Biochemical and functional characterization of Fyn-PAG association and its role in T-cell anergy

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1. Introduction

The immune system protects our body against a broad variety of pathogens and ensures homeostasis within the body. The immune system consists of two parts – the innate immune system and the acquired (or adaptive) immune system.

Innate immunity represents the basic resistance to pathogens. It is the first line of defense, acting immediately after infection. Its action is nonspecific and it does not possess any immune memory. The innate immune system includes also skin and surfaces of mucous membranes as anatomical barriers, various physiological barriers (e.g. low pH within stomach) and soluble factors like lysosyme, interferons, acute-phase proteins and the complement system. The cellular components of innate immunity consist mainly of granulocytes, monocytes and macrophages, specialized cells capable of phagocytosis – ingestion of particles and whole microorganisms.

Adaptive immunity is able to specifically recognize and selectively eliminate foreign microorganisms and molecules. The adaptive immune response is induced only later during infection. Its main properties are specificity (capability of distinguishing even subtle differences among antigens), diversity (generation of a broad spectrum of recognition molecules), memory (second encounter with the same antigen induces a faster and stronger response) and self/nonself recognition (ability to respond only to foreign antigens and tolerate self-antigens). Adaptive immunity consists of two distinct components:

- Humoral response – mediated by antibodies produced and secreted by B lymphocytes
- Cellular response – mediated primarily by T lymphocytes

T lymphocytes are further divided into helper T cells (Th), cytotoxic T cells (Tc), suppressor T cells (Ts) and regulatory T cells (Tregs). Helper T cells express the CD4 coreceptor on their surface. They recognize foreign antigens presented by major histocompatibility complex (MHC) class II molecules on the surface of professional antigen presenting cells (i.e. dendritic cells, B cells or macrophages). Upon activation, Th cells secrete a variety of cytokines and provide help to B cells and cytotoxic T cells leading to their full activation. B cells proliferate and mature into plasma cells producing huge amounts of antibodies. Cytotoxic T cells on the contrary express the CD8 coreceptor and recognize antigens presented by MHC class I molecules expressed on any nucleated cell within the body. Activation of Tc cells induces multiple mechanisms leading to an

apoptosis of the target cell. Ts and Treg cells regulate the activities and the activation status of other T cells.

Every immune cell originates from a pluripotent stem cell in bone marrow. The pluripotent stem cell differentiates initially into either a myeloid stem cell or a lymphoid stem cell, which then give rise to a committed progenitor for each type of immune cell. Immature lymphocytes then mature and become immunocompetent within the primary (central) immune organs – bone marrow (in the case of B cells) and thymus (in the case of T cells). A variety of secondary (peripheral) immune organs exist, which trap antigens in the periphery and provide sites where immunocompetent cells can interact effectively with these antigens. Such secondary immune organs are lymph nodes, spleen and mucosa-associated lymphatic tissues (MALT) within respiratory and gastrointestinal tracts.

1.1. T-cell development

Thymocyte development had been fairly well studied (Shortman and Wu, 1996; Sebzda et al., 1999). T-cell precursors develop, as mentioned above, in the bone marrow and then migrate to the thymus where they gain immunocompetence. First they receive survival and instructive signals to initiate the αβ T-cell developmental pathway. At this stage, they are called double negative (DN1) cells as they express neither the T-cell coreceptor CD4 nor CD8. Various DN stages exist, which are mainly characterized by the expression of CD44 and CD25, with DN1 being $CD44^{+}CD25^{-}$, DN2 $CD44^{+}CD25^{+}$, DN3 $CD44^{-}CD25^{+}$ and DN4 $CD44^{-}CD25^{-}$. The cells then proceed to the DN2 and DN3 stage, for which Notch and interleukin-7 (IL-7) signals are required. The DN3 stage is where the first critical checkpoint takes place, the so called TCR (T-cell receptor) β selection checkpoint. Only cells that have productively rearranged the TCR β chain can develop further. This newly formed TCR β chain pairs with an invariant pre-TCR α chain and this heterodimer is transported together with the CD3 and TCRζ molecules to the plasma membrane. Here the pre-TCR provides survival signals that rescue the cell from apoptosis, initiate allelic exclusion and lead to cell cycle entry and proliferation. Thymocytes downregulate CD25 expression and upregulate expression of the coreceptors CD4 and CD8. Note that this signal is ligand independent as pre-TCR α chain lacks any antigen binding site. T cells that are unable to generate a proper pre-TCR signal are arrested and die via apoptosis at the DN3 stage.

The DN thymocytes then proceed through the DN4 stage to the double positive (DP) stage, expressing both CD4 and CD8, where the second critical checkpoint occurs, TCR αβ selection. This stage requires that a properly rearranged TCR α chain pairs with the previously expressed TCR β chain. As the rearrangements of gene segments are random and α chains pair randomly with β chains, every T cell expresses a unique T-cell receptor with one specificity. The selection of functional TCR-bearing cells is very critical at this point. Therefore, newly formed TCR αβ heterodimers interact with self-peptides presented by MHC molecules expressed on stromal thymic epithelial cells. Each thymocyte will undergo one of three fates. The cells without a functional TCR are not able to generate a positively selecting signal and die by neglect. The cells with too strong interaction with self-peptides/MHC represent potentially autoreactive cells and are deleted via apoptosis (= negative selection). Only the cells with low affinity binding transduce the proper survival signal allowing their further development (= positive selection) (Starr et al., 2003). They selectively downregulate either CD4 or CD8 becoming single positive (SP) thymocytes and can migrate into the periphery. The mechanism responsible for CD4 versus CD8 lineage commitment is not fully clarified. The strength of the signal that these cells receive may play a role, however Notch signaling and Src family kinase Lck involved in the positive signal might also contribute.

However, thymic selection is not a perfect process and some T cells expressing self-reactive TCRs do escape into the periphery. Such cells would then recognize self-peptides, become activated and initiate immune reaction against self tissues leading to autoimmunity. To prevent the development and activation of these potentially destructive T cells, several mechanisms of peripheral tolerance have developed: activation-induced cell death (AICD), anergy, regulatory T cells (Tregs) and T-cell ignorance (Walker and Abbas, 2002).

1.2. Peripheral T-cell tolerance

1.2.1. AICD (Activation-induced cell death) (van Parijs et al., 1998)

Activation-induced cell death is initiated by repeated activation of T cells with their cognate antigen and is accompanied by high interleukin-2 (IL-2) production and cell death. T cells undergoing AICD co-express Fas (CD95) and Fas ligand (FasL) and the consequent engagement of Fas delivers the death-inducing signal. Fas signaling seems to be the major pathway involved

in AICD-mediated peripheral tolerance as mice with defects in either Fas or FasL exhibit defects in AICD and develop a fatal lupus-like systemic autoimmune disease. A similar disease has also been observed in humans with mutations in the Fas protein (Poppema et al., 2004).

Note that AICD is a phenomenon distinct from a passive cell death occurring after inadequate stimulation or the depletion of growth factors. Passive cell death is not receptor mediated and is prevented by CD28 and IL-2 signals, well known survival factors inducing the expression of proteins of the Bcl family. On the contrary, CD28 or IL-2 signaling does not prevent Fas-mediated death, but rather potentiates it. The mechanism behind this is not well understood, but IL-2 is known to enhance the expression of FasL and may promote the association of various proteins with the cytoplasmic domain of Fas that constitute a functional death complex.

1.2.2. Anergy

Anergy is a cellular state in which a lymphocyte is alive but fails to display certain functional responses when optimally stimulated through both its antigen-specific receptor and any other receptors that are normally required for full activation (Schwartz, 1996). Thus, anergic cells are functionally inactivated and are characterized by a block in their ability to produce IL-2 and to proliferate upon subsequent challenge with Ag presented on a mature antigen-presenting cell (APC) (i.e. with full costimulation) (Jenkins et al., 1987). Anergy is not an intermediate step to cell death, but rather persists for a prolonged time. TCR ligand is both necessary and sufficient to induce anergy (Quill and Schwartz, 1987).

1.2.2.1. Models of anergy

There are several different approaches that have been used to generate anergic T cells (reviewed in Schwartz, 1996; Lechler et al., 2001; Macian et al., 2004). Traditionally, anergy is induced by TCR occupancy in the absence of positive costimulation (i.e. CD28) or on the contrary in the presence of inhibitory costimulation (i.e. via CTLA4) (Greenwald et al., 2001). Also addition of IL-10 into a mixed lymphocyte reaction renders T cells anergic (Groux et al., 1996). Alternatively, anergy can be induced by using altered peptide ligands (Sloan-Lancaster et al., 1993) or in the presence of high concentrations of soluble peptides (O'Hehir et al., 1991; LaSalle and Hafler, 1994). Also immature dendritic cells proved to be a potent tolerogenic agent since they express only moderate levels of MHC class II and almost no costimulatory molecules

(Kubsch et al., 2003; Steinbrink et al., 2002). Anergic cells generated with these models possess different degree of unresponsiveness, which usually results from defective Ras signaling and blocked cytokine production (see below). Here I shall describe two commonly used anergy models – anergy induction in the absence of costimulation and the ionomycin-induced anergy.

a) Anergy induced by TCR occupancy in the absence of costimulation

The two-signal model proposes that a T cell requires both antigen recognition via the T-cell receptor and an additional costimulatory signal via CD28 or another costimulatory molecule (Bretscher and Cohn, 1970). TCR engagement alone in the absence of costimulation is insufficient to provide a stimulatory signal and to induce IL-2 production, but instead results in long lasting anergy, i.e. the cells fail to proliferate when restimulated with normal APC and antigen (Schwartz, 2003). Several models have been demonstrated to induce this type of anergy – antigen presentation on chemically fixed APCs, CD3 crosslinking with immobilized antibodies, purified MHC complexes with peptide (Jenkins and Schwartz, 1987; Jenkins et al., 1990; Wolf et al., 1994; Quill and Schwartz, 1987). The defect in proliferation is caused by a block in IL-2 production (Jenkins et al., 1987). Beside IL-2, anergic cells also possess reduced production of IL-3, IFNγ (interferon γ) and GM-CSF (granulocyte and monocyte-colony stimulating factor), whereas IL-4 secretion is unaffected (Jenkins et al., 1987; Trenn et al., 1992; Beverly et al., 1992). Interestingly, anergic cells are unable to proliferate to IL-4 (Chiodetti and Schwartz, 1992) or IL-12 (Quill et al., 1994) mediated signals. Additionally, anergic $CD4^+$ cells cannot provide help to B cells due to their impaired expression of CD40 ligand (Bowen et al., 1995). Anergic $CD8^+$ cells have a block in IL-2 production, but not in TCR-dependent cytotoxicity (Otten and Germain, 1991).

Costimulation. When fully stimulated, naïve T cells produce high amounts of IL-2. However, if only the TCR is triggered without costimulation, the amount of produced IL-2 is low and the cells enter an anergic state. Costimulation given up to 2 hours after TCR triggering is still sufficient to block the induction of anergy (Jenkins et al., 1988; Harding et al., 1992). Thus, costimulation provides not only the second signal needed for proliferation, but it also delivers signals that prevent anergy induction. This “second signal” pathway either prevents the production of the molecular inhibitors responsible for anergy, or the large amount of IL-2 produced upon costimulation prevents the expression of the inhibitors through an IL-2R signaling pathway. Alternatively, the inhibitor may be diluted out after multiple rounds of division that are

induced by IL-2. Costimulation via CD28 causes activation of PI3K (phosphatidylinositol-3-kinase) (Prasad et al., 1994), followed by the activation of JNK (c-Jun N-terminal kinase) (Saez-Rodriguez et al., 2007), which together with MAPKs (mitogen-activated protein kinases) activated by the TCR induces activation of the transcription factor AP-1 and augments IL-2 gene transcription (Su et al., 1994). Additionally, CD28 signaling increases the stability of IL-2 mRNA (Lindsten et al., 1989).

In summary, costimulation is critically important for the decision of the immune system to make a response or not (Janeway, 1992; Matzinger, 1994). Importantly, costimulatory receptors are upregulated during inflammation, infection and under other pathological conditions, therefore sensing their expression levels seems to be an ideal mechanism that enables T cells to make the decision between "non-infectious self" and "infectious non-self" (Medzhitov and Janeway, 2000).

Anergy reversal. The anergic state can be reversed by stimulation with exogenous IL-2 (Beverly et al., 1992). The reversal was demonstrated both on the level of cytokine production (Beverly et al., 1992) and by transcriptional activation of the IL-2 gene (Kang et al., 1992). This IL-2 responsiveness demonstrates that anergic cells are partially activated, in that they express a high-affinity IL-2 receptor upon their surface, and also confirms that it is indeed an unresponsive state instead of non-viability (Macian et al., 2004). The block in anergic cells can be also overcome by stimulation with phorbol ester plus ionomycin (Schwartz, 2003).

b) Ionomycin-induced anergy

In this model, anergy is induced simply by treating T cells with the calcium ionophore ionomycin (Jenkins et al., 1987; Trenn et al., 1992). This causes an influx of calcium without inducing diacylglycerol and thereby leads to the activation of the transcription factor NFAT (nuclear factor of activated T cells) without activating either AP-1 or NFκB. The selective activation of the calcium/NFAT pathway alone resembles the processes occurring in the previous model induced by TCR triggering without costimulation (see also 1.2.2.3.). Activation of NFAT alone leads to the transcription of a new set of genes believed to be responsible for the unresponsive state (Macian et al., 2002; Im and Rao, 2004). Since the ionomycin-induced anergy mimics the characteristics of anergy induced by TCR triggering alone, the ionomycin model is considered to be very similar to the one induced in the absence of costimulation (Jenkins et al., 1987).

1.2.2.2. Clinical applications of anergy

Establishing anergy is of particular interest in the treatment of patients with autoimmunity and after transplantation, as specific tolerance to self-antigen is desired without inducing total immunosuppression. Systemic or mucosal administration of antigens or altered peptide ligands causes TCR stimulation in the absence of costimulation. Tolerance induction depends upon the route of administration and the dose and form of the antigen. Soluble peptide:MHC complexes have been used to induce anergy in autoreactive $CD4^+$ cells of diabetic mice (Casares et al., 2002). In bone marrow transplantation, blockade of CD28-B7 costimulation by CTLA4-Ig induces long-lasting tolerance and extended graft survival (Wekerle et al., 2002).

Genetically modified DCs have also been used to treat allograft rejection and autoimmune diseases (Morel et al., 2003). Thus, tolerogenic DCs were effective in modulating long-term allograft survival (Guillot et al., 2003) and preventing autoimmune diabetes (Feili-Hariri et al., 2003), multiple sclerosis (Menges et al., 2002), myasthenia gravis (Yarilin et al., 2002) and collagen-induced arthritis (Morita et al., 2001).

1.2.2.3. Biochemical characteristics of anergic cells

Here I shall focus on anergy induced in the absence of costimulation, one of the most well studied and best characterized types of anergy. When describing changes within anergic cells though, one should distinguish between the induction of anergy and its maintenance.

Anergy induction. Calcium signaling was shown to be critical for anergy induction (Jenkins et al., 1987; Jenkins et al., 1990). CD28 signaling influences only the pathways of TCR signaling that do not induce calcium flux, thus the lack of CD28 costimulation causes an unbalanced signaling in which the calcium signal predominates (Macian et al., 2002). Anergic cells have been shown to possess increased levels of intracellular calcium (Gajewski et al., 1994; Gajewski et al., 1995). Calcium signaling results in the activation of NFAT, which normally cooperates with AP-1 to induce the expression of genes required for full activation (Hogan et al., 2003). Activation of NFAT alone without other transcription factors, however, leads to the transcription of a completely different set of genes encoding proteins that could function as negative regulators of TCR signaling (Macian et al., 2002; Im and Rao, 2004). Such proteins include phosphatases, proteases and transcriptional repressors. The RNA expression of anergy-associated genes was investigated by the group of Jan Buer using gene array analysis (Lechner et al., 2001).

Interestingly, fusing anergic and non-anergic T cells maintains the anergic phenotype, meaning that anergic T cells indeed express proteins that dominantly suppress TCR activation (Telander et al., 1999).

Maintenance of anergy. There are many factors believed to be important for the maintenance of the anergic state. These factors are upregulated during anergy induction and might be responsible for both inducing and also maintaining the unresponsive state. Some mechanisms interfering with the proper signal progression are found already at the membrane proximal level, i.e. at the level of Src kinases and LAT (Linker for activation of T cells). The Src family kinase Fyn has been implicated in anergy, as Fyn was shown to be upregulated in anergic cells both on the protein level (Quill et al., 1992; Welke and Zavazava, 2002) and in its kinase activity (Gajewski et al., 1994; Gajewski et al., 1995). Additionally, the CD4-Lck complex appears to be displaced from lipid rafts in cells anergized by dimeric peptide:MHC molecules and consequently the recruitment and phosphorylation of ZAP70 is reduced (Thomas et al., 2003). Recently, impaired palmitoylation of the adaptor protein LAT has been observed, leading to defective LAT localization within plasma membrane and its impaired phosphorylation (Hundt et al., 2006). A reduction in ZAP70 and LAT phosphorylation was also shown using *in vivo* anergized transgenic T cells (Utting et al., 2000).

Another mechanism hindering the activation of anergic cells is based on the regulation of IL-2 promotor transcriptional activity. A hallmark of anergic cells is the block in Ras activation (Fields et al., 1996; Rapoport et al., 1993). This leads to a decrease in the activities of ERK and JNK (Li et al., 1996) and consequently to a failure to activate AP-1 (Kang et al., 1992), a transcription factor critical for IL-2 production. Additionally, increased expression of Nil-2a, a negative regulator of AP-1 transactivation, was observed (Becker et al., 1995). Anergic cells also overexpress Tob, a protein enhancing the binding of Smad proteins to the negative regulatory element in the IL-2 promoter (Tzachanis et al., 2001). Additionally, the CREB/CREM (cAMP response element binding protein/cAMP responsive element modulator) repressor complexes bind to the IL-2 promoter in anergic cells (Powell et al., 1999).

Three E3 ubiquitin ligases were also shown to be upregulated in anergic cells – GRAIL (gene related to anergy in lymphocytes), Itch and Cbl-b that specifically ubiquitinate and degrade important signaling proteins like PLCγ1 (phospholipase C gamma 1) and PKCθ (protein kinase C theta) (Anandasabapathy et al., 2003; Heissmeyer et al., 2004). Interestingly, T cells from Itch-

and Cbl-b-deficient mice are resistant to anergy induction (Heissmeyer et al., 2004; Jeon et al., 2004).

Another key feature of anergic cells is the lack of proliferation caused by a block in the cell cycle progression at the G1 to S stage transition (Gilbert et al., 1992). To this end, increased expression of p27kip1 and p21cip1 were found and these inhibitors of the cyclin-dependent kinases (Cdk) were proposed to promote cell cycle arrest at the G1 phase (Boussiotis et al., 2000; Jackson et al., 2001). Surprisingly, anergy still can be induced in p27kip1 and p21cip1 deficient cells (Verdoodt et al., 2003). However, when p27kip1 lacking the Cdk-binding domain is expressed in murine T cells, these cells proliferate under tolerizing conditions suggesting that intact p27kip1 is indeed required for anergy induction (Li et al., 2006).

1.2.3. Regulatory T cells (Tregs) (reviewed in Jonuleit and Schmitt, 2003)

Regulatory T cells are a specific population of T cells with suppressive properties. Two different subsets of Tregs can be distinguished based upon their suppressive mechanisms. Naturally occurring $CD4^+CD25^+$ Tregs were suggested to exert their regulatory activities probably via cell-cell contact, although the membrane molecules responsible have not yet been fully identified. $CD4^+CD25^+$ regulatory T cells are hyporesponsive to TCR stimulation, but they remain responsive to IL-2. However, they need to be activated through their TCR in order to suppress the proliferation of conventional $CD4^+CD25^-$ cells. Once activated, their suppressive ability is nonspecific to the antigen, meaning that suppression is independent of antigen specificity of the responding population. The exact mechanism of their suppressive effects is not clear, but it results in the inhibition of IL-2 transcription.

The second subset consists of Th3 and Tr1 cells, which develop from conventional $CD4^+CD25^-$ cells in the periphery and represent altered states of differentiation rather than a unique cell lineage. Their suppressory properties are independent of cell contact and thus are mediated via soluble suppressive cytokines. Tr1 cells were found to produce large amounts of IL-10, whereas Th3 cells preferentially secrete TGF-β.

1.2.4. T-cell ignorance

Self-reactive T cells can exist in the periphery if their antigen is sequestered from them or is not presented in its immunogenic form. In this case, these autoreactive T cells persist in the

periphery without meeting their cognate antigen and thereby not becoming activated (Walker and Abbas, 2002). Additionally, there are specialized organs that are immunologically privileged, e.g. the brain, eyes, testes and ovaria. These organs must be protected from the consequences of inflammatory damage, which would destroy the microanatomical structure of these organs (Streilein, 1996). Therefore, these organs possess mechanisms to attenuate both the innate and adaptive immune response, e.g. the presence of blood-tissue barriers, reduced migration of dendritic cells due to limited number of draining lymphatic vessels, production of immunosuppressive and anti-inflammatory cytokines like TGF-β and expression of death inducing molecules like FasL (Chen et al., 1998; Griffith et al., 1995).

1.3. Ras proteins

Ras proteins are members of the guanine nucleotide binding protein superfamily. They are highly conserved, ubiquitously expressed and play an important role in signaling pathways activating transcription factors involved in cytokine gene induction in lymphocytes (Downward et al., 1990). Ras is activated in a rapid and sustained manner by the TCR, but also by some cytokines, mainly IL-2, IL-3 and GM-CSF (Satoh et al., 1991; Graves et al., 1992). Ras was originally identified as a proto-oncogene and its mutated forms are found in many human tumors (Bos, 1989). These mutations usually confer resistance to the GTPase-activating proteins or decrease GTP hydrolysis. Additionally, Ras is essential for thymocyte development, as it mediates some of the pre-TCR signals during β selection and is required for positive selection of thymocytes (Swat et al., 1996; Alberola-Ila et al., 1996).

1.3.1. Ras structure and localization

There are three main isoforms of Ras expressed in humans – N-Ras, K-Ras and H-Ras. They are highly homologous, with conserved effector-binding domains, but distinct hypervariable regions constituting the last 23 amino acids, which may be responsible for their distinct localization. Ras proteins contain a CAAX box at their C-terminus, which becomes isoprenylated upon the cysteine residue, the last three amino acids are cleaved off and consequently the new C-terminal cysteine is methylated (Hancock et al., 1989). Isoprenylation is essential for their biological activity as it anchors Ras to the membrane (Willumsen et al., 1984; Hancock et al., 1989). Additionally, H- and N-Ras proteins can be palmitoylated on cysteines adjacent to the

CAAX box (Hancock et al., 1989) and this is presumably responsible for their targeting into lipid rafts. Whereas K-Ras is constitutively located outside of lipid rafts, H-Ras can shuffle laterally within the membrane depending upon its activation state (Prior et al., 2001). Inactive H-Ras sits in lipid rafts, however the activation of H-Ras redistributes it from the lipid rafts into the non-raft membrane by a mechanism requiring its hypervariable region. This redistribution is necessary for the proper activation of H-Ras and interaction with its effectors (Prior et al., 2001).

1.3.2. Ras regulation

Ras exists in one of two forms – either a GDP-bound form that is catalytically inactive or a GTP-bound form, which is active and interacts with its downstream effectors. Ras is able to rapidly cycle between these two forms and this cycling is controlled by the balanced activities of two groups of proteins. Guanine nucleotide exchange factors (GEFs) promote the transition from the inactive GDP-bound form to the active GTP-bound state. This activity is opposed by GTPase-activating proteins (GAPs), which stimulate the intrinsic Ras GTPase activity resulting in hydrolysis of bound GTP to GDP thereby inactivating the protein (see **Figure 1.1.**). Note that in lymphocytes, there are relatively high basal levels of nucleotide exchange onto Ras (Genot and Cantrell, 2000). Importantly, there has been a discrepancy whether Ras activation and signaling occurs at the plasma membrane or rather upon endomembranes of the Golgi apparatus (Perez de

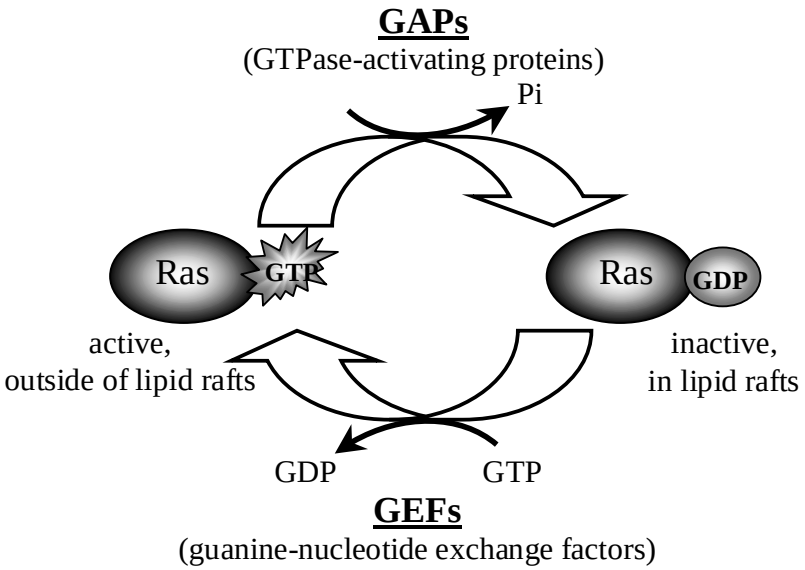


Figure 1.1. Regulation of Ras. Inactive Ras (Ras-GDP) is located in lipid rafts where it is loaded with GTP by GEFs and consequently moves out of lipid rafts. Its GTPase activity is enhanced by GAPs thereby inactivating the protein.

Castro et al., 2004; Rocks et al., 2005). However, a recent approach has enabled visualization of endogenous Ras and has illustrated preferential Ras activation at the plasma membrane (Augsten et al., 2006).

1.3.3. RasGEFs (Ras guanine-nucleotide exchange factors)

There are two RasGEFs known in T cells, Sos (son of sevenless) and RasGRP. Sos forms a complex with the SH3 (Src homology 3) domains of the cytosolic adaptor protein Grb2 (growth factor receptor-bound protein 2) (Cheng et al., 1998). Upon TCR triggering, Grb2 binds to phosphorylated LAT, bringing Sos to the plasma membrane, thereby inducing Ras activation (Zhang et al., 2000). The other GEF, RasGRP, contains a diacylglycerol/phorbol-ester binding C1 domain (O'Ebinu et al., 1998; Tognon et al., 1998). TCR stimulation induces tyrosine phosphorylation of LAT and recruitment and activation of PLCγ1, which hydrolysis phosphoinositide-4,5-bisphosphate (PIP_2) to produce inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (see 1.4.3.). DAG, in turn, is bound by the C1 domain of RasGRP recruiting it to the plasma membrane. However, the contribution of each GEF pathway to Ras activation is not clear. On one hand, the PLCγ1-mediated pathway was found to be required for Ras activation in Jurkat T cells (Yablonski et al., 1998), while on the other hand, experiments with peripheral blood T cells showed Ras activation even in the absence of PLCγ1 activity and DAG production (Izquierdo et al., 1992). Thus, knocking down one or the other GEF directly in primary T cells should help to resolve this issue of Ras activation and this approach is currently being performed in our institute by Dr. Luca Simeoni.

1.3.4. RasGAPs (Ras GTPase-activating proteins)

The ever-growing family of RasGAPs contains 14 members in human so far. The best characterized RasGAPs in T cells are p120RasGAP, Neurofibromin 1 (NF1) and CAPRI. All of them are ubiquitously expressed. CAPRI contains tandem C2 domains, which recruit the protein to the plasma membrane in a calcium dependent manner and thereby CAPRI switches off the Ras pathway following elevated calcium levels (Lockyer et al., 2001). NF1 was originally identified as the protein mutated in patients with neurofibromatosis (Bernards, 1995). It seems that beside Ras regulation, NF1 may also link Ras signaling to tubulin as NF1 was found to interact with microtubules and tubulin (Bollag et al., 1993; Xu and Gutmann, 1997).

p120RasGAP contains two SH2 (Src homology 2) domains and one SH3 domain that mediate its association with other proteins and additionally a PH (pleckstrin homology) and C2 domain (protein kinase C conserved region 2 domain) that are responsible for binding to membrane phospholipids. The central region of the C2 domain is known as a CaLB domain (calcium and lipid binding domain). The CaLB domain interacts with the calcium-dependent phospholipid-binding protein annexin VI in response to increased intracellular calcium and thus may increase p120RasGAP association with the plasma membrane in the presence of elevated calcium (Chow et al., 1999). p120RasGAP was shown to interact with the phosphorylated adaptors p62dok (downstream of kinase) (Yamanashi and Baltimore, 1997) and Sam68 (Src-associated in mitosis) (Guitard et al., 1998; Jabado et al., 1998) upon T-cell stimulation and these interactions are supposed to regulate GAP activity either by changing its conformation or by recruiting GAP to the appropriate location. Furthermore, p120RasGAP associates with p190RhoGAP and thereby contributes to the coordinated downregulation of both Ras and Rho GTPases. Additionally, the function of p120RasGAP may be also regulated through its tyrosine phosphorylation and binding to the Src family kinase Lck (Amrein et al, 1992). Interestingly, mice deficient for p120RasGAP die *in utero* by embryonic day 10 due to defects in vascular and neuronal development, indicating the importance of proper Ras regulation during embryogenesis (Henkemeyer et al., 1995).

Although a downregulation of RasGAP activity has long been observed upon TCR stimulation (Downward et al., 1990; Izquierdo et al., 1992), the molecular details of antigen receptor-mediated GAP regulation are not understood. Additionally, the contribution of GAP regulation to the overall Ras equilibrium has also been ignored for the past few years (Genot and Cantrell, 2000; Cantrell, 2003).

1.3.5. Ras effectors

The best characterized Ras effector pathway is the MAPK (mitogen-activated protein kinase) pathway - Ras-Raf1-ERK cascade (Marshall, 1994). GTP-bound Ras recruits the serine/threonine kinase Raf-1 to the membrane, where Raf becomes activated and in turn phosphorylates Mek, which in turn phosphorylates and activates both ERK1 and ERK2. The main substrate for ERK is the transcription factor Elk-1, which regulates the SRF (serum response factor) controlling c-Fos and Egr expression (Marshall, 1994; Turner and Cantrell, 1997). The Ras-ERK pathway also controls serine phosphorylation of STAT3 (signal transducer and activator of transcription), thus

forming a link between the antigen receptor and the cytokine signaling pathways (Ng and Cantrell, 1997). Additionally, a direct association between Ras and PI3K leading to PI3K activation was observed (Rodriguez-Viciana et al., 1994; Rubio et al., 1997), however the evidence of such an interaction and its physiological role in T cells is obscure.

1.4. T-cell signaling

1.4.1. T-cell receptor (TCR)

Peptide/MHC complexes bind to the variable regions within the extracellular portion of the T-cell receptor. During thymocyte development, the genes encoding the variable region of the T-cell receptor undergo somatic recombination resulting in a unique random combination of gene segments. This ensures that each T cell has a unique TCR specificity. Note that all of the TCRs within one T cell possess a single specificity. The T-cell receptor exists as a multisubunit complex consisting of one α and one β chain, which form the antigen binding subunit that is capable of specific recognition of peptide/MHC. However, these chains are themselves not able to transmit this signal. Instead, additional immunoreceptor associated signal-transducing proteins are required to perpetuate the signal. Therefore, the αβ heterodimer noncovalently associates with CD3 subunits, namely one heterodimer consisting of γ and ε and the other of δ and ε chain. Additionally, two ζ chains pair with the TCR (Weissman, 1994). All of these proteins possess specialized signaling motifs called immunoreceptor tyrosine-based activation motifs (ITAMs), which are dually phosphorylated by a member of the Src family of protein tyrosine kinases, namely Lck. The ITAM sequence is D/ExYxxL$(x)_n$YxxL where n is between 6 and 8 amino acids (Reth et al., 1989). The spacing between tyrosines is believed to be crucial for signaling. CD3-γ, -δ and -ε chains each contain one ITAM, whereas the TCR-ζ chains each contain three of them (Cambier, 1995). Thus the TCR possesses 10 ITAMs in total. Both tyrosines within the ITAM are phosphorylated and serve as binding sites for proteins containing tandem SH2 domains, mainly ZAP70, a member of the Syk family protein tyrosine kinases. The six tyrosines within TCR-ζ chain are sequentially phosphorylated in a highly ordered manner and their complete phosphorylation is dependent upon the strength of TCR occupancy (Kersh et al., 1998). Interestingly, since T cells constantly encounter self-peptide/MHC complexes in the periphery, there is a low level signaling (so called tonic signaling) within T cells, which induces constitutive basal phosphorylation of TCR-ζ chains even in resting state (Pitcher et al., 2003). This

phosphorylation is believed to be mediated by a pool of Fyn kinase constitutively associated with the TCR complex (Samelson et al., 1990; Timson Gauen et al., 1992).

Interestingly, resting T cells were shown to express a mixture of monovalent and multivalent TCR complexes upon the membrane, having two or more (up to 20) αβ ligand-binding subunits (Schamel et al., 2005). This observation seems to provide an answer as to how the T cell maintains high sensitivity and specificity of interaction with pMHC despite low-affinity binding of TCR-pMHC. At low concentrations of pMHC, only the multivalent complexes become phosphorylated, whereas the monovalent receptors are phosphorylated only when the antigen concentration increases. Thus, the multivalent complexes may be responsible for sensing low antigen doses, where they can augment the sensitivity to antigen by increasing the avidity or by spreading the signal through cooperative interactions between different receptors. On the contrary, the monovalent receptors may be responsible for producing the concentration dependent response even at high antigen doses, when the multivalent complexes might be saturated (Schamel et al., 2005). Recently, it has been shown that full TCR activation requires both receptor clustering and conformational changes at CD3 that are mediated by cooperative rearrangements of two TCR-CD3 complexes (Minguet et al., 2007).

1.4.2. Lipid rafts

Lipid rafts are also called glycosphingolipid-enriched membrane microdomains (GEMs) and were originally described as detergent-resistant membranes (DRMs). Lipid rafts are islets within the plasma membrane enriched in glycosphingolipids, sphingomyelin and cholesterol (Brown and London, 1998) and have been found in most cell types studied. Because of the high content of cholesterol and lipids with saturated acyl chains, lipid rafts form a specific ordered liquid phase separated from the less-ordered bulk membrane (Schroeder et al., 1998). This ensures their relative resistance to solubilization by some types of detergents (e.g. Brij 58, Triton X-100, NP-40) and enables their isolation by sucrose density gradient ultracentrifugation (Brown and Rose, 1992).

The lipid raft-associated components on the extracellular side of the membrane are anchored to the outer membrane leaflet via a glycosylphosphatidylinositol anchor. The cytoplasmic side of lipid rafts is associated mainly with heterotrimeric G proteins, Src family kinases and some transmembrane adaptor proteins. The signal responsible for targeting proteins into the lipid rafts

is believed to be fatty acid modification of the protein, mainly acylation with long saturated fatty acids, e.g. myristoylation and/or palmitoylation (Melkonian et al., 1999). Whereas myristoylation occurs co-translationally, palmitoylation is a post-translational event. Palmitoylation is also a reversible process and may occur at any time during a life span of the proteins. This enables that certain proteins can be targeted into the lipid rafts once they are required for the signaling and moved out again (i.e. depalmitoylated) when they are no longer needed. Indeed, the initiation of TCR signaling is accompanied by the aggregation of lipid rafts and the lateral recruitment of the T-cell receptor components towards these aggregates (Xavier et al., 1998; Janes et al., 1999; Kosugi et al., 1999). Merging of the immunoreceptor complexes with the lipid rafts brings the TCR chains closer to the raft-associated Src family kinases and enables phosphorylation of their ITAMs and the initiation of the signaling. Furthermore, the critical adaptor protein LAT is also localized in lipid rafts (Zhang et al., 1998). Therefore, lipid rafts seem to act as signalosomes important for both the initiation and spatial organization of immunoreceptor signaling. However, the exact role and requirement of lipid rafts for TCR signaling is still a lively discussed question.

