a control fusion protein bearing the full-length cytoplasmic domain of the TCR-associated  $\zeta$  chain (Fig. 2*a*), as described previously (4, 31). Significantly, however, an increase in cytoplasmic calcium concentration was also detected when the sIg-CD18 chimera was clustered but not when control protein sIg bearing no intracellular domain or sIg-CD29 was employed. The CD18-dependent  $\text{Ca}^{2+}$ -flux appeared different from the sIg-TCR- $\zeta$ -induced signal both in onset and amplitude. We conclude from these data that the clustered cytoplasmic domain of CD18 is sufficient to induce calcium signaling in TAG-Jurkat cells. It was subsequently analyzed whether the intracellular portion of the  $\alpha_L$  chain (CD11a) bore a similar capacity. Fig. 2*b* shows that this is not the case. Furthermore, co-clustering of CD18 and CD11a did not enhance the calcium signal induced by sIg-CD18 alone (not shown), which led us to conclude that the aggregation-dependent signaling elements of LFA-1 that lead to cytoplasmic calcium mobilization are located exclusively within the CD18 cytoplasmic domain.

We attempted a preliminary characterization of intracellular signaling events induced by sIg-CD18 clustering and of the routes of cytoplasmic calcium mobilization. To this end, the Src kinase inhibitor PP2 was employed in calcium mobilization assays. As shown in Figs. 3, *a* and *b*, the addition of 10  $\mu\text{M}$  PP2 to the medium abrogated both sIg- $\zeta$  and sIg-CD18 mediated signals completely, suggesting that CD18-mediated calcium signaling depends on Src kinase activity.

Is the CD18-dependent calcium signal dependent on intracellular calcium stores? To answer this question, calcium was omitted from the medium, and the  $\text{Ca}^{2+}$ -selective chelator EGTA was added before flux measurement. Fig. 3*c* shows that sIg- $\zeta$  induces a transient calcium flux in the absence of extracellular calcium ions, consistent with the known TCR-dependent calcium mobilization from intracellular stores. However, sIg-CD18 was almost completely incapable of inducing calcium transients in the presence of EGTA. This result hints at differential requirements for the two receptors at a point further downstream in the signaling cascade.

Reporter assays were then employed to study T cell signaling events that are located far downstream and which are known to

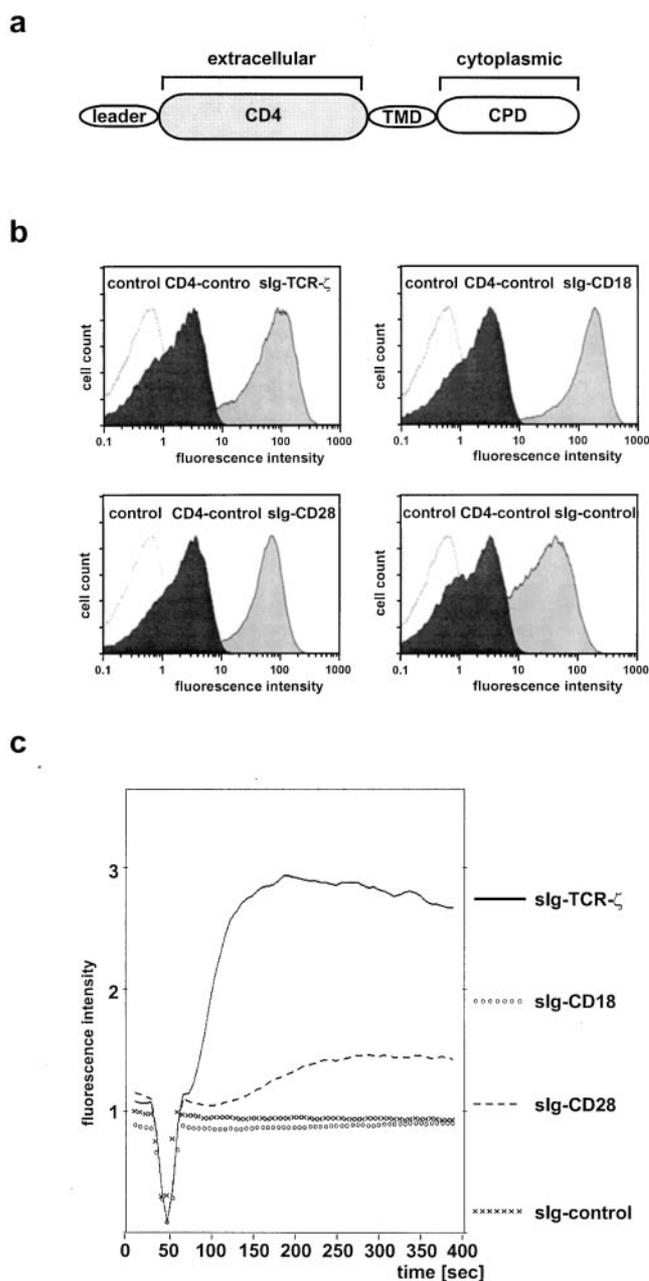


FIG. 5. Reconstitution of sIg-CD18-mediated signal transduction in TCR-negative Jurkat cells by co-expression of a chimeric TCR- $\zeta$  chain. *a*, schematic diagram of the CD4-TCR- $\zeta$  fusion protein employed to reconstitute TCR negative Jurkat cells. Jurkat J.RT3-T3.5 T cells were co-infected with recombinant vaccinia viruses expressing sIg fusion proteins in combination with either native, full-length CD4 (*CD4-control*) (*b*) or alternatively with the CD4-TCR- $\zeta$  chimera (*d*). Cell surface expression of chimeras was analyzed by flow cytometry using anti-human IgG or anti-CD4 antibodies. *c*, co-expression of the sIg chimeras with the CD4-control protein in TCR-negative Jurkat cells revealed similar results as shown in Fig. 3, *e*, calcium signaling after clustering of the sIg-CD18 fusion protein is rescued by co-expression of the CD4-TCR- $\zeta$  chimera. Both types of chimeric proteins could be stimulated independently, as demonstrated by the failure of sIg-control fusion protein to induce intracellular calcium influx after anti-human IgG cross-linking. Additionally, sIg-CD28-triggered calcium signaling is boosted by the co-expression of CD4-TCR- $\zeta$ . *f*, tyrosine phosphorylation of cellular proteins is induced after aggregation of sIg-CD18 in Jurkat J.RT3-T3.5 T cells reconstituted with CD4-TCR- $\zeta$  (fourth lane) but not in cells expressing full-length CD4 (*CD4-control*, second lane). *g*, left panel, tyrosine phosphorylation of a 38-kDa cellular protein is induced by aggregation of sIg-CD18 or sIg- $\zeta$  but not by sIg-CD29 or control chimera (*sIg*). The phosphoprotein likely corresponds to the adaptor protein LAT (right panel).  $\alpha$ -P-Tyr, phosphotyrosine.

