Cytohesin binder and regulator (Cybr) augments T cell receptor-induced NFAT-AP-1 activation through regulation of the JNK pathway.

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Running Title: Cybr regulates TCR signalling

Cytohesin binder and regulator (Cybr also known as CYTIP, CASP, and PSCDBP) is a cytokine-induced gene preferentially expressed in haematopoietic tissues and in T helper (Th) 1 cells. Cybr protein associates with members of the cytohesin family, which are known ADP ribosylation factors GDP/GTP exchange factor (Arf)-(GEFs) and its functions appear to regulate lymphocyte adhesion and cell-cell contact. Here we show that Cybr mRNA and protein levels are increased upon T cell receptor engagement. Cybr expression then influences T cell receptor (TCR)-dependent signalling events such as Nuclear Factor of Activated T cells (NFAT) and AP-1 transcriptional activity. In addition, expression of Cybr results in increased TCR-mediated activation of the Rho/Rac exchange factor Vav and of the JNK-p38 mitogen activated protein kinases (MAPK) signalling pathway.

The effects of Cybr on NFAT and AP-1 are dependent on MAPK activation and enhanced activation of this cascade results in cooperation between the two transcription factors in the regulation of gene expression. These findings provide the first evidence that the adaptor protein Cybr not only regulates lymphocyte adhesion and cell-cell interaction, but also contributes to the regulation of the signalling cascade and of the genetic program downstream of the T cell receptor.

Introduction
Interaction of the T cell antigen receptor (TCR)-CD3 complex with the antigenic peptide presented by the major histocompatibility complex triggers a cascade of signalling events ultimately leading to the regulation of T cell functions such as proliferation and cytokine synthesis 1,2. Activation of protein-tyrosine kinases (PTKs) is an important downstream event after TCR engagement. Upon
antigen ligation, Src family kinases such as Lck and Fyn initiate the phosphorylation of the immunoreceptor tyrosine-based activation motifs of the CD3 chains, which then serve as the docking sites for the engagement of the PTK Syk family member ZAP-70 (70-kDa zeta-associated protein) 3. ZAP-70 in turn phosphorylates the adaptor proteins LAT and SLP-76 which recruit a complex set of proteins including Grb2, SOS, GADs, PLC-γ1, Vav, phosphatidylinositol 3'-kinase (PI3'-K) and Itk 4. In particular, phospholipase-Cγ1 (PLC-γ1) is activated by tyrosine phosphorylation and subsequently hydrolyzes inositol phospholipids into inositol triphosphates (IP3) and diacylglycerol (DAG) 4,5. This step increases Protein Kinase C activity (via DAG) and intracellular calcium concentration (via IP3) 2. The LAT-SLP76 complex regulates PLC-γ1 activation through another pivotal PTK, the Tec family tyrosine kinase Itk 6. These series of events ultimately lead to the activation of the transcription factor NFAT and the AP-1 complex. NFAT activation seems to also require the tyrosine phosphorylation of Vav, the guanine nucleotide exchange factor for the small GTPases Rac, Rho, and Cdc42. In fact, T cells lacking Vav show impaired activation of NFAT, antigen-induced cell proliferation, and cytokine production 7,8. NFAT-mediated gene expression in T cells has been shown to be dependent not only on the calcium signalling pathway, but also on the cooperative recruitment of Fos and Jun, and the generation of an NFAT-AP-1 complex 9, 10. The activation by TCR of the MAPK cascade can influence these events not only by regulating the synthesis of Fos and Jun, but also controlling their transcriptional potential 11. Activation of the MAPK pathway results in phosphorylation of c-Jun and Fos to generate an active AP-1 complex 12-14. Moreover, Vav-mediated complex has also been suggested to link the TCR-induced activation of JNK and p38 15.

We originally described Cybr as a cytokine-inducible gene being rapidly induced in NK cells and peripheral blood mononuclear cells (PBMCs) following IL-2 and IL-12 stimulation 16, 17. It possesses two conserved protein-protein interaction motifs including an N-terminal PSD-95/Dlg/ZO-1 (PDZ) domain and a central coiled-coil (CC) motif 16, which suggest a participation in signalling complex assembly and intracellular trafficking. In fact, the close relative of Cybr in neuronal cells, termed Tamalin or GRASP, is able to form a complex with metabotropic glutamate receptors and contribute to post-synaptic protein trafficking 18, 19. Cybr and Tamalin therefore may represent a new family of molecular scaffold proteins. The CC domain of Cybr has been shown to interact with the CC domain in cytohesin-1 16, 20. This interaction results in Cybr regulation of cytohesin-1’s GEF activity on its substrate, the ARF family GTPases 16. Cybr distribution has been shown to be restricted to the cytosolic regions in several cell types 20-22. Interestingly, in Epidermal Growth Factor-stimulated...
COS-1 cells over-expressing Cybr and cytohesin, Cybr appears to be recruited from the Golgi compartment to membrane ruffles in a cytohesin-dependent manner. Moreover, in Jurkat T cells Cybr has been shown to drive cytohesin away from membrane-associated integrins to the cytosol and thus attenuate lymphocyte adhesion. Using siRNA in dendritic cells, Cybr has been shown to be important for controlling dendritic -T cell interaction.

Here, we show that Cybr expression is increased following TCR engagement and its expression results in augmented NFAT activation. Surprisingly, this is not due to increased Itk-PLCγ activation or to increased Ca2+ flux. Instead it appears to result from NFAT-AP-1 cooperation that is in turn regulated via the Vav-JNK/p38 MAPKs signalling pathways. These results demonstrate that Cybr, besides regulating cell-cell interaction, also participates in the signalling cascade downstream of the TCR and plays a functional role in the control of TCR-dependent gene transcription.