1.4.3. T-cell signaling pathways (see Figure 1.2.)

The signaling pathways activated upon triggering of the T-cell receptor have been extensively studied and are reviewed in many publications (Cantrell, 1996; van Leeuwen and Samelson, 1999; Kane et al., 2000; Samelson, 2002; Cantrell, 2002). Lymphocyte activation is initiated by the T-cell receptor encountering its antigen presented in complex with an MHC molecule. This in turn leads to the activation of Src family kinases, however the exact mechanism as to how Src kinases are activated is not well understood. The current model proposes that a subpopulation of Lck constitutively associated with the coreceptor CD4/CD8 becomes activated upon coreceptor dimerization (Moldovan et al., 2002). These activated Lck molecules then phosphorylate the tandem tyrosine residues of the ITAMs located within the cytoplasmic tail of the CD3 molecules and the zeta chains. Phosphorylated ITAMs provide docking sites for the tandem SH2 domains of the Syk family kinase ZAP70, which is thereby recruited to the plasma membrane and itself becomes activated via phosphorylation by Lck (Chan et al., 1992; Chan et al., 1995). ZAP70 consequently trans-autophosphorylates to achieve full activation. The main substrate for ZAP70 is the transmembrane adaptor protein LAT (Zhang et al, 1998), which functions as a signaling scaffold for the Grb2/Sos complex and the Ca^{2+}-initiation complex. When phosphorylated, LAT

recruits several key signaling molecules containing SH2 domains, such as Grb2, Gads (Grb2-related adaptor downstream of Shc) and PLCγ1 (Zhang et al., 2000). The SH2 domain of Grb2 and Gads is flanked by two additional SH3 domains and these adaptors can thus recruit additional signaling molecules to LAT. Grb2 binds the guanine nucleotide exchange factor Sos, which then contributes to activation of the GTPase Ras. Gads is constitutively associated with SLP-76 (SH2 domain containing leukocyte protein of 76 kDa), which when phosphorylated binds the SH2 domain of the Tec-family tyrosine kinase Itk. Itk then phosphorylates PLCγ1 leading to its activation. Activated PLCγ1 cleaves membrane phosphoinositide-4,5-bisphosphate (PIP_2) to produce the second messengers IP_3 and diacylglycerol.

IP_3 causes the mobilization of Ca^{2+} from intracellular stores into the cytoplasm, where it binds to the calcium-binding protein calmodulin. Calmodulin in turn activates calcineurin, which dephosphorylates the transcription factor NFAT. Upon dephosphorylation, NFAT moves into the nucleus to initiate the transcription of specific genes.

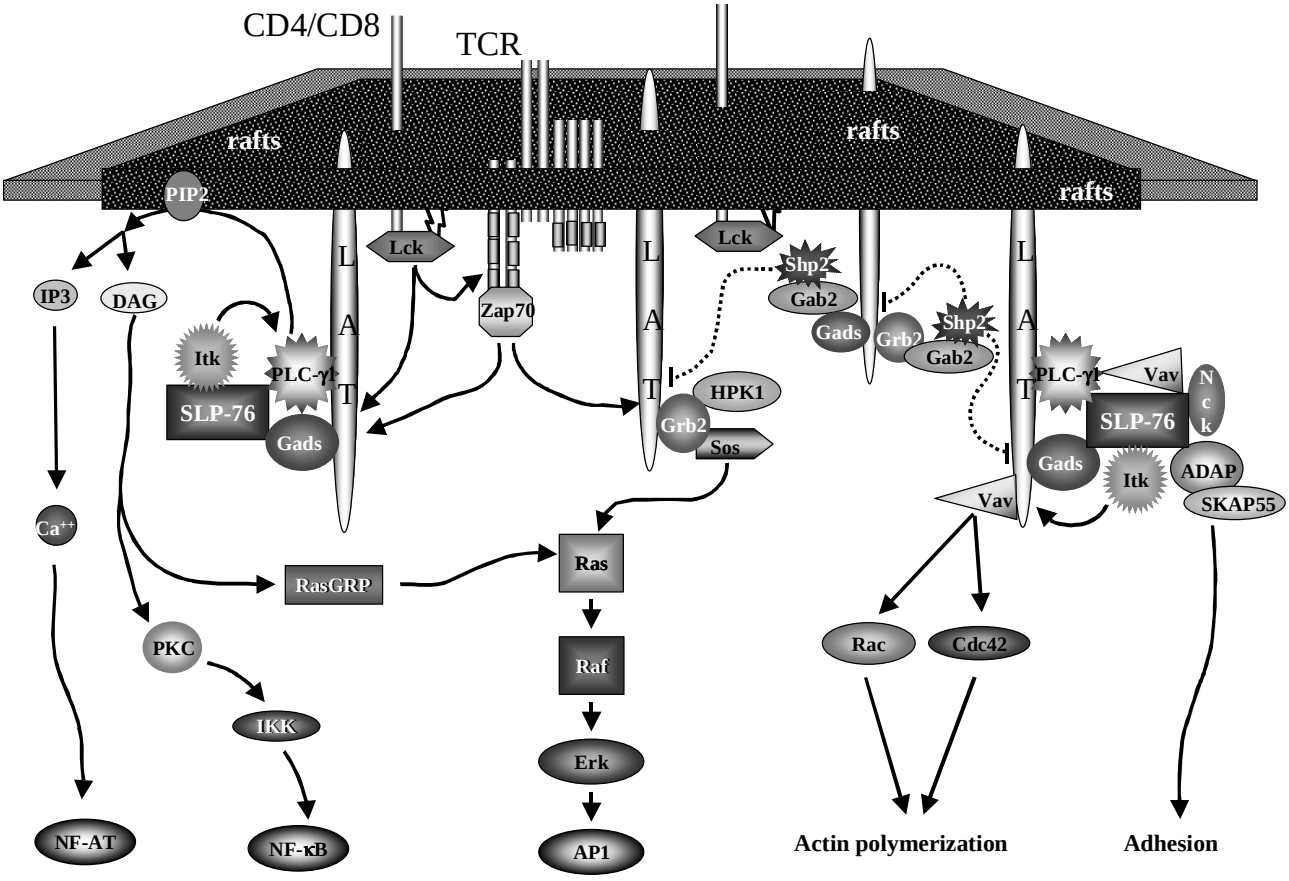


Figure 1.2. T-cell receptor signaling pathways. A scheme of the main signaling pathways activated upon TCR triggering is presented. See 1.4.3. for further details.

Diacylglycerol activates conventional and novel protein kinase C (PKC) isotypes and the guanine nucleotide exchange factor RasGRP. Among the PKC isoforms, PKC theta is of special interest, as it is required for activation of the transcription factor NF-kB. Additionally, PKC theta phosphorylates RasGRP, thereby further enhancing RasGRP activation (Roose et al., 2005). The function of RasGRP appears to be the same as that of the Grb2/Sos complex, namely the activation of the Ras/MAPK pathway leading to the activation of a transcription factor AP-1. At present, it is unclear whether Grb/Sos or the RasGRP pathway is the main contributor to Ras activation.

The Tec-kinase Itk also phosphorylates LAT resulting in the recruitment of the guanine nucleotide exchange factor Vav both to SLP-76 (Wu et al., 1996; Tuosto et al., 1996) and directly to LAT (Perez-Villar et al., 2002). Vav then activates small G-proteins of the Rho family, i.e. Cdc42 and Rac, required for the activation of WASP (Wiskott-Aldrich syndrome protein) and cytoskeletal reorganization (Crespo et al., 1997; Fischer et al., 1998). Additionally, SLP-76 binds the ADAP (Adhesion and degranulation promoting adaptor protein)/SKAP55 (Src kinase associated phosphoprotein of 55 kDa) complex, which then becomes phosphorylated by Fyn. The ADAP/SKAP55 module then recruits GTPase Rap1 to the membrane, thereby increasing integrin affinity, inducing integrin clustering and integrin-mediated adhesion further stabilizing conjugate formation between the T cell and the antigen-presenting cell (Griffiths et al., 2001; Peterson et al., 2001; Kliche et al., 2006), leading to formation of the immune synapse.

1.5. Adaptor proteins

Adaptors are proteins that lack both enzymatic and transcriptional activities. Instead, they participate in the regulation of lymphocyte activation by mediating constitutive and/or inducible protein-protein or protein-lipid interactions via their modular interaction domains. The role of adaptor proteins in lymphocyte signaling has been extensively reviewed (Leo et al., 2002; Horejsi et al., 2004; Togni et al., 2004; Simeoni et al., 2005). Adaptors can be divided into two main groups: transmembrane adaptor proteins (TRAPs) and cytosolic adaptor proteins (CAPs). Upon TCR triggering, CAPs are recruited from the cytosol to the plasma membrane where they bind via their modular domains to other critical signaling molecules like receptors, adaptors and enzymes and thereby enable the formation of the various multiprotein complexes that are required for signal transduction (see 1.4.3.).

1.5.1. Transmembrane adaptor proteins (TRAPs)

TRAPs are integral membrane proteins possessing a short extracellular domain, which does not bind ligand. Their transmembrane domain is followed by a long cytoplasmic tail that lacks any modular protein-protein interaction domains, but contains proline rich regions and/or multiple tyrosine based signaling motifs (TBSMs). The TBSM is a short peptide sequence containing a core tyrosine residue (YxxV/L/I). These residues become phosphorylated by Src and/or Syk family protein tyrosine kinases after antigen receptor triggering and provide binding sites for the SH2 and PTB domains of intracellular signaling and effector molecules, with the binding specificity being determined by the amino acids surrounding the core tyrosine residue. By recruiting these proteins to the plasma membrane, transmembrane adaptors allow the nucleation and formation of membrane associated signaling scaffolds required for the propagation of receptor-mediated signals into the intracellular compartment. Notably, TRAPs have also recently been suggested as potential diagnostic/prognostic markers in hematopathological studies for their distinct expression patterns in different types of human

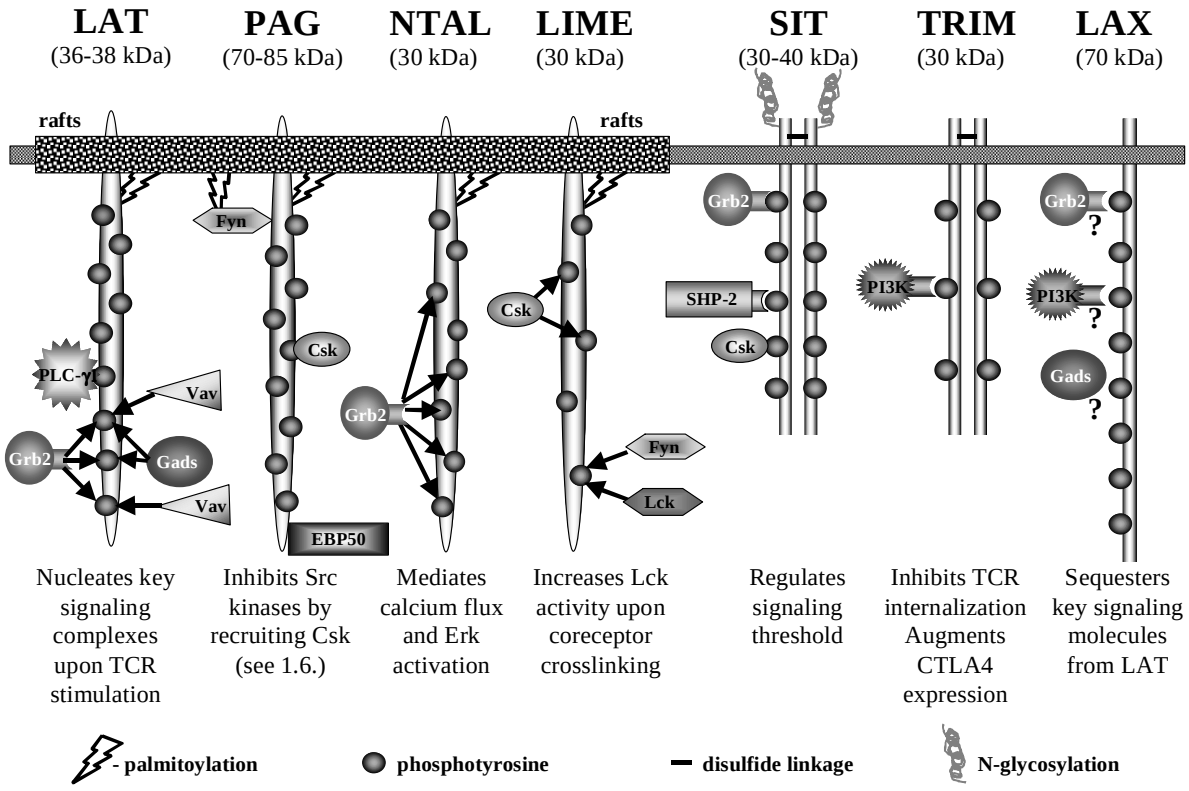


Figure 1.3. Overview of known TRAPs (with their MW). Structure, localization, known interacting partners and the main function for each adaptor are shown. Arrows show known binding sites for the proteins. Binding sites within LAX are unidentified yet.

lymphoid neoplasms (Tedoldi et al., 2006).

So far, seven transmembrane adaptor proteins have been identified – LAT, the T-cell receptor interacting molecule (TRIM), SHP-2 interacting transmembrane adaptor protein (SIT), the phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) also called the Csk-binding protein (Cbp), the non-T cell activation linker (NTAL) also called the Linker for activation of B cells (LAB), the Lck-interacting molecule (LIME), and the Linker for activation of X cells (LAX) (see **Figure 1.3.**).

The TRAPs can be further subdivided into two groups: the TRAPs associated with lipid rafts, which include LAT, PAG, NTAL and LIME, and the TRAPs localized outside of lipid rafts – SIT, TRIM and LAX. The raft-associated TRAPs are monomeric type III transmembrane proteins that possess a palmitoylation motif CxxC juxtaposed to the transmembrane region. This motif becomes palmitoylated and is believed to be responsible for the targeting of these proteins into lipid rafts. The non-raft TRAPs are either monomeric (LAX) or disulfide-linked homodimers (SIT and TRIM) (see **Figure 1.3.**).

1.6. PAG (Phosphoprotein associated with glycosphingolipid-enriched microdomains; also called Csk-binding protein, Cbp) [hereafter referred to as PAG]

1.6.1. Structure and expression of PAG

The adaptor protein PAG is unique among the transmembrane adaptor proteins as it is expressed ubiquitously rather than being restricted to only hematopoietic cells, suggesting a more general function in the regulation of cell activation and differentiation. PAG is strongly expressed in lymphocytes and monocytes and weakly in neutrophils, but the expression of PAG-encoding mRNA was found in almost all tissues examined, with the highest levels in the immune system, developing brain, lung, heart, testis and placenta (Brdicka et al., 2000; Kawabuchi et al., 2000).

PAG is a type III transmembrane protein, meaning that its initial methionine is not followed by a typical signal sequence, but rather the N-proximal amino acids regulate its insertion into the membrane during protein synthesis (Brdicka et al., 2000). PAG consists of a short extracellular domain (16 amino acids), a single membrane-spanning hydrophobic domain (20 amino acids) and a long cytoplasmic tail, in total having 432 amino acids in humans (429 in mouse and 425 in rat). The extracellular part appears to lack any external ligand and no functional significance has been shown so far.

PAG predominantly localizes to the plasma membrane, namely into the lipid rafts or GEMs (Brdicka et al., 2000; Kawabuchi et al., 2000). Its cytoplasmic domain contains a dicysteine motif CSSC juxtaposed to the transmembrane region. This motif was shown to be palmitoylated and this is believed to be responsible for targeting PAG into the lipid rafts. The exact role of this motif and its importance for PAG function is the thesis topic of another PhD student in the lab, Anita Posevitz-Fejfar (Posevitz-Fejfar et al., 2007).

The cytoplasmic tail of PAG contains ten tyrosines, nine of which are found within so called tyrosine-based signaling motifs. These are potential phosphorylation sites for Src kinases and thus potential binding sites for PTB or SH2 domain-containing proteins. Six of them are arranged into three ITAM-like motifs, but with a longer spacing between the tyrosines. *In vitro* GST-SH2 pull-down assays revealed that phosphorylated PAG is capable of binding the tandem SH2 domains of ZAP70 and Syk as well as the SH2 domains of Lck, Fyn, Lyn, Csk, Shc, Vav, RasGAP and PI3K (Brdicka et al., 2000; Durrheim et al., 2001). Additionally, the cytoplasmic domain contains multiple Ser and Thr residues (12 serines and 10 threonines) that are potential sites of phosphorylation by casein kinase 2 and protein kinase B and C. Furthermore, PAG contains two proline rich regions that may bind SH3 domain-containing proteins. The overall acidic nature of PAG and its multiple sites of phosphorylation result in an anomalous binding of SDS and retarded migration on SDS-PAGE leading to an apparent molecular mass of 70 – 85 kDa rather than predicted MW of 47 kDa (Brdicka et al., 2000; Kawabuchi et al., 2000).

1.6.2. Interacting partners of PAG

While it was suggested that multiple proteins could bind to PAG, only three proteins have been reproducibly shown to be associated: Csk (Brdicka et al., 2000; Kawabuchi et al., 2000), Fyn (Brdicka et al., 2000) and EBP50 (Brdickova et al., 2001; Itoh et al., 2002). The interaction between PAG and Fyn was shown to be independent of phosphorylation and thus it was proposed to be mediated via the SH3 domain of Fyn binding to a proline-rich region of PAG (Brdicka et al., 2000). The mapping of the Fyn binding site within PAG is also a topic of the PhD thesis of Anita Posevitz-Fejfar. In contrast, the interaction of Csk (C-terminal Src kinase) with PAG requires tyrosine phosphorylation of PAG by Src family kinases. Mutational analysis has demonstrated that this association is mediated primarily via the phosphorylation of tyrosine 317 of PAG (in human; Y^{314} in mouse and rat), which is then bound by the SH2 domain of Csk

(Brdicka et al., 2000; Kawabuchi et al., 2000). An additional Csk binding site at tyrosine 299 has been suggested (Lindquist, unpublished observation).

Fyn is the main kinase responsible for PAG phosphorylation and thereby mediates Csk recruitment to PAG. Fyn deficient T cells show impaired PAG phosphorylation, Csk recruitment and thus reduced Csk activity towards Lck (Yasuda et al., 2002; Shima et al., 2003; Filby et al., 2007). However other kinases like Lck and Lyn may be involved in PAG phosphorylation (Brdicka et al., 2000; Ohtake et al., 2002).

1.6.3. The PAG phosphatase

The phosphorylation of PAG is a tightly regulated process as it is very rapidly dephosphorylated upon TCR triggering. Although several attempts have been made to identify the PAG phosphatase, they have however not brought clear results. On one hand, CD45, a positive regulator of TCR signaling, appears to be the PAG phosphatase, since CD45 deficient cells have enhanced basal phosphorylation of PAG with basically no detectable decrease upon stimulation (Davidson et al., 2003). On the other hand, PAG still becomes dephosphorylated in Jurkat cells lacking CD45 (Brdicka et al., 2000). Furthermore, CD45 expression is restricted only to hematopoietic cells and thus other PAG phosphatases must exist. One candidate would be PEP (PEST-enriched phosphatase), which can directly associate with the SH3 domain of Csk (Cloutier and Veillette, 1996). PEP is also able to dephosphorylate the activatory tyrosine within Src kinases (Gjorloff-Wingren et al., 1999; Cloutier and Veillette, 1999). However, mice deficient in PEP show normal PAG dephosphorylation upon stimulation (Davidson et al., 2003). Also experiments on protein tyrosine phosphatase PTPα deficient cells excluded this phosphatase as a potential candidate (Maksumova et al., 2005). Additionally, the SH2 domain-containing phosphatases SHP-1 or SHP-2 might contribute to PAG dephosphorylation, especially as SHP-1 was found to be recruited to lipid rafts upon TCR stimulation (Kosugi et al., 2001). PAG was, however, again found to be normally dephosphorylated in stimulated thymocytes from SHP-1 deficient mice (Davidson et al., 2003). Interestingly, SHP-2 was found in a complex with PAG and was shown to influence PAG dephosphorylation. Furthermore, hyperphosphorylation of endogenous PAG and sustained Csk recruitment were observed in SHP-2 deficient cells (Zhang et al., 2004). However, this observation was demonstrated only in fibroblasts and only upon growth factor stimulation. Importantly, as mentioned above, PAG is ubiquitously expressed,

whereas the phosphatases are usually specific to certain cell types. Thus, it appears that several distinct phosphatases could be responsible for PAG dephoshorylation depending upon the cell type. Also note, that, due to tonic signaling (see 1.7.3.), PAG is phosphorylated in resting T cells, but in mast cells is not and becomes phosphorylated only upon FcεRI triggering (Ohtake et al., 2002). Therefore, the PAG phosphatase(s) seem to be also differentially regulated in different cell types.

1.6.4. Function of PAG

The main function of PAG seems to be the recruitment of Csk, a negative regulator of Src family kinases, to the plasma membrane; thereby setting the threshold for activation and keeping cells in a resting state. PAG is constitutively phosphorylated in resting T cells and binds the tyrosine kinase Csk. The activity of Csk increases upon binding to PAG (Takeuchi et al., 2000). Csk in turn phosphorylates the C-terminal inhibitory tyrosine within Src kinases and keeps them under tonic inhibition in the resting state (see **Figure 1.4.**, bottom right panel). Upon TCR triggering, PAG becomes rapidly dephosphorylated by a yet unknown phosphatase at the Csk binding site, leading to the release of Csk. This enables the activation of Src kinases and the initiation of T-cell signaling (see **Figure 1.4.**, top panel). However, when Fyn becomes activated, it re-phosphorylates PAG after several minutes (see **Figure 1.4.**, bottom left panel), recruiting Csk back to the plasma membrane where Csk inhibits Src kinases by phosphorylation of their inhibitory tyrosine. In this way, the Src kinases become inactivated and signaling is shut down (Brdicka et al., 2000; Kawabuchi et al., 2000; Torgersen et al., 2001). Fitting with this model, the overexpression of PAG decreases overall tyrosine phosphorylation and inhibits TCR-mediated proximal events like Ca^{2+} flux (Davidson et al., 2003), downregulates TCR mediated NFAT activation in Jurkat T cells (Brdicka et al., 2000), IL-2 production both in Jurkat T cells (Itoh et al., 2002) and in transgenic mice (Davidson et al., 2003) and causes a block in cell proliferation (Davidson et al., 2003).

Interestingly, this proliferative defect was partially restored by exogenous IL-2 and the production of IL-4 and IFN-γ was not affected by PAG overexpression. On the contrary, mutation of the Csk binding site Y^{317} (respectively Y^{314} in mice) results in a drastic reduction of PAG phosphorylation, complete abrogation of Csk recruitment and the restoration of calcium flux, IL-2 production and cell proliferation (Davidson et al., 2003).

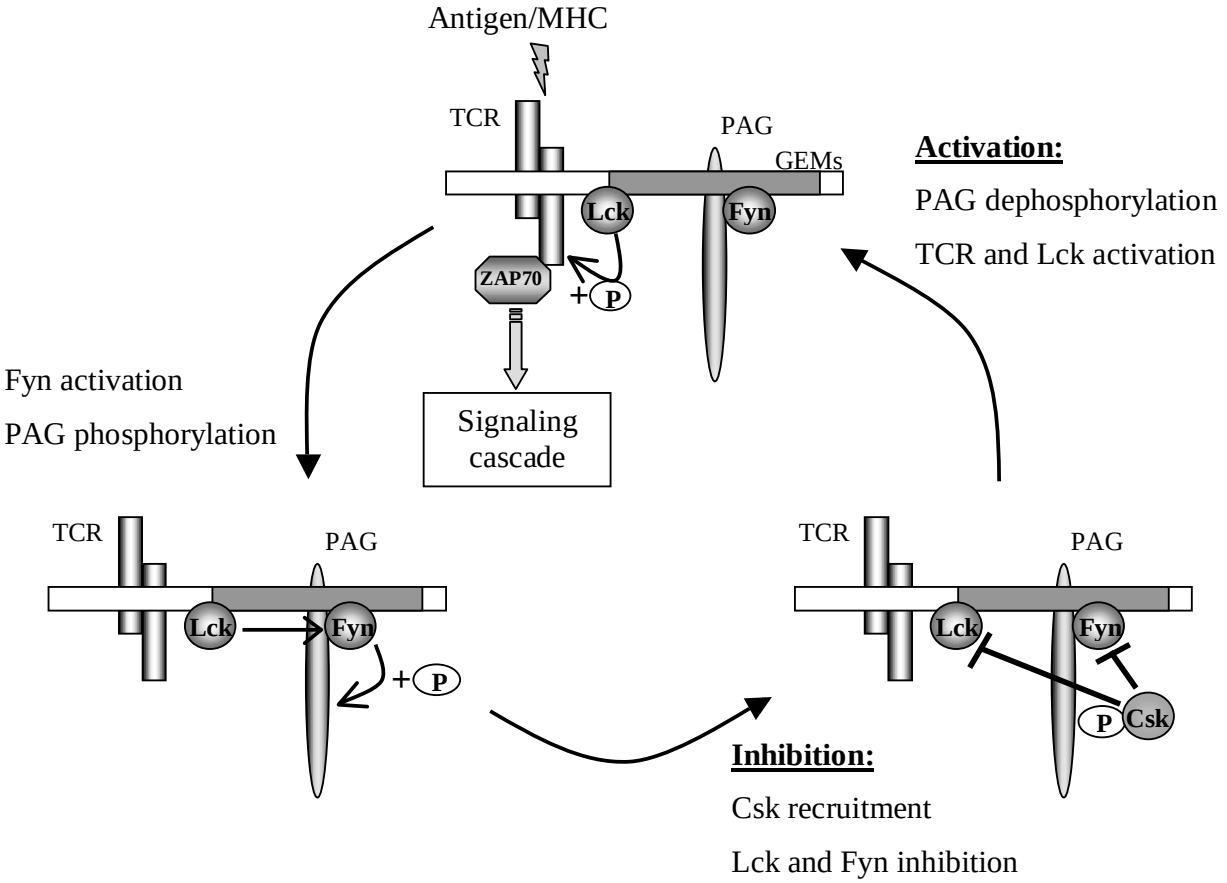


Figure 1.4. PAG regulatory function in T-cell signaling. PAG is phosphorylated in resting T cells and binds Csk (bottom right). Upon activation, PAG becomes dephosphorylated, thereby loosing Csk and allowing activation (top). Fyn then re-phosphorylates PAG, leading to Csk recruitment and inhibition of signaling (bottom left).

Notably, tyrosine 317 is the only tyrosine within PAG for which a functional significance has been shown. The role of the other nine tyrosines has not been clarified. Clearly, there might be more proteins in addition to Csk associated with PAG and thus, PAG may have other distinct functions in addition to the negative regulation of Src kinases. Identification of new binding partners and one novel function of PAG are the outcome of this thesis.

PAG seems to be implemented also in other pathways beside TCR signaling. Experiments with mast cells suggested a role in the negative feedback of FcεRI signaling (Ohtake et al., 2002). Upon FcεRI aggregation, PAG becomes rapidly phosphorylated and recruits Csk, thus inhibiting Lyn kinase activity. Furthermore, the overexpression of PAG leads to an inhibition of FcεRI-mediated cell activation. A role for PAG has also been suggested in cell migration and cell

spreading (Shima et al., 2003). Upon adhesion to fibronectin, PAG becomes phosphorylated and recruits Csk, which in turn inactivates the Src kinases. Knocking down PAG expression leads to impaired cell spreading.

An additional function of PAG is based upon its interaction with the cytoplasmic adaptor EBP50 (ezrin-radixin-moesin binding protein of 50 kDa) also known as NHERF (Na^+/H^+ exchanger regulatory factor) (Reczek et al., 1997; Yun et al, 1997). EBP50 binds through one of its two PDZ domains to the C-terminal VTRL motif of PAG and through its C-terminus to the ezrin-radixin-moesin family proteins, thus linking PAG to the actin cytoskeleton (Itoh et al., 2002; Brdickova et al., 2001). Considering the fact that PAG is located within the lipid rafts, PAG may in this way regulate their mobility. Indeed, the overexpression of PAG reduces the mobility of lipid rafts and inhibits immune synapse formation and subsequent T-cell activation (Itoh et al., 2002). The expression of a mutant PAG incapable of EBP50 binding restored both synapse formation and T-cell activation. This suggests that PAG also regulates the dynamics of the membrane, namely it keeps mobility of lipid rafts low in resting cells, but upon activation the association of EBP50 is lost (by an unknown mechanism) and the rafts become more mobile and thus able to aggregate within the immune synapse (Itoh et al., 2002).

Recently, PAG has been shown to play an important role in *Theileria parva* infection. Transformation of B lymphocytes with this intracellular parasite causes the downregulation of PAG and concomitant loss of Csk from lipid rafts. This in turn enables the constitutive activation of the Src kinase Hck, activation of the transcription factor AP-1 and continuous proliferation reminiscent of leukemic cells (Baumgartner et al., 2003). Histologically, PAG was found expressed also in germinal centers of lymphoid follicles and in follicular lymphomas. Thus, the presence of PAG may potentially be a new marker of follicular lymphomas and its absence a marker of some mantle cell lymphomas (Svec et al., 2005).

In spite of the apparent importance of PAG, two papers characterizing PAG knockout mice have been recently published suggesting that PAG is basically dispensable for T-cell development and function (Xu et al., 2005; Dobenecker et al., 2005). The first paper showed generally normal T-cell development with increased number of thymocytes in PAG deficient mice. Although they observed mild reduction in Csk localization within lipid rafts in cells lacking PAG, the TCR-induced calcium flux, cell proliferation and production of IL-2, IL-4 and IFN-γ were normal (Xu et al., 2005). However, the authors only deleted the last coding exon in the hope

of inactivating the whole PAG gene. But they themselves show expression of a protein in their mice detectable by anti-PAG antisera and claim this to be nonspecific band. This might, however, very well be the truncated form of PAG, which can still partially fulfill PAG function. The second study demonstrated that PAG deficiency had no effect upon embryogenesis, thymic development and T-cell functions *in vivo*. Moreover, Csk recruitment into the lipid rafts was not affected by the loss of PAG and proximal signaling events like the phosphorylation of key signaling molecules was not altered (Dobenecker et al., 2005). Thus it seems, that there is a redundancy among transmembrane adaptor proteins and some are able to compensate for the loss of PAG by taking over its function.

1.7. Src family kinases (SFKs)

1.7.1. Structure and localization of Src family kinases

The Src family consists of 9 members: Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes and Yrk. Among these, Src, Yes and Fyn are mainly ubiquitously expressed. Myeloid cells express Hck, Fgr and Lyn, B cells express Lyn, Fyn and Blk and T cells express primarily Lck and Fyn (Thomas and Brugge, 1997). Src kinases have a common structure consisting of an N-terminal region possessing sites for fatty acid modification, followed by a unique region, an SH3 domain, an SH2 domain, a linker region, a tyrosine kinase domain (SH1) and a C-terminal negative regulatory tyrosine. The N-terminal region is cotranslationally myristoylated on a glycine residue at position 2. This modification enables the kinase to attach to the cell membrane. Furthermore, Src kinases undergo posttranslationally reversible palmitoylation on dual cysteines and this is believed to target them into the lipid rafts. Additionally, Fyn was observed to be methylated at lysine residues within the N-terminal region and this may be required for its function in cell adhesion and spreading (Liang et al., 2004). Interestingly, the localization of Lck and Fyn within plasma membrane is largely different. Whereas Lck was predominantly detected in the non-raft fraction, Fyn in contrast is highly enriched in the raft fractions (Yasuda et al., 2002; Filipp et al., 2003). The unique domain, as well as different binding partners, are likely to contribute to their distinct subcellular localization.

1.7.2. Regulation and activation of Src family kinases

When the crystal structure of Src and Hck was solved, it inspired the model for a common mechanism of Src kinase activation (Xu et al., 1997; Sicheri et al., 1997) (see Figure 1.5.). According to this model, a Src kinase adopts an inactive conformation when its C-terminal inhibitory tyrosine (Y^{505} of Lck, Y^{529} of Fyn) becomes phosphorylated. In this conformation, the inhibitory phosphotyrosine is bound by the SH2 domain, bringing the SH3 domain closer to the linker region located between the SH2 and kinase domain. The SH3 domain forms an additional interaction with the proline-rich region within the linker, further stabilizing the closed inactive conformation (Xu et al., 1997; Sicheri et al., 1997). On the other hand, dephosphorylation of the inhibitory tyrosine releases the structure into a primed state, from which the kinase can then autophosphorylate the activatory tyrosine within the activation loop of the kinase domains leading to its full activation.

However, activatory tyrosine phosphorylation is not the only way to activate an Src kinase. Phosphorylation of other tyrosines have also been demonstrated that enhance kinase activity, although only for Src and only upon growth factor stimulation. In these studies, epidermal growth factor and platelet-derived growth factor selectively induced phosphorylation of a tyrosine within the SH2 domain of Src (Y^{215}) causing a dramatic increase in the kinase activity (Vadlamudi et al.,

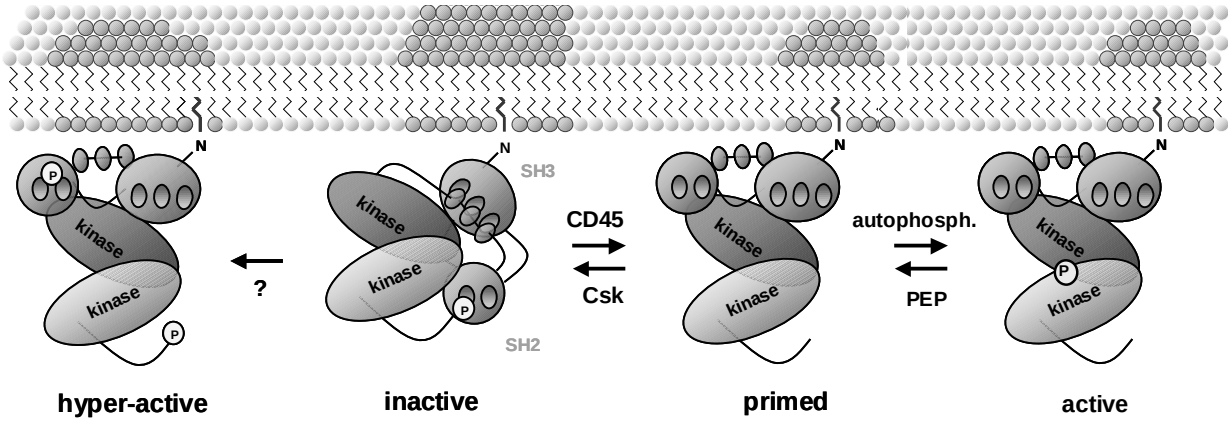


Figure 1.5. Src kinase regulation. The inactive form of SFKs has the linker region bound to its SH3 and phosphorylated C-terminal tyrosine bound to its SH2 domain (middle). This tyrosine can be dephosphorylated by CD45, thereby priming the kinase (right), which can then autophosphorylate, leading to the active state. The phosphorylation of a tyrosine within SH2 domain in addition to the C-terminal tyrosine phosphorylation results in the hyperactive form of kinase (left). For further details see 1.7.2.

2003; Stover et al., 1996). This phosphorylated SH2 domain specifically prevents binding of the phosphorylated C-terminal negative regulatory tyrosine and thus prevents folding of the kinase into an inactive state. This in consequence leads to an over 50-fold increase in the kinase activity (Stover et al., 1996) (see Figure 1.5. hyper-active).