be dependent on intracellular calcium mobilization. sIg-CD18 or a control construct were co-transfected with a luciferase reporter construct driven by the intact promoter/enhancer region of the human interleukin-2 promoter (23). Fig. 3d shows that cross-linking of sIg-CD18 resulted in a 5-fold, specific induction of the interleukin-2 promoter under these conditions. Moreover, co-transfection of a dominant-negative, kinase-deficient Lck construct, but not of intact Lck, abrogated CD18-dependent interleukin-2 promoter stimulation completely. These data are fully consistent with the loss of CD18-dependent calcium induction in the presence of Src kinase inhibitor PP2, and confirm a dependence of CD18 signal transduction on an Src kinase, which is likely Lck (Fig. 3a).

We were interested in determining whether the expression of the TCR was important for sIg-CD18-mediated signal transduction. For these analyses Jurkat J.RT3-T3.5 cells that do not express a TCR on the cell surface were employed (38). Expression of all constructs was highly comparable in TCR<sup>+</sup> TAG-

Jurkat cells and in J.RT3-T3.5 cells (Fig. 5 and data not shown). Fig. 4 shows that Ca<sup>2+</sup> mobilization was induced in J.RT3-T3.5 cells by the sIg-TCR- $\zeta$  chimera as predicted, since this construct is thought to function as a surrogate TCR. The sIg-CD18 fusion protein, however, did not induce cytoplasmic Ca<sup>2+</sup>-influx in the absence of the TCR. We were interested in analyzing whether this deficiency was because of a global inability of co-stimulatory molecules to function properly in the absence of the TCR or the signaling components it might assemble. Therefore, a different fusion protein was employed that bore the intact cytoplasmic domain of CD28, which has previously been implicated in Ca<sup>2+</sup> signaling. The corresponding data are also shown in Fig. 4. It was observed that the CD28 fusion protein was functional both in TAG-Jurkat cells and in J.RT3-T3.5 cells (Fig. 4 and data not shown), although the amplitude of the Ca<sup>2+</sup> flux in J.RT3-T3.5 was on the average lower than in TAG-Jurkat cells (data not shown). These data indicate that the CD28 fusion protein was capable of delivering

