Efficient T-cell receptor signaling requires a high-affinity interaction between the Gads C-SH3 domain and the SLP-76 RxxK motif

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The relationship between the binding affinity and specificity of modular interaction domains is potentially important in determining biological signaling responses. In signaling from the T-cell receptor (TCR), the Gads C-terminal SH3 domain binds a core RxxK sequence motif in the SLP-76 scaffold. We show that residues surrounding this motif are largely optimized for binding the Gads C-SH3 domain resulting in a high-affinity interaction ($K_D = 8\text{--}20\text{ nM}$) that is essential for efficient TCR signaling in Jurkat T cells, since Gads-mediated signaling declines with decreasing affinity. Furthermore, the SLP-76 RxxK motif has evolved a very high specificity for the Gads C-SH3 domain. However, TCR signaling in Jurkat cells is tolerant of potential SLP-76 crossreactivity, provided that very high-affinity binding to the Gads C-SH3 domain is maintained. These data provide a quantitative argument that the affinity of the Gads C-SH3 domain for SLP-76 is physiologically important and suggest that the integrity of TCR signaling *in vivo* is sustained both by strong selection of SLP-76 for the Gads C-SH3 domain and by a capacity to buffer intrinsic crossreactivity.

The EMBO Journal (2007) **26**, 678–689. doi:10.1038/sj.emboj.7601535; Published online 18 January 2007

Subject Categories: signal transduction

Keywords: adaptor protein; protein–protein interaction; quantitative biology; SH3 domain; T-cell signaling

Introduction

Adaptor proteins are signaling molecules that are composed primarily of modular interaction domains through which they

link upstream receptors to specific core elements of the cellular machinery (Samelson, 2002; Jordan *et al*, 2003). The biological properties of adaptors therefore depend on the intrinsic binding affinities of their constituent domains for protein targets, and the extent to which they preferentially recognize specific ligands among the large repertoire of potential binding partners. Some interaction domains have a relatively restricted binding profile, whereas others can potentially bind multiple partners (Mayer, 2001; Pawson, 2004; Li, 2005). In the latter case, an ability to engage several ligands could expand an adaptor's signaling output, but could also result in unwanted cross-talk between pathways that corrupts the fidelity of signal transmission (Figure 1A). Moreover, the specificity with which an interaction domain engages its partners also depends on relative factors, including the cooperative effects of associated domains and the local concentrations of competing protein ligands. It is therefore a challenge to resolve how the individual binding affinities of interacting components in multiprotein signaling complexes relate to the potency and specificity of pathway activation among potentially crossreactive networks of mammalian protein–protein interactions. As such, we still have only a limited understanding of how binding affinity and selectivity, assayed *in vitro*, correlates with the specific activation of signaling pathways in mammalian cells.

To pursue this problem, we have focused on the adaptor protein Gads, a member of a family of proteins, which includes Grb2 and Grap (Liu *et al*, 2001). These proteins all have a central phosphotyrosine (pTyr)-binding Src homology 2 (SH2) domain, flanked by two SH3 domains (Liu *et al*, 2001). Within the TCR signaling pathway, Grb2 and Gads bind similar phosphotyrosine motifs (pY-x-N-x) on the transmembrane docking protein LAT (Liu and McGlade, 1998; Liu *et al*, 2001), while their SH3 domains possess distinct binding specificities, which contribute to their different functions (Liu and McGlade, 1998; Qiu *et al*, 1998; Law *et al*, 1999; Liu *et al*, 1999, 2003; Lewitzky *et al*, 2001; Harkiolaki *et al*, 2003).

Within T cells, Grb2 contributes to Ras activation through an interaction of the Grb2 SH3 domains with the Ras guanine nucleotide exchange factor Sos (Gong *et al*, 2001) while RasGRP is required for sustained Ras signaling (Dower *et al*, 2000; Ebinu *et al*, 2000). Gads, on the other hand, links phosphorylated LAT to the scaffold protein, SLP-76, which binds the Gads C-SH3 domain (Figure 1B). SLP-76, in turn, recruits a number of signaling proteins, including Vav, an Rac guanine nucleotide exchange factor, the adaptor proteins Nck and ADAP, and the Itk tyrosine kinase, that activate pathways involved in gene expression, cytoskeletal rearrangement and cell adhesion (Bunnell *et al*, 2002; Barda-Saad *et al*, 2005). Thymocytes from *Gads*-deficient mice display impaired TCR signaling, due to the uncoupling of SLP-76 from LAT, defects in positive and negative selection, and altered T-cell profiles (Yoder *et al*, 2001; Singer *et al*, 2004; Yankee *et al*, 2004). Thymocytes deficient in either

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Received: 2 August 2006; accepted: 4 December 2006; published online: 18 January 2007

Figure 1 The Gads C-SH3 domain binds with high affinity to an SLP-76 derived peptide. **(A)** Schematic showing pathway control by adaptors. Potential cross-talk to other pathways may occur as a result of the ability of an adaptor protein in pathway X to bind targets in other pathways. **(B)** Schematic of the TCR pathway in which Gads binds to phosphorylated LAT to recruit SLP-76 using the Gads C-terminal SH3 domain. Other RxxK proteins, such as the Gab family of proteins, bind both the Gads and Grb2 C-SH3 domain. **(C)** The structure of the Gads C-SH3 domain in complex with a SLP-76-derived peptide (Harkiolaki *et al*, 2003). **(D)** SPR sensorgram showing the accumulated mass of the SLP-76 (18-mer) peptide (Y-axis) binding to the immobilized Gads C-SH3 domain as a function of time (concentration 0.3–81 nM from bottom). **(E)** Isothermal titration calorimetry binding curve showing the titration of the SLP-76 peptide (100 μ M) into a calorimetric cell containing the Gads C-SH3 domain (5 μ M). The interaction shows a stoichiometry of approximately 1:1.

SLP-76 or LAT also display a block in thymopoiesis at the pre-T3 stage due to defective signaling from the pre-TCR (Koretzky and Myung, 2001). Thus, SLP-76 and LAT play a central role in thymic development and T-cell effector functions and Gads provides an important link to coordinate their activities.

