3. RESULTS

3.1 Activation of human peripheral blood T lymphocytes induces reversible association of cofilin with the actin cytoskeleton

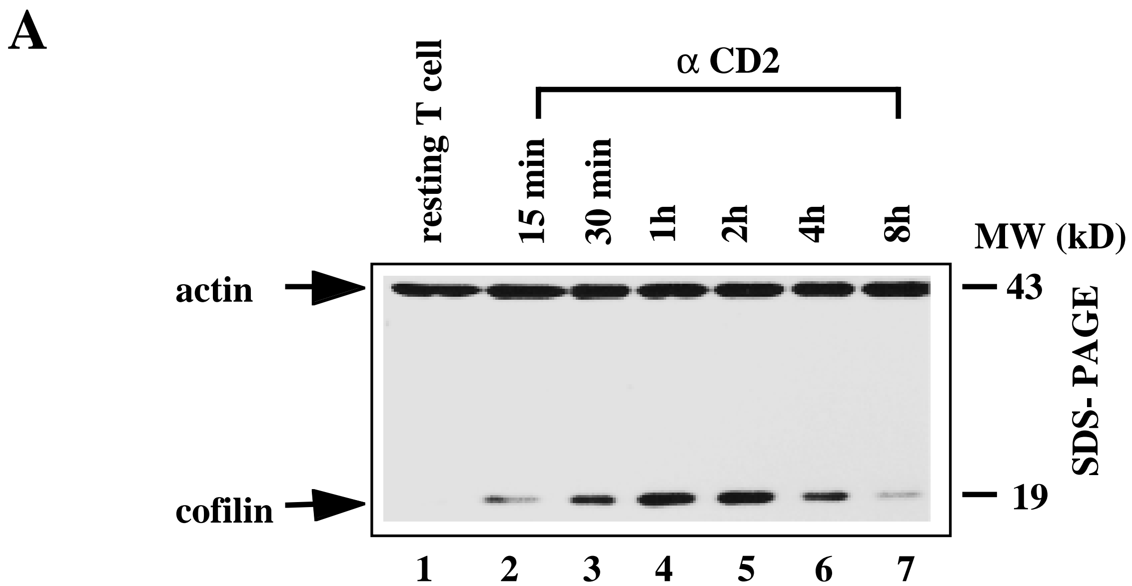
In unstimulated resting human peripheral blood T lymphocytes (PBTs) the small actin binding protein cofilin exists mainly in its inactive phosphorylated form. Following stimulation of accessory receptors, cofilin is dephosphorylated on phosphoserine residues (Samstag et al., 1991; Samstag et al., 1992; Samstag et al., 1994).

To investigate whether the interaction of cofilin with the actin cytoskeleton is regulated through T cell activation, the amounts of cofilin in actin cytoskeletal fractions of PBTs were analysed in unstimulated PBT and at different time points after anti-CD2 stimulation. The cytoskeleton was prepared from the Triton X-100 insoluble material which contains the cytoskeletal matrix as a major component (Gregorio et al., 1992; Marano et al., 1989) and detergent insoluble membrane complexes (Schroeder et al., 1994; Sargiacomo et al., 1993). Cytoskeletal actin and its associated proteins (actin cytoskeletal fraction) were released from this fraction by treatment with cytochalasin B, an agent that interferes with the polymerization of actin filaments and destroys the integrity of the actin cytoskeleton (Preston et al., 1990).

Western-blot analysis showed that cofilin was not detectable in actin cytoskeletal fractions prepared from resting T cells (Fig. 3A, lane 1). Following CD2 stimulation, however, cofilin transiently co-localized with the actin cytoskeleton (Fig. 3A, lanes 2-7). Cofilin was detectable in cytoskeletal fractions as early as 15 minutes after T cell stimulation. The maximum accumulation of cofilin was obtained after 1-2 hours. After 4 hours the amounts of cofilin translocated to the actin cytoskeleton gradually decreased and only minor amounts could be detected 6-8 hours after CD2 stimulation.