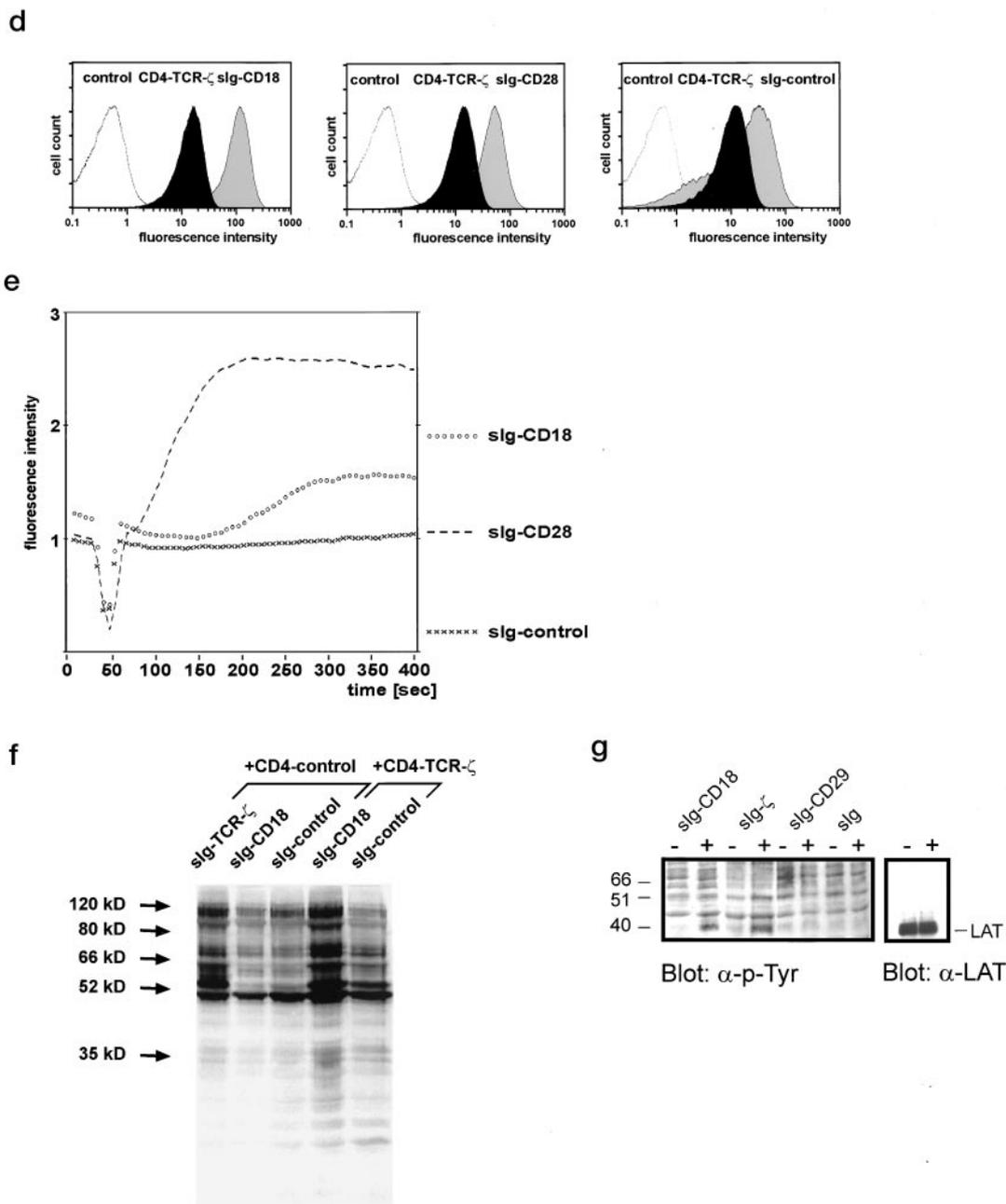
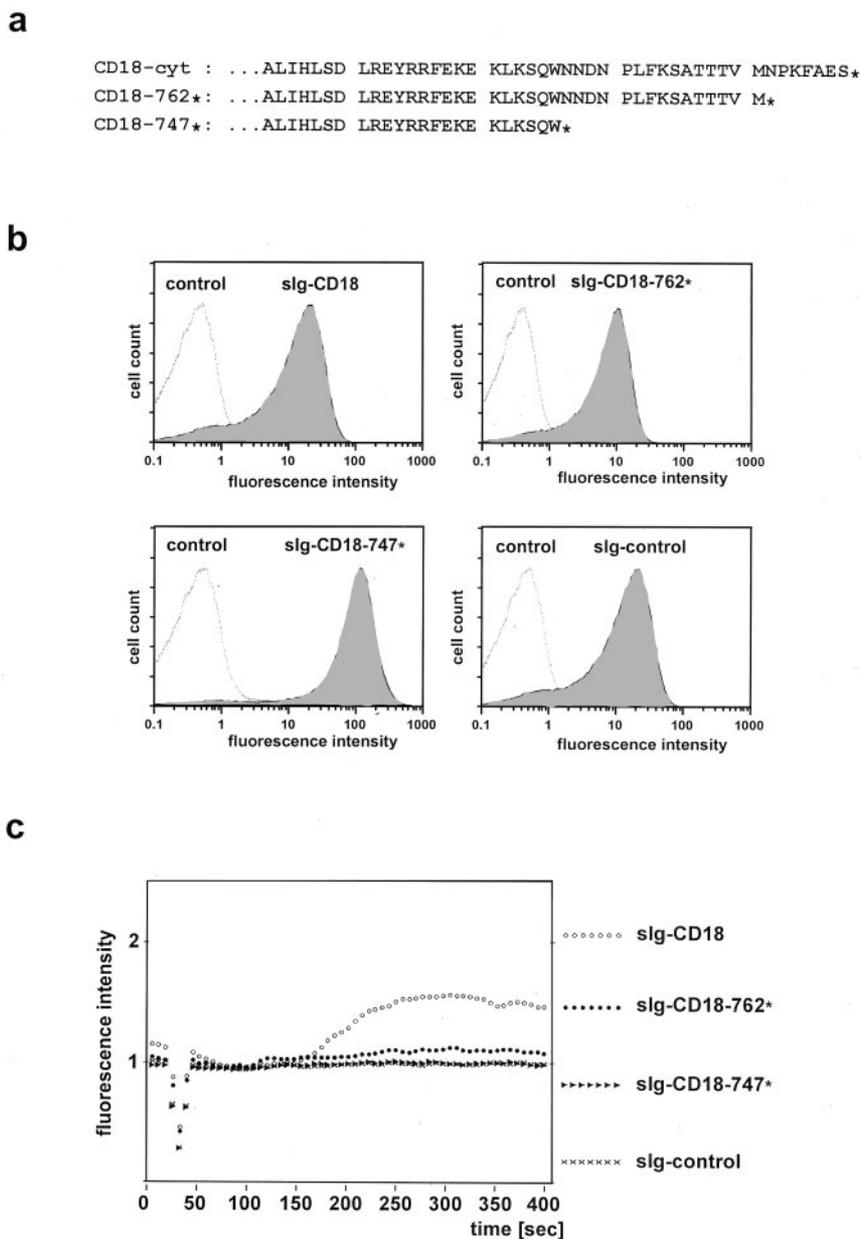


FIG. 5—continued



**FIG. 6. C-terminal truncation of CD18 abrogates calcium signaling.** *a*, sequence alignment of the cytoplasmic (*cyt*) domains of CD18 wild-type and truncation mutants. *b*, flow cytometric analysis of transmembrane CD18 fusion protein expression in TAG-Jurkat cells after infection with recombinant vaccinia viruses. *c*, both CD18 truncation mutants (CD18-762\* and CD18-747\*) failed to induce calcium mobilization.

