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Proximity and orientation underlie signaling by the non-receptor tyrosine kinase ZAP70

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Signaling by the antigen receptor of T lymphocytes initiates different developmental transitions, each of which require the tyrosine kinase ZAP70. Previous studies with agonist and antagonist peptides have indicated that ZAP70 might respond differently to different structures of the TCR–CD3 complex induced by bound peptides. The roles of membrane proximity and orientation in activation of ZAP70 signaling were explored using synthetic ligands and their binding proteins designed to produce different architectures of membrane-bound complexes composed of ZAP70 fusion proteins. Transient membrane recruitment of physiological levels of ZAP70 with the membrane-permeable synthetic ligand FK1012A leads to rapid phosphorylation of ZAP70 and activation of the ras/MAPK and Ca^{2+}/calcineurin signaling pathways. ZAP70 SH2 domains are not required for signaling when the kinase is artifically recruited to the membrane, indicating that the SH2 domains function solely in recruitment and not in kinase activation. Using additional synthetic ligands and their binding proteins that recruit ZAP70 equally well but orient it at the cell membrane in different ways, we define a requirement for a specific presentation of ZAP70 to its downstream targets. These results provide a mechanism by which ZAP70, bound to the phosphorylated receptor, could discriminate between conformational changes induced by the binding of different MHC–peptide complexes to the antigen receptor and introduce an approach to exploring the role of spatial orientation of signaling complexes in living cells.

Keywords: kinase/proximity/signaling/synthetic ligands/ZAP70

Introduction

A role for proximity in intracellular regulatory mechanisms was initially raised by the observation that the src-like tyrosine kinases require myristoylation for transformation (Sefton *et al.*, 1982; Cross *et al.*, 1984; Kaplan *et al.*, 1988). Since myristoylation mediates membrane attachment (Resh, 1994), this observation indicated that the role of myristoylation was to bring the src-like tyrosine kinases into proximity with associated signaling molecules or substrates confined to the cell membrane. Similarly, farnesylation is essential for the function of GTPases such as ras (Willumsen *et al.*, 1984; Buss *et al.*, 1989), presumably to allow the ordered propagation of signaling on the inner membrane surface. A broader role for proximity in signaling was suggested by the identification of scaffolding proteins and anchoring proteins that are required for the function of specific signaling pathways (Mochly-Rosen, 1995; Faux and Scott, 1996). Although these proteins may have roles other than mediating proximity, they are postulated to assemble signaling molecules into an ordered spatial array. Testing the role of proximity in signaling has been difficult. The sequence motifs such as the SH2 domain (Koch *et al.*, 1991; Pawson and Gish, 1992) or covalent modifications such as myristoylation that induce proximity might also activate enzymatic activity or other functions through conformational changes. Intuitively one would think that proximity alone could never mediate a qualitative response, since diffusion would allow random interactions.

One molecule that may be subject to regulation by membrane proximity is the non-receptor tyrosine kinase ZAP70, which has diverse roles in signaling by the antigen receptor. ZAP70 was originally discovered by Weiss and colleagues (Chan *et al.*, 1992) and shown to be related to the Syk family of tyrosine kinases (Chan *et al.*, 1994a). As predicted by the original studies in cell lines, ablation of the *ZAP70* gene in mice results in a profound defect of activation and also defects in positive and negative selection during thymic development (Negishi *et al.*, 1995). In addition, mutations in the human *ZAP70* gene are associated with a failure of normal signaling by T cells in peripheral lymphoid organs and skewed development of subpopulations of T cells (Arpaia *et al.*, 1994; Chan *et al.*, 1994b; Elder *et al.*, 1994).

Previous work suggests that ZAP70 might be responsive to distinct structural features of the MHC–peptide complex bound to the antigen receptor of T cells. In murine lymphocytes ZAP70 becomes associated with the tyrosine-phosphorylated ITAM motifs (Reth, 1989) of the ζ or ε chains of the antigen receptor (Chan *et al.*, 1991, 1992; Wange *et al.*, 1992; Straus and Weiss, 1993). These chains are probably phosphorylated by the src-like tyrosine kinases lck and/or fyn when the antigen receptor binds MHC–antigen complexes on the surface of antigen-presenting cells (Samelson *et al.*, 1985; Reth, 1989; Iwashima *et al.*, 1994). Studies using agonist and antagonist peptides demonstrated that while both were able to induce phosphorylation of TCRζ and recruitment of ZAP70, only the agonist peptides led to signaling and transcriptional activation of the IL-2 gene (Sloan-Lancaster *et al.*, 1994; Madrenas *et al.*, 1995) and presumably other early immune

response genes of T cells. While these observations could be explained by the necessity for signaling mechanisms not dependent on ZAP70, the critical role of ZAP70 in signaling in T lymphocytes raises the possibility that ZAP70 somehow senses the nature of the bound peptide and assumes a configuration that is either effective or ineffective in signaling.

Much of our understanding of signaling pathways has grown out of genetic modifications in the germlines of mice or transfection studies of cells overexpressing constitutively active or dominant negative proteins. While these studies have contributed immensely to our understanding of signaling, such stable genetic modifications set in motion a ricocheting series of actions, reactions and compensations at both the molecular and cellular levels, leading to a new steady-state. Since the outcome of the introduction of these modified signaling molecules is commonly examined many hours or days following their introduction or germline modification, while most signaling molecules are normally only active for seconds or minutes, compensation and indirect effects are inevitable and sometimes impossible to distinguish from direct effects. Such compensatory mechanisms and indirect effects are likely to be consistent with one another, and independent approaches may lead to a coherent steady-state yet not reflect the physiological activity of the protein under study. In an effort to avoid these difficulties and to mimic the evanescent nature of signaling, we have developed an approach to allow real-time analysis of the function of isolated signaling molecules in living cells (Spencer *et al.*, 1993). In this approach we have made use of the importance of induced proximity in activating biological functions (Mochly-Rosen, 1995; Crabtree and Schreiber, 1996; Faux and Scott, 1996). In the following studies we have extended this approach to analyze the roles of configuration or architecture in signaling and applied it to the study of the mechanism of action of the non-receptor tyrosine kinase ZAP70.