**Experimental procedures**

**Cells, plasmids antibodies and chemical reagents**- The human leukemic T-cell line Jurkat, subclone E6 was maintained in standard growth medium (RPMI 1640 medium supplemented with 10% foetal bovine serum, 2mM L-glutamine and 100 U/ml penicillin/streptomycin). Human PBMCs from normal healthy volunteers were purified by Ficoll-Hypaque density gradient centrifugation and incubated in standard RPMI 1640 growth medium overnight before the stimulation.

The cDNA encoding X-press-tagged Cybr in the pEF-HisC mammalian expression vector has been previously described. The NFAT luciferase reporter construct pGL3-NFAT3-Luciferase (pNFAT-Luc) consisting of three direct copies of the murine IL-4 high affinity NFAT site (region -82 to -64 of the murine IL-4 promoter) was a generous gift of Dr James A. Goodrich (University of Colorado, Boulder, CO). The NFAT-IFN luciferase reporter construct was a generous gift of Dr. A. Altman (La Jolla Institute for Allergy and Immunology, La Jolla, CA) and was described previously. pAP-1-Luc (AP-1, 7x) luciferase reporter gene construct was kindly provided by Dr M. Chiariello (CNR, Napoli, Italy). Anti-CD3 OKT3 monoclonal antibody (mAb) was purified from culture supernatants of the corresponding hybridomas by protein G-Sepharose chromatography. Polyclonal anti-CASP Ab was raised in rabbits using the purified C-terminal domain of CASP, amino acids 253 to 350 fused to a (His)6-tag, as immunogen. Polyclonal anti-Itk Ab was generously provided by Dr. Joseph Bolen and Dr. Mike Tomlinson (University of Birmingham, Birmingham, UK). The following antibodies were purchased: Monoclonal anti-X-press (Invitrogen Life Technologies, Paisley, UK); monoclonal anti-phospho-tyrosine 4G10 (Upstate Biotechnology, Lake Placid, NY.); goat anti-mouse (GAM) IgG (Sigma-Aldrich); polyclonal anti-Vav and polyclonal anti-phospho-PLCγ1 (Santa Cruz Biotechnology, CA.); monoclonal...
anti-phospho-ERK, polyclonal anti-phospho-JNK and p38, polyclonal anti-JNK and p38 (Cell Signaling Technology, Beverly, MA); Monoclonal anti-ERK2 (BD Pharmingen) monoclonal anti-Rac1 Ab (Upstate Biotechnology, Lake Placid, NY.), monoclonal anti-phospho-Akt, and monoclonal anti-Akt (Cell Signaling Technology; Beverly, MA).

The JNK inhibitor II (SP600125) was purchased from Calbiochem (San Diego, CA). p38 inhibitor (SB202190) was purchased from Alexis Biochemicals (San Diego, CA). The following reagents were purchased from Sigma-Aldrich: ionomycin, phytohemagglutinin (PHA), Isopropyl-β-D-thiogalactopyranoside (IPTG) and Cyclosporin A (CsA). The transfection reagent METAFASTENE (Biontex, Germany) was utilised for the Jurkat cells experiments according to the manufacturer’s protocol.

**RNA extraction and Reverse Transcriptase-PCR analysis**- Total RNA was prepared by the RNA STAT60 (Biogenesis) isolation system. RT-PCR was performed with 1μg of total RNA using the OneStep RT-PCR kit (Qiagen) and following the manufacturer’s protocol. Polymerase Chain Reaction (PCR) amplification was performed using primer pairs specific for X-press-Cybr (forward primer: 5’ –CTT TAC AAA GGC TCC TGC AAC ACA-3’; reverse primer: 5’ –GGC AAT TAG ATG CAT CAC CAT GAA G- 3’) and for GAPDH (forward primer: 5’ -TGA TGA CAT CAA GAA GGT GG- 3”; reverse primer: 5’ -TTA CTC CTT GGA GGC CAT GT- 3’).

**Cell stimulations and luciferase assays**- For TCR cross-linking experiments, transfected Jurkat cells were transferred to six-well plates, which had been precoated with OKT3. The inhibitors including CsA, JNK inhibitor II and SB202190 at a final concentration of 100ng/ml, 30μM and 20μM, respectively, were added to cells 30 min before OKT3 (10 μg/mL) stimulation. The inhibitors were present during the entire period of cross-linking with OKT3. Cells were then washed once in PBS and lysed in the reporter lysis buffer (Promega). Luciferase activity was measured using a Centro LB 960 Microplate Luminometer (Berthold Technologies). The Promega Dual Luciferase Reporter Assay System was used to normalize the transfection efficiency. Luciferase activity was assayed in triplicate and expressed as the ratio of *firefly* luciferase activity normalized to *Renilla* luciferase.

