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Systematic identification of regulatory proteins critical for T-cell activation

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Abstract

Background: The activation of T cells, mediated by the T-cell receptor (TCR), activates a battery of specific membrane-associated, cytosolic and nuclear proteins. Identifying the signaling proteins downstream of TCR activation will help us to understand the regulation of immune responses and will contribute to developing therapeutic agents that target immune regulation.

Results: In an effort to identify novel signaling molecules specific for T-cell activation we undertook a large-scale dominant effector genetic screen using retroviral technology. We cloned and characterized 33 distinct genes from over 2,800 clones obtained in a screen of 7×10^8 Jurkat T cells on the basis of a reduction in TCR-activation-induced CD69 expression after expressing retrovirally derived cDNA libraries. We identified known signaling molecules such as Lck, ZAP70, Syk, PLCγ1 and SHP-1 (PTP1C) as truncation mutants with dominant-negative or constitutively active functions. We also discovered molecules not previously known to have functions in this pathway, including a novel protein with a RING domain (found in a class of ubiquitin ligases; we call this protein TRAC-1), transmembrane molecules (EDG1, IL-10Rα and integrin α_2), cytoplasmic enzymes and adaptors (PAK2, A-Raf-1, TCPTP, Grb7, SH2-B and GG2-1), and cytoskeletal molecules (moesin and vimentin). Furthermore, using truncated Lck, PLCγ1, EDG1 and PAK2 mutants as examples, we showed that these dominant immune-regulatory molecules interfere with IL-2 production in human primary lymphocytes.

Conclusions: This study identified important signal regulators in T-cell activation. It also demonstrated a highly efficient strategy for discovering many components of signal transduction pathways and validating them in physiological settings.

Background

Activation of specific signaling pathways in lymphocytes determines the quality, magnitude and duration of immune responses. These pathways are also responsible for the induction, maintenance and exacerbation of physiological or pathological lymphocyte responses in transplantation, acute and chronic inflammatory diseases, and autoimmunity. The activation of T lymphocytes is triggered when the T-cell receptor (TCR) recognizes antigens presented by the major histocompatibility complex (MHC) in antigen-presenting cells [1]. Engagement of the TCR by antigen-MHC results in rearrangement of the actin cytoskeleton, induction of gene transcription, and progression into the cell cycle [2,3]. The proximal events of TCR signaling include activation of the Src-family kinases Lck and Fyn, phosphorylation of TCR components, and activation of ZAP70 and Syk tyrosine kinases, as well as recruitment of adaptor molecules (LAT and SLP-76), which in turn couple to more distal signaling pathways including Ras and PLCγ [4,5]. Using classical genetic and biochemical approaches, new components of the TCR signaling pathway are being discovered, albeit at a slow pace. Efficient identification of additional signaling molecules probably requires novel approaches.

Here, we describe our attempt to identify and validate novel signaling molecules specific for T-cell activation. We used up-regulation of the cell-surface marker CD69 in T cells to monitor TCR activation; CD69 as an activation marker has been well validated [6], more recently using T cells deficient in certain key signaling molecules such as SLP-76 and LAT [7,8]. The rationale of this 'functional genomics' screen was to identify cell clones whose CD69 upregulation was repressed following introduction of clones from a retroviral cDNA library. The library clones conferring such repression would then represent immune modulators that function to block TCR signal transduction.

Results

Experimental design

Jurkat Clone 4D9 was selected for low basal levels of CD69 expression and strong induction following TCR stimulation (see Additional data file 1 with the online version of this article for details of the selection and infection procedures). The 'Tet-off' system was adapted for regulated expression of the retroviral cDNA library: cDNA inserts in the retroviral library were cloned behind the tetracycline (Tet) regulatory element (TRE) and the cytomegalovirus (CMV) minimal promoter. Transcription of the cDNA inserts was then dependent on the presence of tetracycline-controlled transactivator (tTA) [9], a fusion of Tet repression protein and the VP16 activation domain, and the absence of tetracycline or its derivatives such as doxycycline (Dox). A derivative of Jurkat clone 4D9 stably expressing tTA, called 4D9#32, was engineered and selected (see Additional data file 1).

As a positive control for this functional genetic screen, we tested dominant-negative forms of ZAP70, which are known to inhibit TCR signaling [10]. We subcloned a kinase-inactive ZAP70 (ZAP70 KI) and a truncated ZAP70, comprising only the two Src homology 2 (SH2) domains and referred to here as ZAP70 SH2 (N+C), into the bicistronic retroviral vector under TRE control followed by the internal ribosome entry site (IRES) coupled to green fluorescent protein (GFP; see Figure 1a). Both ZAP70 SH2 (N+C) and ZAP70 KI inhibited TCR-induced CD69 expression (Figure 1b). Consistent with previous reports using transiently overexpressed ZAP70 constructs [10], the truncated ZAP70 protein inhibited anti-TCR-induced CD69 expression more strongly than the ZAP70 KI protein did (Figure 1b). The CD69-inhibitory phenotype was dependent on expression of dominant-negative forms of ZAP70. When Dox was added before TCR stimulation, there was no inhibition of CD69 expression (Figure 1c, right panels). Fluorescence-activated cell sorting (FACS) analysis of cellular expression of GFP revealed a lack of GFP-positive cells (Figure 1c, left panels), suggesting that the bi-cistronic ZAP70 SH2 (N+C)-IRES-GFP mRNA was not transcribed. A lack of expression of the ZAP70 SH2 (N+C) protein in the presence of Dox was confirmed by western blotting (Figure 1d). Collectively, these results indicated that Jurkat clone 4D9#32 was suitable for screening for inhibitors of anti-TCR-induced CD69 expression.

Screening for cells lacking CD69 upregulation

The scheme to obtain cell clones with a CD69-inhibitory phenotype is shown in Figure 2a. Jurkat 4D9#32 cells were infected with the pTRA-cDNA libraries made from human lymphoid organs such as thymus, spleen, lymph node and bone marrow (see Additional data file 2 with the online version of this article for details of construction and assessment of the pTRA-cDNA libraries). After library infection, cells were stimulated with the anti-TCR antibody C305 overnight. A total of 7.1×10^8 cells were stained with anti-CD69 antibody conjugated to allophycocyanin (APC) and anti-CD3 antibody conjugated to phycoerythrin (PE), and then screened using flow cytometry. There was a significant reduction of the CD3-TCR complex on the cell surface as compared to unstimulated cells, as a result of receptor-mediated internalization, but we were nevertheless able to distinguish the $CD3^-$ population from the $CD3^+$ ($CD3^{low}$ and $CD3^{high}$) populations (see Additional data file 3 with the online version of this article for the distinction between $CD3^-$, $CD3^{low}$ and $CD3^{high}$ cell populations). We consistently observed that more than 2% of the cells had lost TCR-CD3 complex on the surface, causing them to be unresponsive to stimulation and, consequently,