Results

Membrane recruitment can activate the signaling potential of ZAP70

We devised an approach to analyze the role of orientation of signaling molecules, such as ZAP70, using cell-permeable synthetic ligands or chemical inducers of dimerization (CIDs) (Figure 1A and B). This approach makes use of the fact that different linker elements in the CIDs differ in their length, rigidity and preferred orientation and hence allow bound molecules to sample subsets of all possible membrane-associated configurations. In addition, we used ligand-binding domains in fusion proteins that generate different geometries of the associated signaling complex based on the relative positions of the C- and N-termini (Figure 1C). The chimeric molecule SF1ZAP^{wt} was constructed by fusion of the full-length, wild-type murine and/or human ZAP70 cDNA (Chan *et al.*, 1992; Gauen *et al.*, 1994) to one copy of the cDNA encoding the FK506-binding protein FKBP12 (Figure 1C). The membrane-docking construct MF3E consisted of three tandem copies of FKBP12 fused to the myristoylation domain of v-src (Holsinger *et al.*, 1995; Spencer *et al.*, 1995). SF1ZAP^{wt} and MF3E were transiently co-expressed in the human leukemic T cell line TAg Jurkat, along with a construct in which tandem binding sites for the NF-AT transcription factor direct expression of secreted alkaline phosphatase (NFAT SEAP). This plasmid has been shown to initiate transcription at the correct site both in Jurkat cells and in transgenic murine lymphocytes (Durand *et al.*, 1988; Verweij *et al.*, 1990) and, like activation of many early immune response genes in T lymphocytes, requires signals from both the Ras/MAPK pathways and calcium/calcineurin pathways (Clipstone and Crabtree, 1992). Hence it serves as a monitor for both the ras and calcium pathways. Furthermore, since this plasmid replicates in the TAg Jurkat cell line, it assembles into chromatin that is likely to be similar to chromosomal genes (Stillman, 1996). Addition of FK1012 to TAg Jurkat cells that had been co-transfected with MF3E and SF1ZAP^{wt} resulted in dose-dependent induction of NF-AT-SEAP activity to levels comparable with those achieved with either PMA and ionomycin or stimulation through the antigen receptor (Figure 2A). Western blot analysis using an antibody specific for ZAP70 showed that chimeric human and murine SF1ZAP^{wt} were expressed at levels near those of the endogenous protein (Figure 2A and B). In contrast, signaling as manifested by activation of NF-AT-induced transcription was not detected when ZAP70 was homodimerized in the absence of the membrane-docking molecule MF3E (Figure 2A). Since previous studies had found that ZAP70 could not be fully activated by stable membrane association with the transmembrane molecule CD16 (Kolanus *et al.*, 1993), we tested the effects of inducing relatively stable membrane association by directly fusing the c-src myristoylation site to the N-terminus of ZAP70. Transfection of membrane-associated myristoylated ZAP70 resulted in a small but reproducible signal, usually from 20 to 35% of that obtained by transient recruitment with FK1012 (data not shown) or PMA and ionomycin. To determine if the myristoylated ZAP70 or the docking construct could be concentrated in caveoli and perhaps signal as a consequence of this, we examined the localization of the docking construct by confocal microscopy and found that it was uniformly distributed over the inner cell membrane (data not shown). Caveoli in contrast have a focal distribution (Conrad *et al.*, 1995).

To determine the amount of SF1ZAP^{wt} recruited to the membrane-docking molecule MF3E following addition of FK1012, we used fractionated membrane and cytosol preparations from TAg Jurkat cells that had been transiently transfected with human SF1ZAP^{wt} and MF3E. Membrane and cytosolic fractions prepared from stimulated and unstimulated cells were subjected to SDS–PAGE, followed by immunoblotting with an antibody specific for ZAP70. These results demonstrate that SF1ZAP^{wt}, but not endogenous ZAP70, was translocated to the membrane fraction upon addition of FK1012 (Figure 2B). The amount of SF1ZAP^{wt} recruited to the membrane fraction was ~10% of the level of total endogenous ZAP70 (Figure 2B). This degree of membrane recruitment is similar to the level of endogenous ZAP70 found in membrane fractions following TCR stimulation (van Oers *et al.*, 1994; I.Graef and G.R.Crabtree, unpublished results).

When signaling was initiated by FK1012 in cells transfected with the myristoylated docking construct and

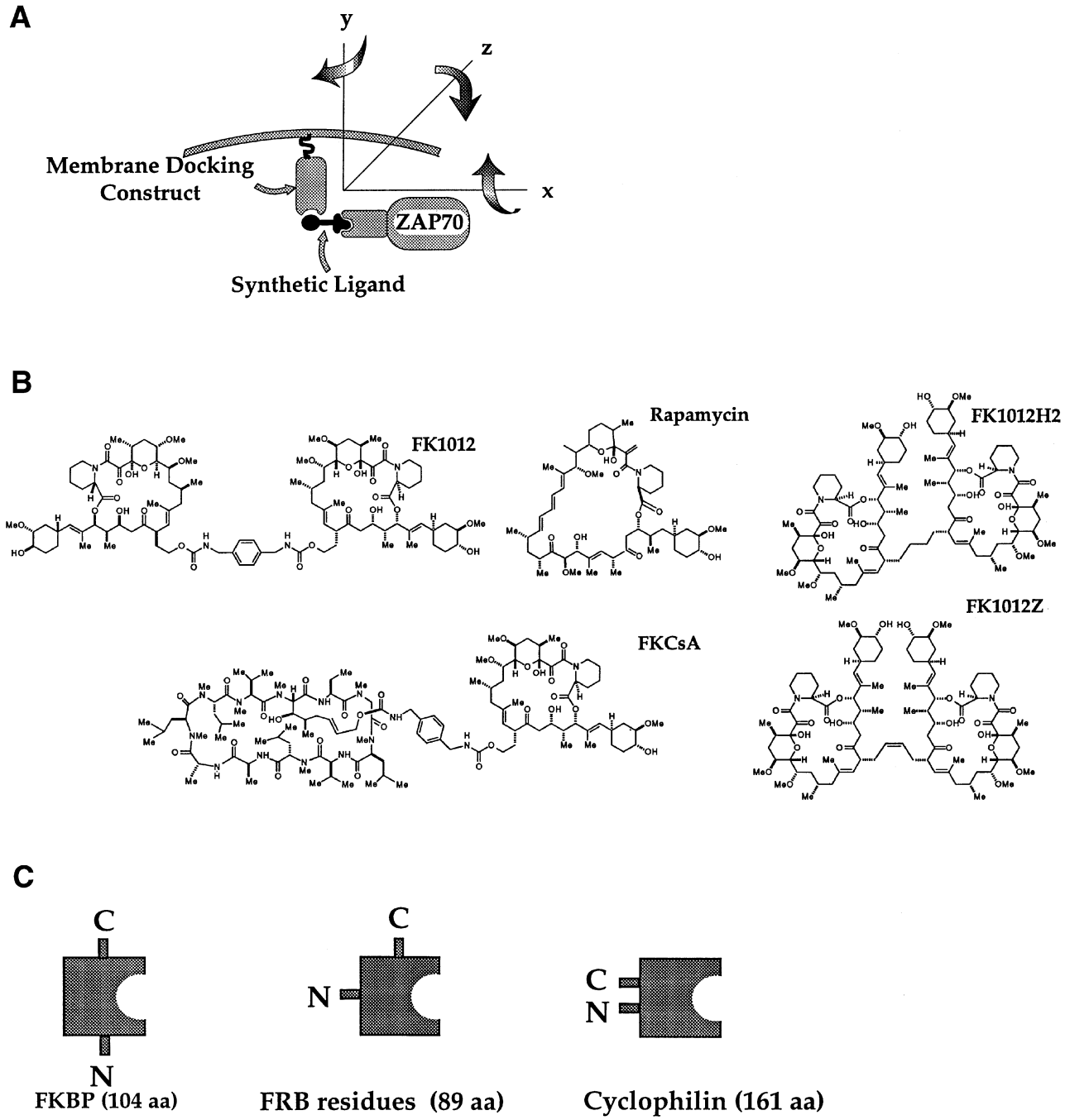

Fig. 1. (**A**) Model of the conformations sampled by ZAP70 upon membrane recruitment with synthetic ligands. (**B**) Molecular structure of synthetic ligands. Me, methyl. (**C**) Schematic representation of ligand-binding domains. FKBP, FK506-binding protein; FRB, FK506-binding region; CpH, cyclophilin A.

chimeric ZAP70^{wt}, the addition of monomeric competing ligand FK506M (Spencer *et al.*, 1993) rapidly blocked signaling with a time course similar to that of blocking signaling with FK506. FK506 inhibits calcineurin (Liu *et al.*, 1991; Clipstone and Crabtree, 1992) and blocks NF-AT nuclear translocation and therefore NF-AT-dependent transcription (Flanagan *et al.*, 1991). FK506M is modified at C21 and hence does not inhibit calcineurin (Spencer *et al.*, 1993). It blocks signaling with essentially identical kinetics as blocking signaling with FK506 (Figure 2C), further indicating that signaling induced by FK1012 is related to recruitment of ZAP70 to the membrane.