Two notable features of the Gads C-SH3 domain interaction with SLP-76 are its relatively high affinity and the unusual sequence of the Gads-binding motif (232 APSIDRSTKPPLDR 245) in SLP-76. This SLP-76 motif contains Arg and Lys residues that are essential for the Gads C-SH3 domain recognition. Solution and crystal structures of the Gads C-SH3 domain have identified a typical SH3 domain fold, formed from two antiparallel β -sheets made up of five β -strands with a small 3_{10} -helix between the β_4 and β_5 strands (Harkiolaki *et al*,

2003; Liu *et al*, 2003; Figure 1C). The binding site for the SLP-76 peptide corresponds to a surface on other SH3 domains that often accommodates proline-based peptides that adopt a polyproline type II helix. However, the SLP-76 peptide has a different binding mode as it forms a right-handed 3_{10} -helix upon binding and contacts at least five sites on the Gads C-SH3 domain (Harkiolaki *et al*, 2003; Liu *et al*, 2003).

Many SH3 domains bind proline-based PxxP motifs, often with moderate micromolar affinities, whereas a subset of SH3 domains, typified by the Gads C-SH3 domain, bind alternate peptide motifs lacking a PxxP core (Mayer, 2001; Zarrinpar *et al*, 2003a; Li, 2005). Gene duplication and subsequent sequence divergence of signaling domains provide an evolutionary mechanism for creating diversity in signaling pathways by permitting modules such as SH3 domains to explore

ligand recognition space, and thereby select domain/ligand combinations with varying degrees of specificity or affinity (Larose *et al*, 1995; Zarrinpar *et al*, 2003b). Here, we find that a high-affinity interaction between Gads and SLP-76 is necessary for optimal TCR signaling and that Gads-dependent signaling declines proportionally with decreasing affinity reaching baseline as the interaction is weakened by two orders of magnitude. Moreover, we demonstrate that the physiological SLP-76 ligand possesses a very high level of selectivity for the Gads C-SH3 domain, which may provide an additional layer of specificity despite an observed tolerance for crossreactivity in TCR signaling. These results provide a quantitative measure of the relationship between SH3 domain binding affinity and signaling output in a defined mammalian system.

Results

High-affinity binding of SLP-76 to the Gads C-SH3 domain is characterized by unique kinetic and thermodynamic signatures

The interaction between the Gads C-SH3 domain and an SLP-76 peptide motif has been previously explored using fluorescence polarization to measure equilibrium binding (Berry *et al*, 2002). To probe the determinants and dynamic properties of this complex more thoroughly, we performed a detailed kinetic analysis of the Gads C-SH3 domain binding to a series of SLP-76-derived peptides using Biacore surface plasmon resonance (SPR) technology. We assessed the binding of a soluble 18-mer peptide (AKLPASIDRSTKPPLDR), corresponding to the Gads-binding region on SLP-76 (residues 228–245), to an immobilized Gads C-SH3 domain. SPR analysis revealed that the SLP-76 peptide bound with a very rapid association rate ($k_{on} = 1.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) and moderate dissociation rate ($k_{off} = 0.12 \text{ s}^{-1}$), yielding an equilibrium dissociation constant, K_D , of 8 nM (Figure 1D; Table I). Reversing the interaction by immobilizing a biotinylated SLP-76 peptide onto a streptavidin-coated chip and flowing the Gads C-SH3 domain over the peptide yielded similar rate and affinity constants (Table I). This indicates a relatively strong interaction, since SH3 domains often exhibit affinity constants in the micromolar range (Kay *et al*, 2000; Mayer, 2001).

To validate the affinity of this interaction, we performed solution-based analysis of the 18-mer SLP-76 peptide binding the Gads C-SH3 domain using isothermal titration calorimetry (ITC). This approach yielded a K_D of 21 nM (Figure 1E), which is in the same affinity range as measured by SPR. Interestingly, the enthalpy of this interaction is high, with a ΔH of -17.4 kcal/mol . This is opposed by a modest

unfavorable entropic component, with a $-\Delta S$ value of -6.7 kcal/mol , consistent with a loss of degrees of freedom upon binding, possibly due to ordering of the RT loop and/or the formation of the peptide's 3_{10} helix upon binding (Liu *et al*, 2003). Overall, these data demonstrate that the interaction between the Gads C-SH3 domain and SLP-76 possesses kinetic and thermodynamic signatures suggestive of a number of highly productive molecular interactions that in aggregate result in a high-affinity interaction between the domain and peptide.

SLP-76 residues outside the core Gads C-SH3 recognition motif contribute to binding kinetics and affinity

The affinity of the Gads C-SH3 domain for the 18-mer SLP-76 peptide was approximately an order of magnitude stronger than we had previously measured, using an 11-residue fluorescein-labeled version of the SLP-76 peptide ($^{232}\text{APSIDRSTKP}^{241}\text{A}$, C-terminal Ala is non-natural) in a fluorescence polarization assay (Berry *et al*, 2002). Based on structural data (Harkiolaki *et al*, 2003; Figure 1C), we speculated that a region C-terminal to the core RxxK motif might contribute to the strength of the interaction. We therefore assayed the ability of SLP-76 peptides of varying lengths to bind the immobilized Gads C-SH3 domain in an SPR assay (Table I; Supplementary Figure S1). Indeed, the K_D of the 11-mer SLP-76 peptide binding to the immobilized Gads C-SH3 domain was almost 10-fold weaker than that of 14, 18 or 21-mer SLP-76 peptides. The kinetics of the longer peptides revealed a consistent two-fold faster association rate and five-fold slower dissociation rate, accounting for the 10-fold stronger affinity constant ($K_D = 6\text{--}8 \text{ nM}$). These data are consistent with a high affinity ($K_D = 19 \text{ nM}$) interaction observed between full-length Gads and a 265 amino-acid fragment of the Gads-binding SLP-76 region by ITC (Houtman *et al*, 2004). We conclude that the region C-terminal to the core RxxK motif modestly increases the association rate of the complex and more significantly stabilizes the interaction once the complex has formed.