**Stimulation, immunoprecipitation immunoblotting and Rac1 pull-down assay**- Cells were washed once in PBS and then re-suspended in 1ml of serum free medium containing OKT3 (1μg/ml), incubated on ice for 30min before being placed at 37°C for an additional 3 min. Cross-linking was achieved by adding GAM (20μl/ml) at 37°C for the indicated times. The stimulations were terminated by the addition of ice-cold PBS. Cells were lysed in ice-cold lysis buffer containing 0.5% Triton X-100 (TTX-100), 50mM Tris-HCL (pH 7.5), 300mM NaCl, 2mM EDTA, 0.4mM
Na₃VO₄, 10µg/ml Leupeptin, 10µg/ml Aprotinin and 2.5µM p1-nitrophenyl p-guanidinobenzoate (NPGB). Lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4°C, and protein concentration was determined by the Bradford assay. For immunoprecipitation experiments, the protein A-Sepharose beads were pre-associated with antibodies for 2h. Lysates were immunoprecipitated for 1h at 4°C. The beads were then washed five times with TTX wash buffer (50mM Tris-HCL, pH 7.5; 300mM NaCl; 0.1% TTX-100; 2mM EDTA, 0.4mM Na₃VO₄; 10µg/ml Leupeptin; 10µg/ml Aprotinin and 2.5µM NPGB). Immunoprecipitates or whole-cell lysates were boiled in 2× Laemmlı buffer, resolved on 10% SDS-PAGE. The E. coli DH5α bacteria expressing the recombinant GST-PBD (GST fused to the Rac binding domain of Pak) were treated with IPTG to induce the expression of GST-fusion protein. The bacteria were then re-suspended in E. coli lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF), and lysed by sonication (Vibra Cell™ Sonics & Materia Inc. Danbury, CT, USA). Following it, the GST-PBD fusion proteins were purified by incubation with the glutathione-coupled Sepharose 4B beads (Pharmacia Biotech Inc., Piscataway, N.J.) for 1 h at 4°C. Cells were washed once in PBS and then suspended in cold lysis buffer containing 1% Nonidet P-40, 50 mM Tris, pH 7.5, and 150 mM NaCl, supplemented with phosphatase inhibitors. Cell lysates were then precipitated with GST-PBD beads at 4°C for 1h. The active, GTP-loaded form of Rac1 was separated using 12% SDS-PAGE gel electrophoresis.

The proteins were transferred electrophoretically to Immobilon™-P PVDF transfer membrane (Millipore). Membranes were blocked and incubated with the relevant antibodies for 2h, followed by incubation with horse-radish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary Abs (BioRad). Immunoreactive proteins were visualized using enhanced chemiluminescence and the ChemiDoc SRS imaging system (BioRad). Membranes were stripped and then re-probed to confirm equal loading.

**Determination of Ca²⁺ flux**

10⁷ transiently transfected Jurkat cells were washed twice with 1x HBSS containing 1mM calcium, 1mM magnesium and 0.5% BSA and resuspended in 1ml of HBSS. Fluo-3™AM and Fura Red™AM (Invitrogen) were added to the final concentration of 5µl/ml and 10µl/ml, respectively. Cells were then incubated for 30 min at 37°C. In order to allow complete de-esterification of intracellular AM esters, cells were diluted 1:5 by adding 4 ml of HBSS plus 1% FCS and incubated for a further 30 min at room temperature. Cells were then washed three times with HBSS plus 1% FCS and re-suspended in 7 ml of Hepes-buffered saline (137 mM NaCl, 5 mM KCl, 1mM Na₂HPO₄, 5 mM glucose, 1 mM CaCl₂, 0.5 mM MgCl₂, BSA 1g/L, Hepes 10 mM, pH 7.4). Cells were maintained in darkness until acquisition. Analyses were performed...
at room temperature on a Cyan™ flow cytometer (DAKO Cytomation). Results were shown as histograms of the fluorescence ratio (Fluo-3/Fura Red) vs. time. A complete run of 380 seconds was recorded. Stimulation with the Ca^{2+} ionophore, ionomycin (1μg/mL) was used as measurement of maximal activity.

**Results**

**Cybr is inducible upon T cell receptor (TCR) triggering.**

We recently identified Cybr as a cytokine-inducible gene preferentially expressed in immune cells. Notably, Cybr expression was elevated in differentiated Th1 cells and low in Th2 cells. In the latter cells, expression was increased upon stimulation with phorbol ester and ionomycin. As this stimulation mimics the effects of TCR ligation we therefore sought to determine whether TCR cross-linking could also augment Cybr expression. Levels of Cybr mRNA were analyzed in PBMCs by RT-PCR upon CD3 cross-linking (Figure 1A). In freshly isolated un-stimulated PBMCs, Cybr was barely detected but was induced after 1 h of stimulation (lane 4). Its level then rapidly decreases after 2 h (lane 5), although became enhanced again after 24 h (lane 8). We also analyzed Cybr mRNA expression in the T cell line Jurkat (Figure 1B). As previously reported, Cybr mRNA in Jurkat appeared to be undetectable, but was observed after 2 h and peaked after 6 h of treatment. Interestingly, in these cells, Cybr mRNA levels remained elevated for 24 h after OKT3 treatment. To further analyse the regulation of Cybr in T lymphocytes at the protein level, freshly isolated human PBMCs were incubated for 3 days in the presence of PHA to induce T cell proliferation. Cells were then left un-stimulated or were stimulated with plate-bound OKT3. The expression of Cybr protein was then detected using a polyclonal Cybr antiserum (Figure 1C). Cybr protein levels were detected at 8 h upon OKT3 treatment and reached maximum levels at about 16h. Furthermore, we immunoblotted whole cell lysates (WCL) from Jurkat cells treated with OKT3 (Figure 1D). The expression of Cybr protein after incubation for 2 h with OKT3 was augmented as compared with un-stimulated Jurkat T cells and was still elevated after 24 h. Together, these experiments revealed that Cybr levels are induced following TCR engagement.