Activation of signaling by ZAP70 recruitment parallels activation by the antigen receptor

To determine if FK1012-mediated recruitment of SF1ZAP^{wt} mimicked physiological activation by antigen receptor stimulation, we compared TCR crosslinking with activation of SF1ZAP^{wt} by FK1012. The time course of activation of signaling was similar in cells treated with

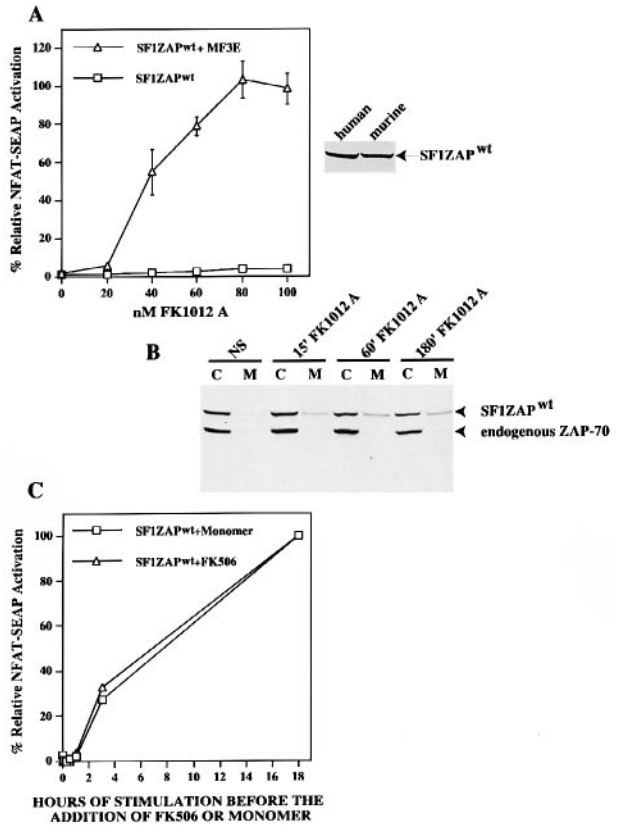

Fig. 2. Activation of T cell signal transduction by recruitment of ZAP70 to a myristilated docking molecule at the cell membrane. (**A**) TAg Jurkat cells were co-transfected with 2 μg murine SF1ZAP^{wt} together with 1 μg MF3E (Spencer *et al.*, 1995) and 2 μg NFAT-SEAP (Bram *et al.*, 1993) or 2 μg murine SF1ZAP^{wt} alone and 2 μg NFAT-SEAP. Twenty-four hours following electroporation, cells were divided equally and duplicates were treated for 18 h with medium alone, 25 ng/ml PMA and 1 μM ionomycin or titrations of FK1012A, and SEAP activity was measured. Data are presented as the percentage of maximal activation obtained by stimulation with 1 μM ionomycin plus 25 ng/ml PMA. Data represent the mean of four independent transfections ± SEM. Protein expression levels of human and murine SF1ZAP^{wt} chimeras were verified by immunoblotting with the 12CA5 mAb specific for the HA epitope Tag (insert). (**B**) Near physiological levels of chimeric ZAP70 are recruited to the membrane fraction. TAg Jurkat cells were co-transfected with 2 μg human SF1ZAP^{wt} and 1 μg MF3E. Twenty four hours post-transfection cells were stimulated with 200 nM FK1012A and membrane and cytosolic fractions were prepared. Cytoplasmic (C) and membrane (M) fractions were analyzed by Western blot with an antibody specific for ZAP70 (UBI). (**C**) Monomeric synthetic ligand inhibits signaling by membrane-recruited ZAP70. TAg Jurkat cells were co-transfected with 2 μg SF1ZAP^{wt}, 1 μg MF3E and 2 μg NFAT SEAP and after 24 h cells were stimulated with 200 nM FK1012A. At the indicated time points a 10-fold molar excess of FK506M monomer (2 μM) (Spencer *et al.*, 1995) or 2 ng/ml of the immunosuppressant FK506 were added. Data are presented as the percentage of maximal activation obtained by stimulation with FK1012A alone for 18 h.

FK1012 or stimulated by crosslinking of the T cell receptor with an antibody specific for the Jurkat TCR (C305, kindly provided by Dr A.Weiss) (Figure 3A). ZAP70 becomes phosphorylated on tyrosine residues that are essential for its *in vitro* kinase activity after antigen receptor engagement (Chan *et al.*, 1995; Wange *et al.*, 1995). FK1012 addition to Jurkat cells co-expressing the myristoylated docking construct MF3E and the chimeric ZAP70, SF1ZAP^{wt}, led to phosphorylation of SF1ZAP^{wt} within 10 min of addition of FK1012 (Figure 3B). This is similar to the time course of ZAP70 phosphorylation induced by anti-TCR antibodies. Antigen receptor stimulation also induces a significant reduction in mobility of the transcription factor NF-ATc on SDS–PAGE, which appears to be related to phosphorylation within the N-terminus of NF-ATc (I.Graef, N.Clipstone and G.R.Crabtree, unpublished results). TCR stimulation as well as membrane recruitment of SF1ZAP^{wt} resulted in a rapid shift in the mobility of co-transfected NF-ATc, which was detectable 15 min after stimulation (Figure 3C).

Previous studies had shown that ZAP70 stably associated with the membrane by covalent fusion to a transmembrane protein failed to signal (Kolanus *et al.*, 1993). Since we also found minimal stimulation upon transfecting cells with myristoylated ZAP70 we determined whether prolonged stimulation via the antigen receptor in Jurkat cells would lead to accommodation of the signaling pathways or silencing of signaling. Jurkat cells stimulated with an antibody to the TCR complex for up to 1 week showed a pronounced defect in their ability to activate NF-AT- and AP-1-dependent transcription (Figure 4A). In addition, phosphorylation of the mitogen-activated protein (MAP) kinases ERK-1 and ERK-2 was also defective in long-term TCR-stimulated cells compared with non-treated cells (Figure 4B). These results parallel previous studies in T and B cells (Wilde and Fitch, 1984; Goodnow *et al.*, 1988; Fields *et al.*, 1996; Li *et al.*, 1996) and indicate that prolonged stimulation through the antigen receptor leads to a refractory state and reduced signaling, possibly by MAP kinase inactivation. This refractory state probably explains why previous studies either failed to detect signaling or found reduced signaling when ZAP70 was stably associated with the membrane.