Multiple residues within the SLP-76 RxxK peptide are optimized for recognition by the Gads C-SH3 domain

We next investigated whether the high affinity of the Gads C-SH3/SLP-76 RxxK peptide interaction correlates with a preferred selectivity of the Gads C-SH3 domain for specific peptide ligands. To examine the specificity with which the Gads C-SH3 domain binds the SLP-76 peptide, we generated a peptide array in which each residue of the 14-mer Gads-binding peptide of SLP-76 was changed, one at a time, to each of the 20 natural amino acids. This array was probed with a

Table I Kinetic binding parameters of Gads C-SH3 domain with SLP-76 peptides of varying length

	Analyte	Immobilized substrate	k_{on} ($\text{M}^{-1} \text{ s}^{-1}$) ^a	k_{off} (s^{-1}) ^a	K_D^b (nM)
11-mer	$^{232}\text{APSIDRSTKP}^{241}\text{A}$	Gads C-SH3	$8.5 (\pm 0.68) \times 10^6$	$0.50 (\pm 0.05)$	59
14-mer	$^{232}\text{APSIDRSTKPPLDR}^{245}$	Gads C-SH3	$1.3 (\pm 0.15) \times 10^7$	$0.10 (\pm 0.01)$	8
18-mer	$^{228}\text{AKLPASIDRSTKPPLDR}^{245}$	Gads C-SH3	$1.5 (\pm 0.13) \times 10^7$	$0.12 (\pm 0.008)$	8
21-mer	$^{225}\text{HKTAKLPASIDRSTKPPLDR}^{245}$	Gads C-SH3	$1.7 (\pm 0.34) \times 10^7$	$0.10 (\pm 0.02)$	6
Gads C-SH3		Biotin-SLP-76 18mer	$9.5 (\pm 0.34) \times 10^6$	$0.06 (\pm 0.0007)$	6

^aValues represent mean rate constants determined by three replicates ($\pm \text{s.e.m.}$).

^bEquilibrium dissociation constant is determined by the ratio between k_{off} and k_{on} ($K_D = k_{off}/k_{on}$).

Note that 11-mer peptide is based on a 10-mer derivative of SLP-76 (residues 232–241). The final Ala is non-natural to SLP-76.

6xHis-tagged form of the SH3 domain, which permitted us to discern more subtle changes in spot intensities compared to previous observations using a GST-fusion (Berry *et al*, 2002). Interestingly, five SLP-76 residues (P233*, D236*, R237*, K240*, P241*; asterisk denotes SLP-76 synthetic peptides as opposed to the full-length protein) are unequivocally optimal for their position within the SLP-76 sequence, such that altering the wild-type residue to any other amino acid strongly impaired the interaction with the Gads C-SH3 domain (Figure 2). SLP-76 I235* is also an optimal residue, but may be substituted with valine with minimal loss of Gads C-SH3-binding. Based on this analysis, at least six of the 13 residues within the SLP-76-binding region make significant contributions to this interaction, consistent with previous observations (Berry *et al*, 2002; Harkiolaki *et al*, 2003; Liu *et al*, 2003). Most remarkable, however, is that a substantial number of residue positions within the SLP-76 peptide sequence are fully optimized for recognition by the Gads C-SH3 domain, demonstrating that the sequence of this peptide motif is highly favorable for an interaction with its physiological binding partner.

The wild-type SLP-76 sequence optimally activates downstream TCR signaling

To assess the role that affinity of the Gads/SLP-76 interaction plays in mediating TCR signaling, we generated a series of SLP-76 peptide variants in which each residue within the Gads-binding epitope was altered to Ala. The resulting SLP-76 peptide variants exhibited binding affinities for the Gads C-SH3 domain that ranged from 4 nM to 44 μ M, as measured by SPR (Table II; Supplementary Figure S2). These affinities are consistent with the relative importance of each SLP-76 residue in binding the Gads C-SH3 domain suggested by the

Gads C-SH3 domain/SLP-76 peptide structure (Harkiolaki *et al*, 2003; Liu *et al*, 2003), and previous, more limited, mutational analysis (Berry *et al*, 2002). To exploit the range of affinities exhibited by these SLP-76 variant peptides, we individually incorporated several of these Ala substitutions into full-length SLP-76 constructs. We transiently expressed these proteins in SLP-76-deficient J14-v29 Jurkat T cells, and measured NFAT activation as an output of TCR activation to assess whether the high affinity between Gads and SLP-76 is necessary for efficient signaling downstream from the TCR.

TCR stimulation of mock-transfected J14-v29 cells did not induce any measurable NFAT activation (Figure 3A). Conversely, TCR stimulation of J14-v29 cells transfected with wild-type SLP-76 induced effective activation of NFAT (Figure 3A and B), consistent with the view that SLP-76 is required for activation of this pathway in T cells (Koretzky and Myung, 2001; Yablonski *et al*, 2001; Kumar *et al*, 2002; Singer *et al*, 2004). When NFAT activation was plotted as a function of $\Delta\Delta G$ for the variant SLP-76 peptide/Gads C-SH3 domain interactions relative to that of wild-type SLP-76 (Figure 3B), the ability of SLP-76 variants to support TCR signaling in transfected J14-v29 cells correlated with their binding affinity for the Gads C-SH3 domain, within the NFAT activation range of 18–28 units. Interestingly, as binding affinity between Gads and SLP-76 was weakened beyond a $\Delta\Delta G$ of approximately 3.5 kcal/mol ($K_D = \sim 3 \mu$ M), no further decrease in NFAT activation was observed. These results suggest that a minimum K_D of approximately 3 μ M represents the affinity threshold at which the interaction between the Gads C-SH3 domain and SLP-76 becomes physiologically relevant *in vivo*. As suggested by previous data, our results indicate that selective ablation of the Gads/SLP-76 interaction weakens, but does not completely abrogate signaling to

Figure 2 Several residues within the Gads-binding SLP-76 sequence are optimized for binding the Gads C-SH3 domain. A peptide array was generated consisting of the SLP-76 peptide (APSIDRSTKPPLDR) in which each of the residues within the SLP-76 peptide (N- to C-terminus: top to bottom) were altered to each of the 20 natural amino acid (left to right).

Table II Kinetic binding parameters of Gads C-SH3 domain with SLP-76 peptide variants

Peptide variant*	k_{on}^a ($M^{-1}s^{-1}$)	k_{off}^a (s^{-1})	K_D^b	$\Delta\Delta G$
Wt	$1.4 (\pm 0.15) \times 10^7$	$0.12 (\pm 0.007)$	8 nM	0
P233A*	$2.0 (\pm 0.12) \times 10^6$	$0.41 (\pm 0.035)$	207 nM	1.93
S234A*	$3.7 (\pm 0.51) \times 10^7$	$0.10 (\pm 0.015)$	4 nM	-0.49
I235A*	$7.3 (\pm 0.18) \times 10^6$	$0.63 (\pm 0.011)$	87 nM	1.45
D236A*	$7.3 (\pm 0.48) \times 10^6$	$0.82 (\pm 0.075)$	202 nM	1.91
R237A*	—	—	12 μ M	4.35
S238A*	$8.3 (\pm 0.24) \times 10^6$	$0.14 (\pm 0.002)$	17 nM	0.43
T239A*	$7.6 (\pm 0.06) \times 10^6$	$0.16 (\pm 0.003)$	21 nM	0.58
K240A*	—	—	44 μ M	5.10
P241A*	$8.0 (\pm 0.70) \times 10^6$	$0.95 (\pm 0.093)$	119 nM	1.60
P242A*	$1.2 (\pm 0.01) \times 10^7$	$0.087 (\pm 0.001)$	7 nM	-0.07
L243A*	$1.2 (\pm 0.02) \times 10^7$	$0.33 (\pm 0.005)$	29 nM	0.76
D244A*	$7.4 (\pm 0.72) \times 10^7$	$0.26 (\pm 0.082)$	4 nM	-0.48
R245A*	$1.6 (\pm 0.22) \times 10^7$	$0.40 (\pm 0.026)$	26 nM	0.69
P233A/P241A*	—	—	5 μ M	3.83