The kinetics of the CD2 induced dephosphorylation of cofilin in NEPHGE-Western blot analysis (Fig. 3B) showed an analogous time course as its interaction with the actin

cytoskeleton (Fig. 3A). In line with previous results (Samstag et al., 1991), cytoplasmic cofilin was dephosphorylated within 15-30 minutes following CD2 receptor stimulation. Maximal cofilin dephosphorylation occured 30 minutes to 2 hours after anti CD2 treatment. Subsequently, the amounts of dephosphorylated cofilin gradually decreased again (Fig. 3B lanes 2-7).



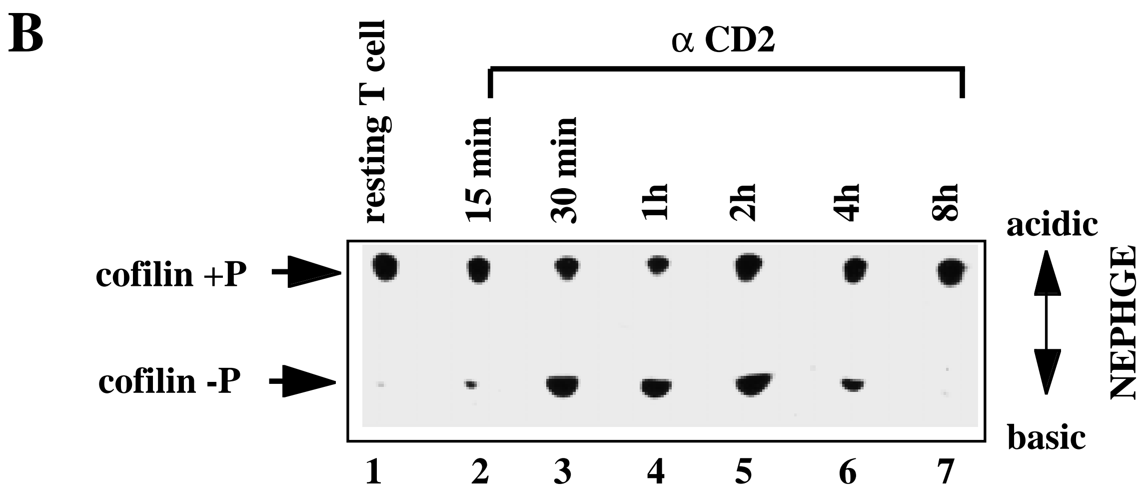


Figure 3

T cell activation induces dephosphorylation of cofilin and its association with the actin cytoskeleton in vivo.
(A) Time course of the association of cofilin with the actin cytoskeleton following CD2 stimulation of freshly isolated human peripheral blood T cells (PBTs) for 15 min to 8 hours. The amounts of cofilin and actin in actin cytoskeletal fractions were determined by SDS-PAGE/Western blot analysis. Unstimulated PBT cells (lane 1), CD2 stimulated PBT cells (lanes 2-7). Data are representative for four independent experiments.
(B) Time course of the dephosphorylation of cofilin following CD2 stimulation for 15 min to 8 hours. The phosphorylation state of cytoplasmic cofilin was examined by NEPHGE-Western blot analysis. The upper band corresponds to the phosphorylated form of cofilin (cofilin+P), the lower band represents the unphophorylated form (cofilin-P). Unstimulated PBT cells (lane 1), CD2 stimulated PBT cells (lanes 2-7).

3.2 In activated T lymphocytes the dephosphorylated form of cofilin associates with the actin cytoskeleton

Both kinetics described above suggest that cofilin dephosphorylation represents a critical step in the activation induced association of cofilin with the actin cytoskeleton. To test this hypothesis, the phosphorylation state of cofilin in cytoskeletal actin fractions of CD2 stimulated PBT cells was analyzed by NEPHGE-Western blot analysis. As shown in Fig. 4 (lanes 2-4), cofilin associated with the actin cytoskeleton exclusively in its unphosphorylated basic form. These data strongly support the view, that activation induced cofilin dephosphorylation is essential for cofilin/actin cytoskeleton interactions *in vivo*.

