4. DISCUSSION

The reorganization of the actin cytoskeleton is an essential prerequisite for T cell activation: signaling by cell surface receptors during the activation of T lymphocytes initiates a cascade of biochemical events that result in profound changes in the organization of membrane proteins. The main driving force of this movement of surface receptors on T lymphocytes is the actin cytoskeleton (as reviewed by Sheets et al., 1995; Bretscher, 1996; W lfing and Davis, 1998).

As yet, little is known regarding the molecular processes which link receptor stimulation directly to the resulting rearrangements of the actin cytoskeleton *in vivo*. The first clues in the elucidation of this important phenomenon came from studies with human primary T lymphocytes where it was observed that cofilin is subject to reversible dephosphorylation on serine residues and that this process is regulated by accessory receptor dependent signals (Samstag et al., 1992; Samstag et al., 1994).

Here the functional role of the small actin binding molecule cofilin which was suspected to link surface receptor signals to the actin cytoskeleton during T cell activation processes has been investigated.

4.1 Reversible association of cofilin with the actin cytoskeleton following activation of human peripheral blood T lymphocytes

In experiments performed with human peripheral blood T lymphocytes, it could be demonstrated that T cell stimulation via surface receptors results in a transient association of cofilin with the actin cytoskeleton. Cofilin/F-actin interactions were not detectable in unstimulated resting T cells, however, occured in T lymphocytes which were activated

through the accessory receptor CD2 or through CD3 x CD28 crosslinking. The activation induced dephosphorylation of cofilin was accompanied by its transient translocation to the actin cytoskeleton with similar kinetics. Since the phosphorylation state of cofilin seems to be important for its binding activity to the actin cytoskeleton, cofilin was exclusively present in its dephosphorylated form in the actin cytoskeletal fraction of activated T cells. That stimulation of accessory receptors on T cells leads to dephosphorylation of cytoplasmic cofilin and its association with the actin cytoskeleton, supports the notion for a role of the actin cytoskeleton in the transmission of outside-in signals through transmembrane receptors.

That the interaction of cofilin with F-actin participates in the regulation of the cytoskeleton is further supported by a number of additional observations. Thus, cofilin localizes to regions of cells characterized by high actin dynamics, including neuronal growth cones, membrane ruffles, cleavage furrows and yeast cortical actin patches (Bamburg and Bray, 1987; Yonezawa et al., 1987; Moon et al., 1993; Nagaoka et al., 1995). Recent studies have shown that cofilin promotes rapid actin filament turnover *in vivo* in the *Listeria* tail (Carlier et al., 1997; Rosenblatt et al., 1997) and in the cortical actin cytoskeleton in yeast cells (Lappalainen and Drubin, 1997). Moreover, binding of cofilin to F-actin leads to alterations in the F-actin filament twist which influences the interaction of other molecules with F-actin (McGough et al., 1997). This alteration of the F-actin structure by cofilin appears to be a novel mechanism through which the actin cytoskeleton is remodeled. Thus, by binding to the actin cytoskeleton cofilin might function as a physiological mediator of receptor-dependent signals leading to cytoskeletal rearrangements. These signals may also be translated into modifications of cytoskeletal-associated molecules in the receptor vicinity (den Hartigh et al., 1992; Gronowski et al., 1993; Lemmon and Schlessinger, 1994).

While representing a key step upstream of the interaction of cofilin with F-actin, dephosphorylation of cofilin alone is not sufficient to induce their association *in vivo.* This conclusion is drawn from the following observations: unstimulated resting T lymphocytes contain a substantial amount of nonphosphorylated cofilin, yet *in vivo* binding of cofilin to F-actin is not detectable. Moreover, PMA or PMA plus ionophore stimulation, which is

believed to bypass early receptor signaling events, leads to an accumulation of unphosphorylated cytoplasmic cofilin, but does not induce the interaction of cofilin with the actin cytoskeleton *in vivo.* Finally, non-phosphorylatible S3A-cofilin mutant proteins expressed in fibroblasts do not associate with actin bundles or stressfibres (Moriyama et al., 1996; Nebl et al., 1996). Thus, additional as yet unknown receptor induced early signaling events appear to be required.

4.2 Involvement of PI3-kinase activity in the cofilin signaling pathway

Early signaling events in T cells are activated by PI3-kinase. This enzyme and its products contribute to cellular stimulation by recruiting signaling molecules to the stimulated receptors (Rameh et al., 1995) and influence actin cytoskeletal rearrangements following stimulation of T lymphocytes through accessory receptors such as CD2 or CD28 (Shimizu et al., 1995; Zell et al., 1996).