*Denotes peptide as opposed to FLAG-SLP-76 expression constructs.—Indicate that rate constants are not available as the dissociation rates were too rapid for monitoring by Biacore. K_D values were instead determined by fitting the binding levels at equilibrium versus peptide analyte concentration to the Michaelis–Menton equation.

^aValues represent mean rate constants determined by three replicates ($\pm$ s.e.m.).

^bEquilibrium dissociation constant is determined by the ratio between k_{off} and k_{on} ($K_D = k_{off}/k_{on}$).

Figure 3 The affinity of SLP-76 for Gads is related to the ability of Gads to enhance NFAT activation in response to TCR stimulation. (A) Jurkat J14-v29 cells were transiently transfected with vector or FLAG-SLP-76 variant constructs and an NFAT luciferase construct. Cells were stimulated by crosslinking anti-CD3 antibody. Cell lysates were monitored for NFAT activation by detection of luciferase activity using a luminometer. Histogram shows NFAT levels in response to anti-CD3 crosslinking for each of the SLP-76 variants. (B) Plot of NFAT levels determined from (A) as a function of the value in the change in Gibb's free energy ($\Delta\Delta G$) associated with the SLP-76 peptide variants compared to wild-type SLP-76 peptide binding the Gads C-SH3 domain (see Table II). $\Delta\Delta G$ was determined by the equation: $\Delta\Delta G = -RT \ln(K_{D(wt)}/K_{D(mut)})$.

NFAT, consistent with the possibility that there are alternative mechanisms to link LAT to SLP-76 (Koretzky and Myung, 2001; Yablonski *et al*, 2001; Kumar *et al*, 2002; Singer *et al*, 2004). However, our data demonstrate that the high affinity of the interaction between Gads and SLP-76 is a necessary feature in determining the efficiency with which the TCR activates downstream pathways, and that compromising the strength of the Gads C-SH3/SLP-76 RxxK interaction proportionally decreases TCR signaling.

SLP-76 binds with high specificity to a small subset of SH3 domains

The preceding data prompted us to examine whether the SLP-76 RxxK peptide has a particular selectivity for the Gads C-SH3 domain among a larger set of SH3 domains, which might be important for optimal TCR-dependent signaling. To explore this issue, we probed a protein array of 147 unique mammalian SH3 domains with an N-terminally biotinylated SLP-76 18-mer peptide. In this assay, the SLP-76 peptide only bound detectably to the Gads C-, Grb2 C-, STAM1 and STAM2 SH3 domains (Figure 4A and B). The relative affinities of the Grb2 C-SH3 domain and the Gads C-SH3 domain for the SLP-76 peptide, measured by SPR ($K_D = 8 \mu$ M versus 8 nM, respectively) (Supplementary Figure S3), are consistent with the relatively lower spot intensity for the Grb2 C-SH3 domain. Interestingly, the affinity of SLP-76 for the Grb2 C-SH3 domain is ~ 3 -fold weaker than the minimal level required to support TCR signaling. STAM1 and STAM2 are endocytic proteins with SH3 domains known to bind RxxK-based peptides found in the UBPY and AMSH polypeptides (Kato *et al*, 2000; Kaneko *et al*, 2003); however, SLP-76 has not been physiologically linked to the STAM proteins *in vivo*, and they might reside in different cellular compartments. Other SH3 domains from the Grb2 family of proteins failed to interact with the SLP-76 peptide, and SPR analysis of the Grb2 N-SH3 domain was consistent with these results (Supplementary Figure S3). Prolonged exposure revealed a faint interaction

A

SH3 domain array configuration

Array I

Amp	Lck	SPCN	Cortactin	MLPK3	Yes1	Abl2	SJHUA	Itk	CRK-D2
Dlg2	EMP55	FGR	SLK	Nebulin	C-src	Fyb-D1	Hck	Vav2-D2	NOF-D1
Vav-D1	Nck1-D3	Y124	PEXD	BTX	RasGAP	PSD95	Tim	HS1	Stam1
BLK	Abl	PLCγ	Riz	PI3b	ITSN1-D1	ITSN1-D2	TXK	GST	

Array II

Abl2B	Gads-D1	Abl2	CCBA	CRKL-D1	C5KP	Nck1-D2	NE-DLG	NOF-D1	OSF
PI3α	SP93	STAC	Tec	TRIP10	PIG2	ARH6	BCA1	BIN1	EFS
GRAP-D1	JIP1	M3KA	My7A	Nck2-D2	Nck2-D3	RHGA	SH31	SNX9	UAS3
Vav2-D1	Vav3-D1	VINE-D1	VINE-D3	C-Src	GST				

Array III

PRMT2	BMX	SPAG1	CACNB2	Crk-D1	Crk-D2	Crk1-D2	Fyb-D2	GAP	Grap-D2
Grb2-D1	Grb2-D2	Gads-D2	ITSN1-D3	ITSN1-D3	JIP2	Lyn	ZO2	MATK	MIA
MY1E	MY1F	NCF1-D1	NCF2-D2	Nck2-D1	NPH1	OTOR	Dlg1	SH3GL2	SH3GL3
SPIN90	TRIO	VINE-D2	C-Src	GST					

Array IV

ITSN2-D1	ITSN2-D2	ITSN2-D3	HIP-55	PACSIN2	LASP-1	ARGBP2-D2	ARGBP2-D3	STAM2	SORBS-D3
ITSN1-D4	Nck1-D1	HIAA0554	NEBL	Csk	SLA	Vav-D2	ARGBP2-D1	SRGAP2	KIAA0456
BZRAP1-D1	GRAF	CXorf9-D1	DDEF2	KIAA0418-D3	KIAA0418-D4	KIAA0418-D5	ARHGEF-9	PSTPIP1	NCF4
NEDD9	EP58	ARHGEF16	KIAA0769-D1	BAIAP2	KIAA0790	PPP1R13B	DFKZp434D-D3	DFKZp434D-D4	PACSIN3
KIAA1139	KIAA1249	DKFZP434D146	TRIO2	SH3-pos	GST				