**Cybr expression enhances TCR-induced NFAT activity.**

Having observed increased Cybr expression upon CD3 cross-linking; we hypothesized that Cybr might be involved in signalling events downstream of the TCR. The NFAT family of proteins are key transcription factors that regulate early gene transcription and cytokine production following TCR stimulation. We therefore evaluated the effect of Cybr on NFAT transcriptional activation upon mitogen stimulation. We utilised the Jurkat cell line which does not express any basal level of Cybr and therefore constituted an ideal model. To evaluate the effects of the presence of Cybr we transiently transfected the pNFAT-Luc vector, which contains
three copies of the region -82 to -64 of the murine IL-4 promoter along with either Cybr plasmid or a control plasmid. As shown in Figure 2A (upper panel), stimulation with PHA or OKT3, induced NFAT-dependent transcription in control cells which was robustly enhanced when Cybr was expressed. Expression of Cybr in the cells was confirmed by analyzing cell lysates by western blot (lower panel). We therefore wondered if the presence of Cybr was able to alter the cellular responses to TCR engagement. Therefore, we evaluated the effects of Cybr expression upon TCR activation at different doses of anti-CD3. In Figure 2B, EV- or Cybr-transfected cells were assayed for the transcriptional activity of NFAT after incubation with increasing concentrations of plate-bound OKT3. Cybr effects were noticeable at doses of OKT3 above 5 μg/mL. Taken together, these data suggest that Cybr can augment TCR-dependent NFAT activation in a dose-dependent manner.

**TCR-dependent Cybr-enhanced NFAT activation is dependent on calcineurin activity but is not mediated by increased ITK or PLC-γ1 activity.**

NFAT activity is controlled by the Ca²⁺-calmodulin-regulated phosphatase, calcineurin, and therefore regulated by the levels of cytoplasmic calcium. The immunosuppressive drug CsA binds to immunophilins and inactivates calcineurin, hence impairing NFAT nuclear import and subsequent activation. In order to verify if Cybr regulation of NFAT occurs via the classical Ca²⁺-calcineurin pathway, we examined the inhibitory effect of CsA on Cybr expressing Jurkat cells. As shown in Figure 3A, OKT3-induced NFAT activation in control cells was totally suppressed by CsA. Notably, the up-regulation induced by Cybr was also inhibited by CsA suggesting the requirement of a CsA-sensitive signalling pathway for Cybr-mediated NFAT activation.

The rapid proximal signalling events following TCR activation require tyrosine kinases of the Src and Syk families, which in concert, lead to the activation of the Tec family tyrosine kinase Itk. In conjunction with other T cell specific adaptor proteins, Itk has been implicated in PLCγ1 activation, a step essential for optimal Ca²⁺/calcineurin/NFAT activity. Since the enhanced TCR-mediated NFAT activation observed with Cybr was CsA sensitive, we then examined whether Cybr’s influence was the result of an augmented activation of the Itk-PLCγ pathway. To explore this, control or Cybr-expressing Jurkat cells were stimulated with OKT3 and cell lysates were immunoprecipitated with an Itk Ab, and tyrosine phosphorylation examined whereas, PLCγ1 phosphorylation was analyzed using a phospho-specific Ab. As shown in Figure 3B (panels i and ii) TCR stimuli induces the phosphorylation of both Itk and PLCγ in Jurkat cells with maximal levels of activation about 3 min after stimulation which quickly declined within 10 min. Interestingly, in the presence of Cybr, Itk and PLCγ1 activation followed similar kinetics with no appreciable differences. Therefore, although both Itk and PLCγ1 are activated by TCR
engagement and lie upstream of NFAT, their activation is not affected by the presence of Cybr. Having observed that Cybr-induced NFAT luciferase activity is calcineurin-dependent but not Itk and PLCγ1-dependent, we then investigated the role of Cybr in TCR-induced Ca^{2+} mobilization. Jurkat cells were loaded with the Ca^{2+}-sensitive dyes, Fluo-3 and Fura-Red, treated with OKT3 and calcium flux then analyzed by flow cytometry. Both control and Cybr expressing cells showed a Ca^{2+} elevation with similar kinetics and amplitude suggesting that Cybr had no effect on TCR-induced Ca^{2+} mobilization. Ionomycin (Figure 3C, panel iii) was used as a positive control.

Cybr enhances CD3-mediated NFAT-AP-1 cooperation. The NFAT family forms complexes with other transcription factors such as NF-kB and AP-1. In particular, AP-1 cooperates with NFAT on composite DNA elements to form an enhancerome complex, and control gene expression. Several studies have shown that promoters for genes, such as IL-2 and IL-4, often contain multiple NFAT sites, many of which are part of composite elements to which NFAT and AP-1 bind cooperatively. The murine IL-4 high affinity NFAT reporter (region -82 to -64 of the murine IL-4 promoter), used in this study contains composite DNA elements for the NFAT-AP-1 complex. We therefore turned our attention to the role of AP-1 in Cybr-mediated TCR-dependent effects. We initially examined the effects of Cybr expression on AP-1 transcriptional activity. Figure 4A shows that, CD3 cross-linking enhanced AP-1 activity in the control cells and that the presence of Cybr increased the basal levels as well as the CD-3 dependent activation of AP-1. We hypothesized that NFAT activation by Cybr was mediated by AP-1 rather than increased binding of NFAT proteins. Therefore, we utilised an NFAT reporter construct containing the distal IFN-γ high affinity NFAT site, which does not include AP-1 binding sites. As shown in Figure 4B, CD3 cross-linking minimally enhanced the NFAT-IFN-γ luciferase activity in the EV-transfected cells. Moreover, the presence of Cybr did not further up-regulate the transcriptional activity. Stimulation of the same cells with PMA plus ionomycin induced an evident activation of NFAT-IFN-γ suggesting that the poor activity observed was dependent on the type of stimulation (Figure 4C). As with CD3 cross-linking, PMA plus ionomycin stimulation in the presence of Cybr also failed to up-regulate the NFAT-IFN-γ transcriptional activity supporting our hypothesis that the activation of NFAT-IL-4 reporter could result in a co-operative interaction between AP-1 and NFAT.