The role of specific orientations of the membrane-recruited ZAP70 in signaling

We examined the requirement for a precise conformation of the recruited ZAP70 using synthetic ligands that orient the signaling domains of fusion proteins differently relative to their signaling partners (Figure 1A and B). These were used in combination with different membrane-docking molecules (Figure 1C) to vary the geometry of the membrane-associated complex. A heterodimeric CID was synthesized made up of FK506 chemically linked to CsA, which we termed FKCsA (Belshaw *et al.*, 1996) and used to recruit chimeric FKBP–ZAP70 to the membrane with a single myristoylated cyclophilin A. Although FKCsA is predicted to sample very different conformations than FK1012A, it was ~30–40% as effective as FK1012A in inducing signaling (Figure 5A). An additional CID was synthesized, FK1012H2 (Figure 1B), in which the distance between the twisted amide surrogate structures (Rosen *et al.*, 1990) that bind FKBP was reduced by several Ångstroms (S.Diver and S.L.Schreiber, unpublished results). FK1012H2 was able to induce signaling by recruitment of FKBP–ZAP70 that was quantitatively and qualitatively similar to FK1012A (Figure 5B). To explore the role of rotational freedom around the twisted amide surrogate structures, a fourth synthetic ligand FK1012Z was synthesized. This molecule is constrained by the Z-*cis* double bond in the linker between the two FK506 structures and hence samples fewer and different configurations than FK1012A and the above molecules. Despite this more

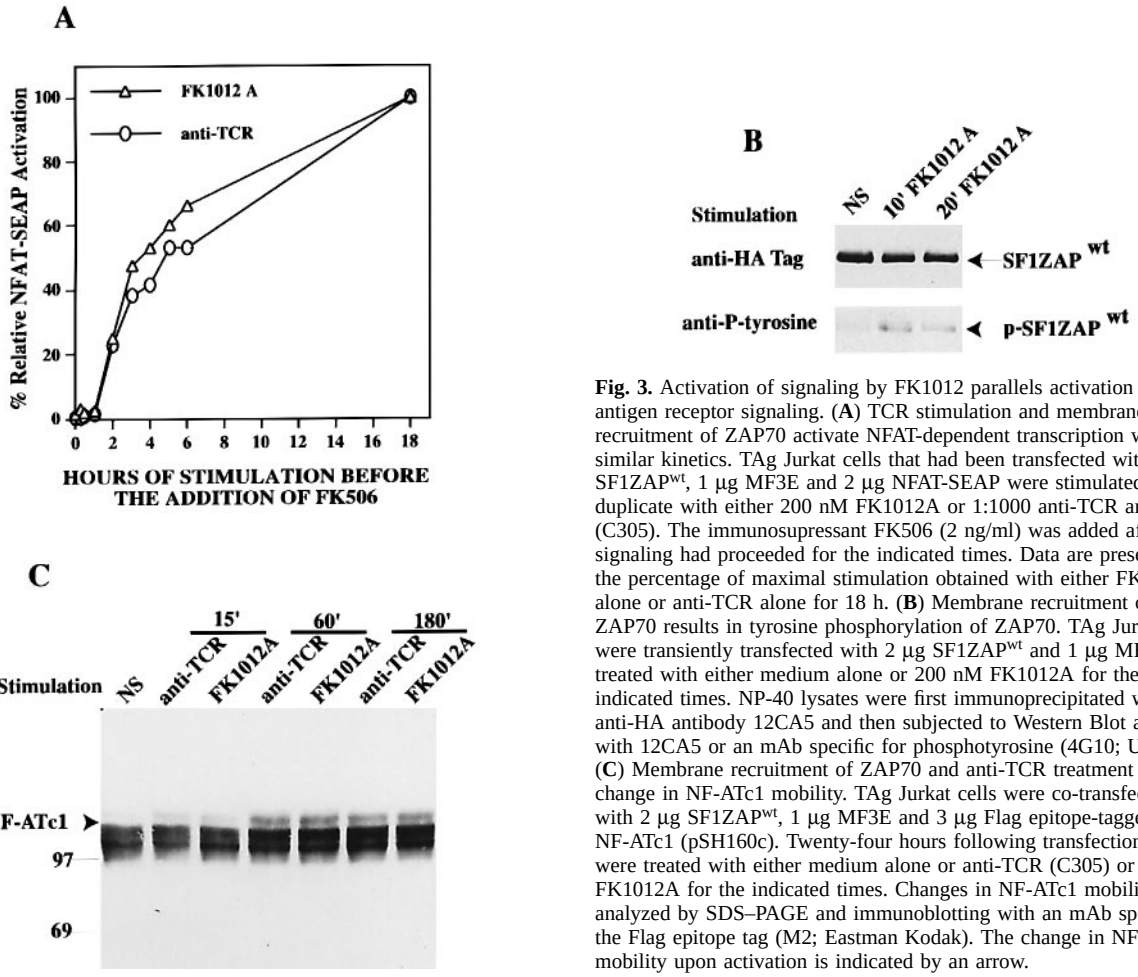

Fig. 3. Activation of signaling by FK1012 parallels activation by antigen receptor signaling. (**A**) TCR stimulation and membrane recruitment of ZAP70 activate NFAT-dependent transcription with similar kinetics. TAg Jurkat cells that had been transfected with 2 μg SF1ZAP^{wt}, 1 μg MF3E and 2 μg NFAT-SEAP were stimulated in duplicate with either 200 nM FK1012A or 1:1000 anti-TCR antibody (C305). The immunosupressant FK506 (2 ng/ml) was added after signaling had proceeded for the indicated times. Data are presented as the percentage of maximal stimulation obtained with either FK1012 alone or anti-TCR alone for 18 h. (**B**) Membrane recruitment of ZAP70 results in tyrosine phosphorylation of ZAP70. TAg Jurkat cells were transiently transfected with 2 μg SF1ZAP^{wt} and 1 μg MF3E and treated with either medium alone or 200 nM FK1012A for the indicated times. NP-40 lysates were first immunoprecipitated with the anti-HA antibody 12CA5 and then subjected to Western Blot analysis with 12CA5 or an mAb specific for phosphotyrosine (4G10; UBI). (**C**) Membrane recruitment of ZAP70 and anti-TCR treatment induce a change in NF-ATc1 mobility. TAg Jurkat cells were co-transfected with 2 μg SF1ZAP^{wt}, 1 μg MF3E and 3 μg Flag epitope-tagged NF-ATc1 (pSH160c). Twenty-four hours following transfection, cells were treated with either medium alone or anti-TCR (C305) or 200 nM FK1012A for the indicated times. Changes in NF-ATc1 mobility were analyzed by SDS–PAGE and immunoblotting with an mAb specific for the Flag epitope tag (M2; Eastman Kodak). The change in NF-ATc1 mobility upon activation is indicated by an arrow.

constrained geometry, FK1012Z induced signaling nearly as well as FK1012A (Figure 5B). As an additional means of exploring the issue of presentation or geometry of the recruited ZAP70, we used the heterodimeric CID rapamycin to recruit ZAP70. Rapamycin binds FKBP through its twisted amide surrogate structure on one side and FRB through the opposite side of the molecule (Choi *et al.*, 1996). Furthermore, the N- and C-termini of the 89 amino acid region of FRB which bind rapamycin protrude from the molecule at right angles, resulting in an orientation of fusion proteins different from either cyclophilin A or FKBP (Figure 1C). Hence, rapamycin produces a relatively rigid connection between FKBP12 and FRB, and would be expected to sample only those structures that are allowed by the flexibility within the FRB–membrane chimeric docking construct and within the FKBP–ZAP70 chimeric protein. Although rapamycin was highly effective in inducing transcription by recruitment of a transcription activation domain (Ho *et al.*, 1996) or by recruiting the src-like tyrosine kinase fyn or the exchange factor Sos to the membrane (Figure 5C and data not shown), it was inactive when used to recruit ZAP70 to the membrane (Figure 5C), despite the fact that ZAP70 was membrane associated in the extracts of these cells (Figure 5C, insert). These observations indicate that while simple proximity is capable of activating the signaling function of ZAP70, the induction of signaling requires a specific configuration of the kinase at the membrane, presumably one suitable for further interaction with downstream targets. These results also demonstrate that the docking construct is not simply taking ZAP70 to a specific membrane compartment, such as caveoli, where signaling is initiated by virtue of localization within a specific membrane domain.