B

Figure 4 The SLP-76 peptide binds with high specificity to a small subset of SH3 domains. (A) In left diagram, protein macroarrays (www.panomics.com) containing GST fusion proteins were probed with N-terminally biotinylated peptides conjugated to streptavidin-HRP and detected by film using chemiluminescence. In right diagram, configuration of protein macroarrays containing a total of 147 unique GST-SH3 domains including Gads C-, Grb2 C-, STAM1 and STAM2 SH3 domains (in purple). Several controls are included on the membranes including GST alone and a class 1b SH3 domain-binding domain. Each domain is spotted twice. (B) Binding of biotinylated RxxK peptides to SH3 domain macroarrays as detected on film using chemiluminescence. RxxK peptide ligands are derived from SLP-76, Gab1, the PxxP ligand from HPK-1 and the mixed PxxP/RxxK peptide from HPK-1 and each incubated with the set of SH3 domain macroarrays (in boxes from left to right).

with the osteoclast-stimulating factor SH3 domain, as the only other *in vitro* binding partner for the SLP-76 peptide. As a positive control, we probed the SH3 domain membranes with a biotinylated peptide from the hematopoietic progenitor kinase (HPK)-1 protein that typifies a type II PxxP peptide (APSENIPPLPKPKFR) (Figure 4B). The HPK-1 peptide detectably bound 53 SH3 domains to varying degrees, including known binding partners of HPK, such as HIP (HPK-interacting protein)-55 (Ensenat *et al*, 1999).

Other mammalian proteins possess RxxK sequences that are implicated as SH3 domain ligands, including the Gab family proteins, HPK-1, UBPY and AMSH (Liu *et al*, 2000; Lock *et al*, 2000; Ma *et al*, 2001; Berry *et al*, 2002; Lewitzky *et al*, 2004). We therefore tested whether the RxxK motifs from Gab1 and HPK1 possess a similarly high degree of specificity as the SLP-76 peptide, using the SH3 domain array. The Gab1 RxxK peptide bound at least 11 SH3 domains, in addition to those recognized by the wild-type SLP-76

peptide (Figure 4B). The murine HPK1 protein also has an RxxK motif that can bind the Gads C-SH3 domain (Liu *et al*, 2000; Ma *et al*, 2001), and is unusual in that it possesses a class II PxxP sequence (PxxPxR) embedded in the RxxK motif (GQPPLVPPRKEKMRGKG, where the PxxPxR is underlined and the RxxK is in bold) (Lewitzky *et al*, 2004; Figure 4B). This peptide combines the structural features of a polyproline type II helix and a 3_{10} helix (Lewitzky *et al*, 2004), and binds the Gads C-SH3 domain with moderate affinity ($K_D = 2.4 \mu\text{M}$) (Lewitzky *et al*, 2004). When used to probe the SH3 domain arrays, this HPK-1 RxxK peptide bound the Gads C-, STAM1 and STAM2 SH3 domains (but not the Grb2 C-SH3 domain), and an additional nine SH3 domains, suggesting that the PxxP sequence may confer expanded reactivity to this RxxK peptide. Overall, these data demonstrate that the SLP-76 RxxK peptide is unusually specific for a small subset of four

SH3 domains out of a possible 147, only one of which (Gads C-SH3) is of sufficient binding affinity and in the appropriate subcellular location to mediate a response to the TCR. The results suggest that the atypical RxxK motif may not by itself be sufficient to provide highly selective SH3 domain recognition, since physiological RxxK-based peptides other than that from SLP-76 have the potential to recognize multiple SH3 domains.

Mutational drift of the SLP-76 peptide results in crossreaction towards other SH3 domains

The preceding data suggest that not only is the Gads C-SH3 domain specifically geared towards the recognition of the SLP-76 RxxK motif (as compared with the Grb2 C-SH3 domain which has a mixed specificity for recognition of RxxK and PxxP ligands, for example, see Supplementary

Figure 5 Engineering of crossreactive SLP-76 RxxK peptide ligands by single amino-acid alterations. (A) Venn diagram representing the high specificity of the SLP-76 RxxK ligand. To explore the effect that crossreactivity of an SH3 domain ligand might have on TCR signaling efficiency, SLP-76 RxxK peptide variants were made in an effort to generate crossreactive SLP-76 ligands whose binding profile included additional SH3 domains. (B) SLP-76 peptides were generated with single amino-acid alterations that correspond to amino-acid residues that are accessible by single nucleotide alterations (S234P, S238R and T239P) and were assessed for their ability to bind the Gads C-SH3 domain. (C) The SLP-76 peptide variants were incubated with the SH3 domain arrays and assessed for their ability to interact with other SH3 domains.

Figure S3), but also that the SLP-76 RxxK motif is highly restricted in its ability to bind SH3 domains. We therefore tested whether there are strict constraints on the sequence of this motif, which are important for its limited access to SH3 domains. We rationalized that if the motif had been selected for a highly specific SH3 domain recognition profile, drift away from the optimal sequence would result in increased crossreactivity (Zarrinpar *et al*, 2003b; Figure 5A). To test this, we generated a number of SLP-76 peptide variants with single amino-acid alterations that are accessible with a single base change in the nucleotide sequence of human SLP-76 (Figure 5B), and used these peptides to probe SH3 domain arrays (Figure 5C). We avoided SLP-76 residues that are key determinants for binding the Gads C-SH3 domain. Two of these variants (S234P and S238R) had affinities towards the Gads C-SH3 domain that were similar to the wild-type SLP-76 peptide (Figure 5B). The T239P variant had a lower affinity towards the Gads C-SH3 domain likely due to a disruption of the 3_{10} -helix formed within the SLP-76 RxxK motif.