Employing the phosphatidylinositol 3-kinase (PI3-kinase) specific inhibitors wortmannin (Fig. 6) and LY294002 (data not shown), the involvement of PI3-kinase activity in the signaling cascade from co-stimulatory receptors to activation of cofilin was examined in peripheral blood T lymphocytes. Wortmannin and LY294002 inhibited the activation induced dephosphorylation of cofilin as well as its association with the actin cytoskeleton following stimulation via CD2 receptors or costimulation by CD3 and CD28 cross-linking, respectively. Thus, these findings suggest that cofilin represents one of the mediators of PI3-kinase function which couples signaling cascades to the cytoskeleton, thereby affecting cytoskeletal restructuring during T cell activation. However, the complete signaling pathway which links PI3-kinase activity to the dephosphorylation of cofilin is as yet unknown.

Note that in Jurkat T lymphoma cells, cofilin is spontaneously dephosphorylated and is permanently associated with the actin cytoskeleton. There, however, wortmannin did not block these events, implying that in this tumor cell line a transforming step distal of PI3-kinase activity has occured, which leads to continuous activation of cofilin through dephosphorylation.

4.3 Blockade of the activation-induced interaction of cofilin with the actin cytoskeleton by cofilin derived peptides *in vivo*

In order to determine the functional significance of the cofilin/actin association for T cell activation, the activation induced *in vivo* interaction of cofilin with the actin cytoskeleton was blocked employing cofilin derived peptides. Employing the Penetratin carrier-system (Perez et al., 1992) these synthetic peptides were efficiently introduced into untransformed human peripheral blood T lymphocytes.

Synthetic peptides derived from the human cofilin sequence corresponding to actin binding residues 1-12 (MAS) and 104-115 (WAP) bound to isolated cytoskeletal actin from PBT cells *in vitro* and were able to competitively inhibit the interaction of cofilin with F-actin *in vivo*. The observed synergistic inhibitory effect of peptides MAS and WAP indicates that these two actin binding sites work in cooperation and are both necessary for efficient interaction between cofilin and F-actin in activated T lymphocytes *in vivo*.

These data are consistent with previous findings, which have suggested that the residues Lys 112 and Lys 114 of the porcine brain cofilin (Moriyama et al., 1992) as well as the N-terminal Ser-3 in chicken ADF (Agnew et al., 1995) and in yeast cofilin (Lappalainen et al., 1997) are essential for its binding to F-actin. Moreover, synthetic peptides derived from the porcine cofilin sequence, corresponding to residues 104-115 (Yonezawa et al., 1991) and 122-128 (Yonezawa et al., 1989), respectively, have been shown to bind to F-actin *in vitro*. However, in the present study, the peptide corresponding to residues 122-128

showed only low binding to cytoskeletal actin from resting human T lymphocytes. One possible explanation for this discrepancy is that the heptapeptide used by Yonezawa et al., (1989) is highly charged (DAIKKKL) and could therefore interact with *in vitro* polymerized actin in a non-specific manner.

Taken together these data suggest that residues 1-12 and 104-115 of human cofilin represent the important sites for F-actin binding *in vivo* in human T lymphocytes.

The peptide blocking system described above allowed to study for the first time functional consequences of the interaction of cofilin with the actin cytoskeleton for immunocompetent primary T lymphocytes in an *in vivo* system.

4.4 Involvement of cofilin in the process of receptor cap formation

Receptor-induced activation of T lymphocytes is accompanied by a clustering of surface receptors into an asymmetric membrane structure referred to as receptor cap. The exact mechanisms of cap formation are largely unknown, but they are believed to involve actin cytoskeletal rearrangements as concluded from earlier experiments with cytochalasin (De Petis, 1974). Therefore, the involvement of cofilin in the process of receptor cap formation in peripheral blood T lymphocytes was investigated.

Cofilin was found to co-accumulate at the site of the CD2 receptor caps along with actin. Pretreatment of T lymphocytes with cofilin derived peptides MAS and WAP completely prevented CD2 receptor cap formation and the co-localization of cofilin. Although previous reports have shown defects in receptor cap formation in Vav and WASP-deficient T cells, this is the first demonstration of a direct relationship between receptor clustering on the T cell interface and the interaction of an actin-binding molecule with the actin

cytoskeleton. These studies could identify cofilin as a key player in the actin dependent structural organization of functional receptor complexes.