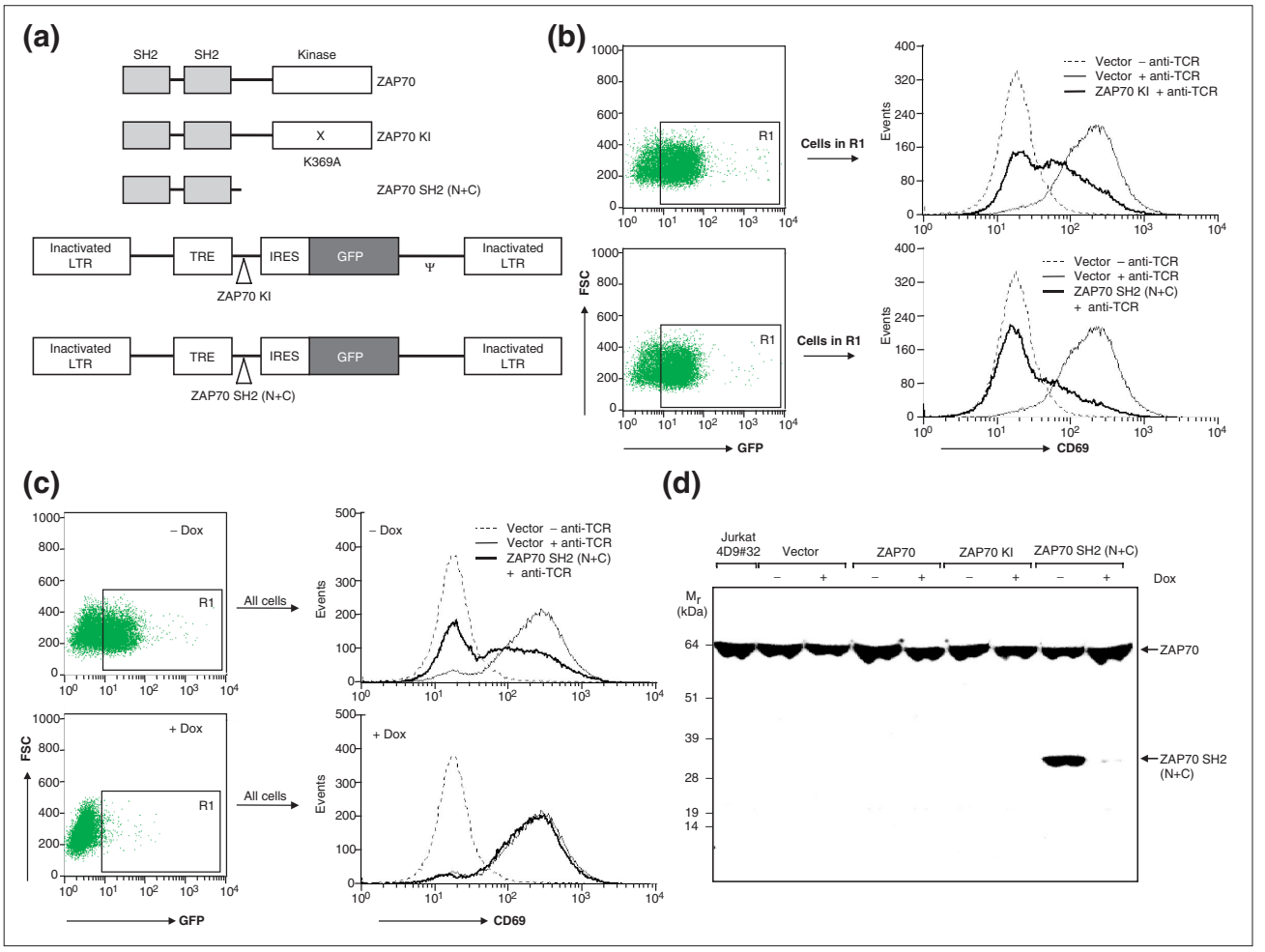
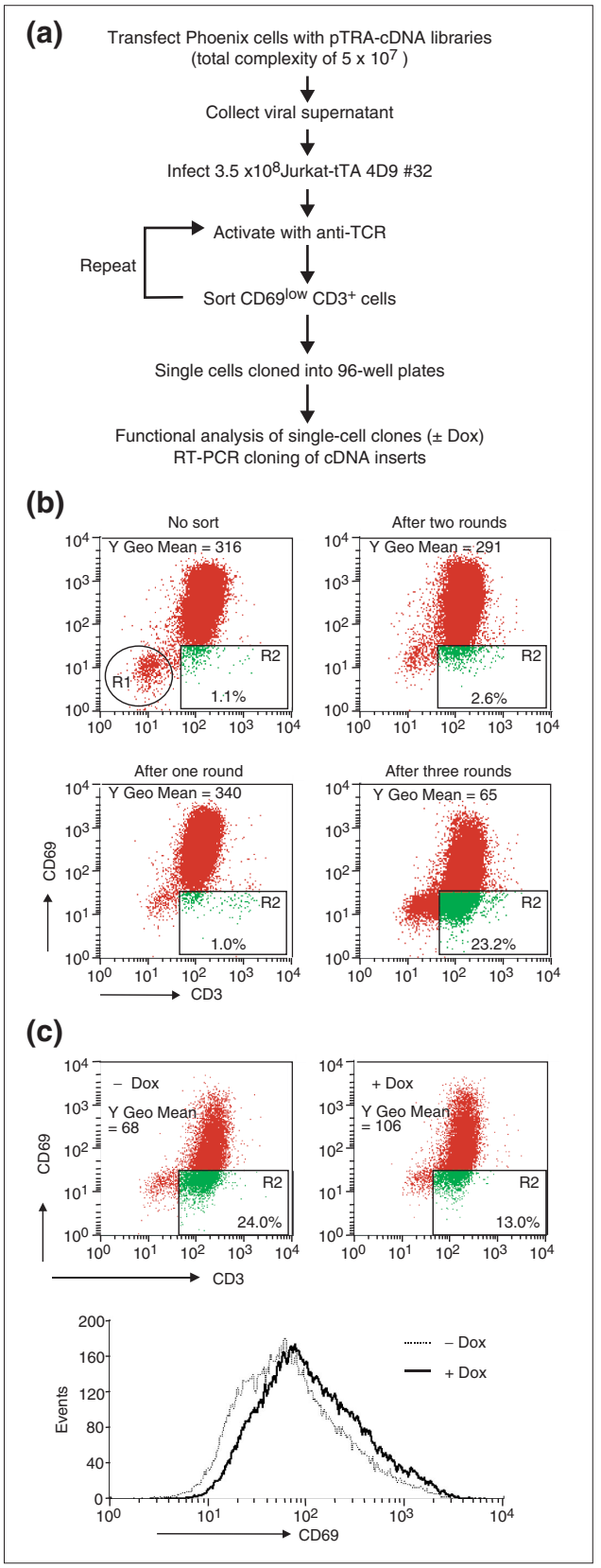


Figure 1

Cell-line and assay development. **(a)** ZAP70 KI and ZAP70 SH2 (N+C) were subcloned in front of the internal ribosome entry site (IRES), followed by GFP, in the Tet-regulated retroviral vector (pTRA-IRES-GFP). **(b)** After infecting tTA-expressing Jurkat (4D9#32) cells with retroviral constructs containing IRES-GFP, ZAP70 KI-IRES-GFP, or ZAP70 SH2 (N+C)-IRES-GFP, cells were left unstimulated or stimulated with anti-TCR antibody for 24 h. CD69 expression was analyzed after gating on the GFP-positive population (infected population, boxed in R1). The dashed line and the thin line on the graphs indicate cells infected with IRES-GFP (vector) before and after TCR stimulation, respectively, and the thick line indicates cells infected with ZAP70 KI-IRES-GFP (top panel) or ZAP70 SH2 (N+C)-IRES-GFP (bottom panel), both after TCR stimulation. **(c)** After infecting Jurkat-tTA (4D9#32) cells with retroviral vector alone or vector containing ZAP70 SH2 (N+C)-IRES-GFP, cells were cultured without (top panels) or with (bottom panels) Dox for 6 days, and then left unstimulated or stimulated with anti-TCR antibody for 24 h. The box R1 indicates GFP-positive cells. CD69 expression was analyzed for the entire cell population. The dashed line and the thin line indicate cells infected with vector before and after TCR stimulation, respectively, and the thick line indicates cells infected with vector containing ZAP70 SH2 (N+C)-IRES-GFP after TCR stimulation. **(d)** The Jurkat-tTA (4D9#32) cells containing different retroviral constructs (shown above the lanes) were cultured in the absence (-) or presence (+) of Dox, and whole-cell lysates were prepared. Lysates were loaded (100 μg per lane) and analyzed by western blotting using anti-ZAP70 antibody (Upstate Biotechnology, Waltham, USA). The top ZAP70 band included endogenous (- and + Dox) as well as retrovirally expressed ZAP70 (-Dox only), whereas the bottom ZAP70 band contained only retrovirally expressed truncated ZAP70 SH2 (N+C).

to have low CD69 expression (circled region R1 in Figure 2b). We therefore collected by high-speed flow sorter only cells with the lowest CD69 expression that still retained CD3 expression. We termed the desired phenotype $CD69^{low}CD3^{+}$ (Figure 2a), and it represented 1% of the total stained cells (boxed region R2 in Figure 2b). The 1% sorting gate also translated as 100-fold enrichment in the first round of sorting. In subsequent rounds of sorting, the sorting gate R2 was always maintained to capture the equivalent of 1% of the control cells that were stimulated but were never flow-sorted. As shown in Figure 2b, we achieved significant enrichment after three rounds of reiterative



sorting; cells with the desired CD69^{low}CD3^{+} phenotype increased from 1% to 23.2% of the population. In addition, the overall population's geometric mean for the CD69 fluorescent intensity was also reduced (from > 300 to 65).