Cybr expression results in augmented JNK and p38 but not ERK MAPKs activation. The AP-1 complex is a homo- or hetero-dimer consisting of various members of the nuclear phosphoproteins Fos and Jun families. The MAPKs: ERK 1/2 (extracellular signal-regulated kinases
1 and 2), p38, JNK/SAPK (c-Jun NH$_2$-terminal kinase/stress-activated protein kinase), ERK 3/4 and ERK 5 directly control the synthesis of Fos and Jun proteins and formation of the AP-1 complex. Moreover, MAPKs activation can regulate AP-1 transcriptional activity. Our data suggested that engagement AP-1 may be required for Cybr-mediated NFAT activation. We therefore decided to investigate whether the presence of Cybr could alter the activation of MAPKs. Activation of endogenous JNK, p38 or ERK-1/2 was evaluated in Cybr expressing Jurkat cells. As shown in Figure 5, the presence of Cybr resulted in a markedly increased activation of both JNK (A) and p38 (B) following TCR stimulation. Moreover, basal levels of phosphorylation of JNK and p38 in Cybr expressing cells were also higher when compared to control cells, which mirrored the higher basal activation of AP-1 we had previously observed. Interestingly, ERK activation was unchanged in the Cybr-expressing Jurkat cells when compared to EV-transfected cells (Figure 5C). Equal loading of JNK, p38, and ERK2 proteins were confirmed by reblotting the membranes with the relevant antibodies. Together, these data suggested that Cybr may act as an intermediate for TCR-induced activation of JNK and p38, but not ERK MAPK in T cells.

**Cybr-mediated increase of the NFAT-AP-1 complex activity is suppressed by JNK and p38 inhibitors.**

To verify the hypothesis that MAPKs could contribute to Cybr-mediated NFAT-AP-1 transcriptional activity, we utilized a JNK inhibitor (SP600125) and a p38 inhibitor (SB202190). Transiently transfected Jurkat cells were pretreated with the SP600125 (30μM) and SB202190 (20μM) for 30 min and stimulated for 6 h with plate-bound OKT3 in the presence of the inhibitors which at these concentrations show selective specificity towards their respective targets. CD3 cross-linking triggered the transcriptional activity of NFAT in EV-transfected Jurkat cells (Figure 6A lane 3) but this activation was dramatically suppressed by the JNK inhibitor (Figure 6A lane 4), indicating that JNK activity was required. Moreover, the Cybr-enhanced of NFAT-AP-1 activity was completely abolished by JNK inhibitor (Figure 6A, lane 8). These results suggest that Cybr-mediated NFAT-AP-1 activation is dependent on JNK. Parallel experiments were performed to analyze the importance of p38 activation. As shown in Figure 6B, treatment with the p38 MAPK antagonist SB202190 caused a suppressive effect, albeit to a lesser extent than with the JNK inhibitor (Figure 6B, lanes 4 and 8). Altogether, our results suggest that the presence of Cybr leads to increased activation of JNK and p38 upon TCR cross-linking. Furthermore, JNK kinase appears to be critical for the Cybr-mediated increase of NFAT and AP-1 transcriptional activity whilst p38 appear to have a contributory role.

**Cybr expression increases Vav phosphorylation**

The Rac GEF Vav enhances TCR
signalling by increasing AP-1 transcriptional activity and, as a consequence, it also regulates NFAT activation. Moreover, Vav can regulate the JNK-MAPK pathway downstream of TCR \(^{25,29}\). We therefore explored the possibility that Cybr could influence Vav activation. As displayed in Figure 7, anti-p-Tyr Western blots of Vav immunoprecipitates showed that in EV-transfected control Jurkat cells, Vav is tyrosine phosphorylated upon TCR cross-linking. Remarkably, in the Cybr-expressing cells, basal levels of Vav Tyr phosphorylation were observed and this phosphorylation was significantly increased after TCR cross-linking when compared to the control cells. These data suggest that the presence of Cybr results in enhanced Vav activation, which in turn augments JNK and NFAT-AP-1 activity.

**Expression of Cybr regulates Rac1 GTPase activity but not Akt phosphorylation.**

To assess if the increased levels of Vav phosphorylation observed in the previous experiments were indicative of augmented enzymatic activity we evaluated the levels of the GTP-bound form of Rac1. As shown in Figure 8 (Panel A), CD3 cross-linking induced Rac1 activation in the control Jurkat cells within 10 min of stimulation and levels rapidly declined to basal levels after 30 min. Expression of Cybr resulted in enhanced and sustained levels of GTP-Rac1 suggesting that the increased levels of Vav phosphorylation paralleled with augmented GEF activity towards its substrates.

PI-3'-K is a key molecule closely coupled with TCR \(^{30,31}\) and it has been suggested to serve as both an upstream regulator and a downstream effector of Vav molecules in a positive feedback loop \(^{32}\). We therefore further investigated whether the presence of Cybr could influence PI-3'-K signaling pathway by analyzing the phosphorylation of the serine/threonine kinase Akt. \(^{33}\) WCL from EV or Cybr expressing Jurkat cells un-treated or stimulated with OKT3 and GAM were analysed using an anti-phospho-Akt antibody. As shown in Figure 8B, Akt appeared to be constitutively activated in these cells and was up-regulated by TCR stimulation resulting in a small increase in its phosphorylation. This also appeared to be unchanged in the Cybr expressing cells. Altogether, these results suggest that Cybr seems to be involved in the activation of Vav and Rac1 GTPase leading to JNK and AP-1 activation but its expression is not required for PI-3'-K/Akt signalling cascade.