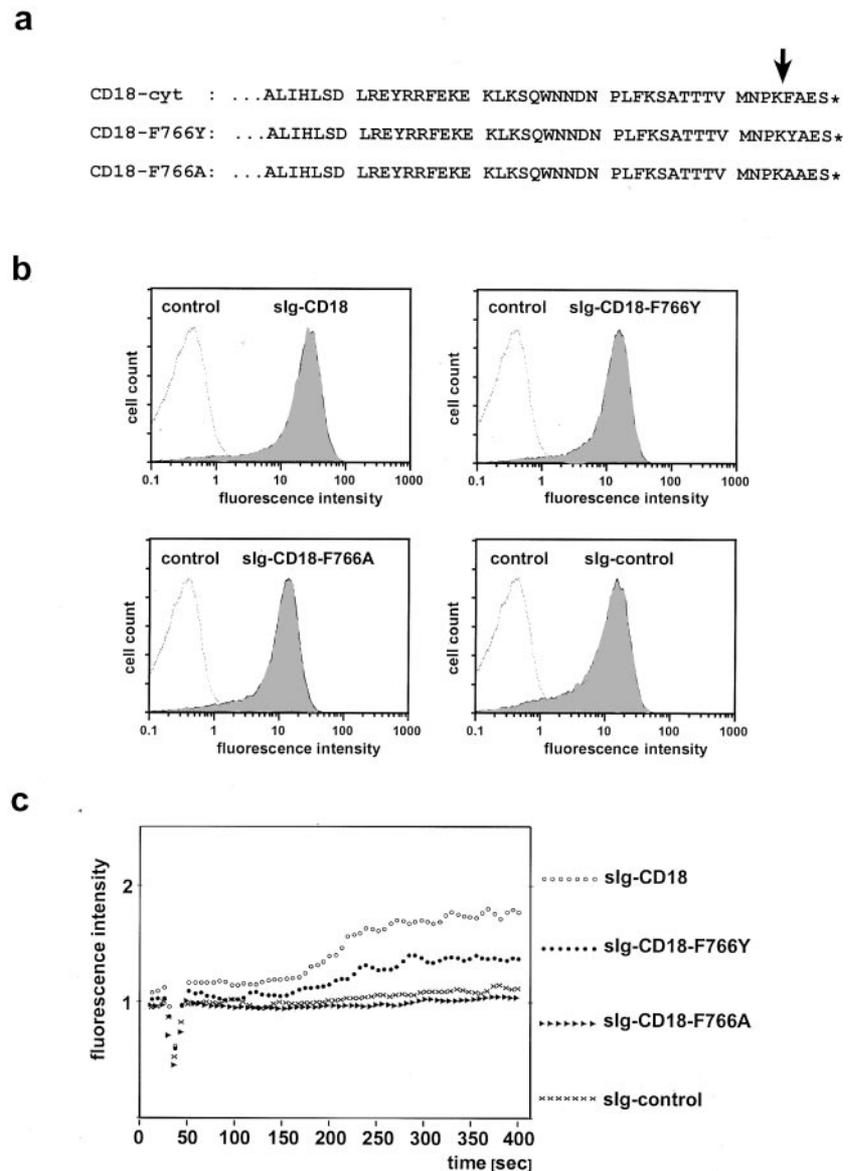
signals that were independent of the TCR to a significant extent, whereas the signal induced by the CD18 chimera strictly required TCR cell surface expression.

These findings prompted us to analyze which components of the TCR were needed for CD18-dependent functions. Therefore, we adapted our system to the simultaneous use of two fusion proteins that could be independently clustered on the surface of the same cell. Fig. 5*a* shows the design of the additional constructs. In this system, the cytoplasmic and transmembrane portions of TCR- $\zeta$  were fused to the extracellular domain of CD4, or alternatively, full-length CD4 was used as a control.

Experiments were performed to determine whether the sIg or CD4 derivatives could be co-expressed and independently manipulated on the surface of J.RT3-T3.5 cells. Fig. 5 shows that this is the case. Co-expression was monitored by flow cytometric analysis using anti-Ig antibodies and anti-CD4 antibody MT151 (Figs. 5, *b* and *d*). Clustering of the CD4-TCR- $\zeta$  fusion protein resulted in  $\text{Ca}^{2+}$  mobilization as expected (not shown). Moreover, sIg-CD18-dependent signal transduction was not reconstituted by co-expression of CD4 (Fig. 5*c*), and

aggregation of an sIg control protein did not result in  $\text{Ca}^{2+}$  mobilization even when CD4-TCR- $\zeta$  was present on the same cell surface (Fig. 5*e*). These data indicate that the antibodies employed targeted the surface chimeras in a highly specific fashion. Therefore, inadvertent antibody-mediated co-aggregation of these molecules could be excluded. It was consequently determined whether the expression of CD4-TCR- $\zeta$  was sufficient to rescue the sIg-CD18-mediated  $\text{Ca}^{2+}$  flux. Fig. 5*e* shows that this was indeed the case. We conclude that the TCR-associated  $\zeta$  chain suffices to promote CD18-dependent  $\text{Ca}^{2+}$  signaling.

Tyrosine phosphorylation of cytoplasmic components is an important event in receptor-mediated T cell activation. Therefore, experiments were performed to determine whether sIg-CD18 was capable of inducing cytoplasmic tyrosine phosphorylation events in J.RT3-T3.5 cells. The results of this experiment are shown in Fig. 5*f*. The *left* lane shows that in the absence of a co-expressed CD4-TCR- $\zeta$  fusion protein only the other TCR- $\zeta$  chimera (sIg-TCR- $\zeta$ ), but not sIg-CD18, was capable of inducing tyrosine phosphorylation of a number of protein bands. This was different, however, when sIg-CD18 and CD4-