Given our experimental design, we expected the expression of retroviral cDNAs and their putative inhibitory effect to be turned off with the addition of Dox. This feature helped us to ascertain that the phenotype was due to expression of the cDNA library rather than to epigenetic changes or spontaneous or retroviral-insertion-mediated somatic mutation(s). To confirm this, we compared anti-TCR-induced CD69 expression in the presence and absence of Dox. As shown in Figure 2c, cells with the CD69^{low}CD3^{+} phenotype decreased from 24.0% to 13.0% with the addition of Dox, demonstrating that a significant number of cells (11%) had lost the CD69^{low}CD3^{+} phenotype when library-cDNA expression was turned off. These data suggested that the CD69^{low}CD3^{+} phenotype in a significant proportion (at least 11% out of 24%, or 45.8%) of cells in this population was indeed caused by expression of the cDNA-library clones.

Functional analysis of single-cell clones

Next, we deposited single cells into 96-well plates in conjunction with the fourth and subsequent rounds of sorting for the CD69^{low}CD3^{+} phenotype. The phenotype of each single-cell clone was characterized by growing the cells in the absence and presence of Dox. A few examples of the Dox-regulatable phenotypes for individual clones are shown in Figure 3a. Dox regulation of CD69 expression was expressed as the ratio of CD69 geometric mean fluorescent intensity in the presence of Dox divided by the CD69 geometric mean fluorescent intensity in the absence of Dox after TCR stimulation; we termed this ratio the 'Dox ratio'. In uninfected or mock-infected cells, Dox had little or no effect on the induction of CD69 expression, with mean Dox

Figure 2
Screen for inhibitors of TCR-activation-induced CD69 expression. **(a)** Cells (3.5 × 10^{8}) were infected with pTRA-cDNA libraries. Single-cells were cloned after at least four consecutive sortings of the CD69^{low}CD3^{+} phenotype. **(b)** Cells (7.1 × 10^{8}) were sorted with high-speed flow sorters (MoFlo) after stimulation and staining with anti-CD69-APC and anti-CD3-PE. The sort gate was set at the equivalent of 1% of satellite control cells that were stimulated but never flow-sorted (shown as R2) to enrich for the CD69^{low}CD3^{+} phenotype. After sorting, the desired cells were allowed to rest for 6 days before another round of stimulation and sorting. **(c)** Cells were split into two populations after the third round of sorting. One half of the cells were grown in the absence of Dox (top left dot-plot) and the other half in the presence of Dox (top right dot-plot). Six days later, CD69 expression was compared following anti-TCR stimulation. The dashed line indicates CD69 level without Dox and the solid line with Dox (bottom graph).

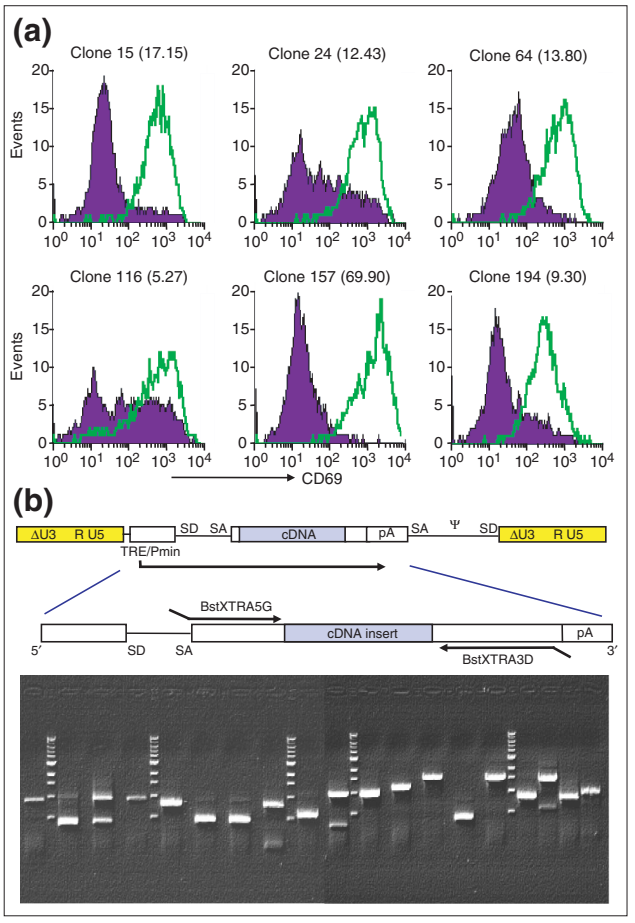


Figure 3
Identification of clones with desired phenotype. **(a)** Individual clones were grown in the presence (open peaks) or absence (filled peaks) of Dox for 6 days and then stimulated to examine CD69 expression by FACS. The 'Dox ratio' was defined as the ratio of CD69 geometric mean fluorescent intensity in the presence of Dox divided by CD69 geometric mean fluorescent intensity in the absence of Dox and is indicated in parentheses following the clone number. **(b)** DNA oligonucleotide primers specific to the library vector (BstXTRA5G and BstXTRA3D, not to scale) were used in RT-PCRs to recover the cDNA inserts from cell clones. The RT-PCR products were analyzed in agarose gel followed by ethidium blue staining. Data from representative clones are shown alongside the 1kb DNA molecular weight ladder (M_r) from New England BioLabs (Beverly, USA).

ratios for individual clones of 1.00 ± 0.25 (standard deviation). We used twice the standard deviation above the mean as a cut-off criterion and regarded clones with a ratio above 1.5 as Dox-regulated clones. Out of 2,828 clones analyzed, 1,323 had a Dox-regulatable phenotype, representing 46.8% of analyzed clones. This percentage was comparable to the percentage based on the overall population (46.8% compared to 45.8%), suggesting that the single-cell clones constituted a fair representation of the entire population. The distribution of Dox ratios among all 2,828 clones is shown in Additional data file 4, with the online version of this article.

The cDNA inserts of selected clones with a Dox-regulatable phenotype were recovered by RT-PCR using primers specific for the vector sequence flanking the cDNA library insert (Figure 3b). Most clones generated only one RT-PCR product, but a few clones generated two or more products. Sequencing analysis revealed that the additional RT-PCR products were usually caused by double or multiple insertions of retroviruses. The results of the cDNA analysis are summarized in Table 1.

Characterization of proteins critical for T-cell activation

As shown in Table 1, we obtained known TCR regulators such as Lck, ZAP70, Syk, PLCγ1, PAG, SHP-1/PTP1C, Csk and nucleolin (reviewed in [11]). The hits with the highest frequency, however, were those encoding the TCR β subunit. This new β chain leads to the assembly of a new TCR complex no longer recognizable by the stimulating antibody C305, because C305 only recognizes the original endogenous Jurkat clonotypic TCR complex [2] (see also Additional data file 5, with the online version of this article).

Among the known T-cell activation regulators, we obtained two ZAP70 hits containing the endogenous ATG initiation codon, missing the catalytic domain and ending at amino acids 262 and 269, respectively (Figure 4a). The deletions closely mirror the positive control for the screen, ZAP70 SH2 (N+C), which ended at amino acid 276 and has been shown to be a dominant-negative protein [10]. Similarly, we obtained a kinase-truncated form of Lck (Figure 4b) that caused inhibition of CD69, mimicking the phenotype of a Jurkat somatic mutant lacking Lck [12]. These clones represent dominant-negative forms of kinases required for T-cell activation. The inhibitory effects of these and other clones were confirmed by subcloning them into the pTRA-IRES-GFP vector, reintroducing into the naïve Jurkat-tTA cells, and comparing the CD69 expression in GFP-positive and GFP-negative cells upon TCR stimulation (Figure 4).