The main proteins regulating the phosphorylation status and thereby the activity of SFKs are the phosphatase CD45 and the kinase Csk, which act antagonistically. CD45 is believed to be a critical positive regulator, which forms a complex with Lck (Schraven et al., 1991) and specifically dephosphorylates the C-terminal inhibitory tyrosine (Hermiston et al., 2003). On the other hand, Csk is able to phosphorylate this inhibitory tyrosine, thereby inactivating the enzyme (Okada et al., 1991; Bergman et al., 1992). The enzyme dephosphorylating the activatory tyrosine was shown to be the PEP (Gjorloff-Wingren et al., 1999; Cloutier and Veillette, 1999). Interestingly, this phosphatase binds to Csk (Cloutier and Veillette, 1996), thereby forming a functional complex that synergistically inhibits the Src kinases by acting upon both critical tyrosines simultaneously (Cloutier and Veillette, 1999). An additional protein tyrosine phosphatase (PTP) involved in the regulation of SFK activity, particularly that of Fyn, seems to be PTPα. PTPα deficient thymocytes exhibit enhanced phosphorylation of both the activatory and inhibitory tyrosines of Fyn, increased Fyn activity, hyperphosphorylation of PAG and reduced proliferation (Maksumova et al., 2005).

Furthermore, SFKs can be activated by interaction of either their SH2 or SH3 domains with other proteins. In this case, this domain is no longer available for the intramolecular interaction and thus the kinase cannot fold into its closed inactive conformation. One such interacting protein is SAP (SLAM-associated protein), which binds the SH3 domain of Fyn leading to its activation (Simarro et al., 2004). Another SFK activator is Unc119 (Uncoordinated 119), which was shown to increase the kinase activity of both Lck and Fyn by binding to their SH3 domain. Since Lck and Fyn are found associated with CD3 and the coreceptor CD4, Unc119 was found complexed with both CD3 and CD4. Recruiting Unc119 to the CD3- and CD4-associated Src kinases may provide the mechanism activating SFKs upon TCR triggering (Gorska et al., 2004).

Since Fyn and Lck are differentially localized within the plasma membrane (Fyn being mainly in the lipid rafts and Lck predominantly outside), the question arises as to how the TCR recruits and activates both proteins. The group of Julius, however, has proposed a model of sequential activation, according to which Lck is initially activated outside of lipid rafts, and then

translocates into the lipid rafts to activate Fyn (Filipp et al., 2003). Supporting this hypothesis is also the observation that Fyn cannot be activated in Lck deficient cells (Filipp et al., 2003).

1.7.3. Function of Lck and Fyn

Src family protein tyrosine kinases play a crucial role in cell differentiation, motility, adhesion, proliferation and survival. In T cells, a fraction of Lck interacts with the coreceptors CD4 and CD8 (Veillette et al., 1988; Barber et al., 1989). This noncovalent interaction is mediated via a dicysteine motif within the unique domain of Lck and two cysteines in the cytoplasmic region of CD4 or CD8 alpha (Turner et al., 1990). It facilitates participation of Lck in the initiation of TCR signal transduction upon antigen recognition. During TCR-pMHC interaction, Lck is recruited into the complex through its association with either the CD4 or CD8 coreceptor (Holdorf et al., 2002). Upon clustering of the coreceptors, Lck molecules transphosphorylate the tyrosine within the activation loop leading to their full activation. Additionally, a pool of Lck interacts with the costimulatory molecule CD28 and this interaction further sustains the activation of Lck (Holdorf et al., 2002). Furthermore, Fyn was shown to directly interact with CD3 subunits and TCR ζ chains (Samelson et al., 1990; Timson Gauen et al., 1992). Thus, active Lck and Fyn coclustered with the receptors are perfectly positioned to phosphorylate the tyrosine residues within the ITAMs of both the CD3 complex and TCR ζ chains. Phosphorylated ITAMs recruit ZAP70, which is then phosphorylated and activated by Lck. The subsequent events of proximal signaling are described in section 1.4.3. Underlining the essential role of Lck in TCR signaling is the finding that Jurkat cells lacking Lck possess a block in ZAP70 phosphorylation and activation, a lack in LAT phosphorylation and calcium flux and no NFAT activation and IL-2 production (Straus and Weiss, 1992; Denny et al., 2000).

Lck was suggested to be involved in a negative feedback pathway allowing the cell to discriminate between self and nonself antigen. Generally, activated Lck induces the phosphorylation of the phosphatase SHP-1, which then binds to the SH2 domain of Lck leading to a downregulation of the activity of the latter and dephosphorylation of TCR-associated ITAMs. However, Lck can also be phosphorylated by ERK on a serine residue, which interferes with SHP-1 binding. Thus, strong binding ligands (i.e. antigenic peptides) induce sufficiently rapid ERK activation to phosphorylate Lck and thus prevent SHP-1 binding. On the contrary, weak ligands (i.e. self peptides) induce a delayed ERK activation, which is simply too slow to

protect Lck from accumulated phospho-SHP-1 and signaling is in this case aborted (Stefanova et al., 2003).

Fyn appears to play a more negative regulatory role in TCR signaling by phosphorylating PAG, thereby mediating Csk recruitment and PAG-Csk interaction (Yasuda et al, 2002; Shima et al., 2003; Filby et al., 2007). The loss of Fyn results in a lack of PAG phosphorylation, a loss of Csk recruitment and reduced phosphorylation of the inhibitory tyrosine of Lck. This then correlates with elevated cytokine production and hyperproliferation (Filby et al., 2007). Reducing Lck levels additionally to Fyn deficiency causes spontaneous T-cell activation *in vivo* and the development of severe autoimmune disorders (Filby et al., 2007). Fyn deficient mice show a reduced presence of naïve $CD44^{low}CD62L^{high}$ T cells in the periphery, presumably due to the inhibition of PAG phosphorylation and Csk recruitment (Yasuda et al., 2002).

Fyn was additionally shown to associate with the adapter proteins SKAP55 (Src kinase-associated phosphoprotein of 55 kDa) (Marie-Cardine et al., 1997) and ADAP (Adhesion and degranulation promoting adaptor protein), also known as Fyb (Fyn binding protein) or SLAP (SLP-76 associated protein) (da Silva et al., 1997). Therefore Fyn also seems to be involved in integrin signaling. Upon TCR triggering, Fyn phosphorylates ADAP and consequently ADAP induces integrin clustering and integrin-mediated adhesion (Griffiths et al., 2001; Peterson et al., 2001). In addition, Fyn contributes to the regulation of cytoskeletal reorganization as Fyn phosphorylates WASP (Wiskott-Aldrich syndrome protein), a critical regulator of the Arp2/3 complex and actin polarization in T cells (Badour et al., 2004). Fyn was also suggested to bind phosphorylated alpha-tubulin and to regulate tubulin cytoskeleton reorganization upon T-cell activation (Marie-Cardine et al., 1995; Martin-Cofreces et al., 2006).

A role for Lck and Fyn in naïve T cell maintenance and survival in the periphery has been suggested, since there is a constant low level of signaling in peripheral T cells and a constitutive low level of TCR ζ chain phosphorylation (Pitcher et al., 2003). As T cells are continuously scanning their environment, their TCRs are constantly encountering self-peptide/MHC complexes inducing weak signaling. Especially Fyn was believed to be responsible for the constitutive low level of zeta chain phosphorylation as Fyn associates with CD3 and TCR ζ chains (Samelson et al., 1990; Timson Gauen et al., 1992). This so called tonic signaling is believed to be responsible for naïve T cell survival and lymphopenic expansion in the periphery. An elegant series of experiments performed by Zamoyska et al. suggested that both Fyn and Lck

(at least one of them) are required for TCR dependent naïve T cell survival in the periphery and Lck, but not Fyn, is essential for TCR dependent homeostatic proliferation (Seddon et al., 2000; Seddon and Zamoyska, 2002a; Seddon and Zamoyska, 2002b).

Finally, Src kinases are required during thymocyte development. Knockout mice deficient for both Fyn and Lck possess an absolute block at the transition from DN3 to DN4 stage during TCRβ selection (Groves et al., 1996; van Oers et al., 1996). Additionally, Lck is primarily responsible for transmitting the positive selection signal during the DP stage and it seems that this signal also determines the fate of CD4 versus CD8 lineage (Hernandez-Hoyos et al., 2000; Legname et al., 2000).

1.8. Csk (C-terminal Src kinase)

Csk is a ubiquitously expressed cytoplasmic protein tyrosine kinase with a structure similar to the SFKs, consisting of an SH3, SH2 and a kinase domain (Nada et al., 1991). However, it lacks both an N-terminal acylation signal and a C-terminal regulatory tyrosine.

Csk is the major negative regulator of Src kinases as it phosphorylates their inhibitory tyrosine at the C-terminus and these adopt the closed inactive conformation (see 1.7.2.) (Nada et al., 1991; Okada et al., 1991; Bergman et al., 1992). Overexpression of Csk results in a dramatic inhibition of TCR-induced protein tyrosine phosphorylation and IL-2 production (Chow et al., 1993). The SH3 and SH2 domains of Csk were found to be absolutely critical for Csk function, therefore Csk probably requires association with other proteins to inhibit SFK activation in the cell (Cloutier et al., 1995). In resting T cells, a portion of Csk is localized within the lipid rafts through its interaction with various membrane proteins (e.g. PAG) and sets an activation threshold for TCR signaling (Brdicka et al., 2000; Kawabuchi et al., 2000). PAG can not only relocate Csk to the lipid rafts, but was also shown to activate Csk and increase its kinase activity up to 6-fold (Takeuchi et al., 2000). Additionally, Csk can be phosphorylated upon a serine residue within the catalytic loop by the cAMP-dependent protein kinase (PKA), resulting in a 2-4 fold increase in Csk activity (Vang et al., 2001), which is additive to the increase caused by PAG binding (Vang et al., 2003). Upon TCR triggering, PAG is dephosphorylated and Csk association is lost, thus enabling activation of Src kinases and proper signaling (Torgersen et al., 2001). In this phase, Csk may be sequestered by another binding partner G3BP (RasGAP-SH3-binding

protein), which is located outside of lipid rafts and augments T-cell activation simply be reducing the amount of Csk in the rafts (Rahmouni et al., 2005).

Csk forms complexes with all three members of the PEP phosphatase family, protein tyrosine phosphatase PEP and PTP-PEST (PTP containing PEST domain) bind to the SH3 domain (Cloutier and Veillette, 1996; Davidson et al., 1997), whereas PTP-HSCF (hematopoietic stem cell fraction derived PTP) binding was shown to be SH2 domain mediated (Wang et al., 2001). As PEP phosphatases are believed to dephosphorylate the activatory tyrosines within the Src kinases (Gjorloff-Wingren et al., 1999; Cloutier and Veillette, 1999), the formation of Csk-PEP complex would constitute an efficient mechanism to inactivate Src kinase-mediated signaling.

The adaptor proteins Dok-1 and Dok-3 were also reported to associate with Csk through its SH2 domain (Neet and Hunter, 1995; Lemay et al., 2000). Dok proteins are efficient inhibitors of immunoreceptor signaling (at least in B cells) as they coordinate the recruitment of three inhibitory effectors, Csk, the phosphatase SHIP-1, and p120RasGAP, to the membrane upon receptor stimulation (Lemay et al., 2000; Tamir et al., 2000). Additionally, Csk was shown to bind to the transmembrane adaptor protein SIT *in vitro* and might contribute to the inhibition of TCR induced NFAT activation caused by SIT overexpression (Pfrepper et al., 2001). Csk is also recruited to another transmembrane adaptor protein, LIME, that is phosphorylated upon CD4 or CD8 crosslinking (Brdickova et al., 2003). Csk can also associate with focal adhesion-associated proteins paxillin, tensin and FAK (focal adhesion kinase), although the physiological relevance of such interactions is unclear at present (Sabe et al., 1994).

Csk deficient mice die in utero because of abnormalities in neuronal development, which result from uncontrolled Src kinase activity (Nada et al., 1993; Imamoto and Soriano, 1993). Conditional inactivation of Csk in thymocytes abrogates the requirement for pre-TCR mediated signals during TCR β checkpoint and also uncouples positive selection from the TCR-mediated signal, presumably because of hyperactive Src kinases, and single positive $CD4^+$ T cells develop and leave into periphery (Schmedt et al., 1998; Schmedt et al., 2001).

Given the lethal phenotype of Csk knockout mice and the fact that PAG is believed to be the main protein recruiting Csk to the plasma membrane, it was expected that PAG deficiency would also have fatal consequences (Hermiston et al., 2002). Therefore it was surprising to find that PAG knockout mice have no severe phenotype (Xu et al., 2005; Dobenecker et al., 2005; see 1.6.4.). However, if one investigates the newborn mice, they indeed possess enhanced Src kinase activity, which is then downregulated at three months of age, clearly demonstrating a

development of compensatory mechanism (J. Lindquist, unpublished observation). For this reason, a simple knockout may not be the ideal approach and therefore we applied RNA interference to knock down PAG expression in primary human T cells in order to demonstrate the importance of PAG as a negative regulator of T cell signaling.

1.9. Aim of the project

The aim of this study was to investigate the alterations in signaling pathways upon induction of an unresponsive state, anergy, in primary human T cells. We were mainly interested in the membrane proximal signaling events with the special focus on PAG, the negative regulator of T-cell signaling. There are three main observations that this project was based on. First, the Src family kinase Fyn was shown to be upregulated in anergic T cells both on the protein level and activity (Quill et al., 1992; Gajewski et al., 1994). Second, Fyn is the kinase responsible for phosphorylation of the transmembrane adaptor protein PAG (Yasuda et al., 2002). And third, most importantly, T cells overexpressing PAG were shown to be unresponsive to any further stimulation via TCR and this is phenotype very similar to anergy (Brdicka et al., 2000; Davidson et al., 2003). Moreover, PAG overexpressing cells produce reduced levels of IL-2 whereas normal amount of IFN-γ and IL-4 (Davidson et al., 2003). The similar alteration in cytokine production has been observed also in anergic T cells (Jenkins et al., 1987; Blish et al., 1999). Therefore we hypothesized that PAG may play a role in the maintenance of the anergic state.

2. Methods

2.1. Antibodies used in this study

Antibody	Species and clone	Application	Source
actin	mouse monoclonal IgG_1 (AC-15)	WB - 1:10,000	Sigma
anti-mouse	rabbit	coating - 10 μg/ml	DAKO
anti-mouse-HRP	goat	WB - 1:10,000	Dianova
anti-rabbit-HRP	goat	WB - 1:10,000	Dianova
CD25-FITC	mouse monoclonal IgG_1 (M-A251)	FACS - 1:10	BD Biosciences
CD28	mouse monoclonal IgM (248.23.2)	coating - 1:1 stimulation - undiluted	hybridoma grown in our laboratory
CD3	mouse monoclonal IgM (MEM92)	stimulation - undiluted	hybridoma from V. Horejsi, Prague, grown in our laboratory
CD3	mouse monoclonal IgG_{2a} (OKT3)	coating - 1:100, 1:10 stimulation - 1:1	hybridoma grown in our laboratory
CD3-PE	mouse monoclonal IgG_1 (UCHT1)	FACS - 1:10	BD Biosciences
CD59	mouse monoclonal IgG_{2b} (MEM43/5)	WB - 1:250	V. Horejsi, Prague
CD69-FITC	mouse monoclonal IgG_1 (FN50)	FACS - 1:10	BD Biosciences
Csk	rabbit polyclonal IgG (C-20)	WB - 1:200	Santa Cruz
Csk-pSer364	rabbit polyclonal	WB – 1:50	K. Tasken, Norway
DGK alpha	rabbit polyclonal	WB - 1:2,000 IP - 5 μl	I. Merida, Spain
DGK zeta	rabbit polyclonal	WB - 1:1,000	T. Judd, Utah
FLAG	rabbit polyclonal	WB - 1:400	Sigma
FoxP3	rabbit polyclonal IgG	WB - 2 μg/ml	E. Schmitt, Mainz
Fyn	rabbit polyclonal	WB - 1:1,000	Biosource

Fyn	mouse monoclonal IgG_1 (1S)	WB - 1:1,000	Biosource
Fyn	mouse monoclonal IgG_2 (Fyn-02)	IP - 0.5 µl	V. Horejsi, Prague
GAPDH-HRP	mouse monoclonal IgG_{2b} (mAbcam 9484)	WB - 1:10,000	Abcam
Grb2	rabbit polyclonal IgG (C-23)	WB - 1:1,000	Santa Cruz
Lamin A	rabbit polyclonal	WB - 1:500	BioLegend
LAT	rabbit polyclonal	WB - 1:1,000 IP - 1 µl	V. Horejsi, Prague
Lck	rabbit polyclonal	IP - 0.5 µl	A. Magee, London
Lck	rabbit polyclonal	WB - 1:1,000	Biosource
Lck	mouse monoclonal IgG_{2b} (3A5)	WB - 1:200	Biosource
Lck	mouse monoclonal IgG_1 (Lck-04)	IP - 30 µl coupled to sepharose	V. Horejsi, Prague
Lck-pY505	rabbit polyclonal	WB - 1:1,000	Biosource
LIME	rabbit polyclonal	WB - 1:1,000	V. Horejsi, Prague
NTAL	mouse monoclonal (NAP03)	WB - 1:1,000	V. Horejsi, Prague
p62Dok	mouse monoclonal IgG_1 (45)	WB - 1:250	BD Biosciences
PAG	rabbit polyclonal	WB - 1:2,000	V. Horejsi, Prague
PAG	mouse monoclonal IgG_{2a} (MEM255)	WB - 1:100	hybridoma from V. Horejsi, Prague, grown in our laboratory
PAG-C6	mouse monoclonal IgG_{2b} (PAG-C6)	IP - 30 µl coupled to sepharose	hybridoma from V. Horejsi, Prague, grown in our laboratory
PAG-pY317	rabbit polyclonal	WB - 1:10,000	produced in our laboratory
pan-Ras	mouse monoclonal IgG_{2b} (Ab-4)	WB - 1:1,000	Oncogene
pTyr	mouse monoclonal IgG_{2b} (4G10)	WB - 1:100	hybridoma grown in our laboratory
RasGAP	mouse monoclonal IgG_{2a} (B4F8)	WB - 1:100 IP - 5 µl	Santa Cruz

Sam68	mouse monoclonal IgG_1 (15)	WB - 1:10,000 IP - 4 µl	BD Biosciences
Src-pY215	rabbit polyclonal	WB - 1:1,000 IP - 4 µl	Abcam
Src-pY418	rabbit polyclonal	WB - 1:1,000	Biosource
Src-pY529	rabbit polyclonal	WB - 1:1,000	Biosource
zeta chain	mouse monoclonal IgG_1 (6B10)	WB - 1:1,000 IP - 3 µl	Sigma

2.2. General reagents for cell culture

RPMI 1640 medium with $NaHCO_3$ and stable glutamine	Biochrom AG
PBS without $Ca^{2+}Mg^{2+}$	Biochrom AG
PBS with $Ca^{2+}Mg^{2+}$	Biochrom AG
FCS	PAN Biotech GmbH
CiproBay 200	Bayer
Phorbol myristate acetate (PMA)	Calbiochem
Ionomycin	Sigma
Interleukin 2	Tebu-bio
Trypan blue	Sigma

2.3. T cell isolation and purification

Reagents and instruments:

Ficoll	Biochrom AG
Heparin	Biochrom AG
Pan T cell isolation kit II	Miltenyi Biotec
AutoMACS	Miltenyi Biotec

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation of heparinized blood collected from healthy volunteers. A ring containing PBMCs formed during the gradient centrifugation. PBMCs were carefully aspirated and washed 3x with RPMI 1640 medium. The cells were rested in RPMI 1640 medium supplemented with 10% FCS for 2 hours in an incubator. T cells were further purified by non-T cell depletion on the AutoMACS machine using the Pan T cell isolation kit II. T cell populations of greater than 95% purity were obtained, as determined by flow cytometry analysis.

2.4. Anergy induction

Reagents and instruments:

24-well flat-bottomed tissue culture plates	TPP

To induce anergy *in vitro*, 24-well flat-bottomed tissue culture plates were precoated with 10 µg/ml rabbit-anti-mouse immunoglobulin in 0.3 ml/well PBS without $Ca^{2+}Mg^{2+}$ overnight at 4°C. After washing the plates three times with 0.5 ml/well PBS, anti-CD3 (OKT3) supernatant diluted to approx. 1µg/ml was immobilized in 0.3 ml/well PBS overnight at 4°C. The plates were washed again three times with 0.5 ml/well PBS and T cells were inoculated at $6x10^5$ cells/ml in 1 ml/well RPMI 1640 medium supplemented with 10% FCS and CiproBay 200 (1:200). Rescued samples received additionally 10^{-9} M PMA to mimic costimulation. The resting sample was kept in culture without stimulus.

After three days of incubation in a humidified atmosphere at 37°C and 5% CO_2, the cells were collected, centrifuged and transferred into new, uncoated 24-well plates again in 1 ml/well fresh RPMI/10% FCS/CiproBay and rested for one additional day. After a total of 4 days in culture, the cells were harvested, cell viability determined by trypan blue staining and the dead cells removed by Ficoll centrifugation.

2.5. Proliferation assay

Reagents and instruments:

96-well round-bottomed tissue culture plates	Costar
[^{3}H]-thymidine	ICN
PHD cell harvester	Inotech AG
liquid scintillator 1450 Microbeta Wallac	Perkin Elmer

To assess the proliferative capacity after anergy induction, the cells were restimulated in 96-well round-bottomed tissue culture plates. The plates were coated with anti-CD3 (OKT3, 100 µl supernatant/well) or with anti-CD3 plus anti-CD28 (100 µl of each supernatant/well) as described above. The cells were then plated at $5x10^4$ cells/well in triplicates and restimulated with either anti-CD3 alone, anti-CD3 plus anti-CD28, anti-CD3 plus exogenous IL-2 (100 U/well), exogenous IL-2 alone or PMA (10^{-9} M) plus ionomycin (0.25 µg/ml).

[^{3}H]-thymidine (0.3 µCi/well) was added for the last 8-10 hours of the three-day incubation and the plates were harvested using the PHD cell harvester. Thymidine incorporation was measured by liquid scintillation and the results expressed as the mean cpm.

2.6. Stimulation of T cells

Buffers:

TBS (per 1 liter) - 8.0 g NaCl
0.2 g KCl
3.0 g Tris
to pH 8.0, dI H_2O to 1 liter final

Resting, anergic and rescued cells coming from the primary stimulation described in section 2.3. were washed once with 1 ml RPMI 1640 without FCS and re-challenged by anti-CD3 (MEM92) stimulation for 2 min at 37°C; 100 µl antibody supernatant was used for $5x10^6$ cells. Stimulation was stopped by adding 1 ml ice-cold TBS, the cells were quickly spun down at 5,000 rpm, 2 min, 4°C and lysed.

2.7. Cell lysis, immunoprecipitation and Western blot analysis

Reagents and instruments:

BSA	Sigma
protein A sepharose CL-4B	Pharmacia Biotech
CNBr-activated sepharose 4B beads	GE Healthcare
Gel electrophoresis system	Bio-Rad
Western blotter Multiphor II	GE Healthcare
nitrocellulose membrane	GE Healthcare
Milk powder	Lasana
Tween 20	Roth
ECL Western Blotting Detection reagents	GE Healthcare
Hyperfilm ECL	GE Healthcare

Buffers:

NP-40 lysis buffer –	1% Nonidet P-40	Sigma
	100 mM NaCl	
	50 mM Hepes pH 7.4	
	5 mM EDTA	
	1% Lauryl maltoside	Calbiochem
	1 mM phenylmethylsulfonylfluoride (PMSF)	

1 mM sodium orthovanadate
50 mM sodium fluoride
10 mM sodium pyrophosphate

Digitonin lysis buffer – 1% digitonin Sigma
100 mM NaCl
50 mM Hepes pH 7.4
5 mM EDTA
1 mM PMSF
1 mM sodium orthovanadate
50 mM sodium fluoride
10 mM sodium pyrophosphate

NP-40 washing buffer – 0.05% NP-40
5 mM EDTA
150 mM NaCl
50 mM Tris pH 7.4

Digitonin washing buffer – 0.05% digitonin
5 mM EDTA
150 mM NaCl
50 mM Tris pH 7.4

Sample buffer – 10% glycerol
60 mM Tris pH 6.8
2% SDS
0.002% bromphenol blue
1% 2-mercaptoethanol

10% SDS-polyacrylamide gel – 4.7 ml H_2O
2.5 ml 40% Acrylamide/Bis solution 37.5:1 Bio-Rad
2.6 ml 1.5 M Tris pH 8.8
0.1 ml 10% SDS
0.1 ml 10% APS
0.004 ml TEMED

Stacking gel – 2.19 ml H_2O
0.375 ml 40% Acrylamide/Bis solution 37.5:1
0.375 ml 1.0 M Tris pH 6.8
0.03 ml 10% SDS
0.03 ml 10% APS
0.003 ml TEMED

Electrophoresis buffer – 10x TGS buffer Bio-Rad

Blotting buffer (per 1 liter) – 5.8 g Tris base
2.9 g glycine
0.37 g SDS
to 800 ml dI H2O, add 200 ml methanol

TBS – see section 2.6.

Cells ($5x10^6$) were lysed in 120 µl ice-cold NP-40 lysis buffer. After 30 min incubation on ice, lysates were centrifuged for 15 min at 13,000 rpm, 4°C. Post-nuclear supernatants were transferred into new tubes containing 30 µl 5x reducing sample buffer and heated for 5 min at 95°C.

For immunoprecipitation, $20x10^6$ cells were lysed in 500 µl NP-40 lysis buffer, 10% of postnuclear lysate was kept for whole cell analysis and the rest incubated together with 1 mg/ml BSA, the immunoprecipitating antibody and 30 µl protein A sepharose for 2-18 h with gentle rotation at 4°C. Note, that some antibodies were produced from hybridoma cells in our laboratory (see section 2.1.). In this case, the antibody (Ab) was purified from the hybridoma supernatant by affinity chromatography on a column filled with protein A sepharose. The antibody was eluted, concentrated to 6 mg/ml and covalently coupled to CNBr-activated sepharose 4B beads. 30 µl of the Ab-sepharose was then used for immunoprecipitation. Immunoprecipitates were washed five times with 1 ml low detergent NP-40 washing buffer and the immunoprecipitated material was released by heating with 30 µl 1x reducing sample buffer for 5 min at 95°C. To detect the Sam68-p120RasGAP-PAG complex, cells were lysed in 500 µl digitonin lysis buffer and the immunoprecipitates washed afterwards with low digitonin washing buffer.

Whole cell lysate or immunoprecipitates were electrophoretically separated on 10% SDS-polyacrylamide gel (30 µl sample/lane) at 130V and transferred onto nitrocellulose membrane at

140 mA/gel for 1 h 15 min. The blots were blocked in 5% non-fat milk in TBS pH 8 for 1 hour. Primary antibodies were diluted in 2% milk-TBS pH 8 and incubated for 1 hour at room temperature with gentle shaking. Blots were washed three times with TBS + 0.01% Tween 20 (5 min each wash), followed by 45 min incubation with appropriate horseradish-peroxidase conjugated secondary antibody. After three additional washing steps with TBS/Tween, blots were developed using ECL Western Blotting Detection reagents and exposed on Hyperfilm ECL.

2.8. Mass spectrometry (MS)

Reagents and instruments:

Non-reducing sample buffer (2x) Bio-Rad

Buffers:

Fixative – 10% acetic acid
30% methanol

To identify the p120 protein recognized by anti-DGK alpha antibody using mass spectrometry, the DGK alpha immunoprecipitates were washed with NP-40 washing buffer as described above and heated with non-reducing sample buffer for 5 min at 95°C. The supernatants were then reduced in a new tube by heating with 10 mM dithiothreitol (DTT) for 5 min, 95°C. The cysteines within proteins were modified by incubation with 55 mM iodoacetamide for 30 min in the dark. Samples were loaded onto large 8% polyacrylamide gel and SDS-PAGE was performed. The gel was then fixed for 30 min and stained with Silver. The bands of interest were excised from the gel, digested with trypsin and subjected to MALDI-TOF-MS, which was performed by Dr. Thilo Kähne in the Institute of Internal Medicine, Magdeburg. The results were then compared to the protein database.

2.9. Subcellular fractionation

Buffers:

Buffer I – 10 mM Hepes pH 7.9
10 mM KCl
0.1 mM EDTA
0.1 mM EGTA

1 mM DTT
1 mM PMSF
2 mM Na_3OV_4
2 mM NaF
10 mM sodium pyrophosphate

Buffer II – 1% NP-40
1% LM
50 mM Tris pH 7.4
170 mM NaCl
1 mM DTT
1 mM PMSF
2 mM Na_3OV_4
2 mM NaF
10 mM sodium pyrophosphate

Cells ($10x10^6$) were lysed in 50 µl buffer I for 20 min on ice. Then 3 µl 10% NP-40 was added and the sample incubated for an additional 10 min. Samples were then centrifuged at 2,000 rpm, 5 min, 4°C. Supernatant represented the cytoplasmic fraction containing both the cytosol and the membranes. The pellet was washed twice with buffer I. The pellet was then lysed in 25 µl buffer II for 1 hr at 4°C with agitation, then centrifuged at 13,000 rpm, 10 min, 4°C. Supernatant represented the nuclear fraction.

2.10. Flowcytometry

Reagents and instruments:

FACS Calibur	Becton Dickinson
Cell Quest Pro software	Becton Dickinson

Cells ($5x10^5$) were stained with FITC- or PE- labeled antibodies against CD3 and surface activation markers CD25 and CD69 for 20 min at 4°C. After one wash with cold PBS, samples were measured on a FACS Calibur and the data analyzed using the Cell Quest Pro software.

2.11. Lipid raft preparation

Reagents and instruments:

80% sucrose	Sigma
dounce homogenizer	Wheaton
ultracentrifuge tubes	Beckmann
Sorval OTD-Combi ultracentrifuge	DuPont Company
rotor TH-660	Sorvall

Buffers:

Brij 58-lysis buffer –	3% Brij 58	Pierce
	50 mM Hepes pH 7.4	
	100 mM NaCl	
	1 mM PMSF	
	5 mM EDTA	
	1 mM sodium orthovanadate	
	50 mM sodium fluoride	
	10 mM sodium pyrophosphate	
MNE buffer –	25 mM MES pH 6.5	Sigma
	5 mM EDTA	
	150 mM NaCl	

To isolate lipid rafts, $50x10^6$ cells were lysed in 0.5 ml Brij 58-containing lysis buffer for 10 min on ice. Lysates were mixed with 0.5 ml ice-cold 80% sucrose in MNE buffer and homogenized with 10 strokes in a dounce homogenizer. Samples were then transferred into ultracentrifuge tubes and overlaid with 2 ml ice-cold 30% sucrose and 1 ml ice-cold 5% sucrose. The sucrose gradients were centrifuged in Sorval OTD-Combi ultracentrifuge, rotor TH-660, at 44,000 rpm (200,000g), 4°C for 20 hours without brakes. The following day, 10 equal fractions, 400 µl each, were collected from the top of the gradient. Fractions containing lipid rafts were detected by spotting 3 µl of each fraction onto a nitrocellulose membrane, which was then blocked in 5% milk/TBS and probed for the localization of the lipid raft-associated marker CD59.

2.12. *In vitro* kinase assay

Reagents and instruments:

Hyperfilm MP	GE Healthcare

Buffers:

Kinase buffer –	50 mM Tris-HCl pH 7.4	
	10 mM $MnCl_2$	
	0.1% NP-40	
	10 µg acid-denatured enolase	Sigma
	10 µCi [γ-^{32}P] ATP	GE Healthcare

Cells ($10x10^6$/sample) were lysed as described in section 2.7. and immunoprecipitated with either 30 µl PAG-C6 sepharose; 0.5 µl Fyn02 and 30 µl protein A sepharose; or 0.5 µl Lck and 30 µl protein A sepharose for 18h at 4°C with gentle rotation. Immunoprecipitates were washed 5x with NP-40 washing buffer (see 2.7.) and 10% taken for Western blotting analysis. Remaining 90% was resuspended in 40 µl kinase reaction buffer containing [γ-^{32}P]-ATP. The reaction was allowed to proceed for 5 min at room temperature and stopped by adding 10 µl 5x sample buffer (see 2.7.) and heating at 95°C for 5 min. Samples were analyzed on 10% SDS-PAGE, the gels were dried and exposed to film for 10 min–6 h at -70°C with intensifying screen.

2.13. cAMP measurement

Reagents and instruments:

cAMP Biotrak Enzymeimmunoassay System	GE Healthcare
ELISA reader Dynatech MR 5000	DPC Biermann GmbH
GraphPad Prism software [version 3.0]	
TMB liquid substrate system	Sigma

Intracellular cyclic AMP levels from $1x10^5$ T cells were determined using the cAMP Biotrak Enzymeimmunoassay System according to the manufacturers instructions. Briefly, the cells were lysed in 200 µl lysis buffer provided. 100 µl of the lysate or of the cAMP standard dilutions ranging from 12.5-1,600 fmol were inoculated together with rabbit anti-cAMP antiserum into duplicate microplate wells precoated with donkey anti-rabbit immunoglobulin. The plate was incubated for 2 hours in fridge and then 50 µl cAMP-peroxidase conjugate was added, which

competes with the endogenous cAMP of the sample or standard for the binding sites of anti-cAMP antiserum. After additional 60 min incubation in fridge, the plate was washed and shaked with 150 µl/well enzyme substrate TMB at room temperature for 60 min. The intensity of the color reaction was read at 630 nm. Data were analyzed using the GraphPad Prism software.

2.14. Transfection

Reagents and instruments:

BTX cuvette, gap size 4 mm	Qbiogene
Gene Pulser II	Bio-Rad
QuickChange^{TM} site-directed mutagenesis kit	Stratagene
Nucleofection kit	Amaxa biosystems
Nucleofector instrument	Amaxa biosystems
12-well-plate	TPP

DNA constructs:

pEF Bos	Mizushima and Nagata, 1990
wt FLAG-PAG	Brdicka et al., 2000
Y317F-FLAG-PAG	Brdicka et al., 2000
Y181F-FLAG-PAG	Brdicka et al., 2000
Y181/317F-FLAG-PAG	Smida et al., 2007
FLAG-LAT	Brdicka et al., 2002
Fyn	Dr. A. da Silva, Boston, USA
pCMS3-EGFP/Renilla	Smida et al., 2007
pCMS3-EGFP/PAG	Smida et al., 2007
siRNA PAG	Invitrogen
siRNA Renilla	Invitrogen

The Y181/317F-FLAG-PAG construct was generated using the QuickChange site-directed mutagenesis kit according to the manufacturers instructions. For RNA interference, the human sequence 5’ GCGAUACAGACUCUCAACATT 3’ corresponding to Shima et al. (Shima et al., 2003) was cloned as shRNA into the vector pCMS3-EGFP. As a control, Renilla siRNA was also cloned into pCMS3-EGFP. All constructs were sequenced to ensure integrity.

Jurkat T cells ($20x10^6$) grown at a density of $2\text{-}5x10^5$ cells/ml were washed once with 20 ml PBS with Ca^{2+} and Mg^{2+} and resuspended in 350 µl PBS. The cells were electroporated in BTX cuvette with 30 µg DNA at 210V, 950 µF using a Bio-Rad Gene Pulser II. 1 ml of prewarmed medium was immediately added to the cells in cuvette, the precipitated DNA was removed and the cells were transferred into 40 ml medium and cultured for 20-24 hours in an incubator. Jurkat cells transfected with siRNA constructs were cultured for 72 hours to ensure protein downregulation.

Primary human T cells ($3x10^6$) were washed once with PBS with Ca^{2+} and Mg^{2+}, resuspended in 100 µl Nucleofector solution and nucleofected with 8 µg siRNA oligonucleotides (of the same sequence as cloned into pCMS3-EGFP) using the Nucleofection kit on a Nucleofector instrument with program U-14. 0.5 ml of prewarmed medium was immediately added to the cells in cuvette and the cells were transferred into 1.5 ml prewarmed medium in 12-well-plate and cultured for 72 hours in an incubator.