The SH2 domains can be replaced by membrane recruitment with synthetic ligands

The SH2 domains and inter-SH2 region of ZAP-70 have been proposed to contribute to the catalytic activity of ZAP70 by inter- or intramolecular interactions (Hatada *et al.*, 1995). The functional significance of the SH2 domains and inter-SH2 region of ZAP70 was addressed by preparing chimeric FKBP–ZAP70 proteins lacking either the N-terminal (SF1ΔNSH2) or both (SF1ΔNΔ-CSH2) of the SH2 domains. SF1ΔNSH2 and SF1ΔNΔ-CSH2 were expressed at levels comparable with the wild-type (Figure 6B) and induced signaling comparable with that observed after activation of the full-length construct SF1ZAP^{wt} (Figure 6A). These results indicate that in this context the SH2 domains of ZAP70 solely direct localization of ZAP70 to the membrane and are unnecessary for activation of kinase activity or other functions through conformational changes induced by binding of the SH2 domains to the ITAM motif.

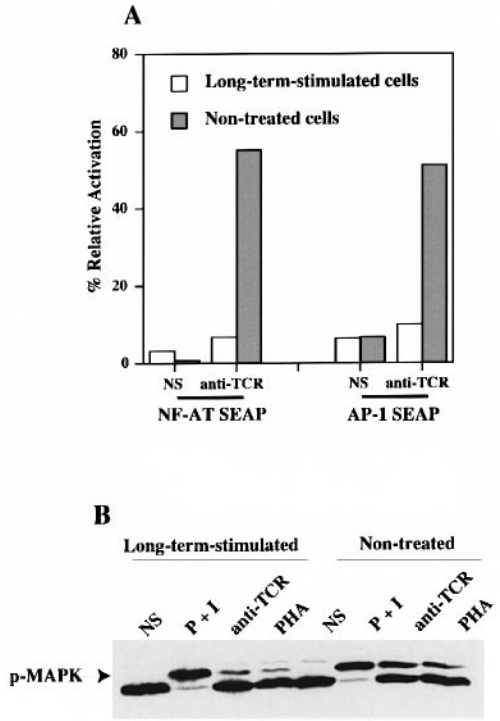

Fig. 4. Accommodation of TCR signaling to prolonged stimulation. (**A**) TAg Jurkat cells were treated for 5 days with an antibody to CD3 (OKT-3; ATCC) or left untreated. Following the long-term stimulation cells were transfected with 2 μg NFAT-SEAP or 2 μg AP-1-SEAP (Spencer *et al.*, 1993). Stimulation and assays for reporter gene activity were performed as described above. (**B**) TAg Jurkat cells were treated for 5 days with an antibody to CD3 or left untreated. After the prolonged stimulation the cells were stimulated with either PMA and ionomycin, anti-CD3 antibody or PHA for 15 min. The cell lysates were analyzed for activation of MAPK, identified by the change in MAPK electrophoretic mobility indicating phosphorylated forms of MAPK. The change in MAPK mobility upon activation is indicated by an arrow.

Requirement for Lck by membrane-associated ZAP70

$p56^{lck}$ is a non-receptor src-like tyrosine kinase that has been implicated in signaling by the TCR and provides essential phosphorylation on both the TCRζ chain and ZAP70 (Perlmutter *et al.*, 1993; Weiss and Littman, 1994). The role of Lck in signaling by FK1012-induced membrane recruitment of $SF1ZAP^{wt}$ was addressed in two ways. Previous experiments (Chan *et al.*, 1995; Wange *et al.*, 1995) had identified Tyr492 and Tyr493 as the tyrosine residues of ZAP70 that undergo *in vivo* phosphorylation following TCR engagement and are phosphorylated by Lck *in vitro*. Furthermore, these residues are necessary for the signaling function of ZAP70 when ZAP70 is transfected into a Syk-deficient B cell line (Chan *et al.*, 1995). Both of these tyrosine residues were mutated to alanine, to generate the chimeric construct $SF1ZAP^{A492,493}$. Membrane recruitment of $SF1ZAP^{A492,493}$ failed to induce activation of a co-transfected NF-AT reporter gene (Figure 7A). Protein expression of $SF1ZAP^{wt}$ and $SF1ZAP^{A492,493}$ was similar, as judged by immunoblotting with an antibody specific for the HA epitope tag (Figure 7A, insert).

To confirm that Lck or a similar src-like tyrosine kinase is required for signaling by membrane-recruited ZAP70, we co-transfected $SF1ZAP^{wt}$ with a catalytically inactive dominant negative form of lck (Lck^{dn} K273R) (Levin *et al.*, 1993) and tested for the ability of $SF1ZAP^{wt}$ to induce NF-AT-dependent transcription after addition of FK1012 in the presence of Lck^{dn}. Lck^{dn} blocked signaling induced by crosslinking the TCR with C305 monoclonal antibody, but showed no effect on signaling, as determined by activation of NFAT-dependent transcription by addition of PMA plus ionomycin, which act downstream of the TCR and Lck (data not shown). Co-expression of Lck^{dn} also completely blocked the ability of membrane-targeted $SF1ZAP^{wt}$ to initiate signaling (Figure 7B). These observations are compatible with the hypothesized role of Lck in ZAP70 activation (Chan *et al.*, 1995).

Requirement for ZAP70 catalytic activity for signaling by membrane-associated Lck

The results above are consistent with a model in which phosphorylation of TCRζ leads to SH2-dependent membrane recruitment of ZAP70, which in turn is activated by phosphorylation by Lck. From these observations we predicted that a ZAP70 with intact SH2 domains but a mutation in the Lck phosphorylation site would be dominant negative. We therefore generated a non-chimeric $ZAP70^{A492,493}$ construct that had Tyr492 and Tyr493 replaced with alanines but lacked FKBP ($SZAP^{A492,493}$). Transfection of $SZAP^{A492,493}$ blocked signaling by anti-TCR antibodies, but a similar construct lacking the SH2 domains ($S\Delta N\Delta CSH2^{A492,493}$) had no effect (Figure 7C). Levels of protein expression were confirmed by Western blotting for the HA epitope tag (Figure 7C, insert). The most parsimonious interpretation of these results is that $SZAP^{A492,493}$ exerts its dominant negative effect by outcompeting endogenous ZAP70 for binding to the phosphorylated ITAM upon receptor stimulation and that recruitment of endogenous ZAP70 to the TCR complex is essential for TCR-mediated activation of NFAT-dependent transcription.

We had previously found that recruitment of a constitutively active Lck (SF1Lck Y505F) could activate signaling (Spencer *et al.*, 1995). We used the dominant negative ZAP70 to determine if membrane recruitment of Lck activated signaling by a mechanism dependent on ZAP70. Signal transduction initiated by SF1Lck was completely blocked by co-transfection of $SZAP^{A492,493}$ (Figure 7D and data not shown), again consistent with data indicating that Lck produces an activating phosphorylation of ZAP70.

Discussion

We have used synthetic ligands or CIDs based on the structures of the macrolides FK506, rapamycin and the cyclic peptide cyclosporin A as probes to analyze the roles of proximity and orientation or configuration in signaling by the the non-receptor tyrosine kinase ZAP70. Our results indicate that while signaling by ZAP70 can be activated by simple membrane recruitment, a specific configuration is probably necessary for its signaling function. In every way tested, signaling by ZAP70 recruitment mimics that of signaling initiated through the antigen receptor.