TCR engagement leads to rapid tyrosine phosphorylation and activation of PLCγ1 [13]. One of our hits contained the pleckstrin homology (PH) domain and the amino- and carboxy-terminal SH2 domains of PLCγ1 (Figure 4c). Significantly, this hit also lacked the crucial tyrosine Y783, which is essential for coupling of TCR stimulation to IL-2 promoter activation. The Y783F mutant is a very potent dominant-negative form of PLCγ1 [14]. Indeed, the original clone encoding the PLCγ1 hit had the highest Dox ratio for CD69 expression among all clones analyzed.

Table 1

Overview of identified molecular targets

Gene	Domain homology	Direction	Accession number*	Relative to ORF*	Frequency*	Phenotype transfer
Known to function in TCR pathway						
TCRβ	Receptor	Sense	Numerous	Partial	46	On hold
ZAP70	Tyrosine kinase	Sense	L05148.1	-147, +787 nt	12	Yes
ZAP70 (long)	Tyrosine kinase	Sense	L05148.1	-21, +809 nt	17	TBD
Syk	Tyrosine kinase	Sense	L28824.1	-27, +1012 nt	2	Yes
Lck	Tyrosine kinase	Sense	U23852.1	-59, +799 nt	4	Yes
PLCγ1	Tyrosine kinase	Sense	NM_002660.1	+1409, +2282 nt	3	Yes
SHP-1/PTP1C	Protein-tyrosine phosphatase	Sense	X62055.1	+472, >+2021 nt	1	Yes
Csk	Tyrosine kinase	Sense	NM_004383.1	-55, +1285 nt	1	TBD
PAG	Transmembrane adaptor	Sense	NM_018440.2	-237, +644 nt	1	Yes
Nucleolin	RNA-binding	Sense	NM_005381.1	-136, +479 nt	1	No
Enzymes and receptors						
TCPTP/PTPN2	Protein-tyrosine phosphatase	Sense	NM_002828.1	-58, +1108 nt	20	Yes
PAK2	p21-activated kinase 2	Sense	NM_002577.1	-50, +339 nt	18	Yes
PAK2 (long)	p21-activated kinase 2	Sense	NM_002577.1	-42, +670 nt	1	Yes
A-Raf-1	Serine/threonine kinase	Sense	X04790.1	-4, +456 nt	5	Yes
EDG1	G-protein-coupled receptor	Sense	NM_001400.2	<-244, +942 nt	4	Yes
EDG1 (long)	G-protein-coupled receptor	Sense	NM_001400.2	<-244, +1037 nt	1	TBD
TRAC-1	RING finger ubiquitin ligase	Sense	NM_017831.1	-254, +510 nt	1	Yes
IL-10Rα	Receptor	Sense	NM_001558.1	+689, +1350 nt	1	Yes
Integrin α_2	Receptor	Sense	NM_002203.2	+3348, +3914 nt	1	Yes
Enolase 1α	Phosphopyruvate hydratase	Sense	NM_001428.1	+703, +1374 nt	2	No
DUSP1	Dual-specificity phosphatase	Sense	NM_004417.2	+817, +1112 nt	1	No
KIAA0251	Pyridoxal-dependent decarboxylase	Sense	D87438.1	nt 2098-2370†	1	No
Adaptors and transcription factors						
Grb7	Adaptor	Sense	NM_005310.1	+1268, +1912 nt	3	Yes
GG2-1	TNF-induced protein	Sense	AF070671.1	-97, +1795 nt	2	Yes
SH2-B	Adaptor	Sense	AF227968.1	+1352, +1960 nt	1	Yes
RERE	Transcriptional factor	Sense	AB036737.1	+914, +1202 nt	3	No
SudD	Serine/threonine rich	Sense	NM_003831.1	-93, +413 nt	1	No
Ku 70	DNA-PKc subunit	Sense	S38729.1	+1026, +2069 nt	1	No
Novel (130 amino acids; no homology)	Unknown	Sense	AC005321.1	nt 33543-33938‡	1	No
Novel signaling molecule	GYF domain	Sense	NM_022574.1	+1, +121 nt	1	No
SCAMP2	Secretory carrier membrane protein	Sense	AF005038.2	-5, +833 nt	1	No
KIAA1228	C2 domain (Ca^{2+}- or IP-binding)	Sense	AB033054.2	nt 1439-2163†	1	No
EST from clone 2108068	LPP20 lipoprotein precursor	Sense	AI357532.1	Novel isoform	1	No
RNH	Ribonuclease/angiogenin inhibitor	Sense	NM_002939.1	-22, +713 nt	1	No

Table 1 *(continued)*

Overview of identified molecular targets

Gene	Domain homology	Direction	Accession number*	Relative to ORF*	Frequency*	Phenotype transfer
Cytoskeleton						
Moesin	Moesin	Sense	NM_002444.1	-93, +1534 nt	1	Yes
Vimentin	Intermediate filament	Sense	NM_003380.1	-98, +374 nt	1	Yes
Others						
Alu repeat					5	On hold
CpG island? (clone 550H1)			AL035420.1		1	On hold
IgG2 heavy chain	Ig superfamily	Sense		Partial	1	On hold
Ig light chain	Ig superfamily	Sense		Partial	1	On hold
18S rRNA		Sense	M10098.1	Partial	2	On hold
28S rRNA		Sense		Partial	1	On hold

*For each identified clone, the GenBank database [51] accession number is given, followed by the first and last nucleotide (nt) positions relative to the initiation codon (ATG being the +1, +2, +3 nts, respectively); Frequency indicates the number of original cell clones expressing the specific hit. †Relative to the EST itself because the start codon is not identified. ‡Relative to the genomic clone itself. Ig, Immunoglobulin; TBD, to be determined.

When introduced into naïve Jurkat cells, this fragment also caused a severe block of TCR-induced CD69 expression (Figure 4c).

In addition to known signaling molecules, we also discovered genes whose sequences had been reported previously but whose involvement in TCR signaling was not documented (Table 1, and see Additional data files 6 and 7 with the online version of this article). EDG1 (endothelial differentiation gene-1) was discovered initially from a set of immediate-early-response gene products cloned from human umbilical vein endothelial cells [15]. EDG1 is a G-protein-coupled receptor (GPCR) with high affinity for sphingosine 1-phosphate (S1P) [16]. Although EDG1 has been reported to link to multiple signaling pathways [17], no role in TCR signaling had been documented. From our genetic screen, we obtained two carboxy-terminal truncation EDG1 mutants. Reintroducing EDG1 Hit 1 into naïve Jurkat cells conferred a CD69-inhibition phenotype (Figure 4d). We believe the EDG1 hits may work as constitutively active forms of the endogenous protein, given that overexpressing full-length EDG1 also caused inhibition of CD69 expression (data not shown).

PAK (p21-activated kinase) proteins are critical effectors that link Rho-family GTPases, such as Cdc42 and Rac1, to cytoskeletal reorganization and nuclear signaling [18,19]. PAK proteins constitute a family of serine/threonine kinases that utilizes the CRIB (Cdc42/Rac interactive binding) domain to bind to small GTPases; members of the family include PAK1, PAK2, PAK3 and PAK4 [19]. Among the four PAK proteins, PAK2 (also known as PAK65 [20] and gamma-PAK [21]) is activated by proteolytic cleavage during caspase-mediated apoptosis [22]. The role of PAK2 in Jurkat T cells has been reported primarily to be in membrane and morphological changes in apoptotic cells [23]. PAK1, on the other hand, has been reported to be involved in T-cell signaling [24,25]. Interestingly, we identified two different truncated versions of PAK2, both lacking the kinase domain, in our functional genetic screens with the fourth highest frequency (after TCRβ, ZAP70 and TCPTP; see Table 1). We further demonstrated that these dominant-negative forms of PAK2 also confer CD69 inhibition when introduced into naïve Jurkat cells (Figure 4e and Table 1).

An interesting adaptor molecule cloned from our genetic screen is Grb7 (Figure 4f). Like Grb2, Grb7 was originally cloned by screening bacterial expression libraries with the tyrosine-phosphorylated carboxyl terminus of the epidermal growth factor (EGF) receptor [26]. The Grb7 family of proteins - Grb7, Grb10, and Grb14 - share significant sequence homology and a conserved molecular architecture [27]. Their functional domains include a proline-rich region, an RA (RalGEF/AF6 or Ras-associating) domain, a PH domain and an SH2 domain. Like other adaptor molecules, Grb7 family proteins function to mediate the coupling of multiple cell-surface receptors to downstream signaling pathways in the regulation of various cellular functions. Our identification of a strong phenotype for the Grb7 SH2 domain in TCR signal transduction suggests that Grb7 may be an important immune-regulatory molecule (Figure 4f).