2.15. Ras activation assay

Reagents and instruments:

GDP	Sigma
IPTG	BTS
Glutathione-sepharose 4B	GE Healthcare
LB Broth	Sigma
Branson sonifier 450	Branson Ultrasonics
GST- Raf1-RBD	Foschi et al., 1997

Buffers:

Starving medium – RPMI 1640 medium supplemented with

0.2% BSA, endotoxin low	Sigma
50 mM Hepes	Biochrom AG

Lysis buffer – 25 mM Hepes
150 mM NaCl
1% NP40
10 mM $MgCl_2$

1 mM EDTA
1 mM PMSF
1 mM sodium orthovanadate
50 mM sodium fluoride

Bacterial lysis buffer – 49.5 ml PBS
25 µl 1M DTT
1 tablet protease inhibitor cocktail Roche
0.5 ml Triton X-100

Cells ($18x10^6$/time point) were washed once with 20 ml starving medium and resuspended in 50 ml starving medium. After 2 hours of starving, the cells were stimulated with anti-CD3 (OKT3) plus anti-CD28 supernatants (1:1, 200 µl each) for 0, 1, 2, 5 and 10 minutes at 37°C. Cells were quickly spun down and lysed in 1 ml lysis buffer supplemented with 1 mM GDP, vigorously vortexed and centrifuged at 13,000 rpm, 5 min, 4°C. 10 % of the postnuclear lysate was kept as a loading control and the rest was used for the pull-down assay.

GST-Raf1-Ras binding domain (RBD) was expressed in bacteria by induction with 1 mM IPTG for 3 hours. The bacteria were then sonicated, lysed and the extranuclear lysate was incubated with glutathione-sepharose (120 µl sepharose in 1 ml of bacterial lysate) for 1 hour with gentle rotation at RT. The Raf1-RBD-sepharose was washed twice with 1 ml cold TBS and as 50% slurry with TBS used for pull-down assay. Active Ras was pulled down with 30 µl Raf1-RBD-sepharose by rotating for 35-45 min at 4°C. Pull-downs were washed twice with 0.5 ml lysis buffer (without GDP), heated in 50 µl 2x reducing sample buffer (see 2.7.) at 95°C, 5 min and separated on 14% SDS-PAGE. The gel was then blotted and stained with anti-Pan-Ras antibody to detect precipitated active Ras.

2.16. Scanning and quantification

Western blots were scanned with an Epson Perfection 4990 Photo scanner. The optical density of the bands was determined using Kodak 1D 3.6 software. The fold induction (FI) was then calculated as the density of the band of interest in proportion to the density of the loading control normalized to the value in resting cells.

e.g. $FI = [(Fyn/Actin)/(Fyn_{rest}/Actin_{rest})]$

3. Results

3.1. Proximal alterations within *Anergic* T cells

3.1.1. Induction of *Anergic* T cells

To prepare anergic T cells, we applied a well established protocol using stimulation of the cells with immobilized anti-CD3 antibodies in the absence of costimulation (Wolf et al., 1994). Note that this is the only system reproducibly shown to induce anergy in naïve T cells *in vitro* (Fathman and Lineberry, 2007). Thus, fresh human PBMCs (peripheral blood mononuclear cells) were isolated from healthy human volunteers by Ficoll gradient centrifugation and T cells were further purified by magnetic separation to more than 95% purity as determined by CD3 versus CD19 staining (Figure 3.1.).

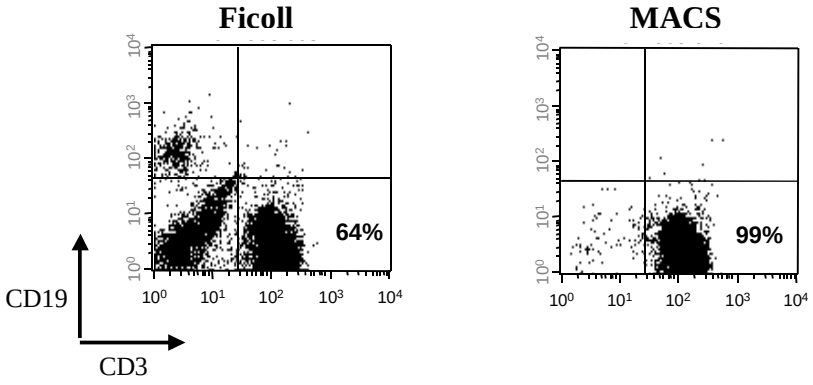


Figure 3.1. T-cell purity. T cells were isolated by Ficoll gradient centrifugation and further purified by magnetic separation (MACS). The purity was determined by staining with CD3 and CD19 antibodies and measuring by flow cytometry. Result from one representative experiment is shown. Note that T cells used in all the experiments were of more than 95% purity. Also please note that all the experiments were performed at least three times and always one representative experiment is shown.

Purified T cells were then divided into three populations. The first population was left untreated and is referred to as *Resting* cells. The second population was inoculated into anti-CD3-coated plastic plates and cultured for three days to induce anergy (= *Anergic* cells). The third population, so called *Rescued* cells, was cultured also in anti-CD3 coated plates, but received additionally the phorbol ester PMA (phorbol myristate acetate) to mimic costimulation and thereby prevent anergy. After three days of culture, *Anergic* and *Rescued* cells proliferated as expected and increased their numbers approximately 3 - 4 fold (Figure 3.2.).

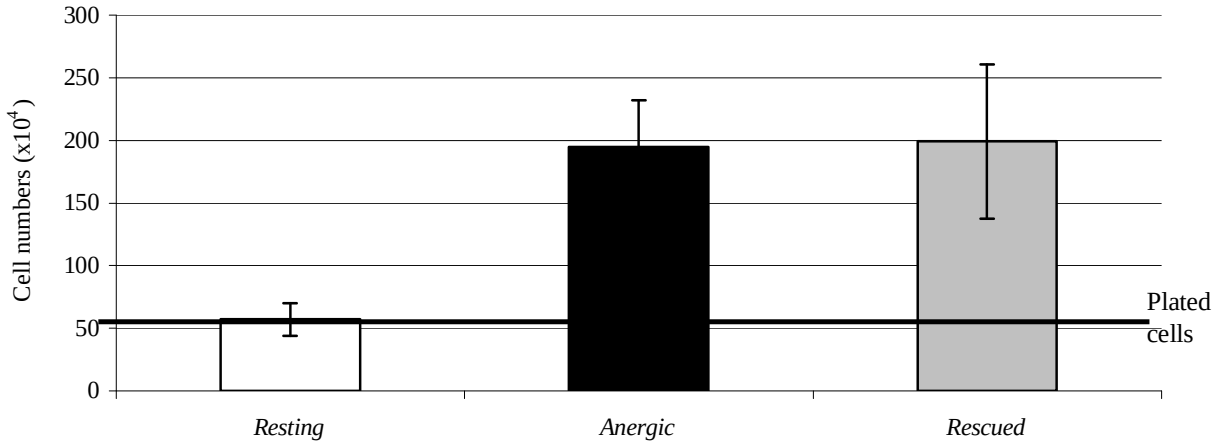


Figure 3.2. *Anergic* and *Rescued* cells proliferate on CD3-coated plates. Purified T cells ($6x10^5$) were inoculated into plastic plates coated with either no antibody (*Resting*), anti-CD3 (*Anergic*) or with anti-CD3 plus PMA (*Rescued*). Averaged cell numbers and standard deviations from 10 experiments after 3 days of culture are shown.

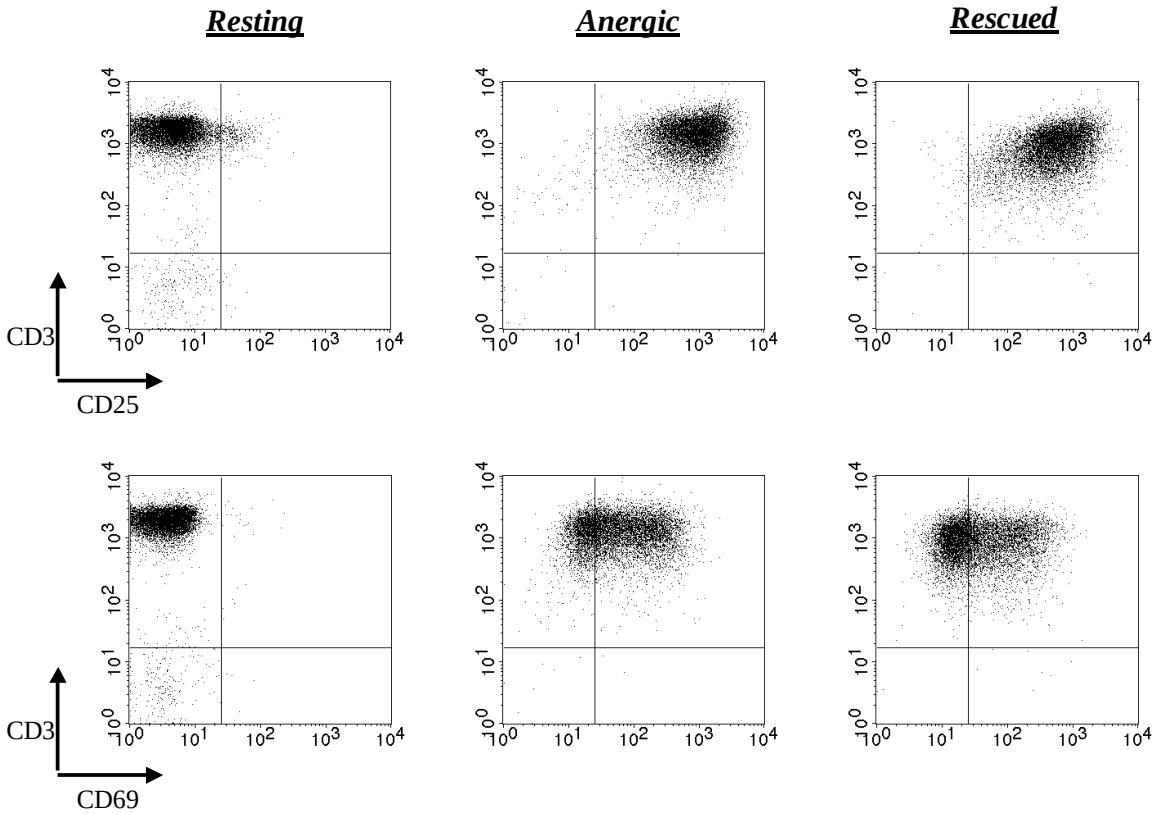


Figure 3.3. *Anergic* and *Rescued* cells upregulate activation markers. Expression of activation markers CD25 (top) and CD69 (bottom) upon *Resting*, *Anergic* and *Rescued* cells after 3 days of culture was analyzed by staining with appropriate antibodies and measuring by flow cytometry. Results from one representative experiment are shown.

Proliferation was accompanied by an upregulation of the surface activation markers CD25 and CD69 as measured by flow cytometry (Figure 3.3.). Whereas the late activation marker CD25 was still expressed at high levels (top panels), the early activation marker CD69 was already being downregulated after three days of stimulation (bottom panels).

Following 3 days of culture, the cells were allowed to rest without stimulus for 1 additional day and then restimulated with no stimulus, anti-CD3 alone, anti-CD3 plus anti-CD28 or anti-CD3 plus IL-2 to demonstrate that the cells are anergic (Figure 3.4.). The cells do not proliferate without stimulus, showing that they are indeed rested. The *Resting* cells will proliferate to all stimuli (white bars), as is the case also of the *Rescued* cells. The magnitude of *Rescued* cell proliferation (grey bars) is much higher than that of *Resting* cells, because *Rescued* cells already possess a preactivated phenotype. On the contrary, *Anergic* cells (black bars) will not proliferate when restimulated with either anti-CD3 alone or anti-CD3 plus anti-CD28. However, this proliferative block is broken by adding exogenous IL-2 to the culture. This is because these cells are not able to produce their own IL-2, indicating that these cells are indeed anergic (Jenkins et al., 1987).

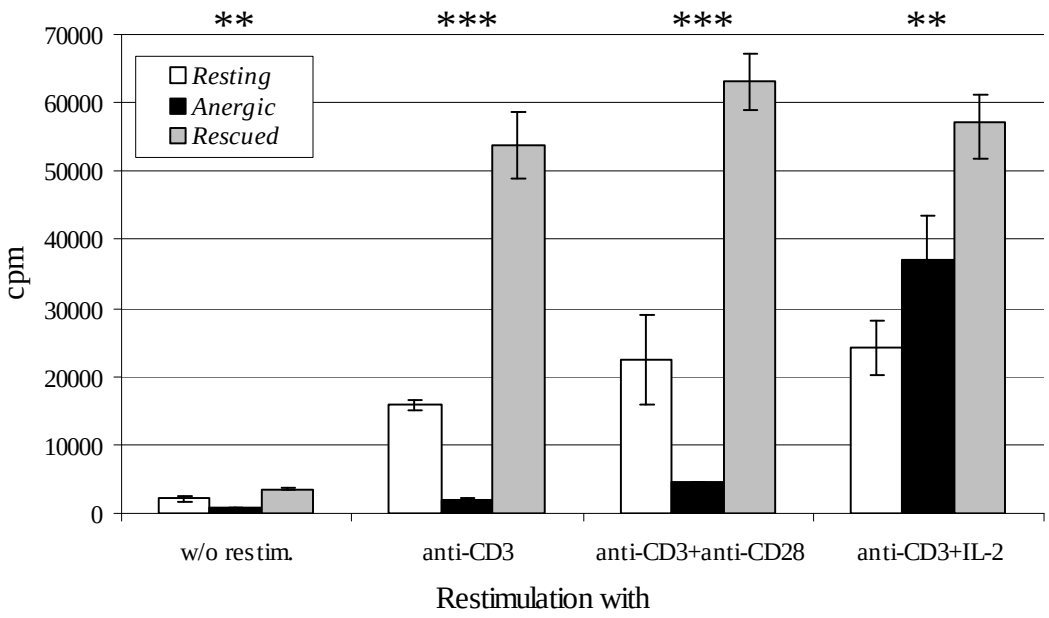


Figure 3.4. *Anergic* T cells are able to proliferate only in response to exogenous IL-2. *Resting* (white bars), *Anergic* (black bars) and *Rescued* (grey bars) cells were restimulated with no stimulus, anti-CD3, anti-CD3 plus anti-CD28 and anti-CD3 plus IL-2 and the proliferation after 72h was assessed by ^{3}H-thymidine incorporation. Average values and standard deviations from triplicate wells of one representative experiment are shown. Data were analyzed using one-way ANOVA (** $p<0.01$, *** $p<0.001$).

However, we also needed to show that we are in fact not inducing the expansion of regulatory T cells (Tregs), which are also unresponsive and would additionally suppress the proliferation of other T cells. Regulatory T cells are known to have a markedly upregulated expression of the transcription factor FoxP3 (Hori et al., 2003; Fontenot et al., 2003), which has become their main characteristic. Therefore, to exclude the possibility of Tregs induction in our culture, we immunoblotted lysates of *Resting*, *Anergic* and *Rescued* cells with an antibody against FoxP3. To determine the basal level of FoxP3 expression in normal cells, lysates of Th cells were used and compared to Tregs, the positive control. As shown in figure 3.5., Tregs indeed possess an increased amount of FoxP3 on the protein level compared to Th cells. On the contrary, there is no increase in FoxP3 expression in any of our cultures, indicating that we are not inducing regulatory T cells.

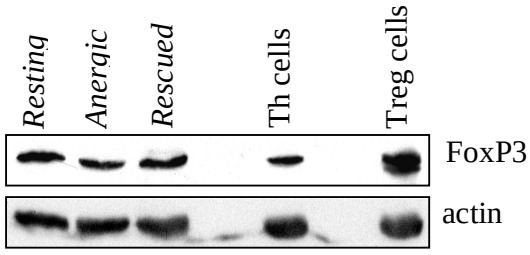


Figure 3.5. Absent induction of FoxP3 in the unresponsive cells. Lysates of *Resting*, *Anergic* and *Rescued* cells were imunoblotted with anti-FoxP3 antibody. Lysates of Th and Treg cells were included as negative and positive control, respectively. Actin staining is shown for equal loading.

3.1.2. Increased Fyn activity and expression within *Anergic* T cells

We next investigated whether our anergic cells also upregulate the level of Fyn protein and/or kinase activity as originally described (Quill et al., 1992; Gajewski et al., 1994; Gajewski et al., 1995). *Resting*, *Anergic* and *Rescued* cells were lysed, Fyn and Lck were immunoprecipitated and *in vitro* kinase assays (IVKs) were performed (Figure 3.6.). An exogenous substrate of Src kinases, acid-denatured enolase, was added to the IVK reactions. We found that both Fyn autophosphorylation and phosphorylation of the substrate enolase were dramatically enhanced in *Anergic* cells compared to the *Resting* sample and approximately 2-fold increased when compared to the *Rescued* cells, meaning that Fyn is hyperactive in anergic cells. (Figure 3.6.A).

The levels of phosphorylation were normalized with respect to Fyn protein levels and are presented as relative ratios. Lck activity, however, was only slightly increased compared to *Rescued* cells as visible by the phosphorylation of enolase (Figure 3.6.B). Lck autophosphorylation was not detected, because the antibody chosen for immunoprecipitation (IP) seems to preferentially recognize the autophosphorylated form of Lck. We additionally determined the expression levels of Fyn and Lck and observed a specific increase in the expression level of Fyn in *Anergic* cells [1:2,6:1,6], but only a marginal increase in Lck expression that was also detected in *Rescued* cells [1:1,7:1,7] (Figure 3.6.C).

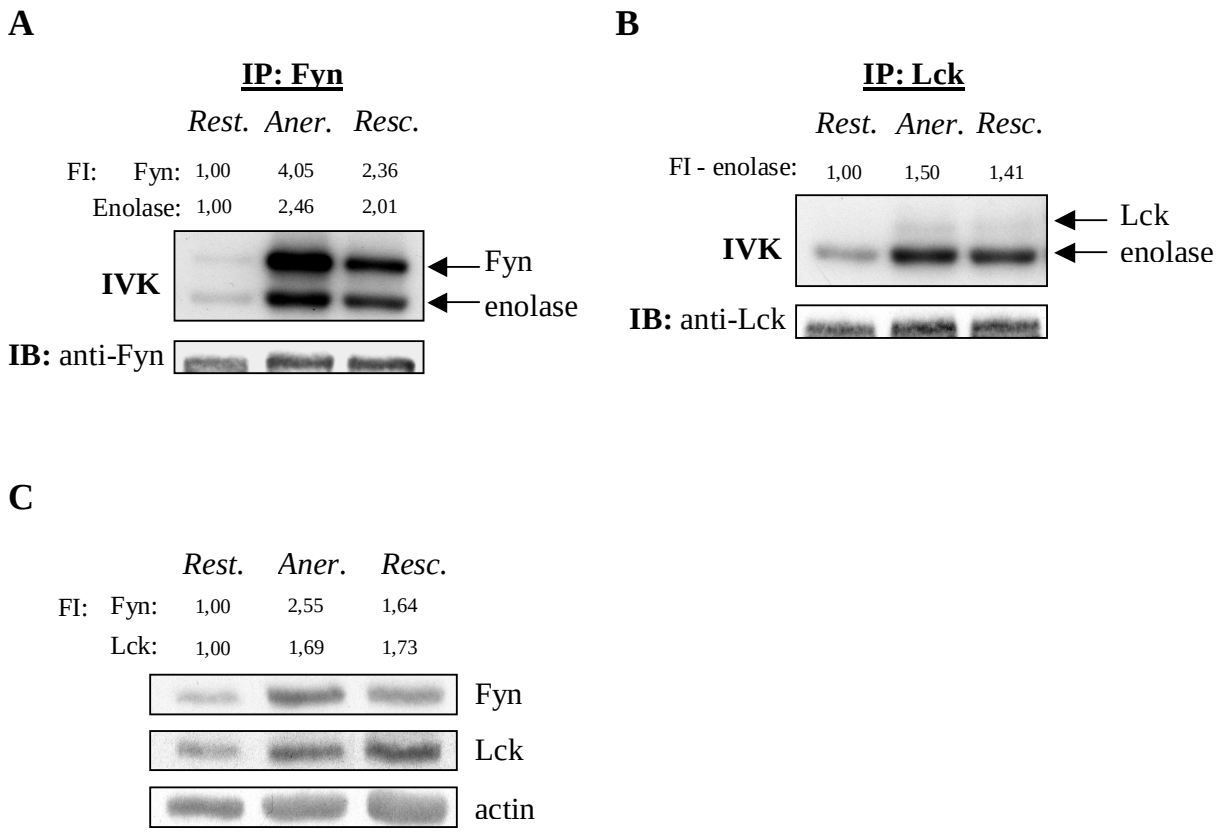


Figure 3.6. *Anergic* cells show markedly enhanced Fyn kinase activity. Fyn (A) and Lck (B) were immunoprecipitated from *Resting* (*Rest.*), *Anergic* (*Aner.*) and *Rescued* (*Resc.*) T cells and *in vitro* kinase (IVK) assays were performed. Phosphorylation was visualised by autoradiography. The amount of precipitated kinase was detected by immunoblotting with specific antibodies. Phosphorylation of Fyn and enolase were normalized to the level of precipitated kinase and are presented here as the fold induction (FI) of the *Resting cells* value. (C) Expression of Fyn and Lck in whole cell lysates is shown and normalized to the amount of actin.

3.1.3. Altered phosphorylation profile in *Anergic* T cells

Due to the increase in SFK activity, we next examined the global tyrosine phosphorylation in whole lysates. *Resting*, *Anergic* and *Rescued* cells were either left untreated (-) or rechallenged with anti-CD3 antibody for 2 minutes (+), lysed and subjected to Western blotting (Figure 3.7.). The basal tyrosine phosphorylation in *Anergic* cells is markedly increased when compared to *Resting* cells and corresponds more to that seen in *Rescued* cells. Whereas the only protein phosphorylation observed in *Resting* cells is that of PAG, Src kinases and the constitutive basal phosphorylation of ITAMs within TCR-zeta chains, *Anergic* and *Rescued* cells show a dramatic increase in phosphorylation at 55 kDa (i.e. phosphorylation of Src kinases), in the range of 60-80 kDa and around 30 kDa. Additionally, *Anergic* cells also react differently upon TCR restimulation and show an increased number of phosphorylated proteins. Further experiments have excluded that the band induced at 30 kDa is either the Lck interacting molecule (LIME) or the non-T-cell activation linker (NTAL), two adapter proteins involved in immunoreceptor signaling (Brdickova et al., 2003; Brdicka et al., 2002) (data not shown). It is important to point out that since both *Anergic* and *Rescued* cells originally received the same stimulus via the TCR, the pattern observed with anti-phosphotyrosine staining is quite similar.

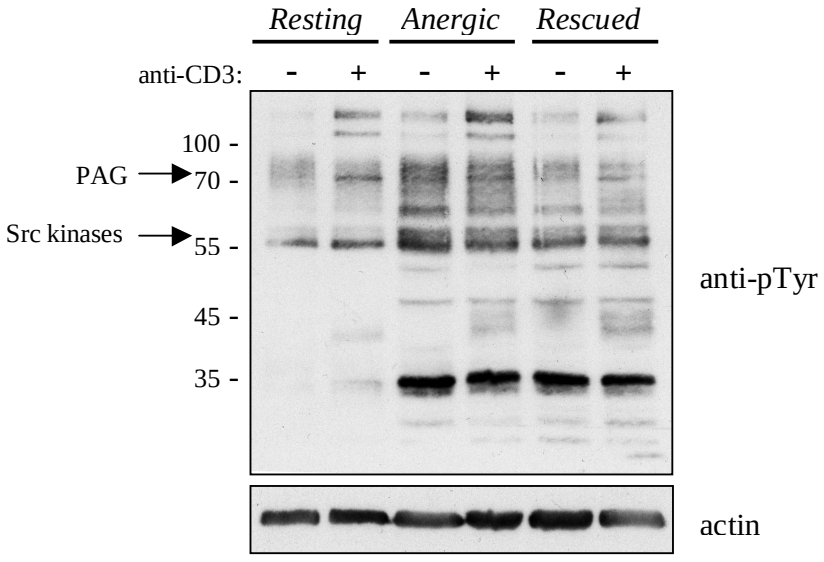


Figure 3.7. *Anergic* cells have altered phosphotyrosine profile. *Resting*, *Anergic* and *Rescued* cells were either left untreated (-) or restimulated with anti-CD3 antibody for 2 minutes (+). The cells were lysed, subjected to Western blotting and probed with anti-phosphotyrosine antibody (4G10) to show changes in overall phosphorylation pattern. Actin staining is shown for equal loading.

The difference between these cells is found more distal in the signaling cascade. *Rescued* cells received additional stimulation via PMA, an analogue of diacylglycerol, which acts directly upon PKC-θ (protein kinase C theta) and RasGRP (Ras guanyl releasing protein) to activate Ras and promote transcription, thereby preventing anergy induction.

3.1.4. Defective proximal signaling in *Anergic* T cells

We next investigated the alterations in proximal signaling caused by the induction of anergy. One of the most proximal events upon TCR triggering is the phosphorylation of zeta chain associated with the TCR complex (see 1.4.3.). This is followed by the recruitment and activation of ZAP70, which in turn phosphorylates the adaptor protein LAT (Zhang et al., 1998). Figure 3.8. shows that the phosphorylation of the zeta chains upon TCR triggering is almost completely abolished in both *Anergic* and *Rescued* cells in comparison with *Resting* cells. Note that *Anergic* cells possess an even more profound defect, having only 50% of the zeta-phosphorylation seen in the *Rescued* sample (compare FI of lane 4 versus lane 6, left panel). However, the phosphorylation of LAT is undetectable in both the *Anergic* and *Rescued* cells. Thus, the proximal signaling appears to be affected to the similar extent in both cell populations, which reflects the fact that both *Anergic* and *Rescued* cells originally received the same stimulus via the TCR.

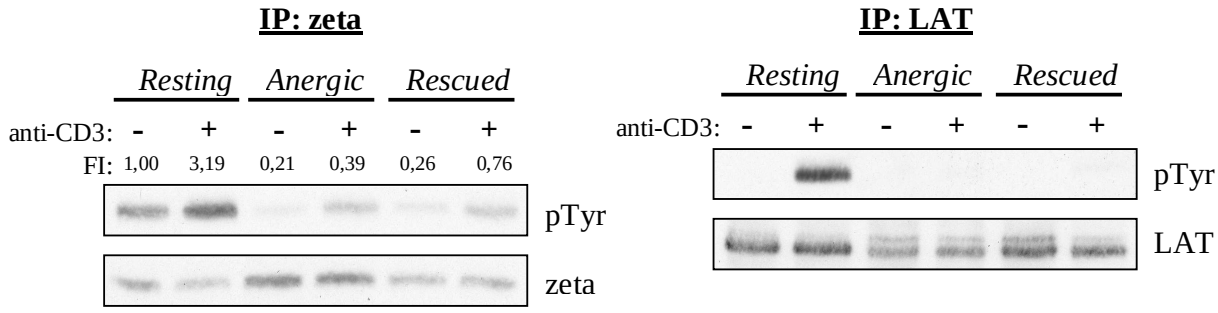


Figure 3.8. *Anergic* cells have abolished phosphorylation of zeta chain and LAT. *Resting*, *Anergic* and *Rescued* cells were either left untreated (-) or restimulated with anti-CD3 antibody for 2 minutes (+). The cells were lysed and zeta chain (left panel) or LAT (right panel) were immunoprecipitated. Western blots of phosphotyrosine staining (4G10) and total protein are shown. The amount of zeta chain phosphorylation was normalized to the total zeta immunoprecipitated and is presented as the fold induction (FI) of the *Resting cell* value.

3.2. Alterations within PAG-associated complex in *Anergic* T cells

3.2.1. PAG-associated kinase activity is enhanced in *Anergic* T cells

Considering the fact that Fyn constitutively associates with PAG and that we found enhanced Fyn activity in anergic cells, we were interested in whether also the activity of the Fyn pool bound to PAG had specifically increased. Thus, we performed *in vitro* kinase assays on PAG immunoprecipitates and found markedly enhanced phosphorylation of the substrate enolase in *Anergic* cells when compared to both the *Resting* and *Rescued* cells (Figure 3.9.). Interestingly, it seems that PAG is the preferred substrate for Fyn as we observed only very minor phosphorylation of enolase in *Resting* cells, despite clear PAG phosphorylation meaning that Fyn was indeed active. Note that Fyn kinase levels coprecipitated with PAG were similar throughout the samples and thus the differences in phosphorylation are indeed caused by increased kinase activity and not by an increased amount of Fyn associated to PAG.

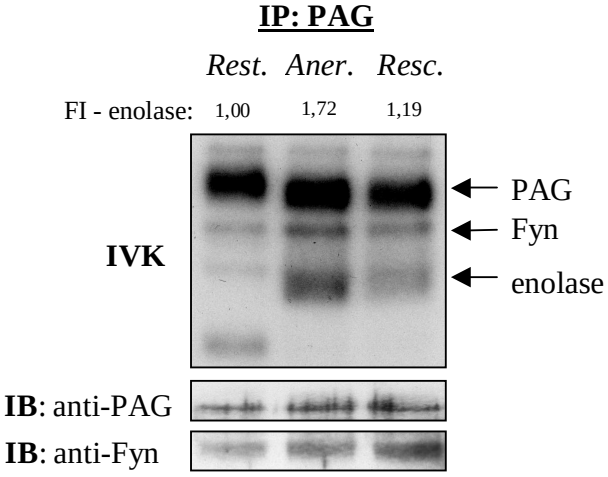


Figure 3.9. *Anergic* cells show markedly enhanced PAG-associated Fyn kinase activity. PAG was immunoprecipitated from *Resting* (*Rest.*), *Anergic* (*Aner.*) and *Rescued* (*Resc.*) T cells and *in vitro* kinase (IVK) assays were performed. Phosphorylation was visualised by autoradiography. The amount of precipitated PAG and coprecipitated Fyn was detected by immunoblotting with specific antibodies. Phosphorylation of enolase was normalized with respect to the level of precipitated PAG and is presented here as the fold induction (FI) of the *Resting cell* value.

3.2.2. PAG is hyperphosphorylated at Y^{317} in *Anergic* T cells

Since Fyn is the kinase primarily responsible for PAG phosphorylation and we observed an increase in PAG-associated Fyn kinase activity, we next investigated PAG phosphorylation and its changes upon restimulation of the cells (Figure 3.10.). Note that in the resting state, PAG is phosphorylated and becomes dephosphorylated by an unknown phosphatase upon TCR stimulation, thereby releasing Csk and allowing T-cell activation (see 1.6.4.). Probing the Western blots of whole cell lysates with a phospho-specific antibody to the Csk binding site (pY317) showed that PAG becomes hyperphosphorylated during anergy induction. An increase in PAG phosphorylation was also observed in *Rescued* cells, but the increase in phosphorylation was not so dramatic as in the case of *Anergic* cells. Upon restimulation, PAG becomes dephosphorylated in all samples, including *Anergic* cells, indicating that the phosphatase is still active. However, in *Anergic* cells the level of PAG dephosphorylation upon restimulation never falls below the level observed in unstimulated *Resting* T cells. On the other hand, upon TCR triggering of the *Rescued* cells, PAG is rapidly dephosphorylated to the same level as in restimulated *Resting* T cells. Also note that the expression of PAG protein does not seem to be altered by the induction of anergy. We have quantified the level of PAG phosphorylation, since the expression of phospho-PAG in the cells is, as we believe, the critical event for the regulation of signaling.

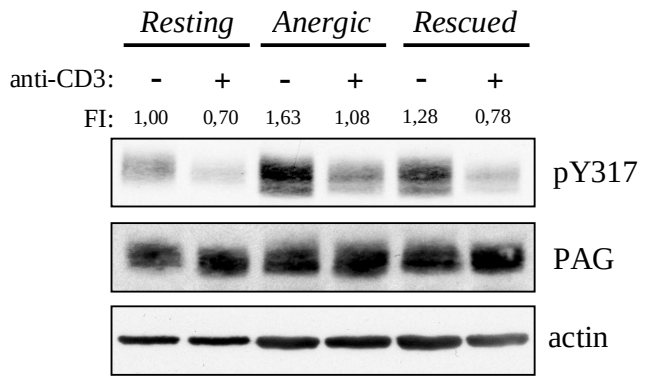


Figure 3.10. PAG is hyperphosphorylated in *Anergic* cells. *Resting*, *Anergic* and *Rescued* cells were either left untreated (-) or restimulated with anti-CD3 antibody for 2 minutes (+). The cells were lysed, separated by SDS-PAGE, subjected to Western blotting and probed with a phospho-specific antibody recognizing pY^{317} of PAG and with an antibody against total PAG. Actin staining is shown for equal loading. The level of phospho-PAG in the cells (determined by Y^{317} phosphorylation) was normalized to actin and is presented as the fold induction (FI) of the *Resting cell* value.

3.2.3. Hyperphosphorylated PAG recruits more Csk in *Anergic* T cells

Having found that PAG becomes hyperphosphorylated in *Anergic* cells, we next wanted to analyze how does this hyperphosphorylation affect the amount of the proteins associated to PAG. Therefore we immunoprecipitated PAG from untreated or restimulated cells and probed by Western blotting for the presence of Fyn and Csk (Figure 3.11.). Here we demonstrate that hyperphosphorylation of Y^{317} observed in *Anergic* cells leads also to an enhanced Csk recruitment to PAG. In *Resting* and *Rescued* cells, Csk association decreases upon TCR restimulation, whereas in *Anergic* cells, Csk is only partially lost, but the Csk level is still increased compared to *Resting* unstimulated cells. Fyn association, which was proposed to be phosphorylation-independent (Brdicka et al., 2000), was largely unchanged in these samples.

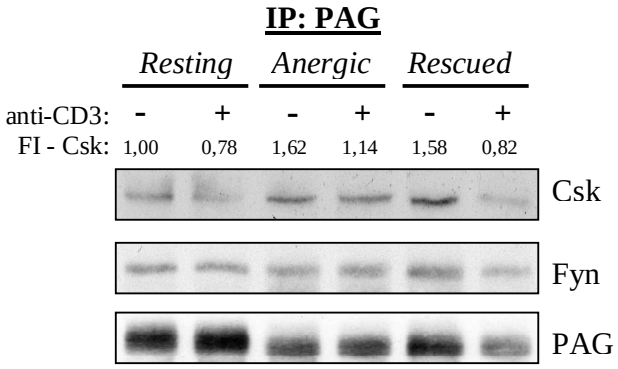


Figure 3.11. Hyperphosphorylated PAG recruits more Csk in *Anergic* cells. *Resting*, *Anergic* and *Rescued* cells were either left untreated (-) or restimulated with anti-CD3 antibody for 2 minutes (+). PAG was immunoprecipitated and the associated proteins detected by immunoblotting with anti-Csk and anti-Fyn antibody. The amount of Csk co-precipitated with PAG was normalized to the PAG level and is presented as the fold induction (FI) of the *Resting cell* value.

3.2.4. Elevated levels of the PAG-Csk complex create an inhibitory environment in *Anergic* cells

Once Csk is recruited by PAG to the plasma membrane, it can phosphorylate the inhibitory tyrosines within the C-terminus of the Src kinases. Since we observe increased Csk association with PAG, we next investigated whether the phosphorylation of the inhibitory tyrosine residues within the SFKs was altered. Thus, whole cell lysates from *Resting*, *Anergic* and *Activated* cells

were probed with phospho-specific antibodies against the inhibitory tyrosine of Fyn (Y^{529}) and Lck (Y^{505}), and against their activatory tyrosine residues Y^{418} or Y^{394}, respectively (Figure 3.12.). Reflecting the increased Csk recruitment, we found a 2-fold enhancement in the phosphorylation of the inhibitory tyrosine within Fyn in *Anergic* cells (Figure 3.12.A, upper panel). We also observed increased levels of Y^{529} phosphorylation in *Rescued* cells, but this change was not as dramatic as that seen in *Anergic* cells. In contrast, the inhibitory tyrosine within Lck is not altered in *Anergic* cells. This we attribute to the fact that little Lck resides within lipid rafts (Yasuda et al., 2002; Filipp et al., 2003) where the PAG-Csk complex is located (Figure 3.12.B, upper panel). Surprisingly, we observed a mild decrease in the phosphorylation status of the activatory tyrosines in both Fyn and Lck in *Anergic* and *Rescued* cells (Figure 3.12.A and B, lower panels). Since both protein tyrosine kinases show substantially increased kinase activity (compare Figure 3.6.), we conclude that autophosphorylation is probably not a direct measure of kinase activity.