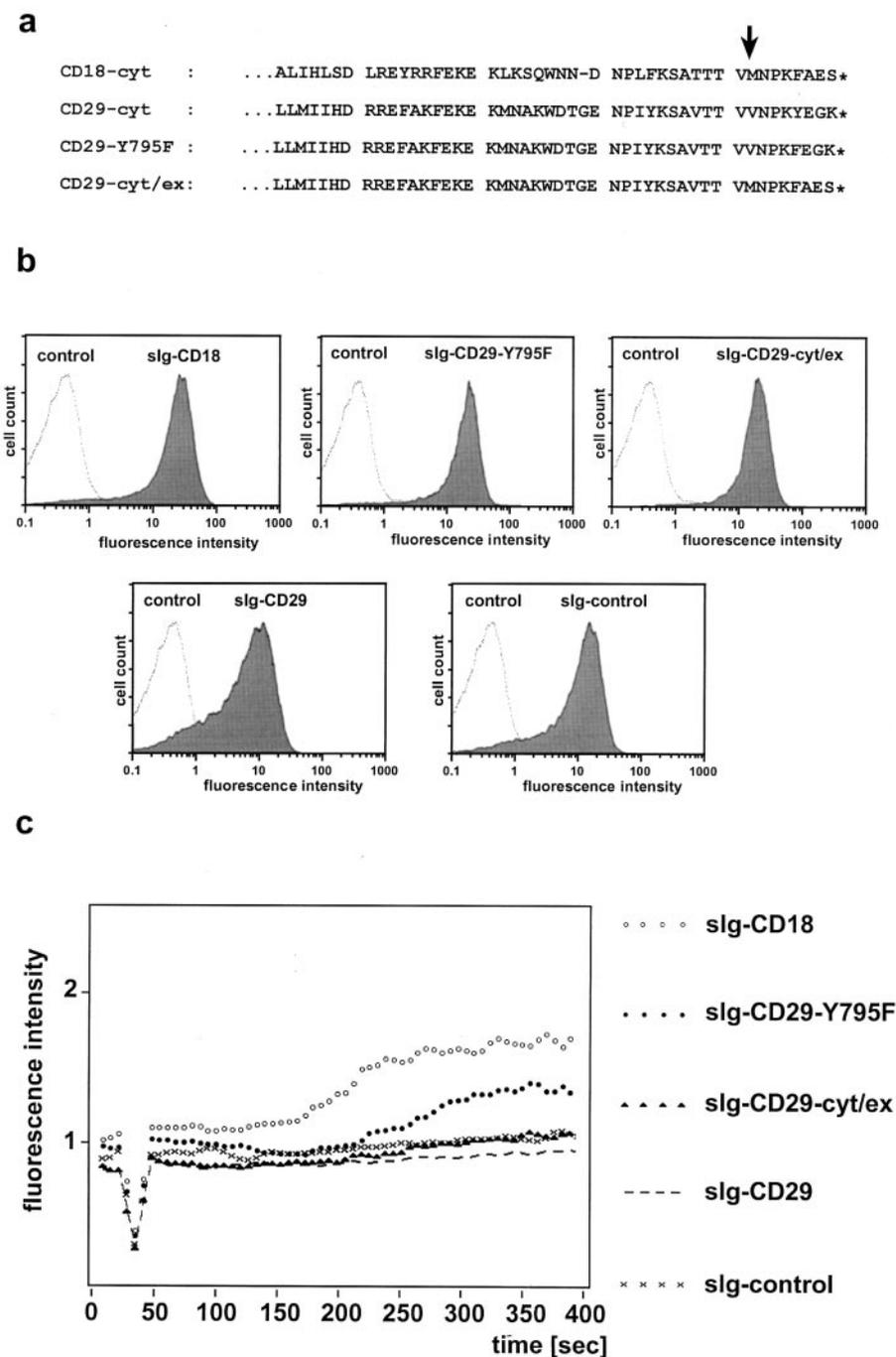
**FIG. 7. Analysis of intracellular calcium influx after cross-linking of CD18 point mutants within the C-terminal NPXF motif.** *a*, sequence alignment of CD18 point mutants. *b*, Jurkat T cells were transfected by recombinant vaccinia viruses expressing sIg fusion proteins of CD18 or CD18 point mutants and analyzed by flow cytometry. *c*, the CD18-F766A mutation completely abrogated the calcium-signaling capacity of the CD18 cytoplasmic tail, whereas cross-linking of the CD18-F766Y chimera resulted in significant reduction of intracellular calcium influx as compared with CD18 wild-type signaling in Jurkat T cells.

TCR- $\zeta$  were co-expressed on the surface of J.RT3-T3.5 cells. After clustering of the sIg-CD18 construct, we observed a tyrosine phosphorylation pattern that was qualitatively similar to that induced by sIg-TCR- $\zeta$ . We conclude that signal transduction by the CD18 cytoplasmic domain progresses intracellularly through tyrosine phosphorylation events and that the TCR- $\zeta$  chain plays an important role in this process. To further corroborate this evidence, Fig. 5g shows protein tyrosine phosphorylation in TAG-Jurkat cells with or without specific surface chimera aggregation. Both antibody-aggregated sIg-CD18 and sIg- $\zeta$  strongly induce phosphorylation of a 38-kDa band, which corresponds well to the T cell receptor-dependent phosphorylation target LAT, which is the predominant T cell activation-induced phosphoprotein of the respective molecular weight range. The 38-kDa phosphoprotein was not detectable in total lysates when sIg-CD29 or sIg alone were employed (Fig. 5g). All these observations are compatible with the notion that CD18 couples to a signaling apparatus that shares important components with the TCR-associated machinery, at least with respect to  $\text{Ca}^{2+}$  signaling.

In the following we dissected the requirements of CD18 cytoplasmic domain elements for  $\text{Ca}^{2+}$  signal transduction. To this end, C-terminal deletion mutants were generated (Fig. 6, *a* and *b*). Fig. 6c shows that deletion of the C-terminal seven

amino acids (sIg-CD18-762\*) abrogated the signal completely. Further deletion (sIg-CD18-747\*) had no effect, confirming that the C-terminal residues were required for the observed function. This C-terminal element bears an NPXF motif, and similar motifs have been implicated in receptor internalization pathways. Interestingly, the cytoplasmic domains of CD18 and CD29 ( $\beta_1$  integrin) display significant differences in this region (Fig. 8a). We, therefore, produced a series of point mutants to test whether these structural differences were responsible for the observed functional specificity. For this purpose, phenylalanine 766 of CD18 was mutated into either alanine or tyrosine by standard molecular biology techniques, and the resulting mutants (Fig. 7, *a* and *b*) were tested for their respective abilities to induce cytoplasmic  $\text{Ca}^{2+}$  mobilization after clustering. Fig. 7c shows that  $\text{Ca}^{2+}$  signaling was completely abrogated when the F766A mutant was employed. A strong reduction of measurable signal was also observed for the F766Y mutant, leading to an unstable flux.