Each of the S234P*, S238R* and T239P* peptides cross-reacted with several novel SH3 domains on the arrays, indicating a decrease in specificity (Figure 5C). For instance, the SLP-76 S234P* peptide variant bound with similar affinity to the Gads C-SH3 domain as the wild-type peptide, as determined by SPR analysis (Figure 5B), but also bound at least 54 SH3 domains on the array in addition to the Gads C-, Grb2 C-, STAM1 and STAM2 SH3 domains (Figure 5C, left panel). The crossreactivity of this peptide may result from the creation of a suboptimal PxxP motif within the binding

epitope of the SLP-76 peptide (KxPxxP or PxxPxxR). However, other peptide variants that generated no obvious PxxP motif, such as SLP-76 S238R*, also resulted in cross-reactivity, although less extensive than S234P* (Figure 5C, middle panel). Of interest, the T239P* mutation abrogated binding to Gads, Grb2, STAM1 and STAM2, as assessed by the SH3 domain array, but resulted in ectopic binding to a number of other SH3 domains. This reduced binding to Gads was consistent with the 100-fold lower K_D of 1.2 μ M of the Gads C-SH3 domain for this ligand as determined by SPR (Figure 5B).

These results suggest that the sequence of the SLP-76 RxxK motif is tightly constrained for selective binding to the SLP-76 RxxK sequence, and that single amino-acid substitutions can lead to a much broader range of SH3 domain recognition. The observation that some SLP-76 substitutions can retain high-affinity binding for the Gads C-SH3 domain yet still crossreact with other SH3 domains indicates that affinity alone also does not necessarily confer very high specificity. Rather, these results, together with the binding results from other RxxK motifs, suggest that while the interaction of SLP-76 with the Gads C-SH3 domain has been selected for high affinity (Figures 2 and 3), the SLP-76 ligand has also been selected to occupy a sequence space that also provides high specificity.

Gads C-SH3 domain crossreactivity is tolerated in TCR signaling, if coupled with high-affinity binding to SLP-76

The mutant SLP-76 peptides with expanded SH3-binding properties allowed us to investigate whether optimal TCR

Figure 6 A crossreactive SLP-76 mutant exhibits wild-type levels of downstream TCR activation. (A) NFAT luciferase assays of anti-CD3 stimulated Jurkat J14-v29 cells transiently expressing crossreactive SLP-76 mutants (S234P and T239P) compared to wild-type SLP-76 and vector alone. (B) Calcium flux assays of anti-CD3 stimulated Jurkat J14-v29 cells stably expressing crossreactive SLP-76 mutants (S234P and T239P) compared to wild-type SLP-76 and vector alone. (C) Anti-M2 FLAG immunoprecipitates immunoblotted for SLP-76, Gads or Lck following SDS-PAGE and transfer to nitrocellulose. Whole cell lysates are shown as loading controls. (D) Model of how multiprotein complexes can contribute to signaling specificity by insulating pathways from cross-talk (left panel) and how crossreactive components might be selected against if leakiness to other pathways subtly corrupts signaling capacity of other pathways resulting in a loss of organism fitness (right panel).

activation requires that the Gads/SLP-76 interaction be highly specific, as well as of high affinity. To investigate whether crossreactivity might interfere with the Gads/SLP-76 interaction through competitive effects within the cell, we expressed FLAG-tagged full-length SLP-76 T239P and S234P variants in SLP-76-deficient J14-v29 Jurkat T cells. Consistent with the importance of a high-affinity interaction between Gads and SLP-76, Jurkat cells expressing the SLP-76 T239P crossreactive variant gave a diminished level of NFAT activation and calcium flux in response to anti-CD3 crosslinking, as compared to cells expressing wild type SLP-76 (Figure 6A and B). This correlates with the observation that an SLP-76 RxxK peptide motif with the T239P substitution has an affinity constant of $1.2\ \mu\text{M}$ for the Gads C-SH3 domain (Figure 5B); this markedly weakened affinity is similar to the measured threshold affinity of around $3\ \mu\text{M}$ that is required before any Gads/SLP-76-mediated enhancement of NFAT activation even begins (Figure 3B). The potential of the T239P SLP-76 variant to crossreact with ectopic SH3 domains is not sufficient to rescue the defect in TCR signaling associated with the decreased affinity for Gads.

In contrast, Jurkat cells expressing the SLP-76 S234P variant, which retains a high-affinity interaction with Gads, activated NFAT in response to CD3 crosslinking to similar levels as Jurkat cells expressing wild-type SLP-76 (Figure 6A). Similarly, calcium fluxing in response to anti-CD3 stimulation also showed wild-type activation levels in the SLP-76 S234P cells (Figure 6B). Thus, despite the potential for the SLP-76 S234P mutant to interact with other SH3 domain-containing proteins, signaling through the TCR-NFAT pathway remains intact. Consistent with this finding, the SLP-76 S234P variant expressed in Jurkat cells associated with Gads to levels comparable to wild-type SLP-76 (Figure 6C), suggesting that even if there were binding of SLP-76 S234P to other SH3 domain-containing proteins, they have no competitive influence on the Gads/SLP-76 interaction. A caveat to this observation is that we do observe an approximate 10–15-fold higher level of ectopically expressed SLP-76 compared to endogenous levels, which could mask a defect in the SLP-76 S234P mutant's ability to activate NFAT. Regardless, we would still expect to observe crossreactive binding of SLP-76 S234P in cells. However, Lck, which interacted with the S234P* peptide on the SH3 domain arrays, failed to coprecipitate with the SLP-76 S234P mutant, suggesting that either SLP-76 S234P and Lck are mutually inaccessible, or that the affinity of the SLP-76 S234P variant for the Lck SH3 domain is much lower than for Gads C-SH3 (Figure 6C). Of note, while Lck has been shown to interact with SLP-76 (Sanzenbacher *et al*, 1999), in our hands, we were unable to observe a stable interaction for either wild-type or the S234P variant. Together, these data demonstrate that the TCR-NFAT signaling pathway remains intact in the presence of an SLP-76 protein containing a cross-reactive RxxK motif provided that the tight interaction between Gads and SLP-76 is not compromised. These results emphasize that a high affinity interaction between Gads and SLP-76 is essential to ensure the efficient activation of pathways downstream of the TCR leading to NFAT activation.

Discussion

To quantitatively test the relationship between binding affinity, specificity and signaling activity of a modular adaptor protein, we examined a biologically important interaction

between the Gads C-SH3 domain and the SLP-76 scaffold in T cells. We find that the Gads C-SH3 domain binds an extended RxxK peptide motif on SLP-76 with a relatively high affinity ($K_D = 8\text{--}20\ \text{nM}$), compared with the majority of SH3 domain-mediated interactions, owing to very fast association rates and relatively slow dissociation rates. Thermodynamic data indicate that the interaction is enthalpically driven, with a modest opposing entropy. Together, these parameters suggest that the high affinity of the interaction results from a number of productive molecular interactions, in support of the exquisite complementarity of the SH3/ligand interface indicated by structural analysis.