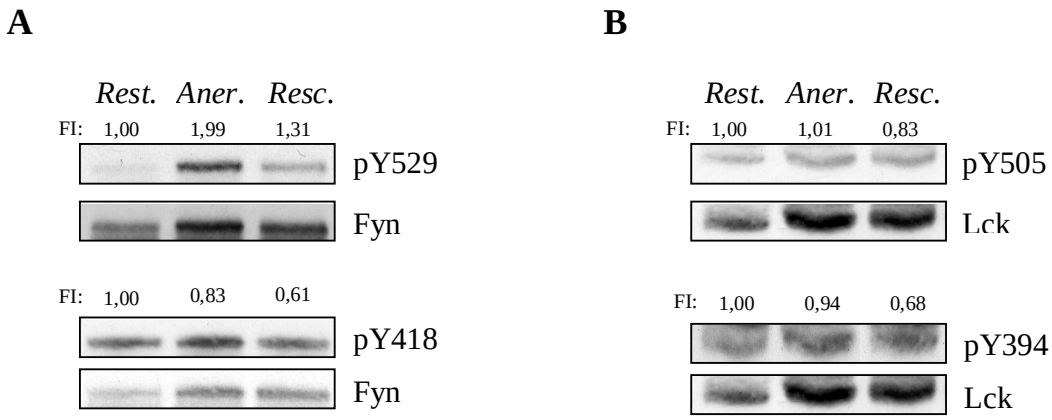


Figure 3.12. Fyn possesses increased inhibitory tyrosine phosphorylation in *Anergic* cells. (A) Whole lysates of *Resting* (*Rest.*), *Anergic* (*Aner.*) and *Rescued* (*Resc.*) T cells were immunoblotted with phosphospecific antibodies against the inhibitory (Y^{529}; upper panel) and activatory (Y^{418}; lower panel) tyrosines of Fyn. The level of phosphorylation was normalized to the total amount of Fyn and is presented as the fold induction (FI) of the *Resting cell* value. (B) The same lysates as in A were probed with phosphospecific antibodies against the inhibitory (Y^{505}; upper panel) and activatory (Y^{394}; lower panel) tyrosine of Lck and the phosphorylation was normalized to the total amount of kinase.

3.2.5. Increased cAMP level and pSer^{364}-Csk in *Anergic* T cells

There are two explanations for the increased phosphorylation of the inhibitory tyrosine within Fyn observed in *Anergic* cells. It may be either due to a decrease in phosphatase activity or on the contrary to an increase in kinase activity, which would result from enhanced Csk recruitment to the lipid rafts and/or increased Csk activity. Although we indeed observed increased amounts of Csk associated with PAG in *Anergic* cells compared to *Resting* cells (Figure 3.11.), the amount was basically equal to that in unstimulated *Rescued* cells. Therefore we presumed that there might be difference in Csk activity between *Anergic* and *Rescued* cells, which is responsible for the specific increase in Fyn inhibitory tyrosine phosphorylation observed in *Anergic* cells. Csk activity was shown to be enhanced upon binding to PAG and additionally upon Ser^{364} phosphorylation through PKA (cAMP-dependent protein kinase). As cAMP levels increase upon TCR triggering, we measured the cAMP levels in our cells (Figure 3.13.A). We indeed found an approximately 2,5-fold increase in cAMP levels in *Anergic* and *Rescued* cells compared to *Resting* cells. Next we investigated the phosphorylation status of Csk using the phospho-specific

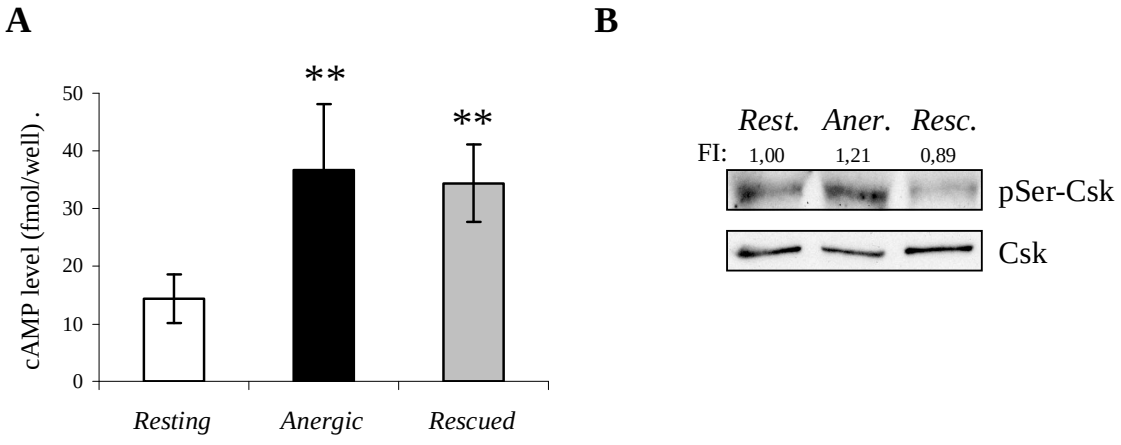


Figure 3.13. *Anergic* cells contain both increased cAMP levels and augmented Ser^{364}-Csk phosphorylation. (A) *Resting*, *Anergic* and *Rescued* cells were lysed and cAMP levels measured using the cAMP Biotrak Enzymeimmunoassay System. The mean values and standard deviations from four independent experiments are shown. Data were analyzed using one-way ANOVA (** $p<0.01$). (B) The level of Ser^{364} phosphorylation within Csk was determined by Western blotting. Total Csk staining is shown for equal loading. The level of phosphorylation normalized to total Csk is presented as the fold induction (FI) of the *Resting* cell value.

antibody against Ser^{364} (Yagub et al., 2003) (Figure 3.13.B). We observed an approximately 20% increase in PKA-dependent phosphorylation at Ser^{364} of Csk in *Anergic* cells, whereas there was a decrease in Ser^{364} phosphorylation in *Rescued* cells. Although it does not seem to be proportional with the elevations of the cAMP levels, the increase in pSer-Csk argues for an increased activity of Csk specifically in *Anergic* cells. However, the increase in Fyn inhibitory tyrosine phosphorylation was much higher (+100%) than the increase in Ser^{364}-Csk phosphorylation and therefore we must conclude that it is also the phosphatase activity of either CD45 or RPTPα that is altered in *Anergic* cells and thus contributing to the differences in Fyn inhibitory tyrosine phosphorylation observed (Figure 3.12.A).

3.2.6. Fyn is dually phosphorylated on its Y^{529} and Y^{215} in *Anergic* T cells

A somewhat puzzling observation is the fact that we observe increased inhibitory tyrosine phosphorylation and, simultaneously, increased kinase activity of Fyn in anergic cells. Normally, one would expect phosphorylation of inhibitory tyrosine to lead to a closed conformation of the kinase and thereby inhibit its activity. However, phosphorylation of the inhibitory tyrosine may, in some cases, lead to a hyperactive state if a tyrosine located within the SH2 domain of the Src kinase is also phosphorylated at the same time (Stover et al., 1996). To investigate whether this mechanism applies to our system, we probed whole lysates from *Resting*, *Anergic* and *Rescued* cells with a phospho-specific antibody to the tyrosine within SH2 domain of the SFKs (i.e. pY^{215}) (Vadlamudi et al., 2003) (Figure 3.14.A). Interestingly, we observed an increase in Y^{215} phosphorylation in both *Anergic* and *Rescued* cells. However the epitope recognized by this antibody is conserved among the SFKs and therefore it is difficult to judge which kinase is responsible for the increased staining. To solve this problem, we decided to immunoprecipitate Src kinases with the anti-pY215 antibody and reprobe for the presence of Fyn (Figure 3.14.B). The phosphorylation of Y^{215} within Fyn is clearly enhanced in *Anergic* cells compared to both the *Resting* and *Rescued* cells and even further increases upon TCR triggering. Reprobing the same blot with a phosphospecific antibody against Y^{529} demonstrates that Fyn phosphorylated on Y^{215} is simultaneously phosphorylated also on its inhibitory tyrosine. This in turn results in the hyperactive state of Fyn observed in *Anergic* cells. Interestingly, Lck does not seem to follow the same kinetic as Fyn and its Y^{215} is rather dephosphorylated upon restimulation of *Resting* and *Anergic* cells.

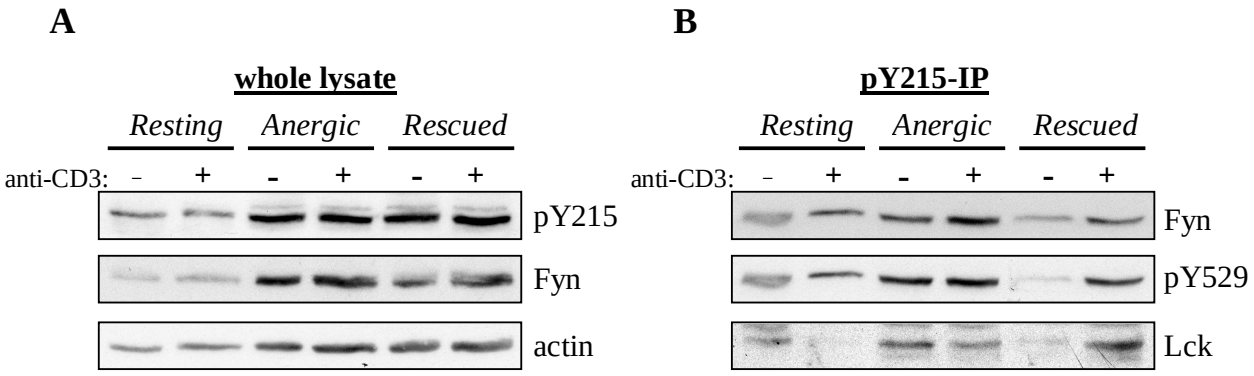


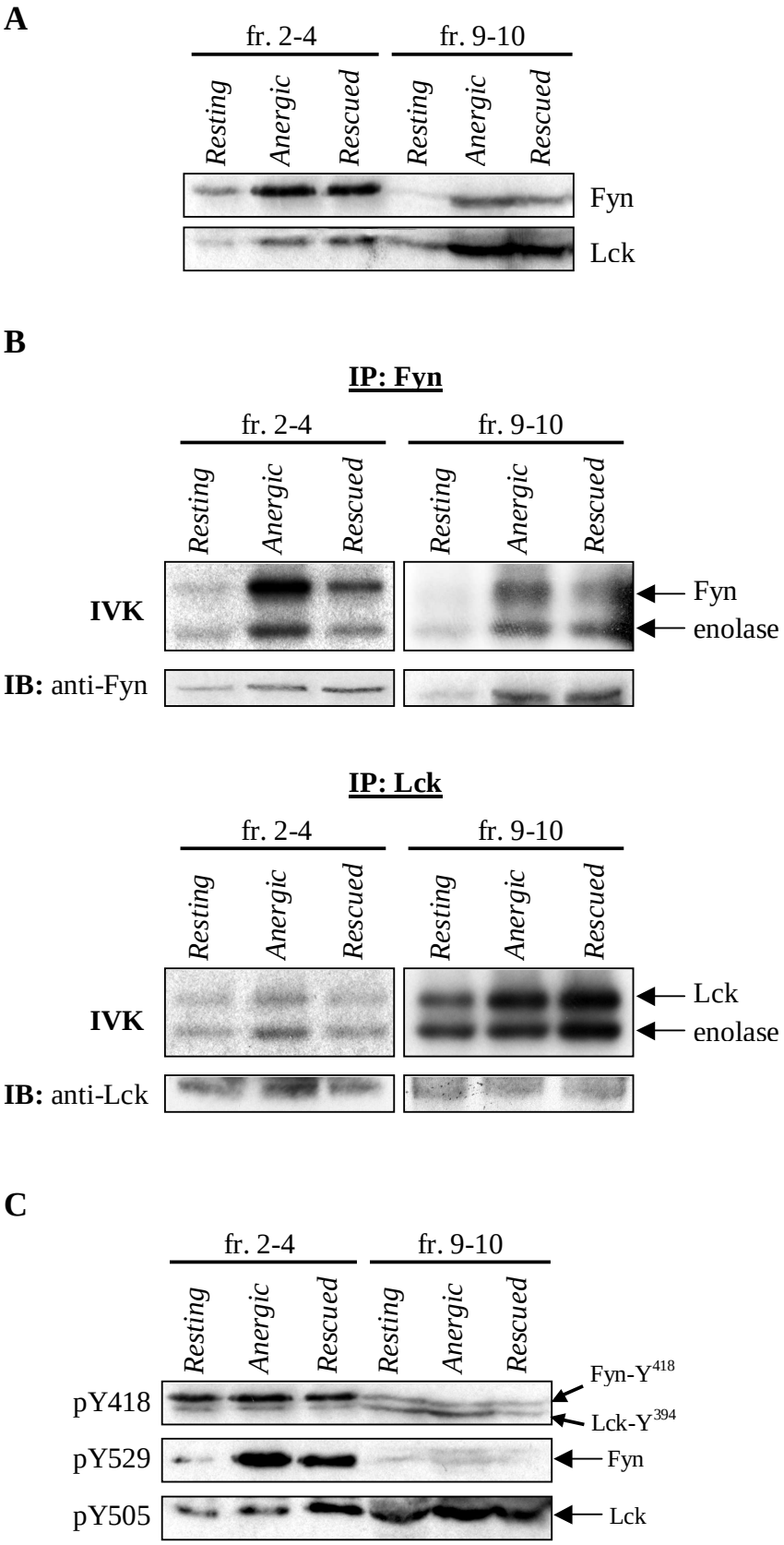
Figure 3.14. *Anergic* cells possess increased phosphorylation of Y^{215} within SH2 domain of Fyn. (A) Whole cell lysates of *Resting*, *Anergic* and *Rescued* cells were immunoblotted with antibodies against phospho-Y^{215}, Fyn and actin. (B) Lysates from A were used for immunoprecipitation with pY215 antibody, samples were resolved by SDS-PAGE, blotted and probed for the presence of Fyn, pY529 and Lck.

3.2.7. Increased Fyn kinase activity and inhibitory tyrosine phosphorylation within the lipid rafts of *Anergic* T cells

Fyn and Lck kinases are differentially localized within the plasma membrane and this spatial distribution is believed to be important for their proper activation and function (Filipp et al., 2003). As we observed a specific increase in both protein level and kinase activity for Fyn in whole lysates of anergic cells, we looked to see whether the localization and/or activity within the lipid rafts and the non-raft fraction had changed. Therefore, we lysed the cells and isolated lipid rafts by sucrose density gradient centrifugation. Aliquots of fractions taken from the gradient were spotted onto nitrocellulose and the raft-containing fractions identified using an antibody against CD59, a known lipid raft-associated protein (data not shown). Pooled raft-containing fractions (fractions 2-4) and non-raft fractions (fractions 9-10) from *Resting*, *Anergic* and *Rescued* cells were then analyzed by Western blotting and the distribution of Fyn and Lck within the various fractions was detected with specific antibodies (Figure 3.15.A). As previously described, Fyn was mainly localized in the lipid raft fractions, whereas Lck is found predominantly outside of the lipid rafts. We observed increased expression of both Fyn and Lck in *Anergic* and *Rescued* cells in comparison to the *Resting* sample. However, this increase was observed in both the rafts as well as the non-raft fractions and no specific shift in the localization of these kinases upon anergy induction was found.

Consequently, Fyn and Lck were immunoprecipitated from either lipid rafts or the non-raft fractions and *in vitro* kinase assays were performed (Figure 3.15.B). We observed that Fyn kinase activity predominates within lipid rafts, whereas the non-raft-fractions had to be exposed for an extended time to obtain a good signal. In agreement with the previously observed Fyn hyperactivity in whole lysates (Figure 3.6.), we again found augmented Fyn kinase activity in both the raft and non-raft compartment of *Anergic* cells (Figure 3.15.B, upper panel). This suggests that the distribution of Fyn activity within the membrane is not altered by anergy induction. Additionally, the localization of Lck activity was not disturbed and moreover, we did not find dramatic changes regarding Lck activity in *Anergic* cells in comparison with other samples (Figure 3.15.B, lower panel).

Since we found a dramatic increase in the inhibitory tyrosine phosphorylation of Fyn in *Anergic* cells, we were further interested whether this change was specific to a certain membrane compartment. Therefore we probed the blots of pooled lipid raft and non-raft fractions with phosphospecific antibodies against the activatory and inhibitory tyrosines of Fyn and Lck (Figure 3.15.C). The phosphorylation of the activatory tyrosine (Y^{418} or Y^{394} respectively) seems to be unchanged for both Fyn and Lck and we also do not observe any remarkable changes in phosphorylation of the inhibitory tyrosine of Lck (Y^{505}), neither in the lipid rafts nor outside. On the contrary, our results clearly demonstrate a specific increase in the phosphorylation of the inhibitory tyrosine within Fyn (Y^{529}) in the lipid rafts of *Anergic* cells, whereas there is hardly any Y^{529} phosphorylation detected outside of lipid rafts. Thus, the dramatic increase in the phosphorylation of the inhibitory tyrosine of Fyn observed in whole lysates of *Anergic* cells (compare figure 3.12.) seems to be preferentially located in the lipid rafts where the bulk of Fyn and the PAG-Csk complex reside.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Anergic
Rescued
pY418
Fyn-Y418
Lck-Y394
pY529
Fyn
pY505
Lck

Figure 3.15. *Anergic* cells possess enhanced Fyn kinase activity and increased inhibitory tyrosine phosphorylation of Fyn in lipid rafts. (A) *Resting*, *Anergic* and *Rescued* cells were lysed and subjected to sucrose density gradient centrifugation. Fractions containing lipid rafts (fr. 2-4) and heavy fractions (fr. 9-10) were pooled, subjected to SDS-PAGE and immunoblotted with antibodies against total Fyn and Lck, respectively. (B) Fyn (upper panel) or Lck (lower panel) were immunoprecipitated from pooled raft-fractions (fr. 2-4) and non-raft-fractions (fr. 9-10) from A and subjected to *in vitro* kinase assay (IVK). Phosphorylation was visualized by autoradiography. The amount of precipitated kinase was detected by immunoblotting with specific antibodies. Note that Fyn IVK and immunoblot from fractions 9-10 were exposed for extended time due to low Fyn localization in these fractions. (C) The blots from A were reprobed with phospho-specific antibody (pY418) recognizing the activatory tyrosine of both Fyn (Y^{418}) and Lck (Y^{394}) and with phospho-specific antibodies against the inhibitory tyrosine of Fyn (pY529) and Lck (pY505). Bands corresponding to Fyn and Lck in pY418 staining are marked.

3.3. PAG forms a novel multiprotein complex, which regulates Ras activation

3.3.1. Increased expression of Sam68 and p120RasGAP

In the previous section, we have identified a mechanism that leads to the hyper-activation of Fyn kinase and consequently to an increased Csk recruitment and alterations in proximal signaling. This mechanism, however, does not explain the connection between increased Fyn kinase activity and the block in Ras activation, a key feature of anergic T cells. If such a link between Fyn and Ras exists, we hypothesized that the candidate protein (or proteins) must be a substrate of Fyn and must possess the ability to attenuate Ras activity, either directly or indirectly, i.e. by recruiting Ras GTPase-activating proteins (RasGAPs). Since we observed an increased phosphorylation of proteins in the range of 60 – 80 kDa in *Anergic* cells (see Figure 3.7.), we hypothesized that the protein should be of this size. Therefore we searched the literature and found two candidates that fulfilled our criteria in addition to p120RasGAP itself – the nuclear/cytosolic protein Sam68 (Src-associated in mitosis of 68 kDa) (Najib et al., 2005) and the cytosolic adaptor protein p62Dok (Downstream of kinase; Dok1) (Yamanashi and Baltimore, 1997). Both of these proteins are phosphorylated by Fyn and were shown to associate with p120RasGAP, the main RasGAP within T cells (Guitard et al., 1998; Jabado et al., 1998; Yamanashi and Baltimore, 1997). We first investigated the expression of these proteins by probing post-nuclear lysates from *Resting*, *Anergic* and *Rescued* cells with antibodies specific for Sam68, p62Dok and p120RasGAP. Interestingly, we found that the expression of both Sam68 and p120RasGAP is enhanced in *Anergic* and *Rescued* cells, whereas p62Dok was not altered (Figure 3.16.A and data not shown).

However, Sam68 seems to be drastically upregulated also in *Resting* cells already after 2 minutes of stimulation (Figure 3.16.A, lane 2). One has to realize though, that these are postnuclear lysates of cells and that Sam68 is an RNA-binding protein that shuttles from the cytosol into the nucleus and back. Indeed, it was shown that, upon stimulation of T cells, Sam68 is rapidly phosphorylated by Fyn (Fusaki et al., 1997) and that Sam68 phosphorylation negatively correlates with its nuclear localization and retains the protein in the cytosol (Wang et al., 1995). To determine whether Sam68 is upregulated in *Anergic* cells or whether only the intracellular distribution of Sam68 is altered, we applied a different fractionation protocol using different lysing conditions to separate the nuclear and cytosolic fractions; note that cytosol in this case

contains both the cytoplasm and the membranes. Using this other fractionation approach, the cytosolic fraction of Sam68 appeared to be unchanged, however we observed a clear increase in Sam68 within the nuclear fraction of *Anergic* and *Rescued* cells (Figure 3.16.B). Thus, it appears that the expression of total Sam68 is indeed increased.

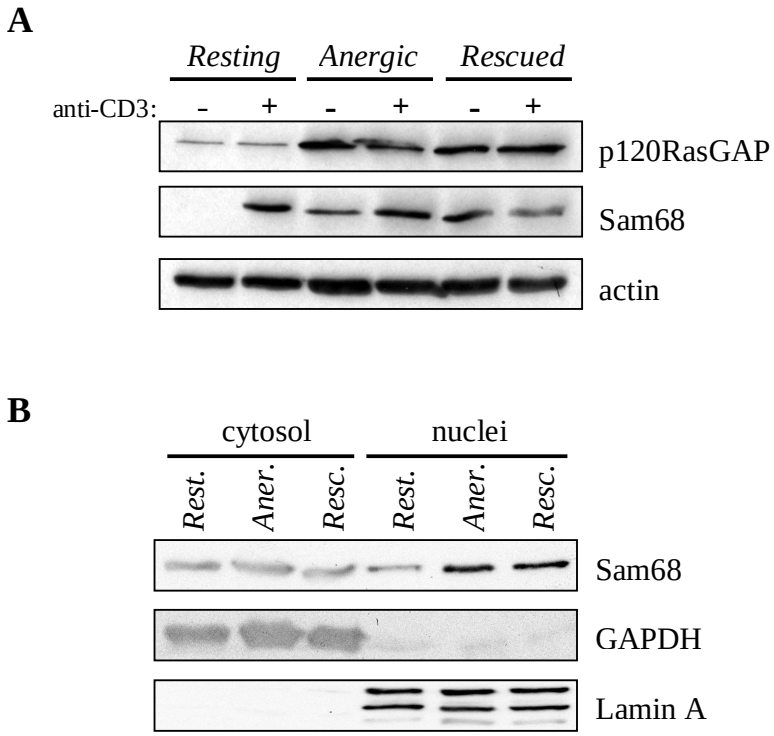

Figure 3.16. Increased expression of Sam68 and p120RasGAP. (A) *Resting*, *Anergic* and *Rescued* cells were either left untreated (-) or restimulated with anti-CD3 antibody for 2 minutes (+). Postnuclear lysates were immunoblotted and probed for the expression of Sam68 and p120RasGAP. Actin staining is shown for equal loading. (B) Cytosolic and nuclear fractions were prepared from *Resting*, *Anergic* and *Rescued* cells and blotted with antibody against Sam68. GAPDH and Lamin A staining is presented as marker for cytosolic and nuclear fraction, respectively.

3.3.2. PAG forms a novel multiprotein complex consisting of PAG, Fyn, Sam68 and p120RasGAP

Since it had been suggested using GST-SH2-pulldown assays that p120RasGAP could directly associate with PAG (Brdicka et al., 2000; Durrheim et al., 2001), we next wanted to investigate whether this interaction occurs also *in vivo*. Additionally, since Sam68 was shown to

associate with p120RasGAP (Jabado et al., 1998), we were also interested to see whether we could find Sam68 in a complex with PAG as well. Therefore, we immunoprecipitated PAG from *Resting*, *Anergic* and *Rescued* cell lysates and looked for the presence of Sam68 and p120RasGAP (Figure 3.17.A). Recall that we have already earlier investigated the association of Fyn and PAG and found this to be unchanged upon anergy induction (Figure 3.11.). Panel A shows that there is a very weak association of p120RasGAP and Sam68 with PAG in *Resting* T cells, but this interaction clearly increases upon prolonged stimulation of the cells (i.e. in *Anergic*

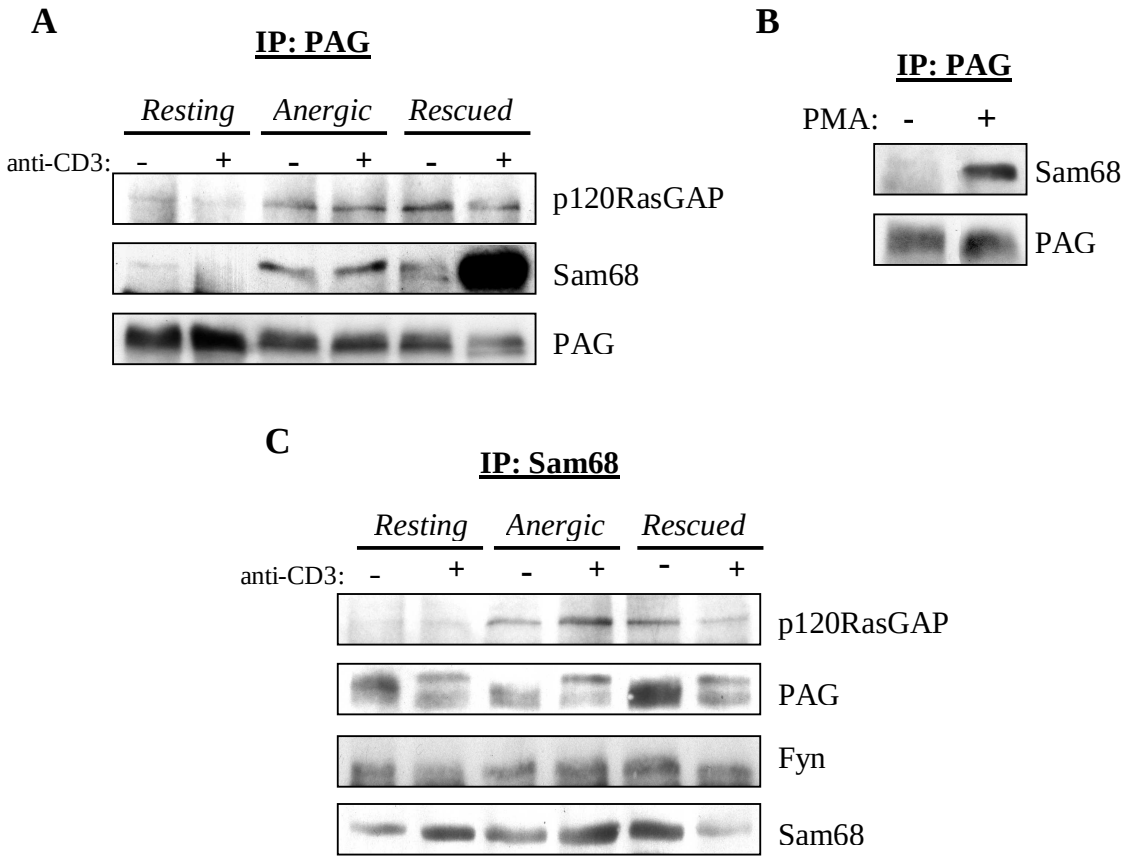

Figure 3.17. Sam68 and p120RasGAP associate with PAG. (A) *Resting*, *Anergic* and *Rescued* cells were either left untreated (-) or restimulated with anti-CD3 antibody for 2 minutes (+). PAG was immunoprecipitated from the lysates and associated proteins detected by immunoblotting with anti-p120RasGAP and anti-Sam68 antibody. The amount of precipitated material is shown with anti-PAG staining. (B) Purified T cells were cultured in the presence (+) or absence (-) of PMA for three days. PAG was immunoprecipitated and associated Sam68 detected by immunoblotting. (C) Samples prepared as in A were used to immunoprecipitate Sam68 and then probed with anti-p120RasGAP, anti-PAG and anti-Fyn antibodies. The amount of precipitated material is shown by probing with anti-Sam68 antibody.

and *Rescued* cells). Restimulation of the cells for 2 minutes appears not to affect the extent of p120RasGAP-PAG interaction. The abundant association of Sam68 upon restimulation of *Rescued* cells is partially the additive effect of PMA treatment itself, as we could also induce some Sam68-PAG association by treating T cells with PMA alone (Figure 3.17.B). Indeed, it was recently shown that the Ser/Thr phosphorylation influences the localization of Sam68 (Paronetto et al., 2006) and thus PMA may prime Sam68 for nuclear export.

To confirm the specificity of these interactions, we performed the reciprocal experiment by precipitating Sam68 and looking for p120RasGAP, PAG and Fyn association (Figure 3.17.C). Sam68 binds p120RasGAP in both *Anergic* and *Rescued* cells, but not in *Resting* state. Surprisingly, Sam68 appears to be constitutively associated with Fyn and PAG in all samples. That we can detect PAG association in Sam68-IP's from *Resting* cells, but not Sam68 in PAG-IP's, may simply result from the inability of the PAG antibody to recognize its epitope within the complex. However, we can clearly detect the complex in *Anergic* and *Rescued* cells when the amount of Sam68 and the phosphorylation of proteins in the cell increase, suggesting that we are at the limit of detectability in *Resting* cells. Also note that the abundant association of Sam68 with PAG in restimulated *Rescued* cells (3.17.A, last lane) was not visible in Sam68 immunoprecipitates (3.17.C, last lane). One explanation would be that Sam68 oligomerizes (Chen et al., 1997) and by precipitating PAG we would then coprecipitate also oligomerized Sam68 bound to it. Whereas if we perform Sam68 IP, the antibody could disturb oligomerization by competing for the same epitope. Alternatively, since we propose that Sam68 is phosphorylated on Ser/Thr after treatment with PMA (see above), such a phosphorylation could affect binding affinity and/or epitope accessibility for the Sam68 antibody towards the native versus denatured protein. Taken together, these results suggest that PAG forms a novel multiprotein complex consisting of PAG, Fyn, Sam68 and p120RasGAP.

3.3.3. PAG negatively regulates Ras activation

In the previous section, we have identified a novel complex containing PAG, Fyn, Sam68 and p120RasGAP. Since PAG is constitutively present in lipid rafts, recruiting p120RasGAP into this compartment would be an ideal mechanism of Ras regulation, since Ras-GDP is also lipid raft-associated (Prior at el., 2001). Thereby, p120RasGAP localized within the lipid rafts would be

perfectly positioned to inactivate any Ras-GTP that is formed before it had the chance to translocate and fulfill its function. Recall that LAT, the adaptor required for recruiting the activators of Ras (i.e. Sos and RasGRP), is also localized within the lipid rafts. To test whether the PAG-associated complex is indeed involved in Ras regulation, we switched to a different system and used the Jurkat T-cell line, since these cells can be easily transfected and consequently Ras assays can be performed, which require large amounts of cells. Thus, Jurkat T cells were transfected with constructs encoding PAG and Fyn, thereby attempting to mimic the situation observed in *Anergic* T cells. Consequently, the cells were stimulated and active Ras was pulled down using the GST-Raf1-RBD (Ras binding domain) construct (Foschi et al., 1997). This construct contains the Ras binding domain of Raf1, the main downstream effector of Ras, which binds specifically only to active Ras. Since the RBD is tagged with GST, one can easily isolate active Ras bound to the GST-RBD with glutathione-sepharose. Total Ras and active Ras in pull-downs were detected by imunoblotting with a pan-Ras antibody (Figure 3.18.). Cells transfected with vector alone activate Ras with normal kinetic peaking at 1 to 2 minutes (Figure 3.18.A). Remarkably, cells expressing PAG together with Fyn demonstrate complete suppression of Ras activation. To prove that this is not simply the result of a disturbed lipid raft organization caused by PAG overexpression, we used another lipid raft marker LAT. The cotransfection of LAT together with Fyn, however, did not block Ras activation (Figure 3.18.A). The Ras kinetic in this sample seems to be slightly faster compared to vector alone, which is not so surprising since LAT recruits Grb2/Sos and PLCγ1, both of which contribute to Ras activation. Panel B shows that the overexpression of Fyn alone does not disturb the Ras kinetic. Cells expressing PAG alone show a more truncated response, although the peak is comparable to the Fyn-transfected cells. Thus, only the cells expressing both PAG and Fyn demonstrate strong suppression of Ras activation (Figure 3.18.B). Interestingly, the ability to block Ras activation appears to correspond with the level of PAG phosphorylation, as indicated by pY317 staining (Figure 3.18.C). These results suggest that a hyper-phosphorylated PAG can recruit other proteins, probably via SH2 or PTB domains, that are able to block Ras activation. However, one could also hypothesize that a hyper-phosphorylated PAG recruits more Csk and thereby suppresses Src kinases activation, blocking LAT phosphorylation and consequently Ras activation. To exclude this possibility, we decided to repeat the Ras assay using the PAG Y317F mutant, which is unable to bind Csk.