Consequently it was analyzed to determine whether the CD29 fusion protein could be induced to couple to the  $\text{Ca}^{2+}$  pathway by exchanging tyrosine 795 (of the corresponding  $\beta_1$  NPXY motif) for phenylalanine (Fig. 8, *a* and *b*). Indeed, it was observed that the sIg-CD29 chimeras became partially functional by this manipulation (Fig. 8c). On the other hand, re-



**FIG. 8. Substitution of the C-terminal tyrosine residue 795 with phenylalanine partially enables calcium signaling triggered by the CD29 cytoplasmic domain.** *a*, sequence alignment of cytoplasmic (*cyt*) tails of CD18, CD29 wild type, CD29-Y795F point mutant, and *CD29-cyt/ex* mutant (in which the four C-terminal residues of CD29 have been replaced with those of CD18) *b*, cell surface expression in Jurkat T cells of sIg chimeras after infection with recombinant vaccinia viruses monitored by flow cytometry. *c*, cross-linking of the CD29-Y795F fusion protein in Jurkat T cells resulted in elevated cytosolic calcium levels compared with the wild-type CD29 chimera.

placement of the C-terminal eight residues of CD29 with the ones of CD18 (*CD29-cyt/ex*) resulted in an inactive construct (Fig. 8c). Taken together, these data indicate that phenylalanine 766 of the CD18 cytoplasmic domain is an important determinant of LFA-1-dependent  $Ca^{2+}$  signaling. However, our data also indicate that the C-terminal amino acid environments of CD18 and CD29 influence the capacity of the homologous Phe or Tyr residues, respectively, to actively engage with the downstream machinery.

We finally attempted to demonstrate that our findings bear significance for more complex T cell activation events. Cytotoxic T cell function was investigated because the requirement for LFA-1 has been very well documented for both cytotoxic T lymphocytes (CTL) and for natural killer cells (39). Allo-recognition-dependent target cell killing of HLA-A24-restricted cytotoxic T cell clone 234 was employed as an experimental

system (40). It was first determined whether 234-mediated killing of BW-LCL, *i.e.* the target cells that express the correct haplotype HLA-A24 alloantigen, was LFA-1-dependent. To this end, anti-LFA-1 antibody MEM-95 was utilized to abrogate LFA-1 binding to ICAM-1 (41). As expected, Fig. 9b shows that 234-dependent killing of BW-LCL was strongly dependent on LFA-1/ICAM-1 interaction since MEM-95 specifically inhibited cell lysis.

sIg fusion proteins were then expressed in 234 cells by recombinant vaccinia viruses, and the infected cells were employed in killing assays (Fig. 9c). The rationale underlying this experiment was as follows. It has well been documented that integrin function may be inhibited by isolated overexpression of  $\beta$ -chain cytoplasmic domains or cytoplasmic domain fusion proteins similar to those employed in our study (42–44). This observation was interpreted as a dominant block that the  $\beta$ -cy-