By measuring the affinities of a series of variant SLP-76 RxxK peptides for the Gads C-SH3 domains, and then determining the ability of full-length SLP-76 carrying such substitutions to support TCR signaling in Jurkat T cells, we established that NFAT activation levels diminished concomitantly with the decreasing affinity between Gads and SLP-76, and identified a threshold K_D of approximately $3\ \mu\text{M}$ beyond which there was no further decrease in NFAT activation. This lower affinity limit likely represents the threshold below which the binding between Gads and SLP-76 is no longer physiologically relevant to TCR-mediated enhancement of NFAT activation. These data establish a direct correlation between the affinity of a mammalian SH3-ligand interaction and the extent to which a biologically significant signaling pathway is activated. They also show that an ~ 400 -fold decrease in SLP-76 affinity for Gads C-SH3 is sufficient to inactivate Gads-mediated signaling, despite the presence of closely related adaptors such as Grb2 in T cells. The biological output of TCR signaling, as measured by NFAT activation, therefore depends on the strength of the Gads/SLP-76 SH3-mediated interaction.

These data indicate that the affinity between an SH3 domain and its ligand can be an important parameter in determining the strength of downstream signaling in a complex mammalian system, and support previous reports showing quantitative relationships between the strength of an interaction between two signaling proteins and downstream responses (Block *et al*, 1996; Marles *et al*, 2004). For instance, Ras/Raf binding affinity *in vitro* correlates with Ras/Raf regulated gene expression in cells (Block *et al*, 1996). Similarly, in the yeast osmosensing pathway, there is a linear correlation between the affinity of the Sho1p SH3 domain for a PxxP motif in the MAP kinase kinase Pbs2p and several cellular outputs from the HOG high-osmolarity response pathway (Marles *et al*, 2004). Our data indicate that the quantitative impact of SH3 domain affinity on signaling is not confined to the simpler yeast system, nor to proline-rich ligands.

The failure of Grb2 to compensate for loss of Gads C-SH3 function may reflect the functional affinity threshold of $3\ \mu\text{M}$ for the Gads/SLP-76 interaction, which is approximately equivalent to the affinity with which the Grb2 N- and C-SH3 domains interact with Sos PXXP motifs, or the Gab1 and SLP-76 RxxK motifs, respectively (Berry *et al*, 2002; Supplementary Figure S3). This supports the argument that a micromolar SH3 domain affinity for SLP-76, typical for many SH3-ligand interactions, is insufficient for this step of the TCR pathway. Consistent with this view, we and others have noted that Grb2 fails to interact with SLP-76, even in the absence of Gads (Cheng *et al*, 1998; Law *et al*, 1999; Berry

et al, 2002; Liu *et al*, 2003), despite the 8 μ M affinity constant between the Grb2 C-SH3 domain and SLP-76 peptide (Supplementary Figure S3). These data argue that the specificity of the interaction between Gads and SLP-76 in T cell derives, in part, from its high affinity, which discriminates against related SH3-containing adaptors. They also indicate that Gads has evolved to fulfill a specialized role within hematopoietic cells, compared to Grb2, which functions in many cells and in response to multiple different signals. Parenthetically, the lower affinity of the Grb2 SH3 domains for individual peptide ligands, as compared with the Gads C-SH3/SLP-76 RXXK peptide, does not mean that full-length Grb2 cannot bind strongly to physiological SH3 ligands, such as Sos. Indeed, in the case of Grb2, this may require multiple molecular interactions between the two proteins, or cooperativity as result of residing in a multiprotein complex localized to the membrane (Sastry *et al*, 1995, 1997; Houtman *et al*, 2004).

The Gads/SLP-76 interaction is not only of high affinity, but is also highly selective. Peptide array analysis indicated that individual residues within the SLP-76 RxxK motif, such as P233*, D236*, R237*, K240*, P241* and I235* are largely optimized for recognition by the Gads C-SH3 domain. The physicochemical properties of these SLP-76 residues are therefore important for their selective recognition by the Gads C-SH3 domain. In a complementary screen, we found that the SLP-76 RxxK peptide possesses high specificity for the Gads C-SH3 domain among a set of 147 SH3 domains. In contrast, a conventional SH3-binding PXXP peptide, other physiological RxxK-containing peptides, and variant SLP-76 RxxK peptides with single amino-acid substitutions displayed varying degrees of crossreactivity towards other SH3 domains. Together, these observations suggest that the SLP-76 peptide, while configured for high-affinity binding to Gads, has also been selected during evolution for high specificity towards the Gads C-SH3 domain.

The properties of the crossreactive RxxK SLP-76 variants suggest an additional layer of specificity, beyond the intrinsic selectivity of the Gads C-SH3 domain for SLP-76. The crossreactive T239P SLP-76 variant failed to signal as well as wild-type likely due to the reduced affinity for Gads that accompanies its expanded recognition of SH3 domains. The SLP-76 S234P variant, in contrast, retained high-affinity binding to Gads, even though the corresponding mutant RxxK peptide was crossreactive with other SH3 domains *in vitro*, and showed similar levels of NFAT activation and calcium fluxing in response to anti-CD3 crosslinking as wild-type SLP-76 (Figure 5A and B). Thus, the exceptional specificity of the wild-type SLP-76 ligand for Gads C-SH3 is not critical for efficient TCR signaling to downstream effectors such as the NFAT transcription factor and calcium flux, as measured in Jurkat cells, provided that the high affinity is maintained.

The crossreactivity of the SLP-76 S234P variant may fail to diminish downstream pathway activation because of the sheer strength of its interaction with the Gads C-SH3 domain, which could out-compete other SH3 domains that might otherwise recognize this mutant SLP-76. In addition, SLP-76 resides within a multiprotein complex, which could provide an insulating effect through cooperative binding and thereby diminish pathway corruption (Houtman *et al*, 2004). The combined effect of multiple protein-protein interactions may contribute to the robustness of this pathway, despite

the presence of potentially crossreactive components (Figure 6D).

An intriguing question then is why the SLP-76 RxxK motif has evolved such a high selectivity for the Gads C-SH3 domain, when TCR signaling as measured in Jurkat cells can tolerate a degree of crossreactivity in the context of a high-affinity interaction. Such crossreactivity may lead to spurious interactions that have more subtle effects on TCR-regulated pathways, which become important in the context of T-cell responses to antigens of widely divergent affinities. Alternatively, increased crossreactivity might perturb SH3-controlled events in other hematopoietic cell types. To address the selective pressures that have yielded the highly selective Gads/SLP-76 interaction, it will be important to investigate the effect of SLP-76 crossreactivity on the behavior of hematopoietic cells in the intact organism.