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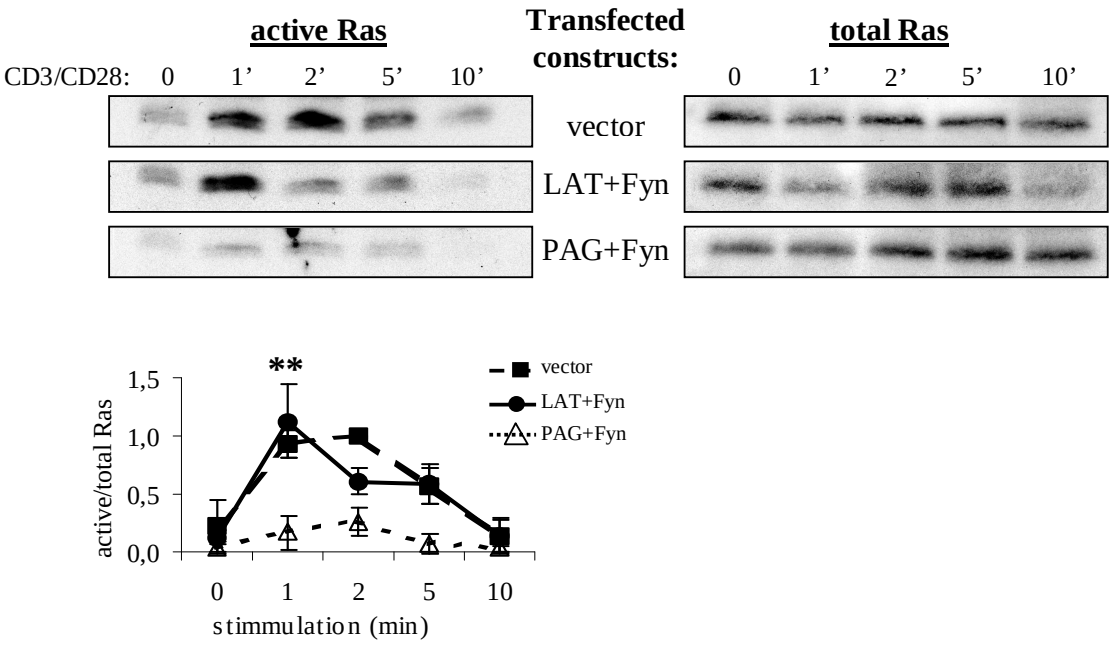
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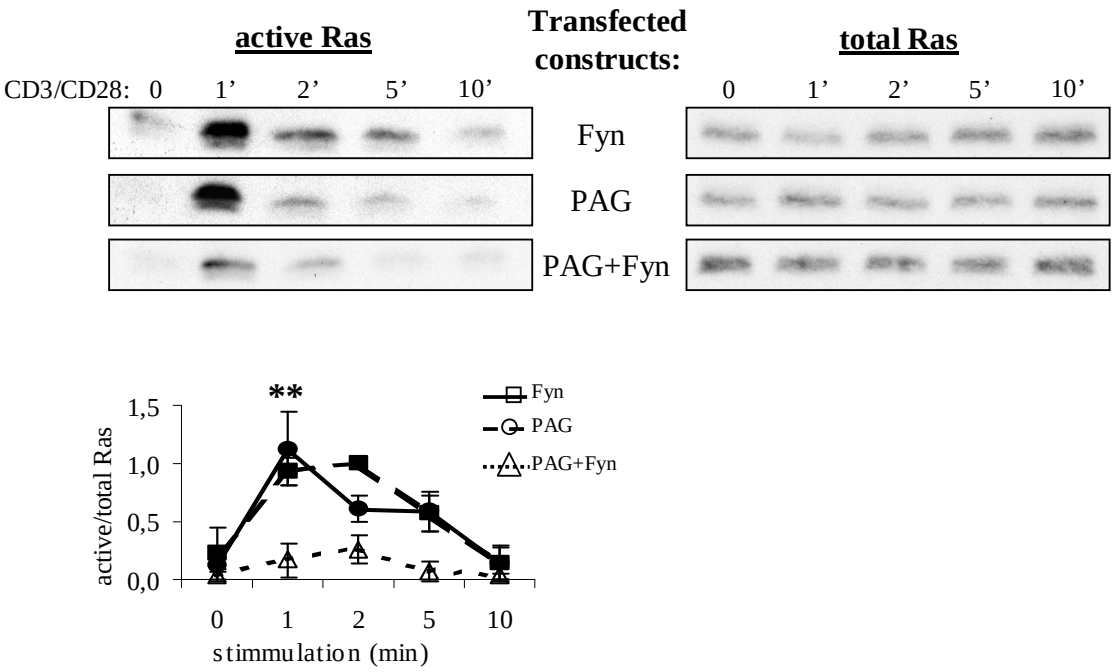
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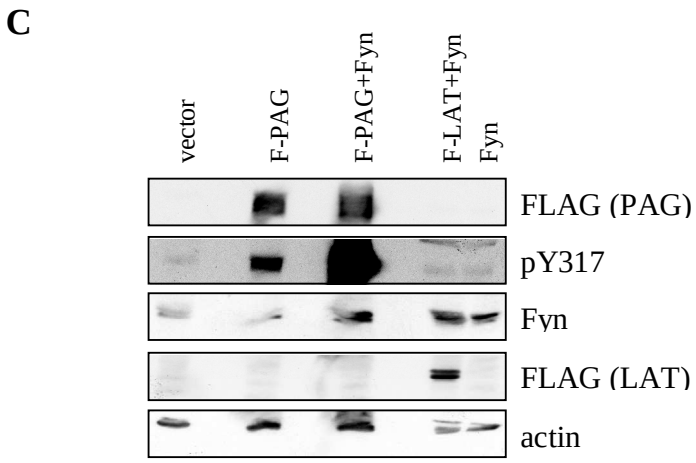


Figure 3.18. PAG overexpression together with Fyn blocks Ras activation. (A) and (B) Jurkat T cells were transfected either with empty vector or with constructs encoding FLAG-PAG, FLAG-LAT and Fyn. Cells were then stimulated with anti-CD3 and anti-CD28 antibodies for the indicated time and lysed. Active Ras was pulled-down using GST-Raf1-RBD (left panels). Total Ras is shown to prove that equal amounts of lysates were used for the assay (right panels). A quantitative analysis of the data is shown under appropriate pull-down assay. (C) Total expression of various constructs and phosphorylation of Y^{317} after transfection is presented here.

3.3.4. Basic characterization of Y317F mutant of PAG

First, we needed to characterize the PAG Y317F mutant to show that the mutated protein functions as expected. Replacing tyrosine 317 with phenylalanine abrogates Csk binding (Brdicka et al., 2000) and thus Csk is no longer recruited to the membrane and cannot phosphorylate the inhibitory tyrosine within the Src kinases. Therefore Jurkat T cells were transfected with Fyn alone or Fyn in combination with either FLAG-tagged wildtype (wt) PAG or the Y317F mutant and the lysates were immunoblotted with a phospho-specific antibody to the inhibitory tyrosine of Fyn (pY^{529}) (Figure 3.19.A). As expected, expression of wt PAG markedly increased the phosphorylation of Y^{529} while the mutated form of PAG did not show this increase. Additionally, we performed PAG immunoprecipitation and can demonstrate that, although both constructs are being phosphorylated, only the wildtype PAG is capable of binding Csk, whereas Y317F mutant has indeed lost its ability to recruit Csk (Figure 3.19.B). Importantly, both the wildtype and mutated form of PAG are able to associate with p120RasGAP as shown in the p120RasGAP immunoprecipitation reprobed with anti-FLAG antibody (Figure 3.19.C). This demonstrates that the loss of Csk binding does not interfere with the binding of p120RasGAP.

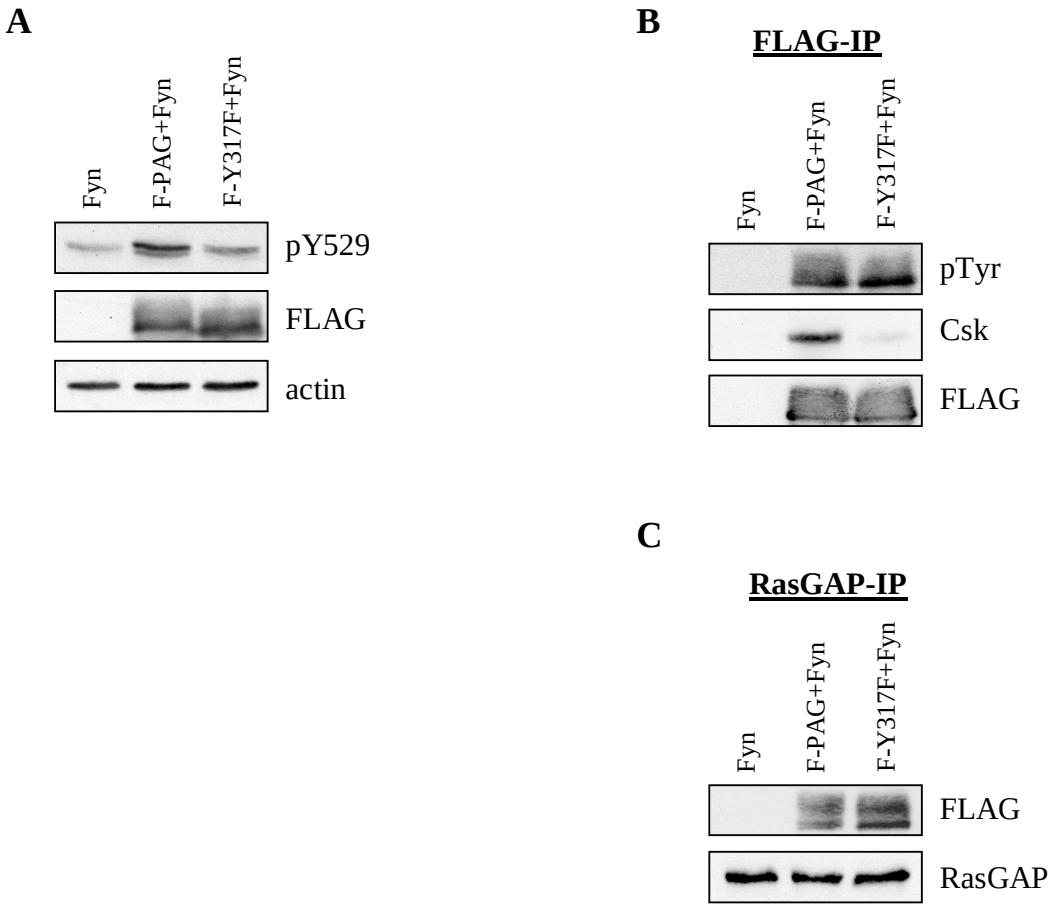


Figure 3.19. Y317F-PAG does not enhance inhibitory tyrosine phosphorylation, but still binds p120RasGAP. (A) Jurkat T cells were transfected with Fyn alone, wt FLAG-PAG plus Fyn or FLAG-Y317F-PAG plus Fyn. The cells were lysed and immunoblotted with anti-pY529 antibody. FLAG staining shows equal expression of the PAG constructs. Actin staining is shown for equal loading. (B) Samples as in A were used for FLAG immunoprecipitation and reprobed with anti-phosphotyrosine and anti-Csk antibodies. FLAG staining shows that equal amount of material was precipitated. (C) Samples as in A were used for p120RasGAP immunoprecipitation and reprobed with anti-FLAG antibody; p120RasGAP staining shows equal amount of precipitated material.

3.3.5. PAG negatively regulates Ras activation independently of Csk binding

Having demonstrated that the PAG Y317F mutant does not recruit Csk, but still binds p120RasGAP, we next investigated how this construct affects Ras signaling. Jurkat T cells were transfected with Fyn alone or Fyn together with either wildtype PAG or the Y317F-PAG construct. The transfectants were stimulated and active Ras was pulled down again with GST-Raf1-RBD. Total and active Ras were then detected by immunoblotting with a pan-Ras antibody

(Figure 3.20.). Cells transfected with Fyn alone show normal kinetic of Ras activation with a peak at 2 minutes, whereas cells expressing wt PAG together with Fyn have a strongly abolished Ras activation similar to the data shown above. Most importantly, if we express the Y317F mutant together with Fyn, we can block Ras activity almost to the same extent as with the wt protein. If we compare the quantitative analysis of the data, it seems that the Y317F mutant can slightly restore the block in Ras activation induced by the wt PAG. Thus, Csk-mediated Ras inactivation may contribute to the total PAG-mediated Ras inhibition, but it represents only a minor pathway. The majority of the effect upon Ras activity must be caused via recruiting another protein, most presumably p120RasGAP.

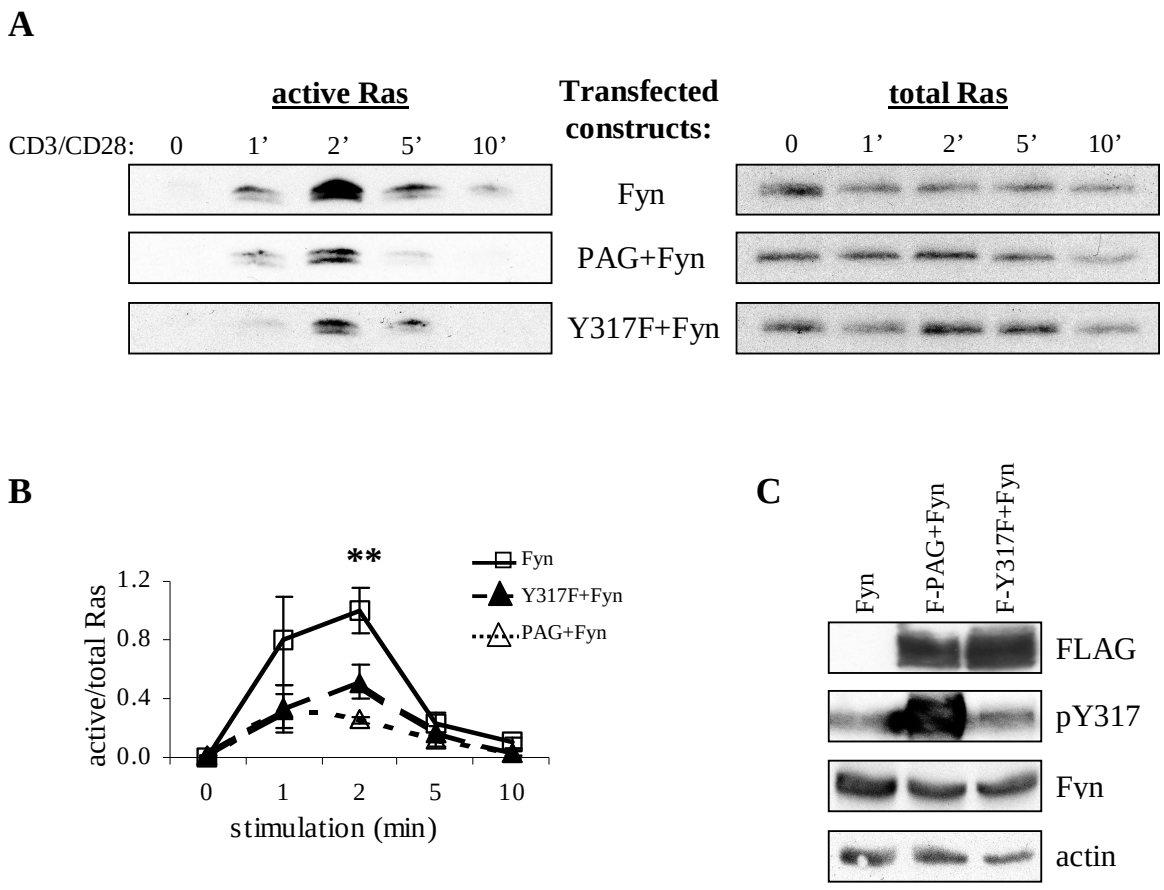


Figure 3.20. PAG negatively regulates Ras activation independently of Csk binding. (A) Jurkat T cells were transfected with constructs encoding Fyn, Fyn plus wt FLAG-PAG or Fyn plus FLAG-Y317F-PAG. Cells were then stimulated with anti-CD3 and anti-CD28 antibodies for the indicated time and lysed. Active Ras was pulled-down using GST-Raf-RBD (left panels). Total Ras is shown to prove that equal amounts of lysates were used for the assay (right panels). (B) A quantitative analysis of the amount of an active Ras compared to a total Ras protein is shown underneath. (C) Total expression of the constructs and phosphorylation of Y^{317} after transfection is presented here.

3.3.6. Y^{181} of PAG is the p120RasGAP binding site

To confirm that PAG is able to inhibit Ras activation by the means of p120RasGAP recruitment, we first needed to identify the binding site for p120RasGAP within the PAG protein and then to use the mutant of this tyrosine for the Ras assay. We took the advantage of having the complete set of FLAG-tagged PAG constructs with single tyrosines mutated to phenylalanine and used these mutants to identify the p120RasGAP binding site. Note that these constructs have been already successfully used for the identification of the Csk binding site (Brdicka et al., 2000). These PAG mutants were transfected into the Jurkat T-cell line and p120RasGAP was immunoprecipitated. IP's were resolved by Western blotting and the blot was probed with an anti-FLAG antibody to detect associated FLAG-PAG mutants (Figure 3.21.). Since the Y181F mutant reproducibly did not coprecipitate with p120RasGAP, we conclude that Y^{181} is the main p120RasGAP binding site.

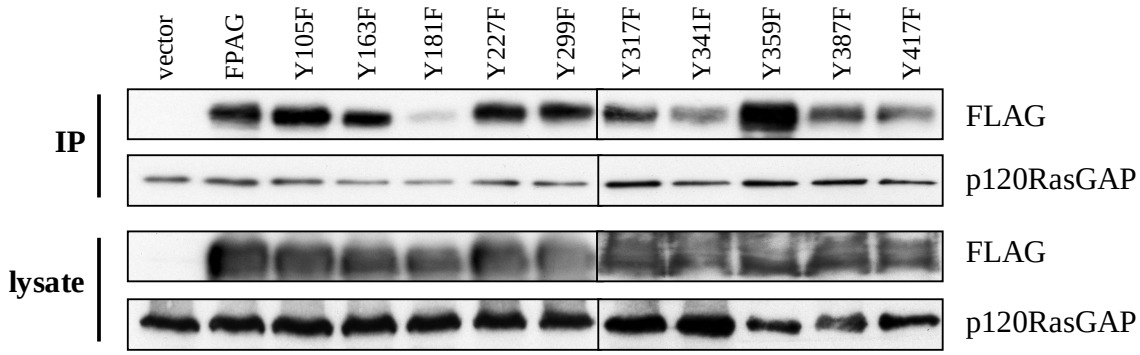


Figure 3.21. Y^{181} of PAG is the p120RasGAP binding site. Jurkat T cells were transfected with constructs encoding individual tyrosine mutants of PAG. The p120RasGAP protein was immunoprecipitated (IP) from cell lysates, IP's were subjected to SDS-PAGE and immunoblotted for the association of FLAG-PAG constructs. p120RasGAP staining shows the amount of precipitated material. Lysates are shown for the expression of FLAG-PAG mutants and p120RasGAP. Note that due to space restrictions, the samples were run in parallel on two gels.

3.3.7. PAG negatively regulates Ras activation also in the absence of p120RasGAP binding

Having identified the p120RasGAP binding site, we could test to see whether the Y181F PAG mutant ablates the block in Ras activation induced upon the expression of either wt or

Y317F PAG. Thus, we transfected Jurkat T cells with constructs encoding Fyn alone or Fyn in combination with either wildtype PAG or the Y181F mutant. Ras, activated upon stimulation, was pulled-down again using GST-Raf1-RBD and detected by immunoblotting with anti-pan-Ras antibody. To our surprise, mutant PAG incapable of p120RasGAP binding was still as efficient in suppressing Ras activation as the wildtype protein (Figure 3.22.). Therefore we had to postulate that the ability of PAG to block Ras activation is still maintained because of the intact Csk binding.

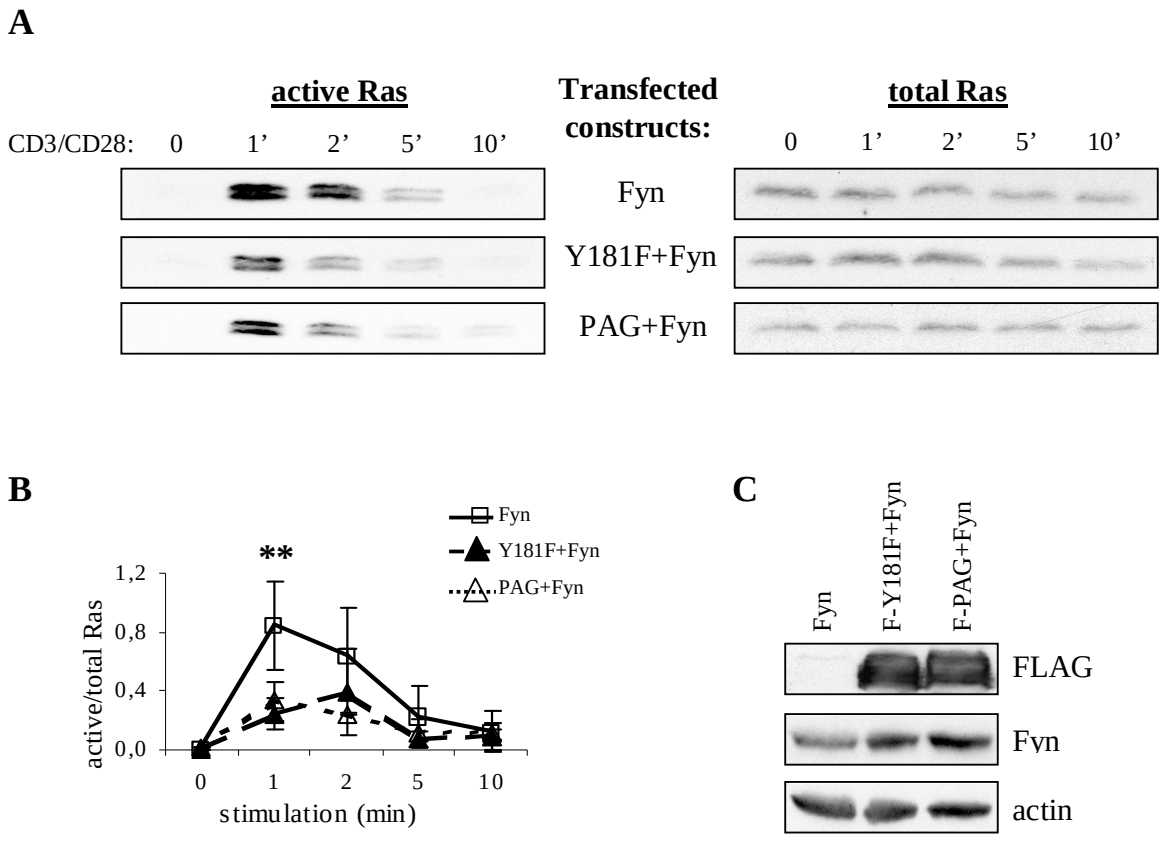


Figure 3.22. PAG negatively regulates Ras activation in the absence of p120RasGAP binding. (A) Jurkat T cells were transfected with constructs encoding Fyn, Fyn plus wt FLAG-PAG or Fyn plus FLAG-Y181F-PAG. Cells were then stimulated with anti-CD3 and anti-CD28 antibodies for the indicated time and lysed. Active Ras was pulled-down using GST-Raf1-RBD (left panels). Total Ras is shown to prove that equal amounts of lysates were used for the assay (right panels). (B) A quantitative analysis of the amount of an active Ras compared to a total Ras protein is shown underneath. (C) Total expression of the constructs after transfection is presented here.

3.3.8. Both Csk and p120RasGAP binding contribute to the block in Ras activation

Since the Y181F mutant was still capable of suppressing Ras activation, we suspected that the Csk-mediated inhibition and the p120RasGAP-meidated inhibition could compensate for each other to ensure proper regulation of Ras. Therefore we had to mutate both tyrosines (i.e. Y181 and Y317) in order to abolish the binding of both Csk and p120RasGAP to PAG. We utilized the Y317F mutant and introduced an additional mutation at Y181 to phenylalanine by site-directed mutagenesis. This double mutant was then transfected into Jurkat T cells together with Fyn. Whereas expression of wildtype PAG with Fyn again lead to a diminished Ras activity, the Y181/317F was able to rescue this block and showed activation of Ras comparable to Fyn alone (Figure 3.23.).

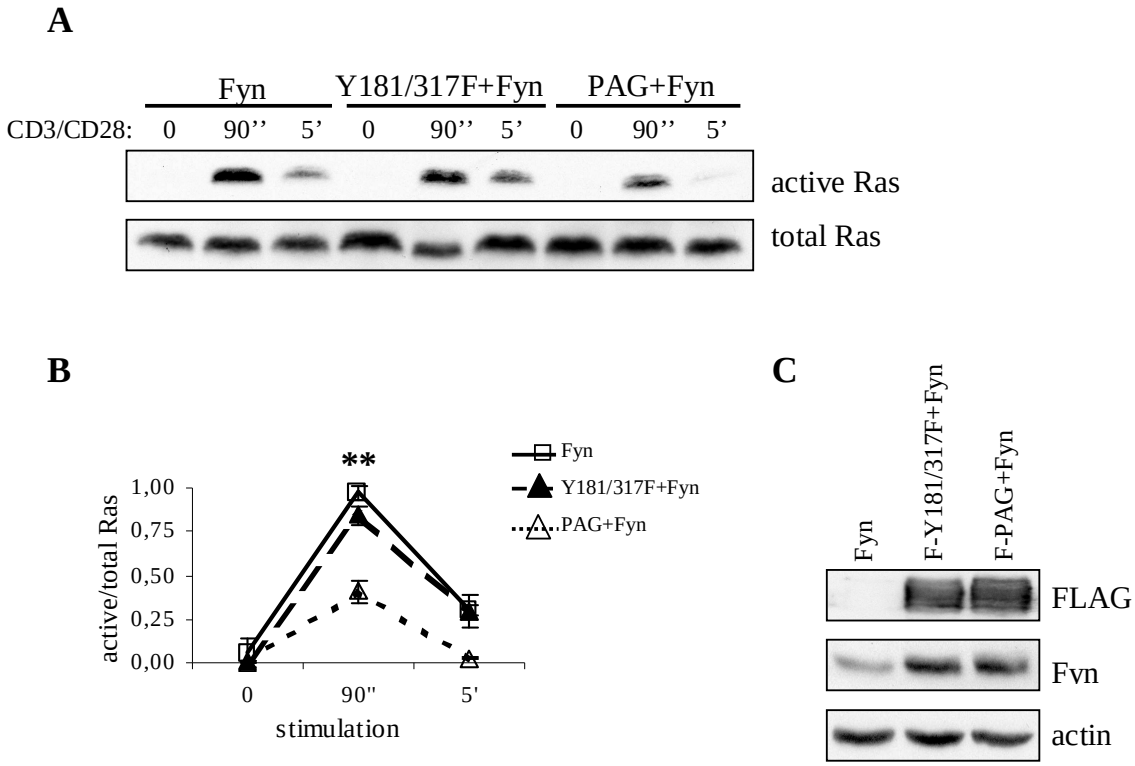


Figure 3.23. Mutation of both Y181 and Y317 ablates Ras suppression by PAG. (A) Jurkat T cells were transfected with constructs encoding Fyn, Fyn plus wt FLAG-PAG or Fyn plus FLAG-Y181/317F-PAG. Cells were then stimulated with anti-CD3 and anti-CD28 antibodies for the indicated time and lysed. Active Ras was pulled-down using GST-Raf1-RBD (upper panel). Total Ras is shown to prove that equal amounts of lysates were used for the assay (lower panel). (B) A quantitative analysis of the amount of an active Ras compared to a total Ras protein is shown underneath. (C) Total expression of the constructs after transfection is presented here.

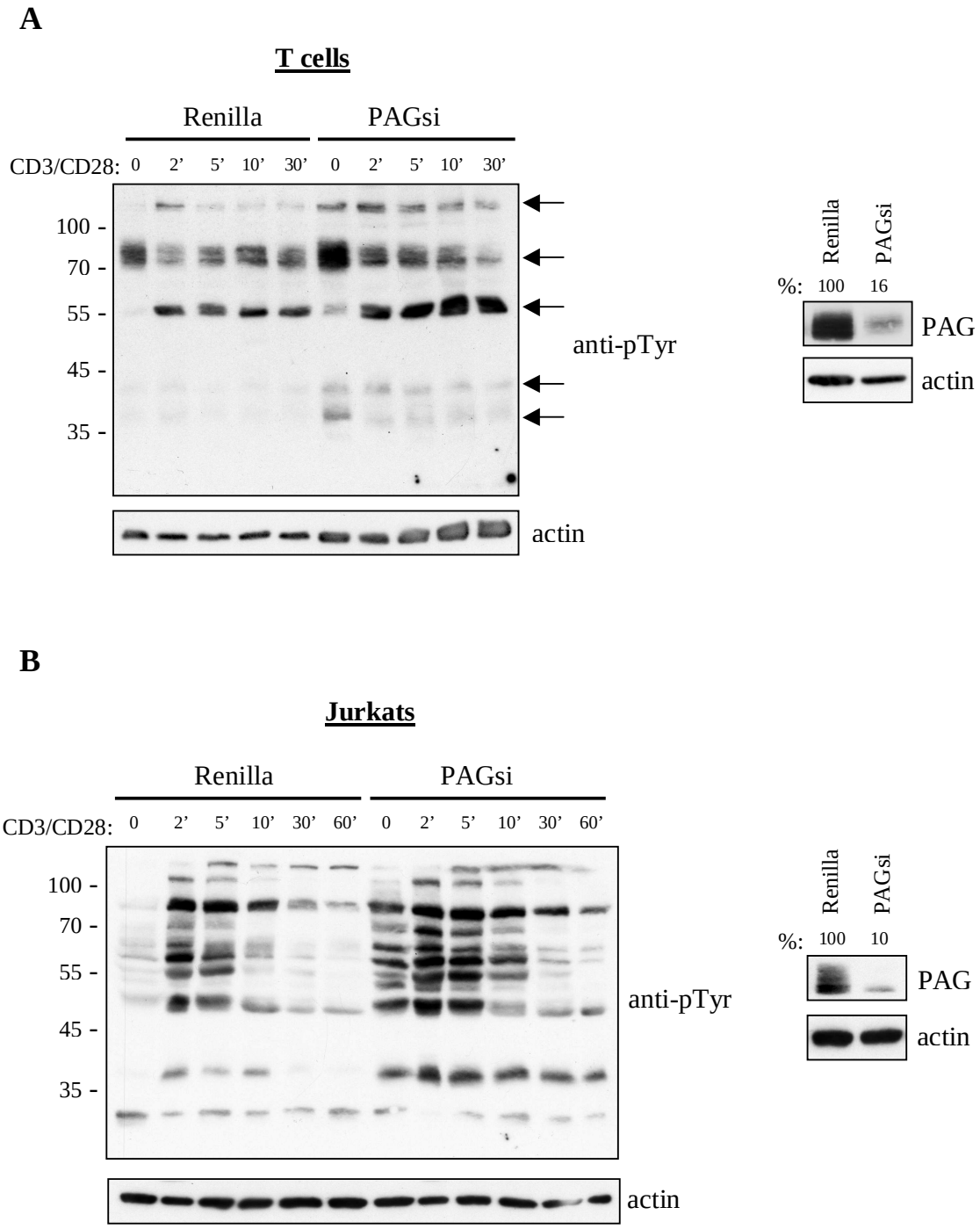
Therefore, in addition to identifying two new PAG associated proteins, i.e. Sam68 and p120RasGAP, we have identified a novel function of PAG. Namely, its ability to negatively regulate Ras by recruiting GAPs into the lipid rafts where they would associate with Ras-GDP rapidly and efficiently inactivating any Ras-GTP that formed before it had a chance to translocate. Additionally, it seems that this pathway may be partially compensated by Csk-mediated Src inhibition and only deletion of both Csk and p120RasGAP binding ablates the ability of PAG to suppress Ras activation.

3.3.9. PAG downregulation leads to enhanced and sustained SFK and Ras activation

The fact that PAG negatively regulates both Src kinases and Ras suggests that PAG is an important negative regulator of cellular activation. However, two recent publications have demonstrated that PAG knockout mice are perfectly normal and do not demonstrate defects in thymic development and/or T-cell function (Xu et al., 2005; Dobenecker et al., 2005). One should hypothesize though, that there might be various compensatory mechanisms developed to regulate Src kinase activity and, upon further investigation, this has indeed turned to be the truth (J. Lindquist, unpublished observation). Therefore, to determine the role and importance of PAG for T-cell signaling, we used RNA interference in both primary human and Jurkat T cells. Primary human T cells were nucleofected with siRNA oligonucleotides against PAG or Renilla using Amaxa's Nucleofection technology, whereas Jurkat T cells were electroporated with vectors encoding shRNA. The cells were kept 72 hours in culture and then stimulated, lysed and subjected to Western blotting to detect phosphorylation pattern upon stimulation (Figure 3.24.). One can clearly see that already unstimulated cells possess enhanced basal tyrosine phosphorylation of several proteins in the absence of PAG. The protein phosphorylation further increases upon stimulation and the activation is sustained in the cells transfected with PAG siRNA. These results suggest that knocking down PAG expression results in markedly enhanced basal Src kinase activity and mainly that it does not become down-modulated later on to shut off signaling.

Additionally, since we have shown that PAG negatively regulates Ras, we investigated the impact of PAG downregulation on Ras activation. Thus, we transfected Jurkat T cells with siRNA against PAG or Renilla, stimulated them, lysed and performed Ras activation assays with the GST-Raf1-RBD (Figure 3.24.C). Here we can show that suppression of PAG expression

leads to a dramatic enhancement (approximately 5-fold) in Ras activation. We believe that these data together clearly demonstrate that PAG is indeed an important negative regulator of both the Src kinases and Ras.



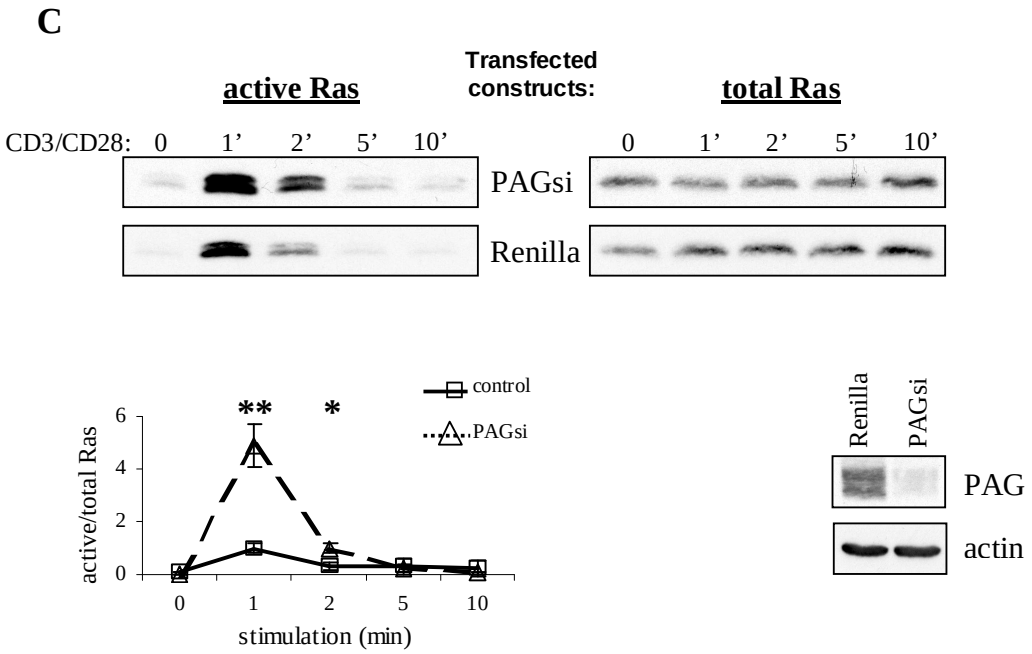


Figure 3.24. PAG downregulation leads to enhanced SFK and Ras activation. (A) Primary human T cells were nucleofected with siRNA oligos against PAG or Renilla control. After 72 hours, the cells were stimulated with CD3/CD28 antibodies, lysed and subjected to Western blotting. Immunoblotting with anti-phosphotyrosine antibody (4G10) shows changes in the phosphorylation signature in these cells. Arrows indicate proteins hyperphosphorylated in the absence of PAG. Actin staining is shown for equal loading. PAG expression in both samples is shown on the right, the amount of PAG was normalized to actin and is presented as percentage of PAG expression in control cells. (B) Jurkat T cells were transfected with pCMS3-EGFP plasmid containing either PAG shRNA or Renilla shRNA and processed as described in panel A. (C) Jurkat T cells were transfected with pCMS3-EGFP plasmid containing either PAG shRNA or Renilla shRNA. Cells were then stimulated with anti-CD3 and anti-CD28 antibodies, lysed and active Ras was pulled-down using GST-Raf1-RBD (left panels). Total Ras is shown to prove that equal amounts of lysates were used for the assay (right panels). A quantitative analysis of the amount of an active Ras compared to a total Ras protein is shown underneath. PAG expression in both samples is shown (bottom right).

3.4. Identification and characterization of IGAP

3.4.1. Expression of DGKs is unchanged in *Anergic* T cells

An interesting issue with regard to anergic cells is that they have increased calcium levels, but simultaneously possess a block in the Ras/MAPK pathway. However, such a situation should not theoretically happen, because PLCγ produces IP_3 and DAG in an equimolar ratio. IP_3 then induces calcium flux, whereas DAG activates PKC and RasGRP leading to the activation of Ras. One explanation would be that diacylglycerol is rapidly metabolized by diacylglycerolkinases (DGK). Therefore we lysed *Resting*, *Anergic* and *Rescued* cells and immunoblotted to see whether the expression of any of the DGK isoforms is enhanced upon anergy induction. However, we did not find any alteration in expression of either DGK alpha (Figure 3.25.A) or DGK zeta (Figure 3.20.B), the two main DGKs present in T cells. However, from these blots we cannot exclude that the activity of either of the DGKs is enhanced in anergic T cells.