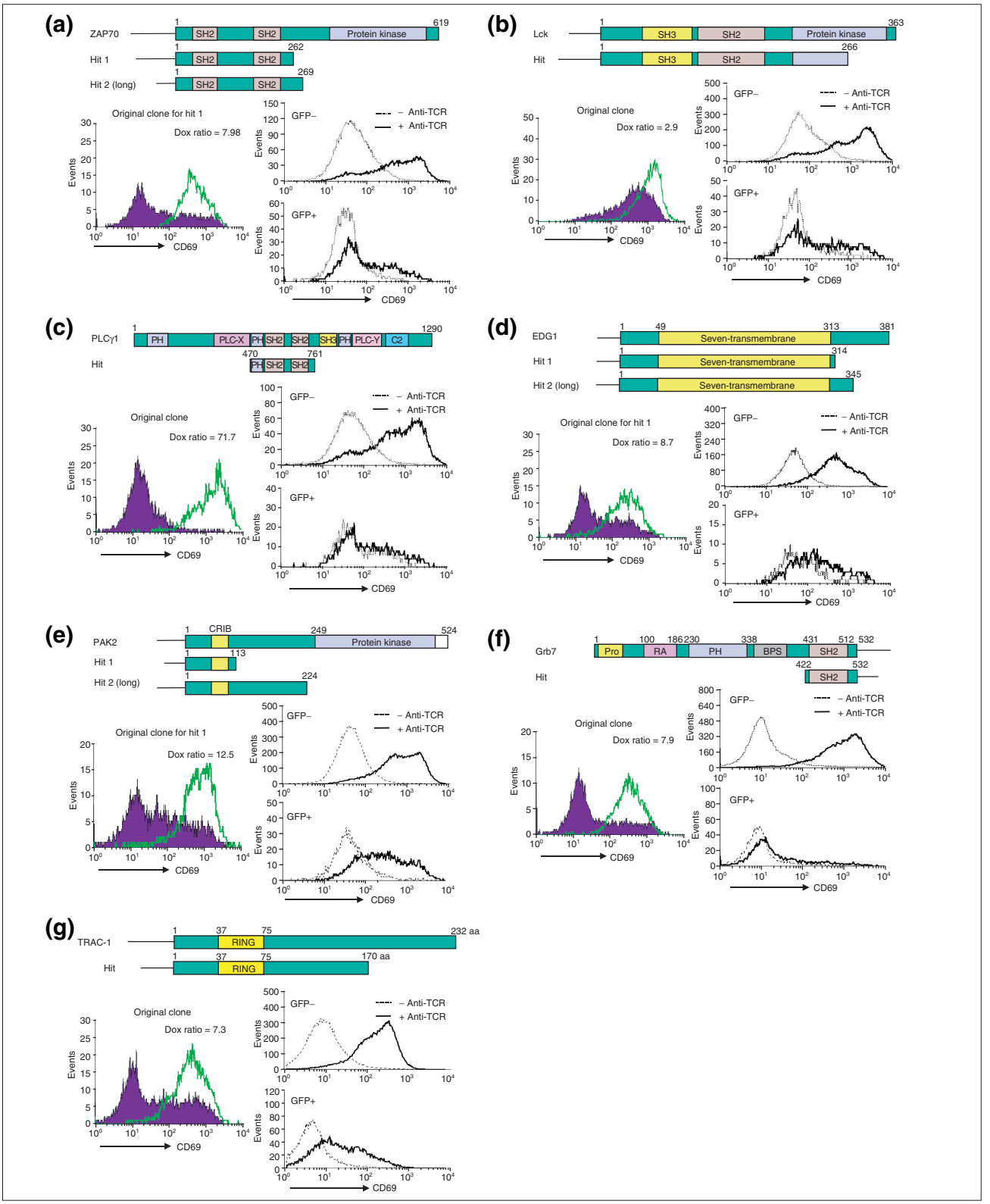


Figure 4 *(see the legend on the next page)*

We also discovered an uncharacterized molecule whose sequence in GenBank was assembled from expressed sequence tag (EST) data. This novel molecule, FLJ20456, was renamed by us as TRAC-1, for T-cell RING protein in activation. As shown in Figure 4g, TRAC-1 has a RING finger domain, which is characteristically found in a class of proteins collectively called ubiquitin ligases or E3s [28]. Members of the Cbl protein family are the best-known E3s involved in the regulation of TCR signaling [29]. T cells manifest enhanced signaling in both *c-Cbl* and *Cbl-b* mutant mice, suggesting that the wild-type function of these proteins is in negatively regulating T-cell activation. More recently, Cbl proteins have been shown to function as RING finger E3s so as specifically to target activated receptors and protein-tyrosine kinases for ubiquitination and therefore to down-regulate their signaling [30]. The TRAC-1 hit we obtained has a truncation in the carboxyl terminus but still retains the intact RING finger domain (Figure 4g). Reintroducing the TRAC-1 hit into naïve Jurkat cells caused strong inhibition of the anti-TCR-induced CD69 expression in infected cells.

For a complete characterization of the functional genetic screen, as well as additional selected hits, see Additional data files 6 and 7 with the online version of this article.

Gene expression in tissues and primary lymphocytes

We studied the expression profiles of EDG1, PAK2, Grb7 and TRAC-1 by northern blot analysis. We detected ubiquitous expression of EDG1 and PAK2 in normal human tissues, including thymus, spleen and peripheral blood lymphocytes (PBL; Figure 5a). Grb7 has strong expression in kidney and placenta, but little or no expression in thymus or PBL by northern blot analysis (Figure 5b). Interestingly, TRAC-1 has a highly specific expression in organs associated with the lymphoid system or hematopoietic system, such as spleen, liver and PBL (Figure 5b). We also detected a faster-migrating band with the TRAC-1 probe in placenta, perhaps representing an alternatively spliced message.

We further examined expression of these selected genes in lymphocyte subsets isolated from healthy human peripheral blood using semi-quantitative RT-PCR. As shown in Figure 5c, EDG1 expression was detected in both T cells (higher expression in $CD4^+$ than in $CD8^+$ T cells) and B cells ($CD19^+$), but not in monocytes ($CD14^+$). Its expression level in T and B cells was not affected upon mitogenic activation. EDG1 was also detected in the brain. PAK2 was detected in resting and activated lymphocytes as well as in the placenta (Figure 5d). Even though Grb7 was not detected in the PBL by northern blot, it was detected in peripheral blood mononuclear cells (PBMC) using the more sensitive RT-PCR method (Figure 5e). Grb7 expression seemed to be slightly increased upon activation. Consistent with the northern blot profile, TRAC-1 was detected only in lymphocytes and not in the placenta (Figure 5f). In summary, all four genes are expressed in the lymphoid system, supporting their potential physiological role in lymphocyte signaling.

Function in primary T lymphocytes

The relevance of the cDNA hits from our screen to the physiological functions of T cells was investigated in primary T lymphocytes. We subcloned the hits into a retroviral vector under the control of a constitutively active promoter embedded in the retroviral long terminal repeat (LTR), followed by IRES-GFP [31]. We then developed a protocol to couple successful retroviral infection to subsequence T-cell activation. As shown in Figure 6a (left panels), fresh PBL contained both T cells and B cells. The combined $CD4^+$ and $CD8^+$ cells represented T cells (about 81% of total lymphocytes in this particular donor). The remaining 19%, which were $CD4^-CD8^-$ cells, were B cells as stained by CD19 (data not shown). Upon culturing with anti-CD3 and anti-CD28 antibodies, primary T lymphocytes were expanded and primary B cells and other cell types gradually died off (Figure 6a, right panels). Importantly, primary T lymphocytes were successfully infected by retroviruses (Figure 6a,b).