Previous studies have suggested that ZAP70 is sensitive to the nature of the peptide bound by the MHC–antigen receptor complex. This conclusion was reached from

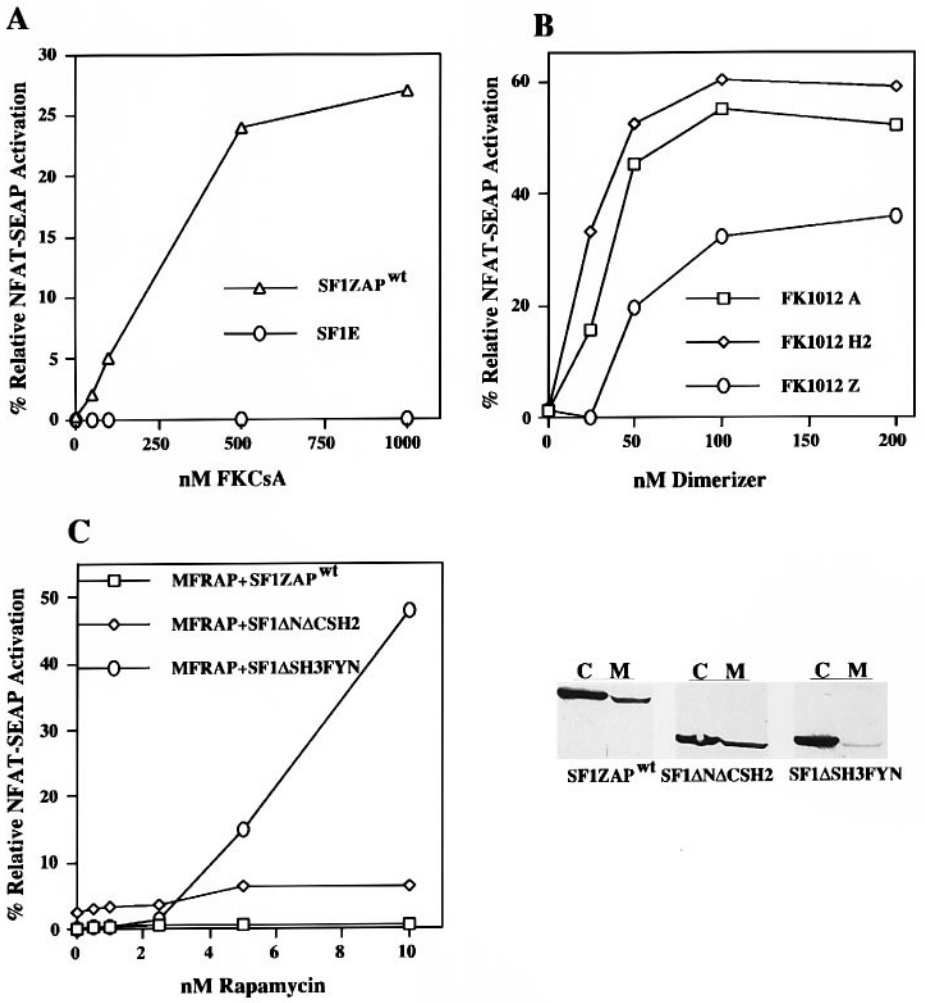

Fig. 5. The role of configuration and the SH2 domains in the activation of ZAP70 by FK1012-induced membrane recruitment. (**A**) FKCsA induces signaling by recruitment of ZAP70 to a myristoylated cyclophilin A. TAg Jurkat cells were transfected with 2 μg SF1ZAP^{wt} plus 1 μg MC1E or 2 μg SF1E plus 1 μg MC1E and 2 μg NFAT-SEAP. Stimulation and assays for reporter gene activity were performed as described above. Data are presented as the percentage of maximal activation obtained by stimulation with 1 μM ionomycin plus 25 ng/ml PMA. Data represent the mean of four independent transfections ± SEM. (**B**) Activation of ZAP70 induced by different CIDs. TAg Jurkat cells were transfected with 2 μg SF1ZAP^{wt} plus 1 μg MF3E and 2 μg NFAT-SEAP. Stimulation and assays for reporter gene activity were performed as described above. Data are presented as the percentage of maximal activation obtained by stimulation with 1 μM ionomycin plus 25 ng/ml PMA. Data represent the mean of four independent transfections ± SEM. (**C**) Orientation sensitivity of ZAP70 but not Fyn. TAg Jurkat cells were co-transfected with 2 μg SF1ZAP^{wt}, 2 μg SF1ΔNΔCSH2 or 2 μg SF1ΔSH3Fyn (Spencer *et al.*, 1995) plus 1 μg MFRB and 2 μg NFAT-SEAP. Stimulation and assays for reporter gene activity were performed as described above. Data are presented as the percentage of maximal activation obtained by stimulation with 1 μM ionomycin plus 25 ng/ml PMA. Data represent the mean of four independent transfections ± SEM. Cell fractionations after stimulation for 2 h with 10 nM rapamycin were performed as described above. Cytoplasmic (C) and membrane (M) fractions were analyzed by Western blot with the anti-HA 12CA5 antibody.

studies of agonist and antagonist peptides, both of which elicit distinct patterns of phosphorylation of the ζ chain of the antigen receptor and result in recruitment of ZAP70; however, only agonist peptides lead to activation of immune response genes, such as IL-2. While the latter results could be explained by a need for additional signaling mechanisms that are independent of ZAP70, our results demonstrate that ZAP70 membrane localization, in specific configurations, is sufficient to activate both the ras and Ca^{2+}/calcineurin pathways leading to IL-2 gene activation. Thus a failure to activate a ZAP70-independent signaling pathway is unlikely to explain the failure of antagonist peptides to activate transcription. Our results support a model in which membrane-associated ZAP70 can be presented to downstream signaling molecules in an effective or ineffective configuration. FK1012A, FK1012Z, FK1012H2 and FKCsA all produce active configurations, while rapamycin, which is equally effective in inducing membrane localization and signaling by other molecules, is unable to present ZAP70 properly to downstream signaling molecules.

Allosteric mechanisms are not likely to explain the activation of signaling by the recruited ZAP70 in our studies, since the crystal structure of FKBP12 alone and FKBP12 bound to FK506 are nearly identical (Van Duyne *et al.*, 1993) and the crystal structures of cyclophilin and cyclophilin bound to cyclosporin are also highly similar (Kallen *et al.*, 1991; Weber *et al.*, 1991). Similarily, intramolecular distortion based on binding of the synthetic ligand to tandemly repeated FKBPs is unlikely to explain our results, since a single FKBP12 with ZAP70 and a single cyclophilin as the docking construct are effective

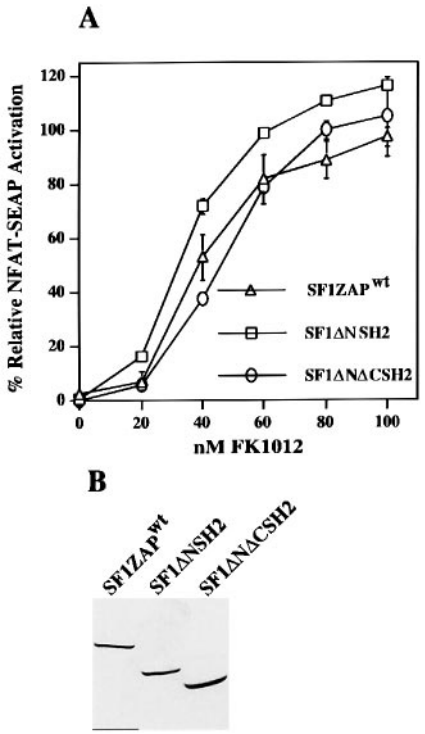

Fig. 6. The tandem SH2 domains can be fully replaced by membrane recruitment. (**A**) TAg Jurkat cells were transfected with 2 μg SF1ZAP^{wt} or 2 μg SF1ΔNSH2 or 1.5 μg SF1ΔNΔCSH2 plus 1 μg MF3E and 2 μg NFAT-SEAP. Stimulation and assays for reporter gene activity were performed as described above. Data are presented as the percentage of maximal activation obtained by stimulation with 1 μM ionomycin plus 25 ng/ml PMA. Data represent the mean of two independent transfections ± SEM. (**B**) Protein expression levels of the SH2 deletion constructs SF1ΔNSH2 and SF1ΔNΔCSH2 in comparison with full-length SF1ZAP^{wt} were verified by immunoblotting with the 12CA5 mAb specific for the HA epitope Tag.

in both recruitment and activation of signaling, indicating that aggregation of ZAP70 is not essential for signaling. The greater effectiveness of an array of ligand-binding regions probably relates to the fact that recruitment by FK1012 is very inefficient, probably crosslinking no more than 5% of transfected molecules (Ho *et al.*, 1996). Since we find that the two SH2 domains can be replaced by FKBP-induced recruitment, our results indicate that the role of the SH2 domains of ZAP70 is to regulate proximity and that allosteric changes induced by interaction of the SH2 domains with the ITAM motif do not detectably affect the signaling potential of ZAP70 *in vivo*.