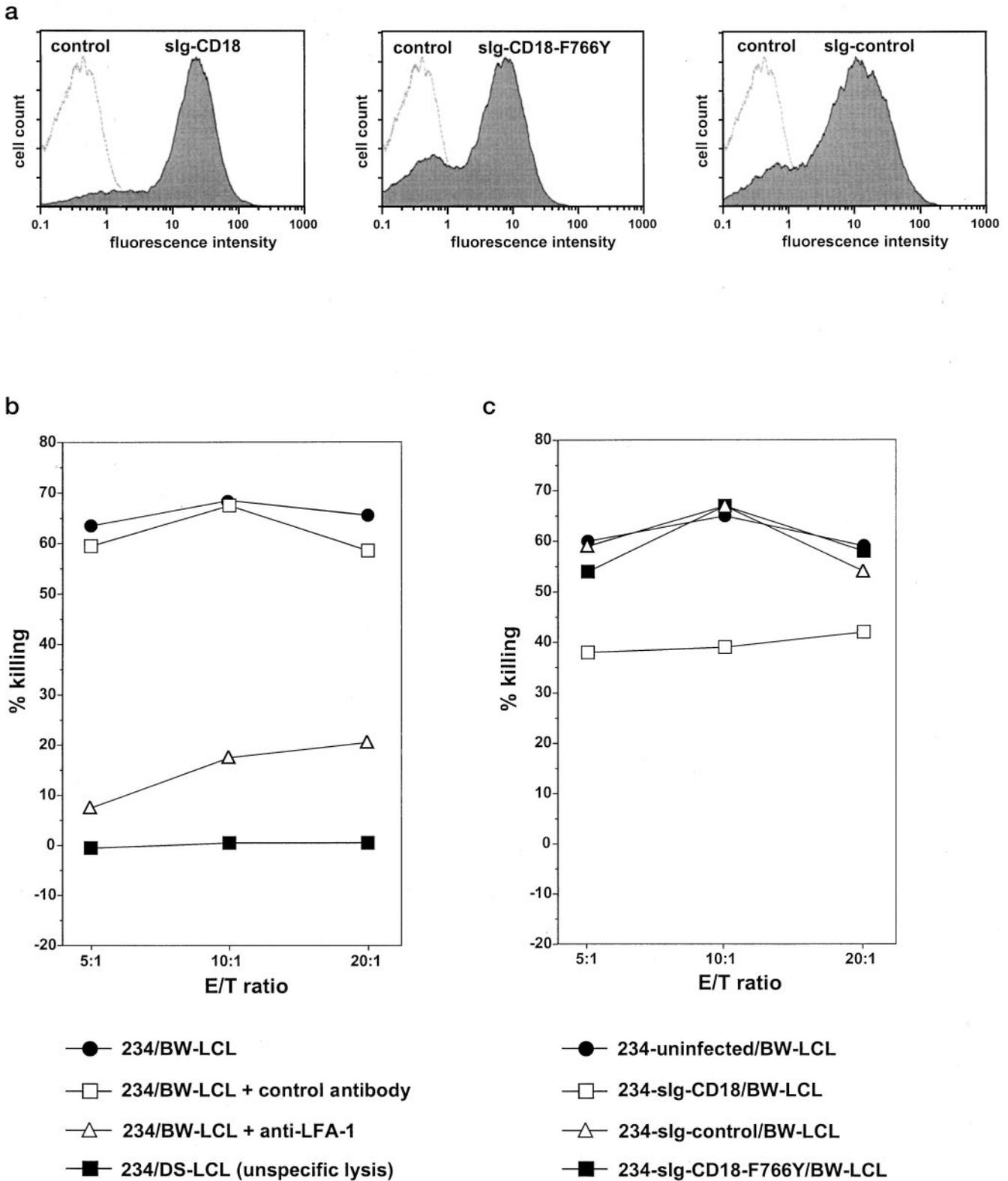


FIG. 9. **Specific inhibition of CTL-mediated cytolysis by interference with LFA-1 function.** CTL 234-mediated killing of HLA-A24+ BW-LCL was monitored by a standard chromium-51 release assay as described under "Experimental Procedures." *a*, flow cytometric analysis of fusion protein expression in 234 CTL. *b*,  $\alpha$ -LFA-1 antibody MEM-95 specifically inhibits 234-mediated lysis of BW-LCL. Control, DS-LCL, HLA-A24<sup>-</sup>. Only uninfected cells were used in this assay; vaccinia virus-infected cells yield very similar results (not shown). *c*, sIg-CD18 but not sIg-control or sIg-CD18F766Y inhibits 234-mediated cytolysis of BW-LCL. In each panel, one representative test of four independently performed experiments is shown.

toplasmic domains exert on the endogenous integrins by titrating important functional, cellular components of the membrane or the cytoplasm. Moreover, this approach has been developed into a functional complementation system in which overexpress-

sion of a cDNA library was utilized to overcome the dominant block exhibited by the  $\beta$ -chain construct, thus leading to the identification of novel components of the integrin "inside-out" signaling pathway (45).

We reasoned that if the sIg-CD18 fusion protein acted in an inhibitory fashion on the cytotoxic potential of 234, this should suffice to document the importance of the cytoplasmic domain of CD18 for the allo-recognition-dependent activation of cytotoxic T cells. Fig. 9 shows that this was indeed the case. sIg-CD18 but not an sIg-control construct significantly inhibited 234-mediated lysis of BW-LCL. Moreover, and importantly, this inhibition was released when the sIg-CD18-F766Y mutant was employed (Fig. 9c). This observation is consistent with the notion that residue Phe-766 of CD18 is involved in signaling events important for CTL activation. Furthermore, these findings are in full concordance with the Jurkat experiments on calcium signaling described above.

#### DISCUSSION

We describe here signal transduction events that are specifically initiated by the cytoplasmic domain of the  $\beta_2$  integrin CD18. Clustering of single-chain fusion proteins was employed to induce changes in intracellular calcium levels. By exploring this system it was found that aggregation of the intact  $\beta_2$ -cytoplasmic domain was sufficient for triggering a calcium signal in TAg-Jurkat cells. Significantly, neither the  $\alpha_1$ -cytoplasmic domain nor the cytoplasmic tail of the  $\beta_1$  integrin CD29 bore this capacity. These results suggest a previously unknown differential ability of specific integrin cytoplasmic domains to stimulate signal transduction events in T cells. The system was chosen because  $\beta_2$  integrins require intracellular activation to facilitate ligand binding. This adhesion-dependent signaling will normally be difficult to discern from the processes initiated by the activation stimulus. Our system circumvents such an activation requirement and, furthermore, operates independently of adhesion and spreading, which in turn might trigger complex signaling systems (*e.g.* through cytoskeletal reorganization). Although changes in cell shape and morphology might contribute to cellular activation in important ways, it is necessary to be able to discriminate among these parameters to determine the minimal requirements for signaling.

The functional elements of integrin cytoplasmic domains have been analyzed in great detail in terms of their relative contribution to cell adhesion and spreading, but their potential roles in signal transduction have not been explored. For the  $\beta_2$ -cytoplasmic domain, these regions can be grouped into membrane proximal and distal functional elements. The binding sites for actin-cytoskeletal linker proteins, such as  $\alpha$ -actinin or filamin (46, 47), are located in the N-terminal portion of the  $\beta_2$  tail in addition to the response element for regulatory protein cytohesin-1, as recently described (41). More distal elements include the 758–60 TTT motif, which was shown to be required for constitutive adhesion of LFA-1 to ICAM-1 in COS cells (48) and for spreading of  $\beta_2/\beta_3$  chimera in an ectopic expression system (49). Our results show that the deletion of the C-terminal seven amino acids abrogates the  $\beta_2$ -dependent calcium signal. Therefore, none of the above-mentioned elements appears to be sufficient for calcium mobilization because they are all present in the non-functional construct CD18–762\*. However, the C terminus of two so-called NPXF motifs is deleted in this mutant. Moreover, mutation of the NPXF-phenylalanine into either a non-conserved alanine or a conserved tyrosine residue abrogated or strongly impeded the abilities of the resulting chimeras to induce a calcium flux. These data strongly suggest that the calcium signaling capacity of the  $\beta_2$ -cytoplasmic domain is largely dependent on the C-terminal NPXF motif. Interestingly, the  $\beta_1$ -cytoplasmic domain of integrin CD29 bears an NPXY motif at the homologous position. This finding prompted us to analyze whether the reversal of a single amino acid in  $\beta_1$  (Y759F) would rescue the calcium signaling

capacity of the  $\beta_1$  fusion protein. Fig. 7c shows that this is partially the case. We conclude from these data that phenylalanine 766 of the distal NPXF motif is a specific determinant of  $\beta_2$ -dependent intracellular calcium mobilization. However, the functionality of this motif appears to be somewhat dependent on the neighboring amino acids. This might be because of specific conformations that the different  $\beta$  tails assume; in fact, the contribution of integrin cytoplasmic tail conformation to function has recently been suggested (50). In an earlier study, F766 was shown to be an important determinant of LFA-1-dependent, constitutive adhesion of COS7 cells to ICAM-1. However, mutation of this residue into alanine had a strong inhibitory effect on adhesion whereas exchange of phenylalanine into tyrosine had yielded no phenotypic changes (48). The involvement of this region in signal transduction apparently is a different one. Firstly, the F766Y mutant bears little capacity to flux calcium and, secondly cytotoxic T cell function was strongly inhibited by the sIg-CD18 fusion protein but not by the F766Y mutant, suggesting that this mutant could not exert a dominant-negative block on the activation of specific cytolysis. Taken together, our data suggest that the distal NPXF has a different function in T cell signal transduction, as compared with COS cell adhesion to ICAM-1 mediated by ectopic LFA-1 expression. It cannot fully be ruled out, however, that some of the observed differences may be because of the cell types employed. The relative contribution of the C-terminal NPXF motif to signal transduction or T cell adhesion, respectively, would thus have to be analyzed in the future.