As seen with Jurkat cells (data not shown), GFP translated by way of IRES was not as abundant as GFP translated using the conventional Kozak sequence (comparing GFP geometric mean from CRU5-IRES-GFP to that from CRU5-GFP). Nevertheless, the percentage infection remained similar (Figure 6b; 32.4% and 31.3% respectively). Insertion of a gene in front of IRES-GFP further reduced the expression level of GFP (Figure 6b), a trend observed with many other

Figure 4 *(see the figure on the previous page)*
Transfer of selected hits from the functional genetic screen to naïve Jurkat-tTA (4D9#32) cells. Diagrams of proteins predicted from the cDNA inserts and those from the corresponding wild-type genes are shown above the histograms. The left panel of histograms shows the phenotype of the original cell clones in the presence (open peaks) or absence (filled peaks) of Dox as analyzed in Figure 3a. The Dox ratio is indicated. The right top and bottom panels of histograms show the phenotypes after expressing the cDNA inserts (followed by IRES-GFP) in a naïve Jurkat-tTA population. After retroviral infection, the Jurkat-tTA (4D9#32) cells were either stimulated with the anti-TCR antibody (solid line) or left unstimulated (dashed line), and analyzed by FACS for CD69 induction after staining with anti-CD69-APC. The top right histogram in each group analyzed GFP-negative cells, which did not express the cDNA hit, whereas the bottom right histogram in each group analyzed GFP-positive cells, which expressed the cDNA hit. The following cDNA hits are shown: **(a)** ZAP70; **(b)** Lck; **(c)** PLCγ1; **(d)** EDG1; **(e)** PAK2; **(f)** Grb7; **(g)** TRAC-1.

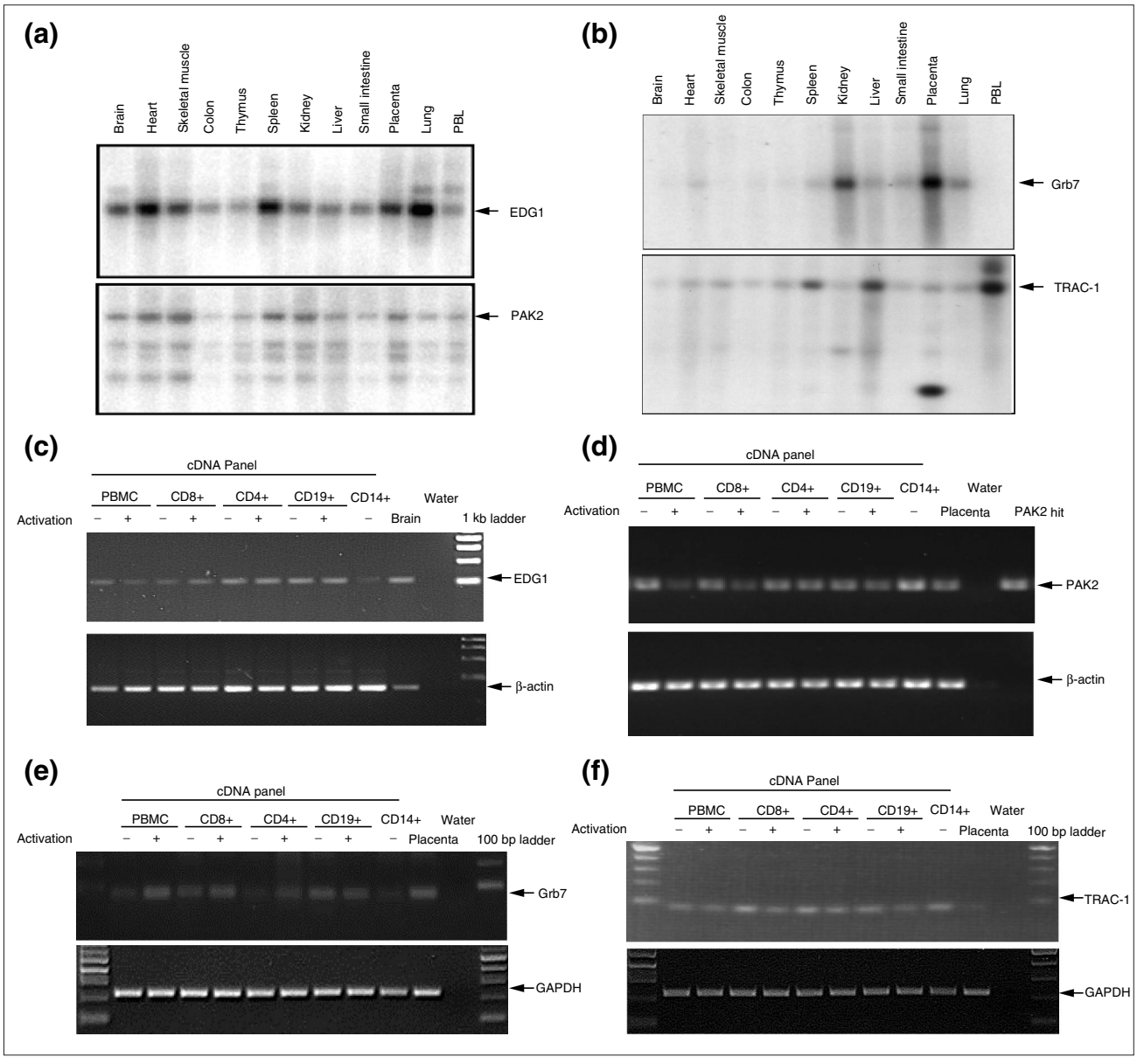


Figure 5
EDG1, PAK2, Grb7 and TRAC-1 expression in normal human tissues and lymphocyte subsets. **(a,b)** Northern blot analysis using multi-tissue blot (Clontech). The following genes are shown: (a) EDG1 and PAK2; (b) Grb7 and TRAC-1. **(c-f)** Semi-quantitative PCR analysis of gene expression in lymphocyte subsets. The cDNA templates were obtained from $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, or $CD14^+$ monocytes (human blood fractions MTC panel from Clontech). Specific target primers or control primers were used in PCR reactions. The following genes are shown: **(c)** EDG1; **(d)** PAK2; **(e)** Grb7; **(f)** TRAC-1.

cell lines (data not shown). After allowing cells to rest for 5 days following infection, we flow-sorted cells into two populations: GFP-negative and GFP-positive. Exact numbers of sorted cells were immediately put into culture. As seen in Figure 6c, resting cells did not produce IL-2, nor did cells stimulated with anti-CD3 alone. Anti-CD3 plus anti-CD28 induced robust IL-2 production in the CIG vector-infected cells (CIG), regardless of the GFP expression (note the different scales of the upper graphs compared to the lower ones).

These observations are consistent with previous reports on freshly isolated primary T lymphocytes and also indicate

that prior culture and retroviral infection did not change the basic properties of these primary T lymphocytes. Addition of anti-CD28 in conjunction with anti-CD3 also led to high IL-2 production from the GFP-negative population of cells infected with CIG-LCK, -PLCγ1, -EDG1 and -PAK2 hits. The GFP-positive population from these cells was, however, significantly impaired in IL-2 production following anti-CD3 and anti-CD28 stimulation (Figure 6c). As expected, the defect caused by the Lck, PLCγ1, EDG1 and PAK2 hits can be completely rescued by stimulation using PMA and ionomycin (Figure 6c). Taken together, these results show that Lck and PLCγ1 play a crucial role in IL-2 production from primary T lymphocytes, consistent with their involvement in membrane-proximal signaling events of T-cell activation. More importantly, our results document for the first time the involvement of the seven-transmembrane molecule EDG1 and the serine/threonine kinase PAK2 in physiological functions of T cells. Together, the results also demonstrate a rapid system for further validating hits from functional genetic screens using primary lymphocytes.