In previous studies covalent linkage of ZAP70 to a transmembrane protein, CD16, did not activate signaling (Kolanus *et al.*, 1993). The approach of linking a cytoplasmic protein to a transmembrane protein may have failed to activate signaling for any of the following reasons: (i) the normally cytoplasmic proteins Lck and ZAP70 were too constrained and sterically inhibited after linking to a transmembrane receptor to signal without the help of an antibody to the extracellular region; (ii) linking ZAP70 to transmembrane proteins may have targeted them to inactive sites where they were unable to come into contact with appropriate signaling molecules; (iii) the chimeric transmembrane proteins may have been subject to endocytosis; (iv) the signaling pathway may have accommodated to the continuous signaling provided by stable introduction of a membrane-linked active tyrosine kinase. The use of synthetic ligands or CIDs to transiently evoke signaling circumvents most of these possible sources of error. We feel that the most likely reason our results differ from those of others is that the antigen receptor signaling pathway is subject to inhibitory feedback regulation. Previous work has shown that B cells become unresponsive to signaling by antigen in transgenic animals expressing the antigen specific for their receptors (Goodnow *et al.*, 1988). These refractory B cells quickly recover signaling when they are transferred to a normal host not expressing the antigen. This mechanism is felt to underlie the mechanism of peripheral tolerance to endogenous antigens. Silencing of signaling has also been observed in T lymphocytes continuously stimulated with antigen (Wilde and Fitch, 1984). Recent data indicate that in a model of T cell clonal anergy the MAP kinase and JNK cascades are refractory to additional stimulation (Fields *et al.*, 1996; Li *et al.*, 1996). We find that chronically stimulated Jurkat cells also show defects in activation of MAP kinase that may underlie the previous observation that stable membrane association of ZAP70 does not appreciably activate signaling. A number of other observations strongly indicate that ZAP70 is subject to negative feedback regulation. Most notably, ZAP70 is normally phosphorylated for only ~5 min following T cell receptor stimulation and then becomes dephosphorylated in the continued presence of ligand (Chan *et al.*, 1991).

Perhaps the most surprising outcome of our studies is that a simple change in localization leads to an apparent all-or-none effect on signaling. We estimate the induction of transcription as a consequence of membrane localization to be >1000-fold. These observations raise the general question of how simple localization could lead to such large changes in activity. Intuitively one would think that random associations would lead to signaling and hence localization would be a poor way of regulating the activity of a protein. However, the probability of an interaction of one molecule with another falls with the cube of the distance between them and this distance effect may be exaggerated in a cell where weak interactions with neighboring proteins or repulsion from the membrane might reduce the number of random associations with signaling partners. The high affinity (0.4 nM) of the FK506–FKBP interaction is probably sufficient to overcome restricted diffusion due to weak random interactions with adjacent molecules. Such random interactions are commonly estimated to be in the range 10–100 μM and are based on surface charge complementarity. Another possible mechanism that could operate with restricted diffusion is replusion of ZAP70 from membranes or essential signaling molecules. In this case the role of the synthetic ligand might be to bridge the distance necessary to allow effective phosphorylation and activation of essential signaling intermediates. The high affinity of the FKBP–FK506 interaction would also be sufficient to overcome most forms of membrane repulsion based on the charge of the recruited protein, thereby creating a high effective concentration of the signaling molelcule and its downstream substrate. While the molecular mechanism underlying qualitative activation of signaling by proximity are open to speculation, our results indicate that this simple mechanism can be used to activate and analyze physio-