In addition to cofilin, the p85 subunit of PI3-kinase and one of the PI3-kinase substrates, namely phosphatidylinositol 4,5-bisposphate (PtdIns(4,5)P_2) translocated to the site of the CD2 caps. In contrast, the Rho GTPases Rac1, RhoA or Cdc42 which are also discussed to be involved in cytoskeletal rearrangement processes (Hall, A., 1994), were not detectable in CD2 receptor caps.

It is tempting to speculate that at least one of the mechanisms which, in addition to its phosphorylation state, regulate the F-actin binding capacity of cofilin relates to its interaction with phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). Several actin-binding proteins including cofilin have PtdIns(4,5)P_2 binding sites, and there is evidence that modulation of the concentration of PtdIns(4,5)P_2 affects the actin configuration (Janmey and Stossel, 1989; Yonezawa et al., 1990; Hartwig et al., 1995). PtdIns(4,5)P_2 binding inhibits the capacity of cofilin to associate with and to depolymerize F-actin (Yonezawa et al., 1991). The level of cellular PtdIns(4,5)P_2 can be reduced through phosphorylation by PI3-kinase. Activation of PI3-kinase as a consequence of surface receptor engagement (Ward, 1996) may, therefore, reduce binding of PtdIns(4,5)P_2 to cofilin thereby facilitating its interaction with the actin cytoskeleton. Such a concept is supported by data demonstrating that PI3-kinase and its products are involved in the regulation of cytoskeletal structures (Kotani et al., 1994; Wennstrom et al., 1994; Rodriguez et al., 1997). The presence of PtdIns(4,5)P_2 as well as the p85-subunit of PI3-kinase in receptor caps supports such a view.

Taken together, the present study sheds novel light on an important phenomenon which links receptor stimulation to the resulting reorganization of the actin cytoskeleton required for receptor clustering of T lymphocytes.

4.5 The interaction of cofilin with the actin cytoskeleton is an important step for T cell activation

A functional relationship between the actin regulated cytoskeletal reorganization and receptor clustering for the induction of a physiological T cell response has first been suggested by studies with actin cytoskeleton poisons, i.e. cytochalasins. Cytochalasins were shown to inhibit T cell proliferation, TCR capping, Ca^{2+} flux and T cell shape changes required for sustaining and facilitating antigen-specific T cell/antigen presenting cell interactions and lymphokine production (Geppert and Lipsky, 1990; Matsuyama et al., 1991; Rozdzial et al., 1995; Valitutti et al., 1995). In this context, it has been proposed that the specific reorganization of the actin cytoskeleton upon cell activation could either enable the formation of coordinated structures composed of receptors and signaling molecules in order to reduce the threshold required for full activation, or prolong essential activating signals, or both (Valitutti et al., 1995).

In addition, recent sudies on actin regulating proteins, such as Vav and WASP, indicated that the assembly of the actin cytoskeleton and capping may play a direct role in mediating signals from the T cell surface receptors to further downstream signaling pathways. The proliferative defects in Vav- and WASP-deficient T cells may result from impairment of these processes (Fisher et al., 1995; Holsinger et al., 1998; Snapper et al., 1998).

However, no information existed regarding actin-binding proteins which link receptor stimulation to the resulting reorganization of the actin cytoskeleton required for cap formation and T cell activation.

The experiments described above led to the hypothesis, that by contributing to the reorganization of the actin cytoskeleton, which is a prerequisite of T cell activation, cofilin could be involved in regulating cellular functions of T lymphocytes. Indeed a series of functional experiments demonstrated that competitive inhibition of binding of activated cofilin to F-actin in untransformed human T lymphocytes by means of cell permeable, non-toxic cofilin derived peptides (MAS and WAP), strongly influenced functional T cell responses. Peptides, MAS and WAP, but not the control peptide WAPQ, exerted potent

inhibitory effects on CD2 induced T cell proliferation, interleukin-2 production, and the expression of the early activation marker CD69. These effects may be due to the inhibition of receptor clustering, resulting in a defect in the activation of downstream signaling pathways. Whether cofilin exerts additional, more direct effects on T cell signal transduction remains to be determined.

Importantly, receptor independent T cell activation by phorbolester and calcium ionophore circumvented the inhibitory effects of cofilin derived peptides on T lymphocyte stimulation. This finding further substantiates the assumption that the cofilin/F-actin interaction represents an essential proximal step in early receptor mediated activation events.