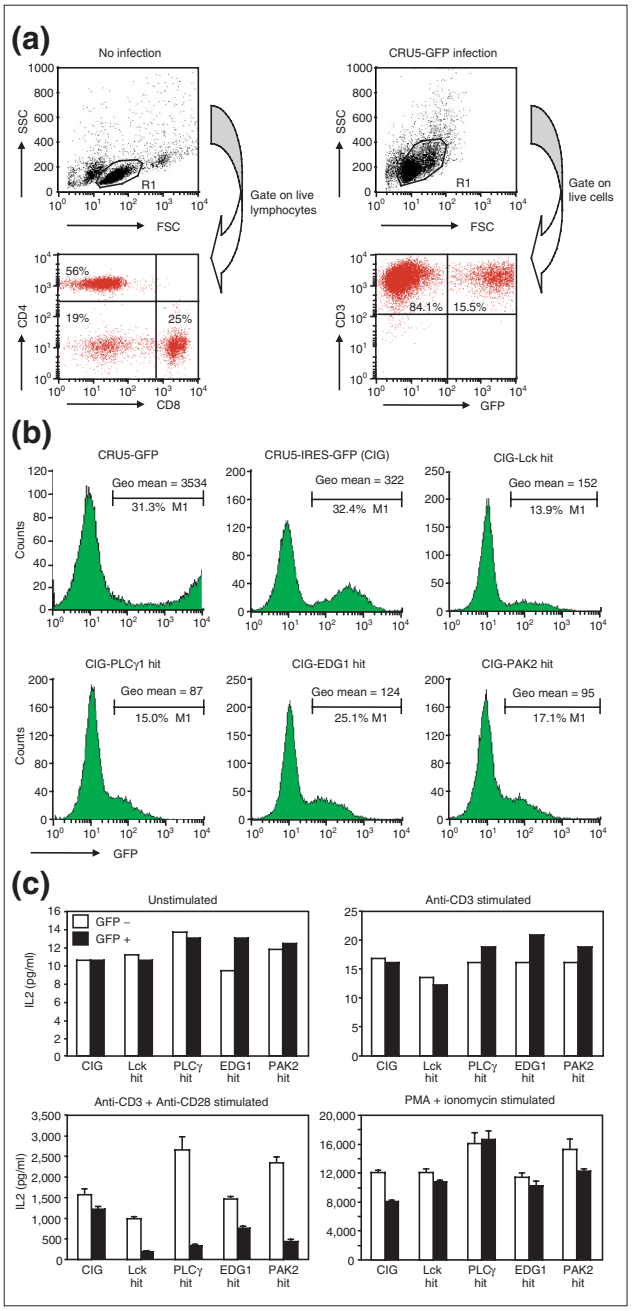


Figure 6
The cDNA hits from the functional genetic screens inhibited activation in human primary T lymphocytes. **(a)** Human PBL were cultured with anti-CD3 and anti-CD28 for 3 days and then infected with the retroviral CRU5-GFP vector, whereby GFP was expressed from the constitutively active retroviral LTR promoter. Cells were stained with anti-CD3-APC, or with anti-CD4-PE and anti-CD8-APC antibodies and analyzed by FACS. The percentage of cells in each quadrant is shown. **(b)** Human primary T lymphocytes were infected with vector alone (CRU5-GFP and CRU5-IRES-GFP or CIG) or with the CIG vector expressing the Lck, PLCγ1, EDG1 and PAK2 hits. The infection rate was monitored by the percentage of GFP-positive cells (marked with M1). The geometric mean of GFP for cells in marker M1 was shown above the marker line. **(c)** Infected primary T lymphocytes were allowed to rest and then sorted to give rise to GFP-negative (open bars) and GFP-positive (filled bars) populations. Equal numbers of cells were cultured without stimulation, with anti-CD3 or anti-CD3 plus anti-CD28 antibodies, or with PMA plus ionomycin. Then, 40 h later the culture supernatants were harvested and assayed for IL-2 production by ELISA. Note the difference in the scales and the standard deviations with cells stimulated with anti-CD3 plus anti-CD28, or with PMA plus ionomycin (lower panels) compared to the upper panels.

Discussion

In this article, we report a large-scale functional genetic screen for inhibitors of TCR signaling. We isolated many known signaling molecules - such as Lck, ZAP70, Syk, PLCγ1 - as novel truncation mutants (probably created during library preparation) with dominant-negative effects. In addition, we also discovered molecules previously unknown to this pathway, including transmembrane molecules (EDG-1, IL-10Rα and integrin α_2), cytoplasmic enzymes and adaptors (PAK-2, A-Raf-1, TCPTP, Grb7, SH2-B and GG2-1), and cytoskeletal molecules (moesin and vimentin; see Table 1). Of note, we also identified a novel molecule, TRAC-1, which had lymphoid and hematopoietic specific expression (Figure 5b).

We showed that EDG1, PAK2, and Grb7, genes originally described in different contexts, are also expressed in lymphocytes (Figure 5). This is not unexpected, since the retroviral cDNA libraries were generated using mRNA from

human lymphoid organs such as thymus, spleen, lymph nodes and bone marrow. Our expression data are generally consistent with those published by other investigators. For example, EDG1 was reported to be expressed in human natural killer cells [32] and dendritic cells [33]. PAK2 is expressed ubiquitously in human tissues [20] and in Jurkat cells [22]. Grb7 has a broad expression in human (pancreas, placenta, kidney, prostate and small intestines) [34]. Grb7 was not easily detectable by northern blot in thymus, spleen and PBL, but its expression was detected in specific lymphocyte subsets (Figure 5e). This indicates that our screen is capable of identifying genes with potentially important roles in lymphocyte activation whose expression is not limited to the lymphoid system. The fact that these genes' expression is not limited to the lymphoid system does not diminish the potential role they could play in lymphocyte activation. For example, the Ras-Raf-MAP kinase pathway is ubiquitously present in many tissues and cell types, as well as conserved evolutionarily, but this pathway has also been shown to be important in lymphocyte signaling.

In the 'post-genomics' era, the novelty of discovery lies in assigning novel functions to gene products. In our screens, for example, we identified two hits representing cytoplasmic truncated versions of EDG-1, a receptor for S1P [16]. Interestingly, FTY720, a potent immunosuppressant in advanced clinical development, has been shown to act through EDG-1 and S1P signaling pathways [35,36]. The fact that truncated EDG-1 proteins were identified in our T-cell activation screen suggests potential intersections of the TCR signaling pathway and the S1P signaling pathway, as well as new insights into the mechanisms of action of FTY720.

Our results also call for attention to potential differences between related family members. For example, PAK-1 (instead of PAK-2), c-Raf-1 (instead of A-Raf-1) and Grb2 (instead of Grb7) have been reported to be associated with the TCR signal transduction pathway [25,37-40]. Our functional genetic screens identified PAK2, A-Raf-1, and Grb7 as important regulators of TCR-induced CD69 expression. It is possible that the dominant-negative proteins we cloned also inhibit other related family members. Alternatively, it is equally possible that the previously reported dominant-negative forms of PAK1, c-Raf-1, and to a lesser extent, Grb2, may have inhibited PAK2, A-Raf-1 and Grb7, respectively. In fact, binding of the human immunodeficiency virus (HIV) Nef protein and subsequent activation of the PAK-related kinase and phosphorylation of its substrate can be readily detected in both infected primary T lymphocytes and macrophages [41]. When the HIV-Nef-associated kinase was characterized carefully, it became clear that this kinase was PAK2 and not PAK1 [42,43]. This example supports the notion that PAK2 could be the more relevant kinase in T-cell signaling. Of course, it is entirely possible that these related family members are not mutually exclusive in participating in the TCR signal-transduction pathway.

In conclusion, we have demonstrated a successful approach for discovering and validating, in a functionally relevant context, important immune regulators on a genome-wide scale. This approach provides a tool for functional cloning of regulators in numerous signal-transduction pathways [44,45]. For example, B-cell activation-induced CD69 expression [46] and, recently, the IL-4-induced immunoglobulin E class switch [47], have also been shown to be amenable to genetic perturbation following introduction of retroviral cDNA or random cyclic peptide libraries. Importantly, the outlined strategy, which requires no prior sequence information of the players involved, does not bias the search to previously known signaling molecules, molecules flagged by DNA-array technologies, or signaling molecules discovered in other contexts. This approach has added to the list of potential players in T-cell biology that have not been identified in other standard pathway-mapping techniques.

Materials and methods

Preparation of cDNA libraries

The mRNA extracted from human lymph nodes, thymus, spleen and bone marrow was used to produce two randomly primed cDNA libraries. For one library (-ATG) inserts were directionally cloned and the second (+ATG) non-directionally cloned and provided with three exogenous ATGs in three frames. The resulting cDNAs were cloned into the pTRA-exs vector [48] for doxycycline-(Dox-) regulatable expression in cell lines expressing the tetracycline transactivator protein (tTA) [9]. The total combined complexity of the two pTRA-cDNA libraries was 5×10^7 independent clones.