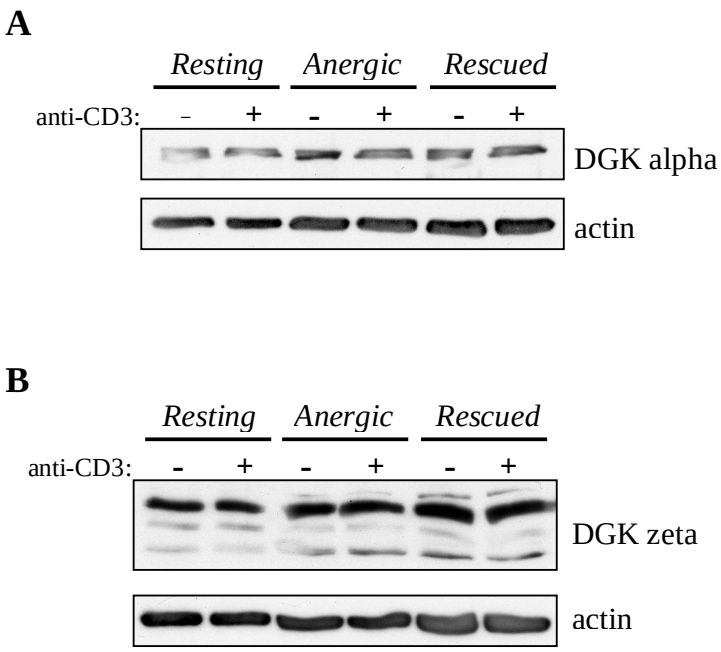


Figure 3.25. Normal DGK expression in *Anergic* T cells. *Resting*, *Anergic* and *Rescued* cells were either left untreated (-) or restimulated with anti-CD3 antibody for 2 minutes (+). Lysates were immunoblotted with anti-DGK alpha (A) or anti-DGK zeta (B) antibody. Note, that DGK zeta exists in several isoforms. Actin staining is shown for equal loading.

3.4.2. DGK alpha antibody cross-reacts with p120 protein

Surprisingly, when probing the lysates with the anti-DGK alpha antibody, we observed a strong induction in the expression of a protein at 120 kDa in *Anergic* and *Rescued* cells (Figure 3.26.A). However, there exists no known isoforms of DGK alpha. Therefore, we used the anti-DGK alpha antibody for immunoprecipitation and tested whether this antibody recognizes the p120 protein also in its native form. By reprobing Western blots of DGK alpha-IP's with anti-DGK alpha antibody, we demonstrate that we can specifically precipitate not only DGK alpha, but also the p120 protein (Figure 3.26.B).

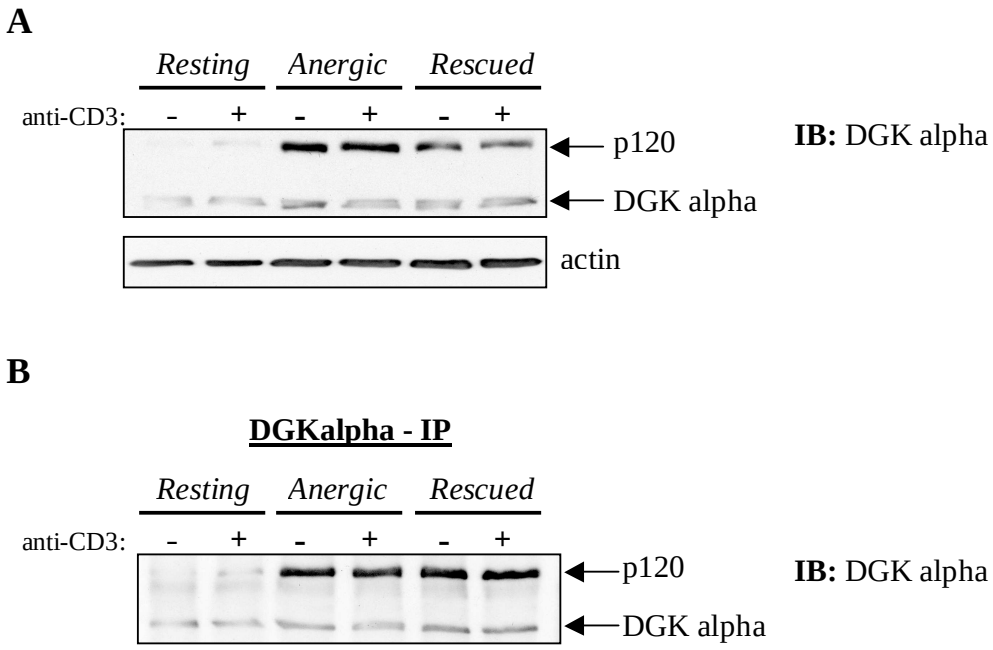


Figure 3.26. Anti-DGK alpha antiserum specifically cross-reacts with induced p120 protein. (A) *Resting*, *Anergic* and *Rescued* cells were either left untreated (-) or restimulated with anti-CD3 antibody for 2 minutes (+) and whole cell lysates were immunoblotted with anti-DGK alpha antibody. Bands corresponding to DGK alpha and p120 are marked. Actin staining is shown for equal loading. (B) Lysates as in A were used for immunoprecipitation with anti-DGK alpha antibody, IP's were subjected to Western blotting and reprobed again with the DGK alpha antibody. Bands corresponding to DGK alpha and p120 are indicated.

Since we could immunoprecipitate p120, we decided to identify this protein. Therefore we prepared a large number of *Rescued* cells and used them for immunoprecipitation with the anti-DGK alpha antibody. *Anergic* cells were omitted since *Rescued* cells can be produced in a higher

amount and also express high levels of p120. As a negative control, resting cells were taken, as they express only very low levels of p120 (see figure 3.26.A). These IP's were loaded onto a large acrylamide gel to ensure better separation and the gel stained with silver to visualize the proteins (Figure 3.27.). We observed a number of bands that were specifically precipitated with the DGK alpha antibody, some were present in all samples (bands 2, 5, 6, 7), but interestingly, others were selectively co-precipitated only in long-term stimulated cells (bands 1, 3, 4).

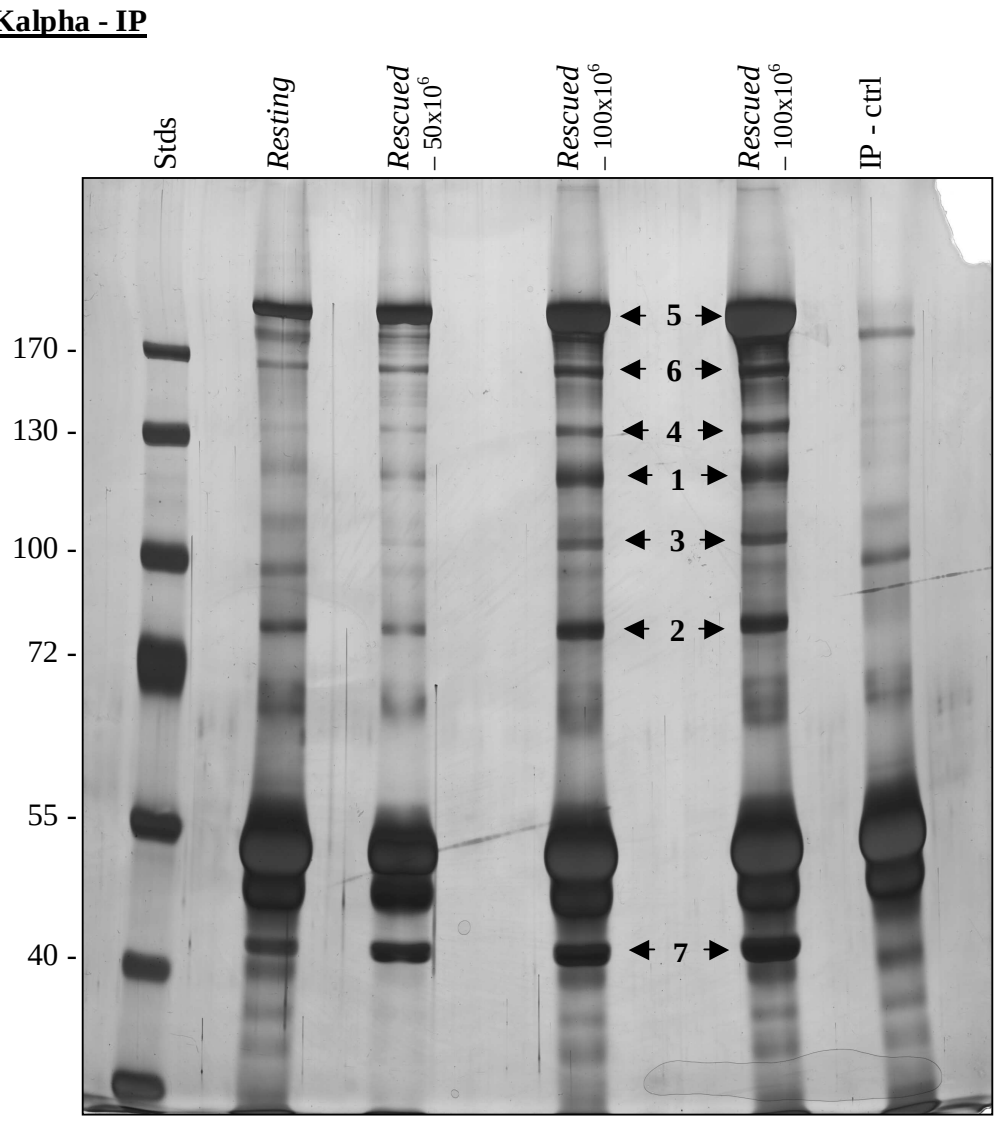


Figure 3.27. Silver staining of DGK alpha-IP's. Lysates of *Resting* and *Rescued* cells ($50x10^6$ or $100x10^6$) were used for immunoprecipitation with anti-DGK alpha antibody. Samples were then loaded onto large 8% acrylamide gel and stained with silver. Bands marked with arrows were cut out and subjected to peptide mass mapping.

3.4.3. Identification of IGAP

Bands 1 – 7 shown in figure 3.27. were excised from the silver gel and subjected to trypsic digestion, which was performed by Dr. Thilo Kähne in the Institute of Experimental Internal Medicine, Magdeburg. The resulting peptides were then analyzed by MALDI-TOF mass spectrometry and the results compared to the protein database to identify the corresponding proteins. The results obtained are shown in Figure 3.28. Band number 2 was identified as DGK alpha, the protein against which the immunoprecipitating antibody was raised. Bands 3 and 4 could not be identified due to low quality of tryptic peptides. Bands 5 and 6 were identified as the non-muscle myosin heavy chain II (MYH9). Band 7 contained actin plus an additional protein that could not be clearly determined. Most interestingly, band 1 (i.e. p120) was identified as a hypothetical protein with the accession number XP_029084, which was originally predicted by automated computational analysis of the human genome.

Band number	Identified protein
1	XP_029084
2	DGK alpha
3	not determined
4	not determined
5	MYH9
6	MYH9
7	Actin + ?

Figure 3.28. List of proteins identified by mass spectrometry. The indicated bands from figure 3.22. were excised, digested with trypsin and subjected to mass spectrometric analysis to identify the proteins.

The SMART algorithm (Schultz et al., 1998) enabled us to model the predicted domain structure of p120 based upon its primary amino acid sequence (Figure 3.29.). To our surprise, p120 contains a RasGAP domain and thus belongs to a family of Ras GTPase activating proteins. Since p120 is expressed in *Resting* T cells only in very small amount, but is drastically induced upon long-term stimulation of the cells, we have named this protein **IGAP** (Inducible GTPase-

activating protein). In addition to the GAP domain, IGAP also possesses one PH and one C2 domain, both of which are believed to be responsible mainly for targeting proteins to the phospholipids within plasma membrane. Moreover, the C2 domain does so in a calcium-dependent manner. Additionally, there are several predicted sites of phosphorylation within the IGAP sequence – multiple serine and threonine residues and several tyrosine residues, two of which are surprisingly arranged into an atypical ITAM sequence. A coiled-coil domain is situated at the C-terminus, which is involved in protein dimerization.

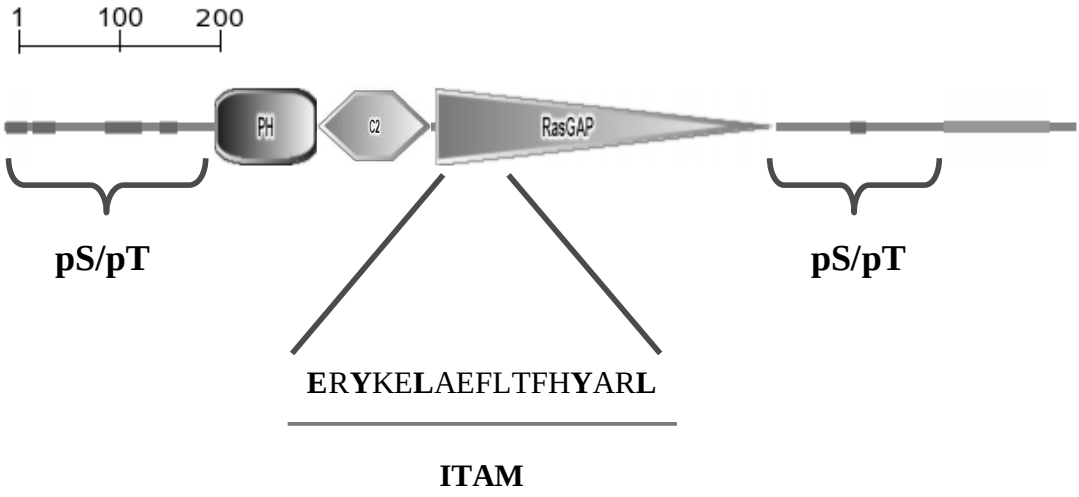


Figure 3.29. Structure and predicted domains of IGAP. The structure and domains of IGAP were predicted based upon the primary amino acid sequence using the SMART tool available on http://smart.embl-heidelberg.de.

When we compare IGAP structure with other known RasGAPs, we realize that IGAP is quite unique among the RasGAPs and is more similar to SynGAP and NGAP, which are, however, not expressed in T cells, but are rather specific for neuronal cells (Figure 3.30.). Compared to the main known GAP in T cells, p120RasGAP, IGAP completely lacks any protein-protein interaction domains like SH2 or SH3.

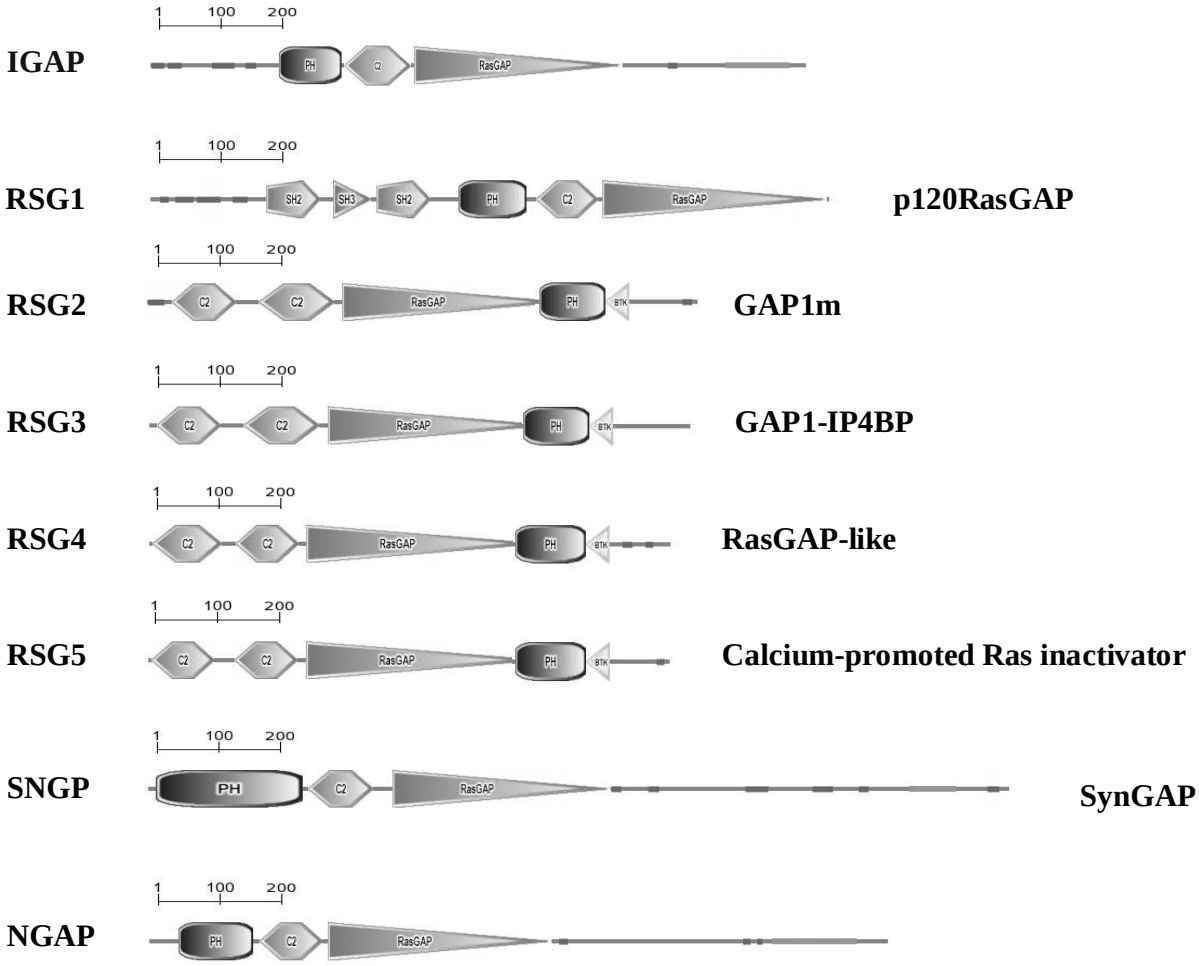


Figure 3.30. Comparison of known RasGAPs.

3.4.4. IGAP is phosphorylated *in vivo*

Since we found an atypical ITAM sequence within the GAP domain, we were interested to see whether IGAP indeed becomes phosphorylated in T cells. Therefore we took the advantage that we could immunoprecipitate IGAP with the anti-DGK alpha antibody (see figure 3.26.B) and reprobed these IP's with anti-phosphotyrosine (Figure 3.31.). We could not detect any phosphorylation in *Resting* cells where there is only minimal expression of IGAP, but we indeed observed IGAP phosphorylation in *Anergic* and *Rescued* cells, which upregulated the IGAP protein.

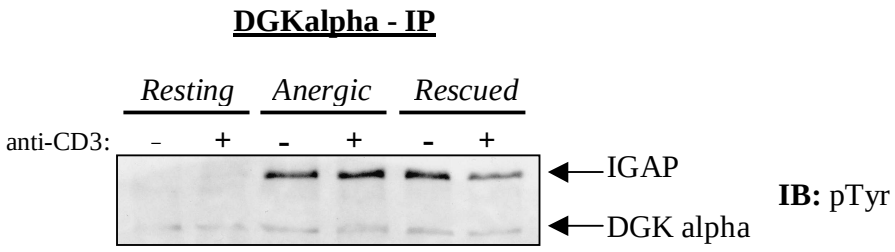


Figure 3.31. IGAP is phosphorylated *in vivo*. Western blot of the DGK alpha-IP from figure 3.26.B was reprobed with anti-phosphotyrosine antibody (4G10). Bands corresponding to IGAP and DGK alpha are indicated.

3.4.5. IGAP is upregulated during long-term stimulation of T cells

Since we originally found IGAP only in cells that had been stimulated for three days and not in resting cells, we wanted to determine more precisely the time kinetic of IGAP induction. Thus, we prepared *Anergic* and *Rescued* cells as always by culturing T cells on CD3-coated plates with or without PMA for three days and resting them for one additional day. Aliquots of cells were taken at each day of culture and lysed for Western blot analysis (Figure 3.32.). Whereas resting T cells express only very little of IGAP, the expression increases during stimulation, with a maximum at day 2. As the cells rest for one day without any stimulus, the level of IGAP expression decreases again (Figure 3.32., last two lanes). There was no difference between *Anergic* and *Rescued* cells with regard to IGAP expression.

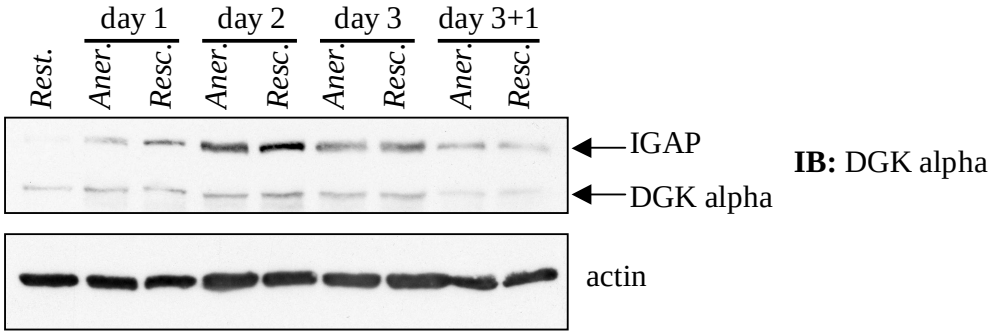


Figure 3.32. IGAP is upregulated during long-term stimulation. *Resting* T cells (*Rest.*) were cultured on anti-CD3-coated plastic plates for three days plus one day of resting with (*Resc.*) or without (*Aner.*) PMA. Aliquots of *Anergic* and *Rescued* cells were taken at each day of culture and probed with anti-DGK alpha antibody. Bands corresponding to IGAP and DGK alpha are indicated. Actin staining is shown for equal loading.

3.4.6. IGAP is predominantly plasma membrane localized

The predicted structure suggests that IGAP contains two domains (PH and C2 domain, see figure 3.29.) responsible for targeting to the membrane phospholipids. In order to investigate the subcellular localization of IGAP, we used Jurkat T cells, which also express endogenous IGAP (data not shown) and can be produced in high amount needed for subcellular fractionation. Jurkat T cells were lysed, their membranes separated from the cytosolic compartment by gradient centrifugation and both membrane and cytosolic fractions were subjected to Western blotting (Figure 3.33.). The transmembrane adapter protein LAT was chosen as a marker for the membrane fraction and the cytosolic adapter protein Grb2 was used to stain the cytosolic fraction. Probing the blot with anti-p120RasGAP antibody showed that this RasGAP is primarily a cytosolic protein. On the contrary, IGAP was detected mainly in the membrane fraction.

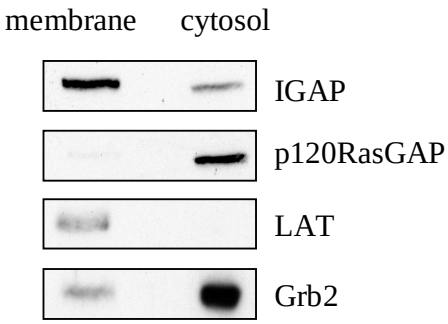


Figure 3.33. IGAP is prelocalized at the plasma membrane. Membrane and cytosolic fractions were isolated from Jurkat T cells and probed with indicated antibodies.

3.4.7. IGAP associates with PAG

Since we identified p120RasGAP in a complex together with PAG, we were further interested to look whether the new protein IGAP can also be found associated to a PAG complex. Therefore we prepared lysates of *Resting*, *Anergic* and *Rescued* cells, immunoprecipitated PAG and looked for the presence of IGAP in these IP's by immunoprobing with anti-DGK alpha antibody (Figure 3.34.A). Surprisingly, we indeed detected IGAP associated with PAG in *Anergic* and *Rescued* cells, whereas there was no IGAP in PAG-IP's from *Resting* sample. Additionally, we performed reciprocal IP's and used the anti-DGK alpha antibody for immunoprecipitation. Also using this

approach, we could detect the interaction of PAG and IGAP in *Anergic* and *Rescued* cells (Figure 3.34.B). Although one could argue that the anti-DGK alpha antibody precipitates both DGK alpha and IGAP and therefore some PAG may have also been associated with DGK alpha and not only specifically with IGAP, we do not find DGK alpha present in PAG-IP's. Thus it seems safe to conclude that immunoprecipitated IGAP was indeed associated with the PAG complex.

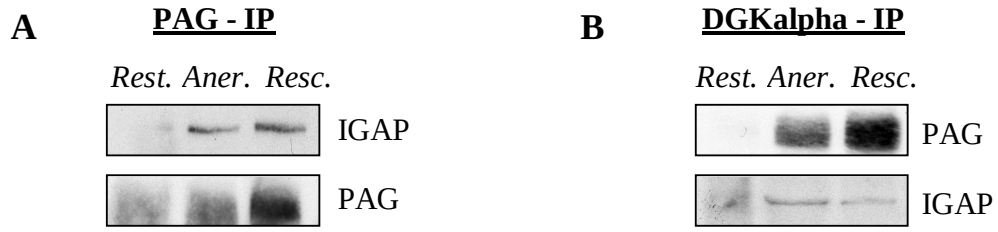


Figure 3.34. IGAP associates with PAG. (A) *Resting* (*Rest.*), *Anergic* (*Aner.*) and *Rescued* (*Resc.*) cells were lysed and PAG was immunoprecipitated. Associated IGAP was detected by immunoblotting with anti-DGK alpha antibody. PAG staining shows the amount of precipitated material. (B) Samples as in A were subjected to immunoprecipitation with anti-DGK alpha antibody and the associated PAG was detected by immunoprobing with anti-PAG antibody. IGAP staining shows the amount of precipitated material.

In summary, we have demonstrated that PAG is able to recruit at least two RasGAPs – p120RasGAP and the novel IGAP. This observation further supports our findings that PAG is a potent negative regulator of Ras GTPases. Whether IGAP is recruited to the same complex of PAG as p120RasGAP or whether two separate complexes exist within the cells is unclear at this moment. What is the exact role of IGAP and whether it can fulfill the same functions as p120RasGAP will be certainly the subject of further investigations.

4. Discussion

4.1. Proximal alterations within anergic T cells (see 3.1.)

In this study, we investigated the signaling alterations underlying anergy, an important mechanism of peripheral tolerance. To perform this, we purified freshly isolated peripheral human T cells and used a well established method of anergy induction, namely culturing the cells on anti-CD3-coated plates. The absence of costimulation drove the cells into anergy, as they no longer proliferated upon subsequent restimulation via CD3 or CD3+CD28 (Figure 3.4.). This is because the CD3-mediated pathway triggered in the primary culture is alone not sufficient to induce the production of sufficient amounts of IL-2. And indeed, the addition of exogenous IL-2 to the culture induced dramatic proliferation of these cells during the secondary stimulation, showing that these cells are indeed anergic. Corresponding to previous findings, we observed a dramatic upregulation of the Src family kinase Fyn in anergic T cells, both at the kinase activity and the protein level, whereas only a marginal increase in the activity and expression level of Lck was observed (Figure 3.6.). Moreover, Lck expression and activity were also enhanced in rescued cells and therefore the Lck upregulation appears to be more related to the proliferation and activation of the cells. Therefore, it seems that Fyn is the main kinase playing an important role in anergic T cells.

Interestingly, we found an enhanced overall tyrosine phosphorylation in anergic T cells (Figure 3.7.), whereas phosphorylation of key signaling molecules, i.e. TCR zeta chain and LAT was completely abolished (Figure 3.8.). This means that there is no general increase in phosphorylation of all proteins that would correspond to the enhanced activity of Src kinases. Instead, the SFK activity is targeted towards specific proteins, which may play an important role in maintaining the anergic state, whereas proteins required for activation and proliferation of the cell are kept inactive (i.e. unphosphorylated). Interestingly, Lck is believed to play the main role in phosphorylating signaling molecules during activation of the cell, whereas Fyn is not so critical for activatory signal progression as it rather phosphorylates more specific proteins. Since Fyn was shown to be specifically upregulated in anergic T cells, the main role of Fyn may be to phosphorylate the proteins responsible for anergy. Note that a large portion of Lck is associated with the coreceptor CD4 or CD8 and the costimulatory molecule CD28 and that these pools of Lck only become activated upon co-triggering of these receptors. CD3 crosslinking alone therefore does not activate these pools of Lck and the positive signal may be “overridden” by the

Fyn-mediated phosphorylation of anergy-promoting factors. Based upon the apparent molecular weight, we propose that the proteins specifically hyper-phosphorylated in anergic T cells are mainly Src kinases and Cbl. Importantly, both Src kinases and the E3 ubiquitin ligases (e.g. Cbl, Itch, GRAIL) are indeed believed to contribute to the maintenance of anergy (Quill et al., 1992; Mueller, 2004). Certainly an interesting approach would be an immunoprecipitation using an anti-phosphotyrosine antibody followed by mass spectrometry as this would enable the identification of other proteins hyperphosphorylated in anergic T cells. This would then lead to a better understanding of the mechanism(s) responsible for the block in signaling pathways. Currently, we are trying to identify pp30, which is the most abundant phospho-protein in anergic and rescued T cells (Figure 3.7.). Since pp30 is equal in both anergic and rescued cells, we propose that the phosphorylation (and possibly also the upregulation) of this protein is associated with the activation and proliferation of the cells and is not specific to anergy.

Note that since both the anergic and rescued cells originally received the same stimulus via the TCR, the phosphorylation profile and the proximal signaling appear to be very similar in both populations. The difference is that the rescued cells received additionally PMA, which acts more distal in the signaling cascade, where it mimics costimulation and overcomes the block in anergy. Alternatively, we could have used stimulation of the cells with CD3 plus CD28 to produce activated cells as the positive control. However, CD28 costimulation may also lead under certain circumstances to anergy and the final outcome of anergy versus activation depends probably upon the proper amount of CD28 antibody used for stimulation (Schwartz, 2003). Additionally, using the stimulation with CD3 plus PMA clearly demonstrates that it is presumably indeed the DAG-mediated pathway that is not activated upon CD3 crosslinking alone, thereby resulting in anergy. As we demonstrate, simple reconstitution of DAG in the CD3-stimulated T cells by its analogue PMA enhances the DAG-mediated signaling and this is sufficient to prevent anergy induction (compare figure 3.4.).

4.2. Alterations within the PAG-associated complex in anergic T cells (see 3.2.1. – 3.2.5.)

Since PAG overexpressing T cells behave very similar to anergic T cells, i.e. they are both unresponsive to TCR stimulation and fail to produce IL-2, we hypothesized that PAG might be upregulated either on the protein level and/or on the phosphorylation level in anergic T cells. Although we did not observe any increase in PAG expression, we can show that, in anergic T cells, the activity of the Src family kinase Fyn associated to PAG is markedly enhanced leading

to the hyperphosphorylation of PAG, determined by anti-Y^{317} phospho-specific antibody (Figure 3.9. and 3.10.). Please note that PAG possesses 10 tyrosines and we have found that whereas some are dephosphorylated (mainly Y^{317}), others become phosphorylated only upon TCR triggering (J. Lindquist, unpublished observation). Therefore it would be rather difficult and potentially misleading to interpret total PAG phosphorylation by performing PAG-IPs and probing with a pan-phosphotyrosine antibody (e.g. 4G10).

This hyperphosphorylation could also be the result of decreased PAG phosphatase activity. However, upon stimulation of the cells, PAG becomes rapidly dephosphorylated in all samples and, in fact, the extent of dephosphorylation is largest in anergic T cells, meaning that the phosphatase is still active. Importantly, the level of phospho-PAG in anergic cells never decreases below that of unstimulated resting cells (Figure 3.10.). Consequently, we also observed an increased recruitment of Csk to PAG in anergic cells (Figure 3.11.). This in turn leads to the increased phosphorylation of the inhibitory tyrosine of Fyn within the total Fyn pool (Figure 3.12.). Thus it seems that hyperphosphorylation ensures that PAG may still fulfill its inhibitory function even upon restimulation of anergic cells and we propose that PAG may, in this way, contribute to the defects in proximal signaling. Until now, we have investigated only the changes in phosphorylation for total Fyn, however it would be interesting to look at specific pools of Fyn within the cell and compare Fyn tyrosine phosphorylation and kinase activity for the fraction associated to PAG with the fraction bound to TCR zeta chain. Since we observed a dramatic decrease in zeta chain phosphorylation, we predict that the associated kinase activity must also be strongly reduced.

Interestingly, a phenotype very similar to the one that we observe in anergic T cells has also been found in PTPalpha knockout mice (Maksumova et al., 2005). PTPalpha deficient thymocytes show increased phosphorylation of several proteins and enhanced Fyn kinase activity. Since PTP alpha localizes in the lipid rafts, it regulates the raft-associated Fyn. The hyperactive Fyn in the rafts in turn induces a hyperphosphorylation of PAG and increased association of Csk with PAG. Consequently, the inhibitory tyrosine within Fyn is more phosphorylated. Importantly, the PTPalpha deficient thymocytes elicit reduced proliferation and impaired IL-2 production upon stimulation with CD3 alone or CD3 plus costimulation and this is a phenotype very similar to anergy. Moreover, stimulation with PMA plus Ionomycin, i.e. with stimulus that normally breaks anergy, induces proliferation and IL-2 production also in PTPalpha deficient thymocytes comparable to the wildtype cells. This data clearly confirms our own

observations and our hypothesis that increased Fyn activity together with increased Csk recruitment to hyperphosphorylated PAG may constitute an effective mechanism to block T-cell signaling.

Opposite to our results, the group of Andre Veillette has very recently demonstrated an increased Fyn recruitment to PAG whereas Csk association was unchanged in murine anergic T cells (Davidson et al., 2007). They have however used the ionomycin-induced model of anergy, where they have first preactivated the cells with CD3 and CD28 antibodies and then expanded them with IL-2 before applying ionomycin to induce anergy. Hence, they were investigating anergy induced in murine effector T cells, whereas we used human naïve T cells. Due to these major differences it is difficult to directly compare the results obtained from two distinct systems. Unfortunately, they did not investigate the kinase activity of Fyn associated to PAG and/or PAG phosphorylation upon anergy induction and thus it is difficult to speculate why the increased recruitment of Fyn did not lead to an increased phosphorylation of PAG and consequently also to enhanced recruitment of Csk in their anergic cells. However, using various transgenic mice, they have shown that the PAG-Fyn complex plays an important role in the maintenance of anergy, mainly by increasing calcium flux without activating the Ras-MAPK pathway; although the authors could not find the mechanism responsible for this effect. We complement this finding with the observation that the PAG-Fyn complex can also actively inhibit proximal signaling and Ras activation by hyperphosphorylating PAG, thereby leading to enhanced recruitment of other critical proteins like Csk and p120RasGAP, two important negative regulators of SFKs and Ras, in order to prevent cell activation and proliferation (see also 4.5.). Indeed, we can show using PAG siRNA that knocking down PAG expression leads to an unbalanced hyperactivation of both Src kinases and Ras (see 4.7.).

4.3. Fyn is dually phosphorylated on Y^{529} and Y^{215} in anergic T cells (see 3.2.6.)

An interesting and also somewhat confusing observation is that we find increased Fyn activity simultaneously with the increased phosphorylation of its inhibitory tyrosine (compare Figure 3.6. and 3.12.). Normally, the phosphorylated inhibitory tyrosine binds to the SH2 domain of Fyn and thus one would expect that its hyperphosphorylation should lead to a decreased kinase activity. However, it is known from the crystal structure that another intramolecular interaction is required for the inactive state of the kinase, namely the binding of the SH3 domain to the linker region between the kinase domain and SH2 domain (see section 1.7.2.). In fact, there is a growing body

of evidence to suggest that interfering with the SH3-linker interaction is sufficient to activate the kinase (Simarro et al., 2004; Gorska et al., 2004). The interaction of Fyn with PAG was demonstrated to be phosphorylation independent in human T cells and therefore mediated by binding of the SH3 domain in Fyn to a proline-rich region within PAG. Conversely, Fyn association appears to be phosphorylation dependent in murine T cells (Davidson et al., 2007). Since the amount of Fyn associated to PAG is not enhanced in our anergic T cells (Figure 3.11.), the strength of interaction with PAG would have to be more stable in anergic cells, thereby preventing the folding of the kinase into the inactive conformation. However, this would concern only the pool of Fyn associated with PAG. Since we also observed hyperactivity of Fyn in whole lysates, we searched for additional mechanisms that would apply to total Fyn. We found two publications proposing that the phosphorylation of the inhibitory tyrosine of chicken Src leads to a hyperactive state of the kinase if a tyrosine within its SH2 domain (Y^{215}) is also phosphorylated (Vadlamudi et al., 2003; Stover et al., 1996). It was proposed that the inhibitory tyrosine cannot bind to the SH2 domain because of the charge repulsion between phosphorylated Y^{215} and a conserved glutamate residue at + 4 position relative to the inhibitory tyrosine within the C-terminus (Src chicken [P00523]: 525-PQpYQPGE-531). Since the sequence around Y^{215} of Src is conserved also among the SFKs, we tested whether this mechanism could also apply to Fyn. We can indeed clearly show that the phosphorylation of Fyn at Y^{215} is remarkably increased in anergic cells compared to both the resting and rescued cells and increases even more upon restimulation. Importantly, Y^{215} phosphorylation clearly correlates with inhibitory tyrosine phosphorylation and therefore we believe that this dual phosphorylation of Fyn in anergic cells results in its opened hyperactive conformation. One may also hypothesize that if the phosphorylated inhibitory tyrosine does not bind to the SH2 domain in this hyperactive state, both the SH2 domain and the C-terminal phosphotyrosine are free and provide binding sites for other proteins that may be additionally recruited to the Fyn complexes, e.g. to the PAG-Fyn complex. Such an amplificatory mechanism has been also proposed for the complex of Lck with LIME (Lck-interacting molecule) (Brdickova et al., 2003).