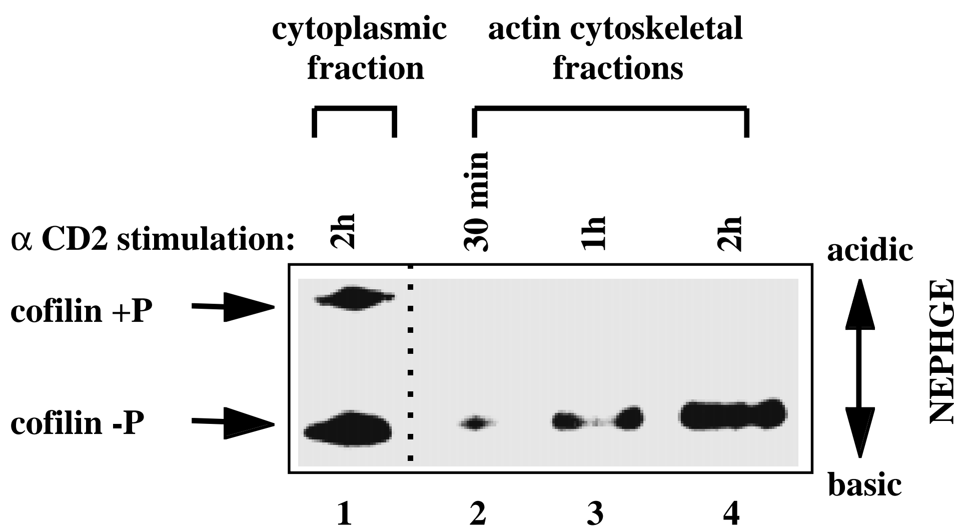


Figure 4

The dephosphorylated form of cofilin associates with the actin cytoskeleton
PBTs were stimulated via CD2 for the indicated times. The phosphorylation state of cofilin in the actin cytoskeletal fractions (lanes 2-4) was visualized by NEPHGE-Western blot analysis. Lane 1 shows, as a control, the phosphorylation state of cofilin in the Triton X-100 soluble cytoplasmic fraction of CD2 stimulated PBT cells (2 h).

3.3 Cofilin co-immunoprecipitates with cytoskeletal actin in activated T lymphocytes

To confirm that cofilin binds to cytoskeletal actin *in vivo*, an actin specific antiserum was used to precipitate actin from actin cytoskeletal fractions of resting and activated T cells, respectively. Co-precipitation of cofilin was then detected employing a biotinylated cofilin specific antiserum. Significant amounts of cofilin are present in immunoprecipitates of cytoskeletal actin from CD2 activated PBT cells (Fig. 5, bar 2), but not from unstimulated PBT cells (Fig. 5, bar 1). Interestingly, in the continuously growing malignant T lymphoma

line Jurkat in which a spontaneous dephosphorylation of cofilin occurs (Samstag et al., 1996) a strong association of cofilin with the actin cytoskeleton was found in the absence of external stimuli (Fig. 5, bar 3).