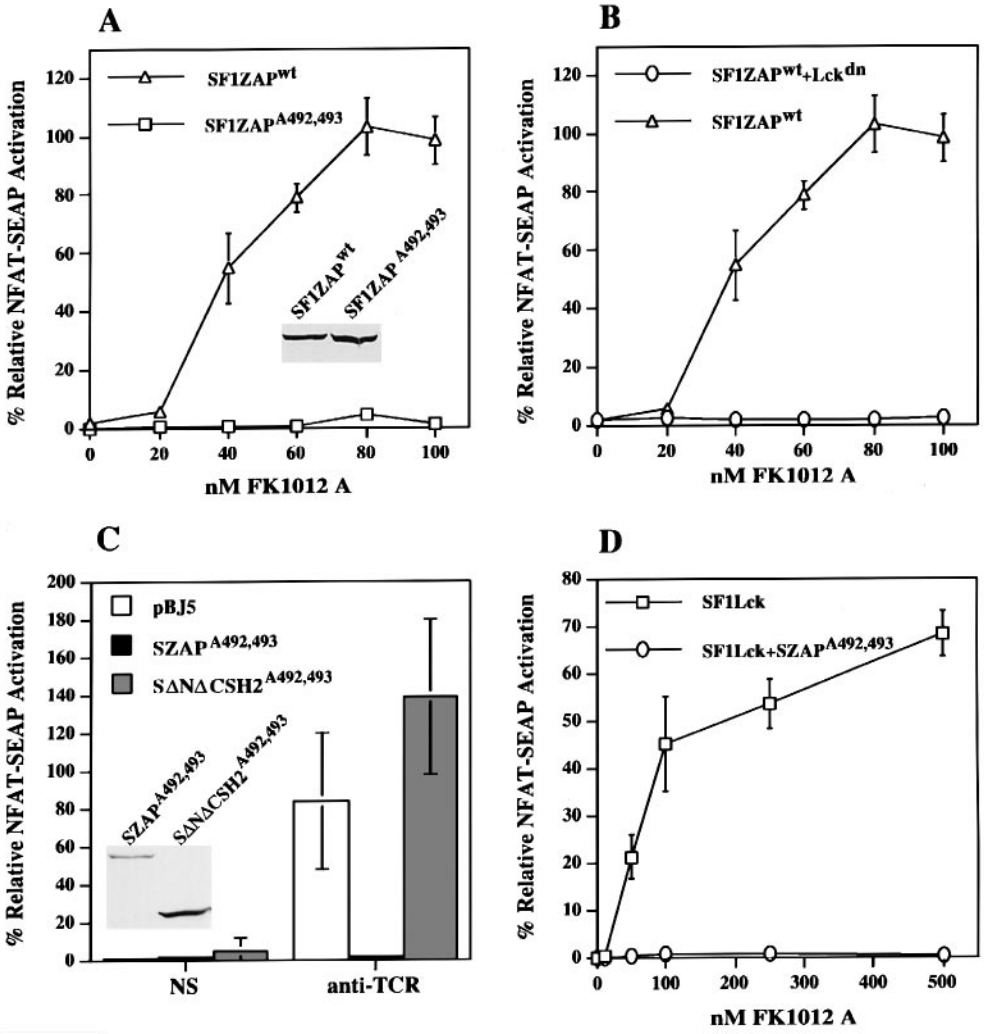

Fig. 7. Lck and its putative phosphorylation site on ZAP70 are required for signaling after membrane recruitment of ZAP70. (**A**) The Lck phosphorylation site is required for signaling by membrane-recruited ZAP70. TAg Jurkat cells were transfected with 2 μgSF1ZAP^{wt} or 4 μg SF1ZAP^{A492,493} together with 1 μg MF3E and 2 μg NFAT-SEAP. Stimulation and assays for reporter gene activity were performed as described above. Data are presented as the percentage of maximal activation obtained by stimulation with 1 μM ionomycin plus 25 ng/ml PMA. Data represent the mean of four independent transfections ± SEM, which were performed in parallel. Levels of protein expression of the SF1ZAP^{wt} and SF1ZAP^{A492,493} chimeras were verified by immunoblotting with the 12CA5 mAb specific for the HA epitope tag (insert). (**B**) Dominant negative Lck blocks signaling by membrane-recruited ZAP70. TAg Jurkat cells were transfected with 2 μg SF1ZAP^{wt}, 1 μg MF3E and 2 μg NFAT-SEAP together with either empty expression vector pBJ5 or with 4 μg Lck^{dn}. Stimulation and assays for reporter gene activity were performed as described above. Data are presented as the percentage of maximal activation obtained by stimulation with 1 μM ionomycin plus 25 ng/ml PMA. Data represent the mean of four independent transfections ± SEM, which were performed in parallel. (**C**) Dominant negative ZAP70 blocks signaling induced by TCR stimulation. SZAP^{A492,493} blocks anti-TCR signal transduction. TAg Jurkat cells were transfected with 3 μg SZAP^{A492,493} or 3 μg SΔNΔCSH2^{A492,493} and 2 μg NFAT-SEAP. Stimulation and assays for reporter gene activity were performed as described above. Data are presented as the percentage of maximal activation obtained by stimulation with 1 μM ionomycin plus 25 ng/ml PMA. Data represent the mean of four independent transfections ± SEM. Levels of protein expression of the SZAP^{A492,493} and SΔNΔCSH2^{A492,493} chimeras were verified by immunoblotting with the 12CA5 mAb specific for the HA epitope tag (insert). (**D**) Dominant negative ZAP70 blocks activation of NF-AT-dependent transcription by membrane recruitment of constitutively active Lck. TAg Jurkat cells were transfected with 3 μg SF1Lck (Spencer *et al.*, 1995) plus either 3 μg SZAP^{A492,493} or 3 μg empty vector, together with 1 μg MF3E and 2 μg NFAT-SEAP. Stimulation and assays for reporter gene activity were performed as described above. Data are presented as the percentage of maximal activation obtained by stimulation with 1 μM ionomycin plus 25 ng/ml PMA. Data represent the mean of three independent transfections ± SEM.

logical signaling pathways independently of the natural mechanisms of compensation that are called into play by constitutively active molecules.

Materials and methods

Expression plasmids

Murine ZAP70 cDNA was kindly provided by Dr A.S.Shaw (Gauen *et al.*, 1994). The eukaryotic expression construct murine SF1ZAP^{wt} contains the full-length murine ZAP70 cDNA linked in-frame at the N-terminus to one copy of human FKBP12. An *Xho*I site was introduced immediately 5′ of the second codon of murine ZAP70 by PCR. A *Sal*I site was introduced at the 3′-end by PCR, thereby deleting the stop codon. The resulting PCR was subcloned into the *Sal*I site of SF1E (Spencer *et al.*, 1993). SF1ΔNSH2 was generated by PCR using murine ZAP70 cDNA as a template. An *Xho*I site was introduced immediately 5′ of nt 580, thereby deleting amino acids 1–156. SF1ΔNΔCSH2 was made by PCR using murine ZAP70 cDNA as a template. An *Xho*I site was introduced immediately 5′ of nt 862, thereby deleting amino acids 1–251. SF1ZAP^{A492,493} was made by replacing Tyr492 and Tyr493 with alanine using PCR-directed mutagenesis and subsequently subcloned into the *Sal*I site of SF1E. Human ZAP70 cDNA (Chan *et al.*, 1992) was a generous gift from Dr A.Weiss. The eukaryotic expression construct human SF1ZAP^{wt} contains the full-length human ZAP70

cDNA linked in-frame at the N-terminus to one copy of human FKBP12. Human SF1ZAP^{wt} was amplified by PCR using human ZAP70 as template. All ZAP70 constructs contain a C-terminal influenza hemagglutinin (HA) epitope tag. In all cases nucleotide sequences of wild-type and mutated cDNAs were verified by DNA sequencing. MC1E consists of the myristylation targeting domain, residues 1–14 from c-src, linked to cyclophilin A. MFRB consists of the myristylation targeting domain, residues 1–14 from c-src (Ho *et al.*, 1996). An *Eco*RI fragment encompassing the coding sequence of murine Lck in pKS was inserted into the *Eco*RI site of the eukaryotic expression vector pBJ5 (Takebe *et al.*, 1988). A *Bgl*II–*Pfl*MI fragment of SF1ΔKLck (K273R) (Spencer *et al.*, 1995) was inserted into the murine Lck pBJ5 expression construct after removal of the corresponding wild-type fragment to generate the eukaryotic expression construct Lck^{dn}. The constructs SF1Lck, SF1E and MF3E have been described elsewhere (Spencer *et al.*, 1995). The NF-ATc1 eukaryotic expression construct pSH160c (kindly provided by Dr S.Ho) contains the human NF-ATc1 cDNA (nt 243–2751) (Northrop *et al.*, 1994) tagged at the N-terminus with a Flag epitope linked in-frame with the second codon. The reporter plasmids NFAT-SEAP and AP-1-SEAP have been described elsewhere (Bram *et al.*, 1993).

Reporter gene assays

Cells were harvested 24 h after transfection and aliquoted in duplicate into 96-well flat bottom microtiter plates (2×10^5 cells/well in 100 μl complete medium) and stimulated with various combinations of ionomycin (1 μM), phorbolmyristate (PMA, 25 ng/ml), dilutions of the CID or a monoclonal antibody to the Jurkat T cell receptor (C305, kindly provided by Dr A.Weiss) in a final volume of 200 μl. Secreted alkaline phosphatase was measured 18 h after stimulation, as described previously (Berger *et al.*, 1988).

Western blots

To monitor protein expression, cells were lysed 36 h after transfection in RIPA buffer; Extracts were analyzed by SDS–PAGE and electrotransferred to nitrocellulose. Blots were probed with monoclonal antibody (mAb) 12CA5 (Babco, Richmond,CA) specific for the HA epitope tag or anti-Flag mAb M2 (Eastman Kodak Company) and horseradish peroxidase-conjugated rabbit anti-mouse IgG (Zymed). Proteins were visualized by ECL (Amersham).

Cell fractionation

TAg Jurkat cells were transfected by electroporation with 1 μg MF3E and 2 μg human SF1ZAP^{wt}. Thirty six hours after transfection cells were stimulated with the CID or left untreated. Cells were lysed by freeze-thawing in hypotonic buffer (10 mM Tris, pH 7.5, 5 mM MgCl and protease and phosphatase inhibitors). The post-nuclear fraction was ultracentrifuged (100 000 *g* for 40 min) to separate membranes and soluble cytosol fractions. Equal amounts of membrane and cytosol fractions were separated by SDS–PAGE, transferred onto nitrocellulose and immunoblotted with mAb specific for ZAP70 (Upstate Biotechnology)

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