It should be stressed that peptides did not only mediate [a]suppressive effects but in some cases even up-regulated cellular responses. For example, a strong enhancement of the production of γIFN was observed. Since γIFN is a stress inducible cytokine, MAS and WAP peptides might exert cellular stress. This stress could result from defects in the regulation of the actin cytoskeleton by blocking the cofilin/F-actin interaction by the use of cofilin derived peptides. Recently, cofilin has been shown to be a major component of the intranuclear and cytoplasmic actin rods (termed stress fibers), which are formed in cultured fibroblast cells exposed to a variety of cell stresses (i.e. heat shock, dimethylsulfoxide treatment, or treatment with cytochalasins) (Nishida et al., 1987; Ohta et al., 1989).

The increased expression of MHC II molecules by treatment with cofilin derived peptides is likely to represent a secondary effect due to the increase in γIFN production, which is well known to induce the up-regulation of MHC II molecule expression (Wallach et al., 1982; Basham et al., 1984; Skoskiewicz et al., 1985; Hobart et al., 1997). If this is the case, then signaling through the γIFN receptor would have to be independent of cofilin/F-actin interactions.

Taken together, the data presented so far clearly demonstrate that cofilin is critical for the readout of at least some receptor-mediated signals that lead to activation of primary T

lymphocytes. The identification of a functional role of cofilin in T cell activation as a central and as yet missing link between surface receptor engagement and coordinated reorganization of the actin cytoskeleton adds substantial new information to the understanding of the central molecular processes which are crucial in effective T lymphocyte activation in the immune response.

4.6 Involvement of cofilin in CD2-mediated activation induced programmed cell death

In T cells CD2 triggering does not only induce cellular proliferation but also, depending on the differentiation state of the responding cell, apoptosis. Apparently, overlapping signal transduction pathways are involved in both type of responses. Several studies have shown that one of the key events in activation induced cell death (AICD) is the expression and interaction of CD95/CD95L (Fas/FasL) and that interference with the CD95/CD95L system inhibits apoptosis (Ju, S.T et al., 1995; Alderson et al., 1995). Therefore, CD95/CD95L is one of the systems by which specific clonal expansion is self-controlled. In the D798.18 T cell clone, the involvement of the CD95/CD95L interaction in CD2-induced apoptosis could be demonstrated by blocking studies employing a CD95 antibody. The cofilin derived peptides MAS and WAP specifically prevented CD2-induced programmed cell death in the human T cell clone D798.18. In contrast to this, apoptosis induced by CD95 receptor triggering was not affected by these peptides, suggesting that the interaction of cofilin with F-actin is essentially involved in the CD2-, but not in the CD95-signal transduction pathway.

Importantly, the expression of CD95 ligand following CD2 receptor triggering was found to be strongly inhibited by the treatment with the MAS and WAP peptides. This finding suggests that the inhibitory effects of cofilin derived peptides on CD2-induced apoptosis are due to the inhibition of CD95 ligand expression.

Although the *in vivo* importance of these results remains to be established, these data provide evidence for an involvement of cofilin not only in the physiological control of T cell activation, but also in modulating lymphocyte death. The signal transduction pathways leading to either T cell proliferation or activation induced apoptosis are still not completely understood, but they share common signaling events like activation of lck, fyn and Zap 70, followed by an increase in the cytosolic calcium concentration (Odaka et al., 1990; Mercep et al., 1989; Shi Y.F. et al., 1989; Ucker et al., 1989).

Taken together, the data presented here clearly demonstrate that cofilin is critical for the readout of receptor-mediated signals that lead to cellular responses of T lymphocytes.

To study, for the first time, functional consequences of the cofilin/actin interaction in immunocompetent T cells, the use of membrane permeable nontoxic peptides and primary untransformed T cells was crucial. In continuously proliferating transformed T lymphocytes, e.g. Jurkat, cofilin activation is uncoupled from receptor stimulation. There cofilin exists permanently in the dephosphorylated, actin bound state and it is not possible to reverse this association by synthetic peptides. Given that cofilin represents an essential protein for cell survival (Iida et al., 1993; Moon et al., 1993; Abe et al., 1996), conventional approaches to address this question (e.g. cofilin knock-out mice, cofilin antisense-RNA expression or transfection of dominant negative cofilin c-DNA-constructs) cannot be applied either.

The present study suggests that cofilin is important in inducing receptor mediated signal transduction and actin cytoskeletal changes which are required for T cell activation leading to either T cell proliferation or programmed cell death. Future experiments to characterize the requirements for these cofilin mediated processes should aid in the understanding of how and to what extent receptor mediated signal transduction pathways and actin reorganization are integrated within signals leading to biological responses.