Cell lines

Phoenix A cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin. Human T-cell leukemia line Jurkat was obtained from Novartis (Vienna, Austria) and was cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin and streptomycin. Clone 4D9 with an optimal CD69 induction was obtained after sequential FACS-sorting for low basal CD69 expression and high induction of CD69 expression following TCR stimulation. To produce the Jurkat-tTA cell line, Clone 4D9 was infected with a reporter construct which expresses Lyt2 driven by a tetracycline responsive element (TRE) and a retroviral construct, CtTA1H, which constitutively expresses tTA [48]. The Jurkat-tTA cell clone 4D9#32 was obtained by sorting for high Lyt2 expression in the absence of Dox and low expression of Lyt2 in the presence Dox (10 ng/ml).

Transfection and infection

Phoenix A packaging cells were transfected with retroviral vectors using calcium phosphate for 6 h following standard protocols [49]. After 24 h, supernatant was replaced with complete RPMI medium and virus was allowed to accumulate for 24 h at 32°C. Viral supernatant was collected, filtered through a 0.2 μM filter and mixed with Jurkat cells or human primary T lymphocytes at a density of 5×10^5 cells per ml. Cells were spun at room temperature for 3 h at 2,500 rpm, followed by overnight incubation at 37°C. Transfection and infection efficiencies were monitored by FACS. Functional analysis was carried out at least 2 days after infection.

Stimulation

For CD69 upregulation experiments, Jurkat cells were split to 2.5×10^5 cells per ml 24 h prior to stimulation. Cells were spun and resuspended at 5×10^5 cells per ml in fresh complete RPMI medium in the presence of 300 ng/ml C305 (anti-Jurkat clonotypic TCR) hybridoma [2] supernatant, 100 ng/ml OKT3 (anti-CD3), 100 ng/ml SpvT3 (anti-CD3), or PMA (5 ng/ml) for 20-26 h at 37°C, and then assayed for surface CD69 expression.

Antibodies and flow cytometry

Jurkat cells or human peripheral blood lymphocytes were stained with FITC-conjugated monoclonal anti-mouse CD8α (Lyt2), APC-conjugated mouse monoclonal anti-human CD3, anti-human CD8, or anti-human CD69 antibodies, and PE-conjugated mouse monoclonal anti-human CD3 or anti-CD4 antibodies (all from Caltag, Burlingame, USA) at 4°C for 20 min and analyzed using a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, USA) with the CellQuest software. Fluorescent-activated cell sortings were performed on the MoFlo instruments (Cytomation, Fort Collins, USA).

Genetic screens

Phoenix A packaging cells were transfected with pTRA-cDNA libraries. Supernatant containing packaged viral particles was used to infect 3.5×10^8 Jurkat-tTA cells with an efficiency of 52% based on parallel infection with TRA-GFP [48]. After 4 days of cDNA expression, library-infected cells were stimulated with 300 ng/ml C305 for 20-30 h, stained with APC-conjugated anti-CD69 and PE-conjugated anti-CD3, and 1% of total cells with the desired $CD69^{low}CD3^+$ phenotype were isolated using MoFlo. Sorting was repeated multiple times with a 6-day rest period between stimulations until the population was significantly enriched for the desired $CD69^{low}CD3^+$ phenotype. Single cells were deposited to 96-well plates and expanded in the presence and absence of Dox, stimulated and analyzed for CD69 upregulation.

Isolation of cDNA inserts

PCR primers were designed to specifically amplify the inserts from pTRA-cDNA libraries. The primers contained flanking *Bst*XI sites for subsequent cloning to the pTRA-IRES-GFP and CRU5-IRES-GFP vectors [48,50]. BstXTRA5G: 5′-TTGCAGAACCACCACCTTGGGCTCTTAACCTAGGCCGATC-3′. BstXTRA3D: 5′-TTGCAGAACCAATTTAATGGCGGCCAGTCAGGCCATCGTCG-3′. RT-PCR cloning was achieved with kits from Clontech (Palo Alto, USA) or Life Technologies (Carlsbad, USA). The gel-purified RT-PCR fragments were sequenced as well as digested with *Bst*XI for subcloning into the retroviral pTRA-IRES-GFP or CRU5-IRES-GFP vectors.

Semi-quantitative PCR analysis

Human Blood Fractions MTC panel (Clontech) with normalized, first-strand cDNA preparations from RNA of various purified cells were used as templates. $CD19^+$ cells were activated with 2 μl/ml pokeweed mitogen for 4 days, mononuclear cells with 2 μl/ml pokeweed mitogen and 5 μg/ml concanavalin A for 3 days, $CD4^+$ cells with 5 μg/ml concanavalin A for 3-4 days, and $CD8^+$ cells with 5 μg/ml phytohemagglutinin for 3 days. The following primers were used to amplify various cDNA fragments: EDG1: forward primer 5′-GCAAGAACATTTCCAAGGCCAGCC-3′, reverse primer 5′-GGGTGTGGGATGTACAGGGCATCC-3′, 35 cycles; PAK2: forward primer 5′-CGGAGAACTGGAAGATAAGCCTCC-3′, reverse primer 5′-AAAGCCAACATGGATGGTGTGCTC-3′, 35 cycles; Grb7: forward primer 5′-ATGCCCACTGACTTCGGTTT-3′, reverse primer 5′-GATCCGAAGCCCCTTGTGT-3′, 40 cycles; TRAC-1: forward primer 5′-TTACACCAGCCTGTCCGGA-3′, reverse primer 5′-CAGACTGGTAGCAATACAGGAACG-3′, 35 cycles.

Commercially available primers were used for GAPDH (PerkinElmer, Wellesley, USA) and β-actin (Clontech), 25 cycles. The PCR products were then electrophoresed on agarose/ethidium bromide gels.

Northern blot analysis

Human Multiple Tissue Northern Blots were purchased from Clontech. The following probes were used: EDG1, base pairs 1-1,023 of its open reading frame; PAK2, base pairs 1-341 of its open reading frame; Grb7, base pairs 1,268-1,599 of its open reading frame; and TRAC-1, base pairs 1-509 of its open reading frame.

Culture and infection of primary T lymphocytes

Commercially available primary blood mononuclear cells (PBMC; AllCells LLC, Berkeley, USA) were cultured in RPMI + 10% FCS for 1-2 h in tissue culture flasks to allow macrophages and other adhering cells to settle down. The suspended cells were cultured with anti-CD3 (30 ng/ml)

and anti-CD28 (100 ng/ml) for 2 days to allow T cells to expand and other cell types to gradually die off. These primary T lymphocytes were infected with 1 ml retroviral supernatant in a 24-well plate. One day after infection, 1 ml spent medium from the bulk culture was added to each well. The cells were further expanded for a few days with addition of fresh RPMI + 10% FCS. Such an expansion also allowed the cells to return to the resting state with low CD69, CD25, and CD40L expression. Cells were then sorted by FACS, on the basis of GFP expression, directly into a round bottom 96-well plate coated with anti-CD3 alone, anti-CD3 + anti-CD28, or not coated. To the uncoated wells, PMA (5 ng/ml final) and ionomycin (1 μM final) were added. Then, 40 h later, supernatants were harvested for IL-2 measurement using commercial reagents (R&D Systems, Minneapolis, USA).

Additional data files

The following are provided as additional materials with this article online: details of the selection and infection of Jurkat clone 4D9 (Additional data file 1); construction of the pTRA-cDNA libraries and assessing the efficiency of infection (Additional data file 2); distinction between $CD3^-$, $CD3^{low}$ and $CD3^{high}$ cell populations (Additional data file 3); distribution of Dox ratios among the 2,828 single-cell clones analyzed (Additional data file 4); details of clones with TCRβ hits (Additional data file 5); a summary of the genetic screen for inhibitors of TCR-induced CD69 expression (Additional data file 6); characterization of additional hits from the T-cell activation screen (Additional data file 7); correlation of the CD69 inhibitory phenotype with the cDNA expression level (Additional data file 8).

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