**Discussion**

The cytokine-inducible gene Cybr (also known as CYTIP, CASP and PSCDBP) belongs to a newly identified family of scaffold proteins containing protein-protein interaction domains including an N-terminal PDZ domain and a central CC motif \(^{16,20,21}\). Its close relative, Tamalin-GRASP has recently been shown to participate in signal transduction events downstream of the metabotropic glutamate receptors and regulates the expression of these receptors \(^{18,19,34}\). Cybr itself appears to be involved in integrin signalling and the regulation of cell function.
adhesion\textsuperscript{20,22}. However, whether Cybr was involved in other receptor-mediated events was not known. Cybr mRNA was found to be elevated in differentiated Th1 cells and increased in purified NK cells, following cytokine stimulation but also upon mitogenic stimuli such as PHA/PMA or PMA plus ionomycin\textsuperscript{16,17}. Here, we have shown that Cybr mRNA levels are enhanced following TCR engagement. Since we initially characterized Cybr as an IL-2 and IL-12-inducible gene the biphasic expression we observed in PBMCs was expected as a result of the TCR-dependent cytokines production\textsuperscript{35,36}. Notably, Cybr protein expression was maintained for up to 24 h suggesting a relative stability of the Cybr protein.

The increased Cybr levels suggested that it may regulate TCR-driven events and using a reporter construct we showed that Cybr expression results in augmented NFAT transcriptional activity. Our results showed that the effects of Cybr were abolished by CsA, suggesting a role for Cybr upstream of calcineurin but surprisingly, the presence of Cybr did not alter either Itk or PLC\textgamma1 phosphorylation or the intracellular calcium flux which follows TCR engagement. AP-1 transcriptional activity can also be suppressed by CsA and several reports have indicated that CsA exerts its immunosuppressive effects through targeting both the calcineurin-dependent NFAT pathway and the calcineurin-independent activation of JNK and p38\textsuperscript{9,27}. Furthermore, NFAT-mediated gene expression in T cells is dependent on the calcium flux pathway, but can also be controlled by the cooperative recruitment of Fos and Jun, and the generation of an NFAT-AP-1 complex\textsuperscript{9,10,14,23}.

Our data showed that expression of Cybr resulted in augmented transcriptional activity of AP-1 suggesting that Cybr would not directly influence the activation of the Ca\textsuperscript{2+}-sensitive NFAT transcription factor pathway but rather regulate NFAT activation by positively regulating AP-1 activity and co-operation of both transcription factors in controlling gene transcription\textsuperscript{13}.

Downstream of the TCR the MAPK pathway and in particular activation of JNK, controls phosphorylation of c-Jun and Fos and has been shown to be essential for the formation of the AP-1 complex\textsuperscript{11-14}. We observed that Cybr expression resulted in enhanced JNK and p38 activity and inhibition of these MAPKs suppressed Cybr-dependent NFAT enhancement suggesting that Cybr effects were indeed mediated by MAPKs/AP-1-dependent signals.

The protooncogene Vav functions as a GEF for Rho-like small GTPases involved in cytoskeletal reorganization and cytokine production and plays a crucial role in T cell development and activation\textsuperscript{37}. Vav enhances basal and TCR-dependent transcriptional activation of the IL-2 and IFN-\gamma promoters and its activity has been shown to be dependent on its tyrosine phosphorylation. Moreover, Vav has been placed upstream of JNK and p38 activation in the TCR signalling cascade\textsuperscript{15}. Interestingly, Vav over-expressing cells display several
similarities with Cybr-transfected cells. Vav mediates NFAT activation but does not influence TCR-induced proximal PTKs activation or the cytoplasmic calcium levels \(^{29}\). Instead, Vav is more important for augmenting the TCR-induced JNK/AP-1 cascade upstream of NFAT activation \(^{25}\). We have shown that Cybr expression enhances TCR-dependent phosphorylation of Vav therefore linking these molecules in the regulation of TCR signalling and suggesting that Cybr may function as a positive regulator of TCR-mediated Vav/JNK/AP1-NFAT activation (Figure 8).

The mechanisms by which Cybr influences Vav remain unknown. In co-precipitation experiments, we failed to observe a direct association between Cybr and Vav (data not shown). Consequently, it appears that the effects of Cybr on Vav are indirect. Vav protein contains eleven potential tyrosine phosphorylation sites and also putative serine/threonine phosphorylation sites \(^{38}\). Several tyrosine kinases have been involved in Vav activation including the Src (Lck, Fyn) and Syk families (ZAP-70, Syk) \(^{39}\). It is possible that Cybr could interact with one or more of these kinases as it has been shown for Tamalin thereby facilitating Vav phosphorylation \(^{34}\). TCR stimulation also results in activation of the PI3’-K and production of PI-3,4,5-P\(_3\). Several studies have suggested that PI3’-K acts as an upstream regulator of Vav. The pleckstrin homology domain of Vav binds PI-3,4,5-P\(_3\) accelerating its docking to the plasma membrane \(^{8}\). Therefore, an alternative possibility is that Cybr may serve as a scaffold protein for the assembly of a multiple complex bringing Vav into proximity with PI3’-kinase products. Indeed, we and others have shown that Cybr associates with the integrin binding protein cytohesin-1, another GEF and also a target of PI-3,4,5-P\(_3\) \(^{16, 20, 21}\). Cybr has been suggested to regulate cytohesin-1 translocation from the membrane to the cytosol \(^{20, 22}\). Similarly, following TCR engagement, Cybr may modulate Vav cellular localisation.

Vav proteins contain various protein-protein interaction domains including a calponin homology domain, an acidic region, a pleckstrin homology domain, a cysteine-rich domain, and three Src homology domains \(^{40, 41}\). Cybr itself possesses two protein-protein interaction domains, a PDZ domain and a CC domain and similar to Tamalin could be involved in the formation of a large protein complex. In fact our data shows that the presence of Cybr results in increased Vav enzymatic activity. Vav GEF activity on the small GTPases Rac1 and Cdc42 regulates JNK and p38 MAPKs activation \(^{15}\) and our experiments have demonstrated that Rac1 activation following TCR engagement is augmented upon expression of Cybr. Notably, we have previously shown that Cybr can increase GTP loading of ARF GTPases, it therefore appears that expression of this scaffold molecule can affect several family of GTPases.