In summary, we present a system to test the importance of SH3 domain affinity and selectivity in a complex mammalian signaling pathway. We find a striking correlation between the affinity with which the Gads C-SH3 domain binds the SLP-76 RxxK motif, and the efficiency of signaling downstream from the TCR. The Gads C-SH3 domain and the SLP-76 RxxK motif have evolved an exceptional mutual selectivity. We also find that this pathway is tolerant to increased SLP-76 crossreactivity, in the context of high-affinity binding to Gads C-SH3, indicating that the cell has developed mechanisms to buffer such crossreactivity, as an additional potential means of maintaining signaling specificity.

Materials and methods

Cell lines, transfections, and cell culture

Details are provided as Supplementary data.

Protein expression and purification

The Gads C-SH3 domain (residues 257–322) and Grb2 C-SH3 domain (residues 152–218) were expressed as 6xHis-tagged proteins in *Escherichia coli* BL21 cells and purified by FPLC using nickel chelating column followed by size exclusion chromatography. Purity was assessed by Coomassie stained SDS-PAGE gels. Details are provided as Supplementary data.

Peptide synthesis

Peptides were synthesized on an Applied Biosystems 431A peptide synthesizer using standard 9-fluorenyl methoxycarbonyl (Fmoc) solid phase chemistry as previously described (van der Geer *et al*, 1996). Additional details are provided as Supplementary data.

Kinetic binding analysis using surface plasmon resonance (SPR)

Biosensor experiments were performed at 25°C on a BIACORE 3000 (Biacore AB, Uppsala). The Gads C-SH3 domain or Grb2 C-SH3 domain was immobilized to a level of 250 response units (RU; 250 pg/mm²) using amine-coupling chemistry onto a CM4 chip. Binding RUs were recorded and globally fitted to a 1:1 mass transport binding model using BIAEVALUATION 4.0.1 (Biacore AB). Additional details are provided as supplementary data.

Isothermal titration calorimetry (ITC)

Calorimetry experiments were performed using a Microcal VP-ITC instrument (Microcal, Northampton). Stock solutions of proteins and peptides were diluted into filtered and degassed 10 mM NaP, pH 7.5 150 mM NaCl. The domain (5 μ M) was loaded into the 1.3 ml sample cell and the peptide (100 μ M) into the injector. There was little or no detectable heat of dilution as determined by the absence of additional heat after binding saturation as well as from control experiment in which peptide was titrated into buffer alone. Data were fitted using the software Origin 5.0 (Microcal) for stoichiometry (*N*), binding constant (*K_A*), and binding enthalpy (ΔH). The

K_A determined from the best-fit curve was used to determine the free energy (ΔG) and the entropy (ΔS) was determined by using the following thermodynamic relation: $\Delta G = \Delta H - T\Delta S = -RT \ln K_A$ where R is the gas constant and T is the absolute temperature.

Peptide and SH3 domain protein arrays

Peptide arrays were generated using an Intavis Multiprep instrument (Intavis, Germany) using Fmoc-based chemistry according to the manufacturer's instructions. Peptide array membranes were probed with purified 1 μ M his-tagged Gads C-SH3 domain and detected by blotting with an antipeptide His primary antibody (Qiagen) and anti-mouse HRP secondary antibody followed by chemiluminescent detection. The SH3 domain arrays were purchased from Panomics Inc. (Fremont, CA), blocked using the Panomics blocking mix and probed using 10 μ M biotinylated peptide, which was premixed with streptavidin-HRP and detected according to the manufacturer's instructions.

Calcium flux

Intracellular free calcium measurement was performed by loading 10^7 cells with 2 μ g/ml of Indo-1 and 4 mM probenidol at 37°C for 45 min protected from light. Baseline cellular calcium levels were measured for 30 s and then cells were stimulated with the C305 antibody (anticlonotypic TCR on Jurkat cells). Ionomycin was added to the cells at the 6 min mark as a positive control. Calcium mobilization was detected by flow cytometry using an LSR Flow Cytometer (BD Biosciences). Data were then analyzed using Flow Jo software (Tree Star Inc), and histograms represent the mean ratio of FL5/FL4 fluorescence for over time.

Transient transfection and NFAT luciferase assays

For transient transfections 2×10^7 Jurkat cells were electroporated with 40 μ g of the indicated plasmids in electroporation buffer (120 mM KCl, 0.5 mM CaCl₂, 10 mM K₂HPO₄ (pH 7.4), 25 mM HEPES (pH 7.5), 2 mM EGTA (pH 7.5), 5 mM MgCl₂) with a Gene Pulser (BioRad) set at 250 V and 960 μ F. Cells were cultured for 24 h, harvested and processed as described below. For NFAT-luciferase reporter assays, 2×10^7 Jurkat cells were electroporated with 20 μ g

NFAT luciferase reporter construct, together with 40 μ g of empty pEF vector or 40 μ g wt-SLP-76 or other SLP-76 mutants. To control for differences in transfection efficiency, empty pEF vector was used to equalize the amount of DNA used in each transfection and equivalent expression was confirmed by Western blot analysis (data not shown). At 24 h after transfection, 5×10^5 cells were stimulated using anti-CD3 UCHT1 antibody (BD Pharmingen) for 16 h at 37°C and lysed. Luciferase activity was quantified with a luminometer as previously described (Fang and Koretzky, 1999) and expressed in arbitrary units relative to cells treated with ionomycin and PMA (maximum stimulation).

Immunoprecipitation and Immunoblotting

20×10^6 Jurkat T cells were lysed and clarified lysates were incubated for 60 min with 5 μ l of a 20% slurry of anti-M2 Flag agarose beads (Sigma). Beads were then washed and boiled in SDS-PAGE loading buffer. Eluted proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked and then incubated for 1 h in blocking buffer containing the appropriate primary antibody. Membranes were then washed with TBS-Tween and incubated with appropriate secondary antibodies conjugated to horseradish peroxidase for 1 h, then rinsed with TBS-Tween and visualized by enhanced chemiluminescence and autoradiography.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

BTS was funded by a fellowship from the Cancer Research Institute (New York, USA). Work in the authors' laboratories was funded by the Canadian Institutes for Health Research (CIHR; TP and CJM), the National Cancer Institute of Canada (TP), Genome Canada (TP), and the National Institutes of Health (GAK and JSM). TP is a distinguished investigator of the CIHR. We wish to gratefully acknowledge the technical assistance of Dr Gerald Gish.

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