NPX(F/Y) motifs have also been implicated in receptor internalization. Specifically, the cytoplasmic tail requirements for endocytosis of LFA-1 have recently been analyzed. Based on this study, the determinant for the internalization of the  $\beta_2$  integrin lies further N-terminal in the  $\beta_2$ -cytoplasmic domain and, thus, does not overlap with the C-terminal NPXF motif (51).

Recent evidence suggests that platelet function *in vivo* is dependent on  $\beta_3$ -integrin signaling through NPXY and NXXY motifs. Interestingly, in the case of the  $\alpha_{IIb}\beta_3$  receptor this loss-of-function correlates with abrogation of receptor tyrosine phosphorylation (52). Thus, in different contexts both NPXF and NPXY motifs may contribute to specific signaling events.

We observed that the ability of the CD18 fusion protein to promote calcium mobilization is dependent on the expression of either an intact TCR or the  $\zeta$  chain fusion protein. These results suggest that LFA-1 acts on elements that are utilized or organized by TCR- $\zeta$ , or it acts through the  $\zeta$  chain itself. It may be possible that co-clustering of the receptors is mediated through links between their cytoplasmic portions. However, initial experiments (not shown) on TCR or  $\zeta$  chain co-aggregation after clustering of the integrin chimera do not support this idea. Intriguingly, one study has shown that the  $\zeta$ -associated tyrosine kinase ZAP-70 functions in an LFA-1 to LFA-1 adhesion regulation pathway important for cell invasiveness, but a direct link between the molecules has not been established (53). In light of our observations it is possible that the T cell receptor complex and LFA-1 coordinately relay information important for both cell activation and migratory functions. Several groups have recently shown that integrin-mediated matrix adhesion and growth factor receptors coordinately regulate cell proliferation (54–56). The underlying mechanisms are poorly understood, but it was suggested that extracellular matrix enhances PDGF-dependent responses by increasing the association of SHP-2 with platelet-derived growth factor receptor  $\beta$  (57). In the light of our results, one is tempted to hypothesize that  $\beta_2$  integrin signaling may also provide a means of modulating ITAM (immunoreceptor tyrosine-based activation motifs)

dependent immunoreceptor function. It should further be noted that signaling of other receptors in T cells (CD2, CD4) had been shown to display similar requirements for the presence of TCR functional elements (58, 59).

It is currently not known at which level the NPXF motif of the  $\beta_2$ -cytoplasmic domain and the TCR-associated  $\zeta$  chain functionally interact. Recently, a transcription factor termed JAB1 had been found to interact with the  $\beta_2$ -cytoplasmic domain; furthermore, this protein was translocated to the nucleus clustering of LFA-1 (60). It is presently not known, however, whether this interaction is important for T cell activation and, if so, whether it affects calcium signaling. Moreover, the precise binding site for JAB1 within the  $\beta_2$ -cytoplasmic domain has not yet been determined.

Substantial evidence supports the notion that Src family kinases are important downstream effectors of integrin signaling (61, 62). Furthermore, the Lck kinase in T cells plays a critical role in T cells with respect to ITAM phosphorylation, and its subsequent downstream interaction with ZAP-70 is critical for phospholipase C $\gamma$  activation and calcium signaling (63). However, convincing molecular links between Src kinases and integrin cytoplasmic domains have not yet been determined. Our results support the contention that functional interaction occurs between the C-terminal NPXF motif of the  $\beta_2$  tail and Src kinases in T cells. Moreover, there is evidence for functional interaction of LFA-1 with the cytotoxic T cell surface receptor DNAM-1 (64). DNAM-1 has been shown to be phosphorylated on tyrosine residues after aggregation of LFA-1, and this process appears to involve the Fyn kinase. Interestingly, we found that the sIg-CD18 chimera did not signal in the absence of extracellular calcium (Fig. 3c), nor did it induce phospholipase C $\gamma$  phosphorylation. On the other hand, induction of a 38-kDa phosphotyrosine was observed, consistent with the activation of LAT, a major phosphorylation target of ZAP-70 in T cells (65). We were unfortunately not capable of proving this point directly, since immunoprecipitation of LAT from TAG-Jurkat cells failed under the conditions used. These findings point to distinct similarities but also to differences between  $\beta_2$  integrin and T cell receptor-induced signaling pathways. Significantly, Takata *et al.* (66) describe differential signaling routes to calcium mobilization in DT40 B cells; these authors concluded that the Src kinase Lyn might regulate Ca<sup>2+</sup> mobilization through a process independent of inositol 1,4,5-trisphosphate generation. It is possible that CD18 couples to a similar pathway in T cells.

Our data provide a specific and testable hypothesis on the role of  $\beta_2$  integrin-mediated signaling events in T cell activation. This may now be verified in more complex systems, which allow the analysis of heterodimeric molecules. In the course of this study we have attempted to reconstitute wild-type and mutant  $\beta_2$  chain expression in  $\beta_2$ -allo-specific leukocyte adhesion deficiency T cell clones (not shown). However, this model system did not in principle allow visualization of calcium signaling induced by the allotype and was therefore not useful for our purposes. Therefore, reconstitution of wild-type and mutant  $\beta_2$  chains in  $\beta_2$  knock-out animals and subsequent analysis of their T cell function *in vivo* appears as a plausible direction for future work.

Taken together our findings suggest that  $\beta_2$  integrins specifically contribute to Ca<sup>2+</sup> signaling in T cells through an NPXF motif of the  $\beta_2$ -cytoplasmic domain. These data further support and extend an emerging general theme of signal integration in cell growth regulation operating through functional interactions of integrins with growth factor receptors.

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**Calcium Signaling through the  $\beta_2$ -Cytoplasmic Domain of LFA-1 Requires Intracellular Elements of the T Cell Receptor Complex**  
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