Note that the paper from Maksumova et al. mentioned above also shows increased activity of Fyn simultaneously with the increased phosphorylation of the inhibitory tyrosine in PTPalpha deficient thymocytes (Maksumova et al., 2005). They propose that Fyn is kept in its active conformation, because it is dually phosphorylated on both the activatory and the inhibitory tyrosine. However they did not investigate the phosphorylation status of Y^{215}. It is tempting to

speculate that PTP alpha may be the phosphatase responsible for the dephosphorylation of Y^{215} and its deficiency leads to Y^{215} hyperphosphorylation and consequently to increased Fyn kinase activity.

So far Y^{215} has only been shown to be phosphorylated in Src upon growth factor receptor stimulation and the phosphorylation is probably mediated by the receptor tyrosine kinase. We are the first to show that Y^{215} phosphorylation occurs also in the case of Fyn and that this takes place in human T cells *in vivo*. Furthermore, we predict that this mechanism might play a role in TCR-mediated signaling and especially in T-cell anergy. Currently, we do not know the kinase responsible for Y^{215} phosphorylation. This tyrosine may either undergo autophosphorylation as is the case for the activatory tyrosine or other kinases may be involved. Certainly, this will be the subject of further investigations.

4.4. Increased Fyn kinase activity and inhibitory tyrosine phosphorylation within the lipid rafts of anergic T cells (see 3.2.7.)

Since lipid rafts play an important role in T-cell signaling, we looked whether the results found in whole lysates of anergic cells are specifically enhanced within the lipid rafts. We found that Fyn and Lck upregulation occurs in both the lipid raft and non-raft membrane compartments of anergic cells. Also the distribution of their kinase activities seems to be unchanged and the increased Fyn activation is detected both in the rafts and outside, with much stronger activity in the raft fraction. The enhanced phosphorylation of the inhibitory tyrosine of Fyn within anergic cells is located almost exclusively within the lipid rafts, where most of the Fyn and PAG-Csk complex are located. Importantly, the localization of PAG is not altered upon anergy induction and PAG is still targeted into the lipid rafts to the same extent as in resting and rescued cells. That also explains why the PAG-Csk complex has a stronger impact upon Fyn, which is mainly situated in the rafts, whereas only a minor effect upon a non-raft kinase Lck.

Notably, it was recently demonstrated that another transmembrane adaptor protein LAT is displaced from the lipid rafts in anergic T cells (Hundt et al., 2006). Since LAT is not palmitoylated in anergic T cells, it cannot be targeted into the rafts and therefore does not function properly. Since palmitoylation is necessary also for other adaptor proteins to be targeted into lipid rafts, one would expect that the lack of palmitoylation and the dislocation would be common also to the other raft-associated transmembrane adaptors. However, we know that treating T cells with polyunsaturated fatty acids (PUFA) also displaces LAT from lipid rafts,

whereas PAG is not dislocated (Zeyda et al., 2002). Thus it seems that PAG is more stably inserted within the rafts by an unidentified mechanism probably involving the transmembrane and/or the extracellular part of PAG. Additionally, protein-protein interactions can be responsible for stable PAG localization within lipid rafts, e.g. its constitutive association with Fyn that is mainly present in the rafts. This stable insertion may ensure that PAG is not so easily displaced from the rafts, e.g. upon PUFA treatment or anergy induction. However, we were unable to show displacement of LAT from the lipid rafts in anergic T cells in our system. This discrepancy might be due to the different systems investigated, as we induced anergy in primary naïve human T cells with immobilized anti-CD3 antibodies, while they applied the ionomycin-induced model of anergy induction using activated murine transgenic T cells (Hundt et al., 2006).

4.5. PAG forms a novel multiprotein complex (see 3.3.1., 3.3.2.)

When searching for the possible link connecting Fyn activity and PAG hyperphosphorylation to the block in Ras activation, we found two proteins whose expression is upregulated in anergic T cells, Sam68 and p120RasGAP (Figure 3.16.). Sam68 was originally identified also as KH domain containing, RNA-binding, signal transduction associated 1 protein (KHDRBS1). By immunoprecipitating either PAG, Sam68 or p120RasGAP, we are the first to demonstrate the formation of a multi-molecular complex consisting of PAG, Fyn, Sam68 and p120RasGAP (Figure 3.17.). The fact that we do not see any detectable difference in p120RasGAP association to PAG between anergic and rescued cells may be again attributed to the fact that rescued cells received the same stimulus as anergic cells (namely immobilized anti-CD3 antibody) and were rescued from anergy by adding PMA, which acts only more downstream on the level of DAG and therefore downstream of PAG. In fact, PMA directly stimulates RasGRP1, a GEF for Ras. The signal delivered by PMA is presumably much more robust than the intrinsic activity of p120RasGAP, thus leading to Ras activation and proliferation of rescued cells. Moreover, we investigated only the association of p120RasGAP with PAG, but not its activity. Interestingly, p120RasGAP was shown to be phosphorylated by receptor tyrosine kinases or transformed Src kinase and this tyrosine phosphorylation was suggested to modulate its GAP activity or interaction with other proteins (Molloy et al., 1989; Kaplan et al., 1990; Liu and Pawson, 1991). Since we find p120RasGAP in a complex with PAG and Fyn and we have observed increased Fyn activity associated with PAG in anergic cells, it is tempting to speculate that Fyn could phosphorylate p120RasGAP in this complex, leading to its activation and consequently Ras

inhibition in anergic T cells. Alternatively, PAG-associated Csk might also phosphorylate p120RasGAP. Since we observed enhanced amount of Csk associated with PAG specifically in anergic cells, the PAG-associated p120RasGAP may be more active in these cells.

The next important step is to identify the binding sites of the individual proteins and to find out how the complex is assembled. The binding of p120RasGAP to PAG appeared to be phosphorylation-dependent as it increased when we induced maximal phosphorylation, i.e. upon pervanadate treatment of the cells (data not shown). Previously, it was shown by an *in vitro* GST-SH2 pull-down assay that the N-terminal SH2 domain of p120RasGAP is capable of binding to PAG. Therefore we utilized the set of individual PAG YΔF mutants that had been previously made in our laboratory to identify which tyrosine might be critical for p120RasGAP binding. By transfecting these PAG mutants into Jurkat T cells and looking for the presence of PAG in p120RasGAP immunoprecipitations, we found out that Y^{181} is the main p120RasGAP binding site within PAG. Surprisingly, we do not observe any decrease in p120RasGAP association to PAG upon restimulation of anergic cells for 2 minutes (Figure 3.17.A), although it induces maximal dephosphorylation of the Csk binding site, Y^{317}. Thus it seems that the tyrosine binding p120RasGAP is dephosphorylated with a different time kinetic than the Csk binding tyrosine and one would have to do the whole time course of stimulation in order to characterize the dynamics of p120RasGAP-PAG association. In addition, we need to generate a phospho-specific antibody against Y^{181} to fully characterize the kinetics of its phosphorylation and dephosphorylation. One should point out that attempts to identify the PAG phosphatase are until now very inconsistent (see 1.6.3.). The fact that different tyrosines are dephosphorylated in a different time manner suggests that there may be not only one phosphatase, but rather several phosphatases with distinct specificities for individual tyrosine-based signaling motifs.

Sam68 most probably does not bind directly to PAG as it lacks any protein-binding domains. Instead it possesses several tyrosines and proline-rich motifs and therefore can bind to either Fyn or p120RasGAP (Fusaki et al., 1997; Guitard et al., 1998; Jabado et al., 1998). Since Fyn binds to PAG presumably via its SH3 domain (Brdicka et al., 2000), its SH2 domain is free to bind phosphorylated Sam68. Additionally, p120RasGAP could be the direct Sam68 binding partner as it possesses two SH2 domains and whereas the N-terminal SH2 was suggested to bind PAG, the C-terminal domain binds preferentially to Sam68 (Brdicka et al., 2000; Durrheim et al., 2001; Sanchez-Margalet and Najib, 2001). The phosphorylation-dependent interaction of Sam68 with the complex would also explain why we could not detect the Sam68-PAG complex in PAG

immunoprecipitates from resting cells, possessing low Fyn activity. Namely, the complex stability may be increased only when the Fyn kinase activity is upregulated by the induction of anergy. The role of Sam68 in this complex is unclear. It may function simply as an adaptor further stabilizing the binding of p120RasGAP. Beside its adaptor function (Najib et al., 2005), Sam68 also belongs to the STAR (signal transduction and activation of RNA) family of RNA binding proteins (Lukong and Richard, 2003). It is postulated that Sam68 binds specific RNAs and their release into the cytosol enables their translation. Thus, Sam68 may enable the cells to become anergic by releasing RNAs for anergy-promoting factors. Additionally, Sam68 is phosphorylated by Fyn and this negatively correlates with RNA binding and leads to the relocalization of Sam68 into the cytosol (Hartmann et al., 1999; Wang et al., 1995). Therefore, increased phosphorylation by Fyn and recruitment into the cytosol towards the PAG complex might mediate enhanced shuffling from the nucleus and more rapid release of RNAs needed for translation of various factors important for anergy.

The fact that we found an upregulation of Sam68 expression in anergic T cells may provide an explanation for the proliferative block attributed to anergy. Sam68 was shown to regulate the cell cycle progression and the expression of cyclin D1, which is required for the transition into S phase (Barlat et al., 1997). Importantly, the overexpression of Sam68 results in decreased levels of cyclin D1 and E and in cell cycle arrest in the G1 phase (Taylor et al., 2004). Notably, anergic cells were also shown to possess a block in the cell cycle at G1/S transition (Gilbert et al., 1992). Since recent studies have questioned the role of p27kip1 and p21cip1 as candidates responsible for the block in cell cycle progression (Verdoodt et al., 2003; Li et al., 2006), it will be interesting to see whether Sam68 deficient mice possess a defect in anergy induction.

4.6. PAG negatively regulates Ras activation (see 3.3.3. – 3.3.8.)

Using the GST-RBD pull-down assay, we can demonstrate that PAG is a novel negative regulator of Ras signaling. Transfection of wildtype PAG and Fyn into the Jurkat T-cell line leads to the hyperphosphorylation of PAG, the same phenotype as we observe in anergic T cells, and this results in an almost completely abolished activation of Ras upon TCR stimulation (Figure 3.18.). We can additionally show that this effect is not dependent upon Y^{317} and therefore is not due to the enhanced recruitment of Csk and reduction of SFK activation. In fact, the Y317F mutant of PAG only minimally restores Ras activation and thus, the Csk-mediated pathway might not play the major role in the downregulation of Ras activity (Figure 3.20.). Interestingly, murine

fibroblasts deficient for the phosphatase Shp2 possess a block in Ras and ERK activation, presumably because of PAG hyperphosphorylation and increased Csk recruitment (Zhang et al., 2004). However, the expression of Y314F-PAG mutant in Shp2 deficient cells also only partially restored ERK activation (Zhang et al., 2004). In addition, T cells from Y314F-PAG transgenic mice demonstrate a reduction in ERK activation compared to control cells; although the mechanism could not be found (Davidson et al., 2007). Therefore it seems that the block in Ras activation is induced mainly by recruiting another protein to hyperphosphorylated PAG and we propose that such a protein is p120RasGAP. However, mutation of p120RasGAP binding site within PAG is not sufficient to restore the block in Ras activation (Figure 3.22.). Thus it seems that both Csk and p120RasGAP participate in the inhibition of Ras signaling. Therefore the presence of either of these mechanisms is sufficient to maintain Ras inactive. Indeed, deletion of both the Csk and p120RasGAP binding sites restores Ras activation comparable with the control cells (Figure 3.23.). Since Ras signaling is initiated in the lipid rafts and active Ras-GTP must translocate out of the rafts to interact with its effectors, formation of a multimolecular complex including p120RasGAP within the rafts would be an effective mean of inhibiting Ras before it had the chance to translocate (Figure 4.1.).

4.7. PAG downregulation leads to enhanced and sustained SFK and Ras activation (see 3.3.9.)

Since PAG knockout mice had been published with no apparent phenotype (Xu et al., 2005; Dobenecker et al., 2005), we had difficulties to convince reviewers that PAG is indeed such an important negative regulator of SFKs and Ras as our data would suggest. Because preliminary experiments had suggested the development of a compensatory mechanism in PAG knockout mice (J. Lindquist, unpublished observation), we decided to investigate the role of PAG directly in human T cells. Downregulation of PAG expression both in Jurkat and in primary human T cells using RNA interference lead to a dramatic upregulation of SFK activity as measured by overall protein tyrosine phosphorylation (Figure 3.24.A, B). In addition, the activity of Src kinases was sustained during stimulation, clearly reflecting the lack of a critical regulatory mechanism. When investigating Ras activation, we found that the loss of PAG results in a 5-fold induction of Ras activity compared to control cells (Figure 3.24.C). This clearly indicates that PAG is indeed an important negative regulator of both SFKs and Ras in human T cells. These results also confirm that there are differences between mouse and human, including development

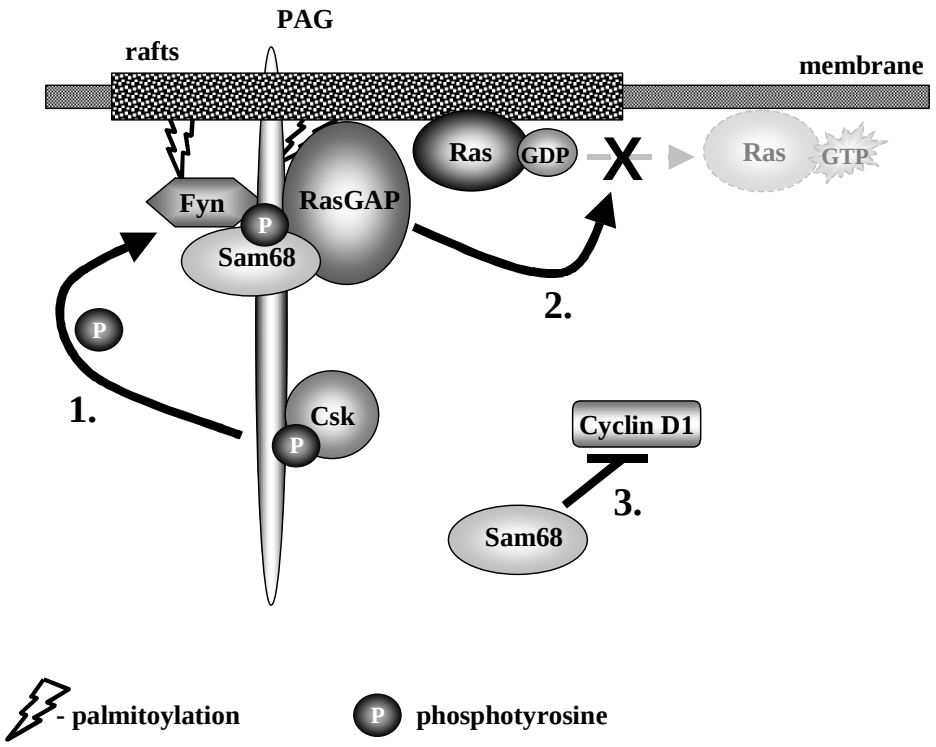


Figure 4.1. Schematic model of PAG function in T-cell anergy. 1. PAG is hyperphosphorylated by Fyn leading to increased recruitment of Csk. Csk in turn phosphorylates the inhibitory tyrosine within Fyn. However, Fyn becomes phosphorylated additionally on its Y^{215} within the SH2 domain, leading to the hyperactive state of Fyn. **2.** Hyperphosphorylated PAG recruits p120RasGAP into lipid rafts, where it stimulates the intrinsic GTPase activity of Ras thereby preventing its activation and translocation out of rafts. **3.** Additionally, PAG binds Sam68, which may further stabilize the complex and release mRNAs for anergy-promoting factors. Increased Sam68 expression can reduce cyclin D1 levels and thereby prevent transition of the cell cycle from G1 to S phase.

of alternative compensatory pathways in knockout mice, and this should always be taken into consideration. Therefore, it is impossible to generalize mouse data onto the human system or vice versa, but rather the data should always be verified by performing the appropriate experiment.

Interestingly, mutation of the Csk and p120RasGAP binding sites within PAG leads to a Ras activation equal to empty vector transfected cells (Figure 3.23.), whereas removal of the total PAG protein induces 5-fold higher Ras activation than the control cells. Clearly, when the mutant form of PAG is expressed in Jurkat T cells, there is still endogenous PAG present that attenuates Ras activation. Only if we downregulate endogenous PAG expression by siRNA, Ras becomes

hyperactive. On the other hand, there might be also additional mechanisms by which PAG can block Ras and thus, these pathways are still present in the mutant lacking both Csk and p120RasGAP binding. Their existence and affect upon Ras would then only become apparent when the whole protein was lost. This also means that these other pathways must be dependent upon the recruitment of proteins to tyrosines other than Y^{317} and Y^{181} and/or they might be recruited to phosphoserines or phosphothreonines or in a phosphorylation-independent manner to the proline-rich regions within PAG. We propose that one such protein recruited to PAG that could negatively regulate Ras might be IGAP, the inducible GTPase-activating protein that we have newly identified (see 4.9.).

4.8. Expression of DGKs is unchanged in anergic T cells (see 3.4.1.)

Anergic T cells are known to have normal calcium signaling but abolished activation of ERK and JNK pathway. Since activated PLCγ1 produces both IP_3 and DAG in an equimolar ratio, it is not clear how calcium flux should be activated by IP_3 without the MAPK cascade being simultaneously activated via DAG. One hypothesis would be that DAG is immediately converted into phosphatidic acid by diacylglycerolkinases. Therefore we hypothesized that the expression of either DGK alpha or zeta, the two main DGK isoforms expressed in T cells, would be enhanced in anergic cells. However, we did not find upregulation of either of these isoforms in our system (Figure 3.25.). This is in contrast to the very recent publications implicating DGK alpha in the regulation of anergy (Olenchock et al., 2006; Zha et al., 2006). These two papers showed that overexpression of DGK alpha in murine T cells leads to reduced activation of ERK and JNK, reduced production of IL-2 and diminished proliferation. Additionally, DGK alpha deficient T cells still produced IL-2 under anergizing conditions and thus it was concluded that DGK alpha deficiency impairs anergy induction. These results are however not so surprising, if one imagines that DGK alpha removal should enhance the DAG levels in the cells and thus lead to the upregulation of the whole DAG-Ras-MAPK pathway, which may in fact out compete the downregulation of Ras activation implemented by anergy-promoting machinery. Only the group of Gajewski showed that the expression of DGK alpha is indeed upregulated in anergic T cells and that the pharmacological inhibition of DGK alpha in these cells could rescue the production of IL-2 (Zha et al., 2006). The fact that we do not observe an upregulation of DGK alpha in our anergic cells may simply result from the differences between murine and human T cells as there

are clearly many differences. Additionally, the use of different DGK alpha antibodies with distinct affinities and epitopes may also give slightly different results. We also cannot exclude that the activity of any DGK isoform be enhanced in anergic cells, therefore one should test DGK activity, e.g. by assessing the rate of DAG conversion *in vitro*.

4.9. Identification and characterization of IGAP (see 3.4.2. – 3.4.7.)

While investigating the expression of DGK isoforms in anergic T cells, we observed a band at 120 kDa specifically cross-reacting with the anti-DGK alpha antibody (Figure 3.26.), which we identified as a predicted hypothetical protein with the accession number XP_029084 (Figure 3.27. and 3.28.). Since this protein is induced upon long-term stimulation of T cells and has a putative GAP domain, we named this protein IGAP (Inducible GTPase-activating protein). IGAP differs from p120RasGAP, the main RasGAP in T cells, in that it lacks SH2 and SH3 domains. Its predicted structure is rather similar to the neuronal GAPs expressed primarily in the brain, SynGAP and NGAP (Figure 3.29. and 3.30.). Surprisingly, IGAP possesses within its GAP domain an ITAM, which is usually found in immunoreceptors. We found that IGAP indeed becomes tyrosine phosphorylated (Figure 3.31.) and we propose that this may regulate its activity and/or accessibility to its interacting partners and substrates. IGAP is expressed only at low levels in resting T cells, but is dramatically upregulated upon prolonged stimulation of the cells. Therefore we hypothesize that IGAP may play role in shutting down signaling and the activation status of the cells. Resting cells do not need IGAP since they need to be activated, however once they are activated and have performed their effector function, signaling must be shut down. Therefore they upregulate IGAP, which may inactivate Ras and thereby terminate AP-1 activation. Once IGAP is not needed anymore, it may be degraded. That would explain why we see a downregulation of IGAP levels when we rest the cells for one day (Figure 3.32.). Whereas IGAP might diminish AP-1 activation, other transcription factors like NFAT and NFkB may remain active. This way, IGAP may switch the transcriptional program within the cell so that a new set of genes would be transcribed leading to the expression of proteins that would alter the fate of the cell. In this scenario, IGAP might affect the development of memory cells or regulate expression of anergy-promoting factors. Interestingly, the main pool of IGAP is localized at the plasma membrane, whereas p120RasGAP is primarily cytosolic in unstimulated cells (Figure 3.33.). Thus, IGAP is already prelocalized in the proximity of Ras and might more efficiently

inactivate Ras signaling. Importantly, there are three main isoforms of Ras, which differ in their subcellular localization. Therefore, IGAP may only be selective for a specific Ras isoform and thereby may have partially distinct effect compared to other RasGAPs expressed in T cells.

Finally, we can show that IGAP is able to associate with PAG (Figure 3.34.). The nature of this association is not clear yet, since IGAP lacks any protein-protein interaction domains. The interaction is therefore most probably not direct, but rather mediated via another yet unidentified partner. One good candidate would be Fyn, which constitutively binds to PAG and could additionally bind the phosphorylated ITAM within IGAP via its SH2 domain. Additionally, since p120RasGAP is recruited to PAG through its SH2 domain, it still has a free SH3 domain that could bind the multiple proline-rich domains within IGAP. Interestingly, there has been an unexpected observation recently that the C2 domain of PKCδ is able to directly bind to a phosphotyrosine (Benes et al., 2005). Since IGAP also possesses a C2 domain, we must consider also the possibility that IGAP binds directly to the phosphotyrosine within PAG via its C2 domain.

Taken together, we conclude that PAG functions as a potent negative regulator of Ras signaling through recruiting two GAPs to the multiprotein complexes at the membrane and thereby situating them to the proximity of their substrates to provide rapid and efficient Ras inactivation.

5. Conclusion

Anergy is an important mechanism of peripheral tolerance preventing self-reactive T cells from becoming activated and thereby the development of autoimmune disorders. Despite many attempts to identify signaling alterations responsible for the unresponsive phenotype of anergic cells, the real molecular mechanism still remains unresolved. The hallmarks of anergic cells are the upregulation of Fyn kinase and the defect in Ras activation. In human T cells, a pool of Fyn is constitutively associated with PAG and was shown to be responsible for PAG phosphorylation. Importantly, we found that PAG-associated Fyn possesses enhanced kinase activity and this leads to hyperphosphorylation of PAG in human anergic T cells. Consequently, PAG recruits more Csk and although a portion of Csk is lost upon restimulation of the cells, there is still a remarkable amount of Csk bound to PAG that leads to enhanced phosphorylation of the inhibitory tyrosine within Fyn. This mechanism might then contribute also to a block in proximal signaling, which was attributed to anergic cells.

Additionally, we have described a novel mechanism of Fyn kinase regulation. We have shown here that Fyn becomes phosphorylated not only on its C-terminal inhibitory tyrosine, but at the same time also on a tyrosine within its SH2 domain and we propose that this leads to its opened hyper-active conformation in human anergic T cells.

Furthermore, we have identified a novel multiprotein complex consisting of PAG, Fyn, Sam68 and p120RasGAP and have demonstrated an important inhibitory role of PAG on Ras activation. We have also shown that its ability to regulate Ras is dependent on both Csk and p120RasGAP association and only the deletion of both binding sites completely ablates PAGs impact on Ras signaling. Using RNA interference, we could demonstrate that suppression of PAG expression leads to an unbalanced upregulation of both Src kinase and Ras activity resulting from the loss of a critical negative feedback loop.

Finally, we have identified a new protein, which we called IGAP, as it appears to be a GTPase-activating protein that is induced only upon activation of T cells. We hypothesize that IGAP may be needed by the activated T cells to shut down their activation status in order to terminate the immune response. We could show that, contrary to p120RasGAP, IGAP is already prelocalized at the plasma membrane and that one mechanism of its regulation might be mediated via its association to PAG.

In conclusion, we have established PAG as a potent negative regulator of Ras activation recruiting two RasGAPs and we propose that this regulatory mechanism may play a role in anergy. Thus, PAG is involved in the regulation of both Src kinases and Ras, two important oncoproteins implicated in many forms of cancer. Therefore it is tempting to speculate that there might be alterations in PAG expression and/or various PAG mutations found in certain types of cancer that might be responsible for dysregulation of cellular signaling leading to a pathological transformation of the cell.

6. Zusammenfassung

Anergie ist ein Mechanismus der peripheren Toleranz, der eine Aktivierung von autoreaktiven T Zellen verhindert und damit der Entstehung verschiedener autoimmuner Erkrankungen vorbeugt. Trotz vieler Versuche, die modifizierte Signalwege zu definieren, welche den aresponsiven Phänotyp der anergischen Zellen verursachen, bleibt der molekulare Mechanismus bisher weitgehend unklar. Die wichtigste Merkmale der anergischen Zellen sind sowohl eine erhöhte Fyn-Kinase-Aktivität und -Expression als auch ein Block in der Aktivierung des kleinen G-Proteins Ras. In humanen T Zellen ist ein Teil von Fyn konstitutiv an PAG gebunden, der auch für die PAG-Phosphorylierung verantwortlich ist. Interessanterweise sind T Zellen mit einer erhöhten PAG-Expression nach T-Zell-Rezeptor-Stimulation aresponsiv, ein Phänotyp, der Anergie sehr ähnelt. Auf diesen Grundlagen basierend war es Ziel der vorliegenden Dissertation, die Rolle von PAG für die Aufrechterhaltung von Anergie zu untersuchen. In anergischen humanen T Zellen konnten wir zeigen, dass die PAG-assoziierte Fyn-Kinase eine erhöhte Aktivität besitzt. Dies führt zu einer Hyperphosphorylierung von PAG. Nachfolgend bindet PAG mehr Csk. Trotzdem ein Teil von Csk nach der Stimulation der anergischen T Zellen seine Assoziation zu PAG verliert, bleibt es noch mehr Csk an PAG gebunden als in unstimulierten oder aktivierten T Zellen. Dies resultiert in einer erhöhten Phosphorylierung des inhibitorischen Tyrosins von Fyn. Dieser Mechanismus kann einen Block in den proximalen Signalwege verursachen, der ein wesentliches Merkmal der anergischen Zellen darstellt.

Zusätzlich haben wir hier einen neuen Mechanismus für Fyn-Kinase-Regulation beschrieben. Wir haben belegt, dass die Fyn-Kinase nicht nur auf ihrem C-terminalen inhibitorischen Tyrosin phosphoryliert wird, sondern gleichzeitig auch an einem Tyrosin, das sich innerhalb der SH2-Domäne befindet. Wir behaupten, dass diese duale Phosphorylierung eine offene Struktur der Kinase bewirkt und so eine Hyperaktivierung von Fyn in humanen anergischen T Zellen herbeiführt.

Weiterhin haben wir einen Multiproteinkomplex entdeckt, der aus PAG, Fyn, Sam68 und p120RasGAP entsteht. Darüber hinaus haben wir eine wichtige Rolle von PAG für die Inhibition der Ras-Aktivierung nachgewiesen. Hierzu konnten wir zeigen, dass das Vermögen von PAG, Ras-Aktivität zu regulieren, von seiner Fähigkeit, sowohl Csk als auch p120RasGAP zu

rekrutieren, abhängt. Nur wenn beide Bindungsstellen für Csk und p120RasGAP eliminiert sind, hat PAG keinen Effekt auf die Ras-Signalwege mehr. Mit Hilfe von RNA-Interferenz ist es uns gelungen, zu zeigen, dass die Herunterregulierung der PAG-Expression zu einer unbalancierter Hochregulation von sowohl Src-Kinasen als auch Ras-Aktivität führt, der ein Verlust eines kritischen negativen Rückkopplungsmechanismen zugrunde liegt.

Zusätzlich haben wir ein neues Protein entdeckt, das wir IGAP benannt haben, da es sich um ein GTPase-aktivierendes Protein handelt, welches erst nach einer Stimulation der T Zellen exprimiert wird. Wir vermuten, dass IGAP erst in aktivierten Zellen benötigt wird, um ihre Aktivation und Proliferation einzustellen und somit die Immunantwort zu beenden. Im Gegensatz zu p120RasGAP ist IGAP schon auf der Zellmembrane vorhanden und kann so effektiver seine Wirkung auf Ras-Proteine ausüben. Zusätzlich haben wir IGAP im Komplex mit PAG gefunden und schlagen vor, dass diese Assoziation einen Einfluss auf die IGAP-Regulation haben könnte.

Zusammengefasst haben wir PAG als einen potenten negativen Regulator der Ras-Aktivierung dargestellt, der zwei RasGAP Proteine rekrutieren kann. Wir vermuten, dass dieser regulatorischer Mechanismus auch eine wichtige Rolle in der Ausbildung und Aufrechterhaltung von Anergie spielen könnte. PAG ist also an der Regulation sowohl von Src-Kinasen als auch Ras beteiligt. Beide sind wichtige Onkoproteine, deren Defekte mit einer Vielzahl von Krebserkrankungen assoziiert sind. Aus diesem Grund spekulieren wir, dass verschiedene Änderungen in PAG-Expression bzw. Phosphorylierung und/oder verschiedene PAG-Mutationen in bestimmten Arten maligner Erkrankungen zu finden sind, die eine Dysregulation der zellulären Signalwege verursachen und somit zu einer pathologischen Transformation von Zellen führen.

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8. Abbreviations

AICD	activation-induced cell death
AP-1	activator protein 1
APC	antigen-presenting cell
C2 domain	protein kinase C conserved region 2 domain
cAMP	cyclic adenosine monophosphate
CAPRI	calcium-promoted Ras inactivator
Cbl	casitas B-lineage lymphoma
CD	cluster of differentiation
Cdk	cyclin-dependent kinase
Csk	C-terminal Src kinase
CTLA4	cytotoxic T lymphocyte antigen 4
DAG	diacylglycerol
DC	dendritic cell
DGK	diacylglycerolkinase
DN	double negative
Dok	downstream of kinase
DP	double positive
EBP50	ezrin-radixin-moesin binding protein of 50 kDa
ERK	extracellular signal related kinase
FI	fold induction
FoxP3	forkhead box P3 protein
Gads	Grb2-related adaptor downstream of Shc
GEMs	glycosphingolipid-enriched membrane microdomains
GM-CSF	granulocyte and monocyte colony-stimulating factor
Grb2	growth factor receptor-bound protein2
GST	glutathione S-transferase
IB	immunoblotting
IFN-γ	interferon gamma
IGAP	inducible GTPase-activating protein
IL	interleukin

IP	immunoprecipitation
IP_3	inositol-1,4,5-trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
IVK	*in vitro* kinase assay
JNK	c-Jun N-terminal kinase
LAT	linker for activation of T cells
Lck	lymphocyte-specific protein tyrosine kinase
LIME	Lck-interacting molecule
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MW	molecular weight
NF1	neurofibromin 1
NFAT	nuclear factor of activated T cells
NFkB	nuclear factor kappa B
p21cip1	cyclin-dependent kinase inhibitor 1
p27kip1	kinase inhibitor protein 1
PAG	phosphoprotein associated with glycosphingolipid-enriched microdomains
PAGE	polyacrylamide gel electrophoresis
PEP	PEST-enriched phosphatase
PH domain	pleckstrin homology domain
PI3K	phosphatidylinositol-3-kinase
PKCθ	protein kinase C theta
PLCγ1	phospholipase C gamma 1
PMA	phorbol myristate acetate
PTB domain	phosphotyrosine-binding domain
PTPα	protein tyrosine phosphatase alpha
pTyr	phosphotyrosine
RasGAP	Ras GTPase-activating protein
RasGEF	Ras guanine-nucleotide exchange factor
RasGRP	Ras guanyl-releasing protein
RBD	Ras binding domain

Sam68	Src-associated in mitosis of 68kDa
SDS	sodium dodecyl sulphate
SFK	Src family kinase
SH2	Src homology 2
SH3	Src homology 3
SHP	SH2 domain-containing phosphatase
SHIP	SH2 domain-containing inositol 5‘ phosphatase
shRNA	short hairpin RNA
siRNA	short interfering RNA
SIT	SHP-2 interacting transmembrane adaptor protein
SLP-76	SH2 domain containing leukocyte protein of 76 kDa
Sos	son of sevenless
Syk	spleen tyrosine kinase
TBSM	tyrosine based signaling motif
Tc	cytotoxic T cell
TCR	T-cell receptor
TGF-β	transforming growth factor beta
Th	helper T cell
TRAPs	transmembrane adaptor proteins
Treg	regulatory T cell
WB	Western blotting
ZAP70	zeta-associated protein of 70kDa

9. Curriculum Vitae

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1994 – 1997	Faculty of Medicine, Masaryk University, Brno, CZ
1997 – 2002	Faculty of Science, Masaryk University, Brno, CZ
	Mgr. (~M.Sc., Dipl.-Biol.) in Molecular Biology/Genetics
2002 – 2007	Doctoral candidate in the Institute of Immunology, Otto-von-Guericke University, Magdeburg, Germany

10. Publications and presentations

Smida M, Posevitz-Fejfar A, Horejsi V, Schraven B, Lindquist JA. A novel negative regulatory function of the phosphoprotein associated with glycosphingolipid-enriched microdomains: blocking Ras activation. Blood 2007;110:596-615

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Simeoni L, **Smida M**, Posevitz V, Schraven B, and Lindquist JA. Right time, right place: the organization of membrane proximal signaling. Semin. Immunol. 2005;17:35-49

5-8 Sept. 2007	37th Annual Meeting of the German Society for Immunology, Heidelberg, Germany – poster presentation
6-9 Sept. 2006	Joint meeting of European national society of immunology, Paris, France – poster presentation
10-12 Nov. 2005	9th Joint meeting in signal transduction – Receptors, mediators and genes, Weimar, Germany – oral presentation
21-24 Sept. 2005	Joint 36th annual meetings of the German society of immunology and the Scandinavian society for immunology, Kiel, Germany – poster presentation
20-23 Oct. 2004	Joint Annual Meeting of the German and Dutch Societies for Immunology, Maastricht, the Netherlands – poster presentation

11-16 Sept. 2004 6th EFIS Tatra Immunology Conference, Tatranske Zruby, Slovakia – poster presentation