Numerous PDZ domain-containing proteins that interact with cell surface receptors have been shown to be critical for T cell activation \(^{42, 43}\).
Moreover, some PDZ domain-containing proteins either possess or bind specifically to GEF proteins involved in the signal transduction processes of small GTPases 18, 44. It will be important to define whether Cybr’s influence on Vav phosphorylation is linked to Cybr’s ARF regulatory activity. In Th1 cells, the NFAT-AP-1 complex is required for cytokine gene induction. In particular, IL-2 production is dependent on the presence of AP-1 whereas NFAT but not AP-1 is required for IL-4 production and Th2 differentiation 45. We have previously reported that Cybr expression is elevated in differentiated Th1 cells. Differential expression of Cybr mRNA between Th1 and Th2 cells occurs within the first 3 days of differentiation (data not shown). Interestingly, here we have shown that Cybr levels are increased within a few hours of stimulation. It is therefore possible to speculate that Cybr protein is important for the regulation of signalling cascades which are responsible for the complex events downstream of cytokine and T cell receptor leading to T helper cell differentiation.

In conclusion, we have shown that Cybr, a molecule previously known to be induced by cytokines and to regulate cell-cell adhesion, is also involved in TCR-mediated signalling. It is induced by TCR engagement and its functions include regulation of AP-1 and NFAT transcriptional activity. This is achieved through enhancement of the Vav/JNK-p38 signalling cascade. Given the importance of the NFAT-AP-1 complex in the regulation of cytokines production in Th1 cells and Cybr’s selective expression in this cellular subset, it will be important to further investigate the role that this molecule has in T cell differentiation and in coordinating TCR signalling.

References:


**Footnotes:**

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The abbreviations used in this manuscript are: Cybr, cytohesin binder and regulator; Arf, ADP ribosylation Factor; TCR, T cell receptor, PDZ, PSD-95/Dlg/ZO-1; PTK, protein tyrosine kinase; ZAP-70, 70-kDa zeta-associated protein; PLC, phospholipase C; IP3, inositol 3 phosphate; DAG, diacylglycerol PI3'-K phosphatidylinositol 3'-kinase; NFAT, nuclear factor activated T; Ab, antibody; MAPK, mitogen activated protein kinase; PBMC, peripheral blood mononuclear cells; NPGB p1-nitrophenyl p-guanidinobenzoate; CC, coiled-coil; GEF, guanine exchange factor; CsA, cyclosporin A; GAM, goat-anti mouse; ERK, extracellular signal regulated kinase; JNK/SAPK, c-Jun NH2-terminal kinase/stress-activated protein kinase; WCL, whole cell lysate.

**Figure legends:**

**Figure 1.** TCR engagement induces expression of Cybr in Human PBMCs and Jurkat cells. $10^7$ human PBMCs (A) or $10^7$ Jurkat T cells (B) were stimulated with OKT3 for the indicated times. Total mRNA was extracted, and RT-PCR performed with 1μg of total RNA using primer pairs specific for human Cybr (upper panel) or the house keeping gene GAPDH (lower panel). Cybr plasmid and Hela cells mRNA were used as controls (lane 2 in panel A and lane 7 in panel 1B). (C) WCLs derived from human PBMCs which were incubated for 3 days in 2% FCS RPMI medium containing 1μg/ml PHA or (D) Jurkat cells, stimulated with plate-bound OKT3 for the indicated times, were analysed by immunoblotting with a polyclonal anti-Cybr Ab
The membrane was stripped and rebotted with a monoclonal anti-ERK2 Ab to verify equivalent protein loading (lower panel).

**Figure 2.** Cybr expression enhances TCR-induced NFAT activity. Jurkat T cells were transiently transfected with: pNFAT-luc and pRLSV40 Renilla luciferase, with either an empty vector (X-press-EV) or X-press-Cybr; (A) after 24 h, the cells were stimulated with PHA or plate-bound OKT3 for 6 h and harvested. (B) The indicated concentrations of OKT3 were used to stimulate cells for 6 h and cell lysates were assayed using the Dual Luciferase Reporter Assay System. The values are the means ± S.E. from triplicates and data are representative of a series of three independent experiments. Samples of the same lysates were analyzed for the expression of Cybr by immunoblotting with anti-X-press mAb (A lower panel).

**Figure 3.** Cybr-enhanced activation of NFAT is dependent on calcineurin activity but is not mediated by Itk and PLC-γ1 activation or increased cytosol Ca2+. (A) Jurkat T cells were transiently transfected with pNFAT-luc plus pRLSV40 Renilla luciferase, with either X-press-EV or X-press-Cybr. After 24 h CsA (100ng/ml) was added for 30 min before stimulation OKT3 (6h). Lysates were assayed using the Dual Luciferase Reporter Assay System. The values are the mean ± S.E. from triplicate values. The data are representative of a series of three independent experiments. (B) Jurkat T cells were transfected with empty vector or Cybr plasmids. After 48 h OKT3 and GAM were added for the indicated times. (i) Cell lysates were immunoprecipitated with polyclonal anti-Itk Ab and immunoblotted with anti-phosphotyrosine Ab (upper panel). (ii) WCLs were analyzed with the anti-phospho-PLCγ1 polyclonal Ab (upper panel). The membranes were reprobed with the relevant Abs to verify equal loading (lower panels). (iii) WCLs were prepared and immunoblotted to confirm Cybr expression. (C) Fluo-3 and Fura Red were used to monitor changes in intracellular calcium in (i) EV- or (ii) Cybr-transiently transfected Jurkat T cells. The fluorescence ratio analyzed by flow cytometry. Fluo-3/Fura Red vs. time is shown for a complete run of 380 seconds. Base-line unstimulated measurements were followed by CD3 cross-linking. (iii) Ca2+ flux in cells stimulated with ionomycin 2μg/mL.

**Figure 4.** Cybr expression augments AP-1 transcriptional activity but does not affect the transcriptional activity of an NFAT-IFN-γ reporter construct. Jurkat T cells were transiently transfected with pAP-1-luc (A) or pNFAT-IFN-γ-luc (B and C) plus pRLSV40 Renilla luciferase, together with X-press-EV or -press-Cybr. After 24 h, the cells were stimulated with OKT3 (A and B) or with PMA+ionomycin (C) for 6 h. Cells lysates were assayed using the Dual Luciferase Reporter Assay System. The values are the means ± S.E. from triplicate experiments. The data are representative of a series of three independent experiments.
Figure 5. Cybr effect on MAPKs phosphorylation. Jurkat T cells were transfected with either X-press-EV or X-press-Cybr plasmids. After 48 h the cells were activated by incubation with OKT3 followed by GAM for the indicated times. WCLs were immunoblotted with (A) anti-phospho-JNK-, (B) anti-phospho-p38 or (C) anti-phospho-ERK, respectively (upper panels). Equal loading of JNK, p38 or ERK proteins was confirmed by reblotting with the relevant Abs (lower panels). (D) WCLs were prepared and immunoblotted to confirm Cybr expression.

Figure 6. Inhibition of JNK and p38 results in decreased NFAT-AP-1 activation. Jurkat T cells were transiently transfected with pNFAT-luc plus pRLSV40 Renilla luciferase together with either X-press-EV or X-press-Cybr. After 24 h, the cells were pre-treated with SP600125 (A) and SB202190 (B) for 30 min at a final concentration of 30μM and 20μM, respectively. The cells were stimulated with OKT3 for 6 h in the presence of inhibitors. Cell lysates were assayed using the Dual Luciferase Reporter Assay System. The values are the mean ± S.E. from triplicate experiments. The data are representative of a series of three independent experiments.

Figure 7. Cybr effects on Vav Tyr phosphorylation. Jurkat cells were transfected with either X-press-EV or X-press-Cybr plasmids. After 48 h the cells were treated with OKT3 and with GAM for the indicated time. (A) Cell lysates were immunoprecipitated with polyclonal anti-Vav Ab and immunoblotted with anti-p-Tyr Ab (upper panel). The membrane was stripped and reprobed with polyclonal Ab to Vav to verify equal loading (lower panel). (B) WCLs from the same samples were immunoblotted to confirm Cybr expression.

Figure 8. Cybr effects on Rac1 GTPase activity and Akt phosphorylation. Jurkat T cells were transfected with either X-press-EV or X-press-Cybr plasmids. After 48 h the cells were activated by incubation with OKT3 followed by GAM for the indicated times. (A) Cell lysates were precipitated with GST-PBD and the active, GTP-loaded form of Rac1 was detected by immunoblotting with the anti-Rac1 Ab (upper panel). As loading control for Rac1 proteins, 50μg of WCLs from the same cells were prepared and immunoblotted with anti-Rac1 Ab (middle panel). (B) Akt phosphorylation was analyzed by immunoblotting 50μg of WCLs with anti-phospho-Akt Ab (upper panel). Equal loading of Akt proteins was confirmed by reblotting with anti-Akt Ab (middle panel). Cybr expression was confirmed by immunoblotting with a Cybr antiserum (lower panels).

Figure 9 Cybr participates in TCR signalling. Cybr expression in T cells regulates Vav phosphorylation and enhances JNK and p38 MAPKs activation upon CD3 cross-linking. In turn AP-1 transcriptional activity is augmented which results in NFAT-AP-1 cooperation.
Figure 1

(A) 

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Cybr

1  2  3  4  5  6  7  8  9

GAPDH

(B) 

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Cybr

1  2  3  4  5  6  7  8

GAPDH

(C) 

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Anti-Cybr Ab

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Anti-ERK2 Ab

(D) 

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Anti-Cybr Ab

1  2  3  4  5

Anti-ERK2 Ab
Figure 2

(A) Luciferase Light Ratio

- EV
- Cybr

(B) Luciferase Light Ratio

- EV
- Cybr

Concentration vs. Luciferase Light Ratio for different treatments.
Figure 3

(A) Luciferase light ratio

(B)

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IP: Itk Ab

Blot: P-Tyr Ab

Blot: Itk Ab

WCL:

Blot: P-PLC γ Ab

Blot: PLC γ Ab

WCL:

Blot: Cybr Ab

(i) (ii) (iii)
Figure 5

(A) WCL
Blot: p-JNK

Blot: JNK

(B) WCL
Blot: p-p38

Blot: p38

(C) WCL
Blot: p-ERK

Blot: ERK2

(D) WCL
Blot: Cybr
Figure 7

(A) IP: Anti-Vav Ab

(Blot
Anti-β-Tyr Ab

(B) WCL

Blot
Anti-Cybr Ab

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Figure 8

(A) OKT3 + GAM

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(B) OKT3 + GAM

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Figure 9
Cytohesin binder and regulator (Cybr) augments T cell receptor-induced NFAT-AP-1 activation through regulation of the JNK pathway
Qian Chen, Alan Coffey, Sylvain G. Bourgoin and Massimo Gadina

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