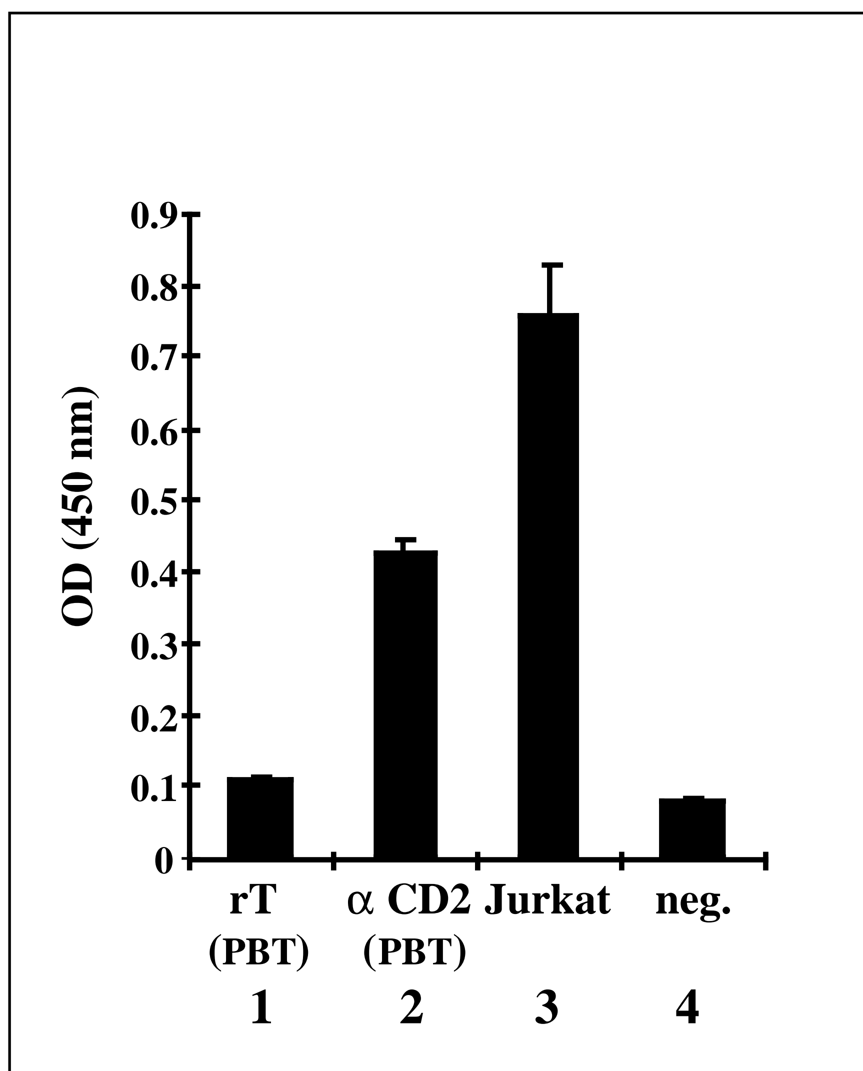


Figure 5

Cofilin co-precipitates with cytoskeletal actin in activated T cells.
Actin was immunoprecipitated from actin cytoskeletal fractions of resting PBT cells (bar 1), CD2 stimulated PBT cells (bar 2) and Jurkat cells (bar 3). Bar 4 shows a control precipitation (no actin antiserum) from CD2 stimulated PBTs. Co-precipitation of cofilin was determined in a sandwich ELISA system employing a biotinylated cofilin specific antiserum. The same amounts of protein were applied to all samples.

3.4 The PI3-kinase inhibitor wortmannin prevents dephosphorylation of cofilin and its association with the actin cytoskeleton in peripheral blood T lymphocytes but not in Jurkat T lymphoma cells

Given that PI3-kinase has been reported to influence actin cytoskeletal rearrangements following stimulation of T lymphocytes through accessory receptors such as CD2 or CD28 (Shimizu et al., 1995; Woscholski et al., 1994), it has been investigated whether PI3-kinase activity is involved in the signaling cascade from receptor stimulation to cofilin dephosphorylation and its subsequent translocation to the cytoskeleton. To this end, human peripheral blood T cells were activated by CD2 stimulation or CD3 x CD28 crosslinking, respectively, for 1 hour in the presence or absence of wortmannin, a specific and irreversible inhibitor of PI3-kinase activity (Wolscholski et al., 1994). Fig. 6 shows that, comparable to CD2 stimulation (Fig. 6A, lane 5 and Fig. 6B, lane 5), crosslinking of CD28 with the TCR/CD3 complex also results in dephosphorylation of cofilin (Fig. 6A, lane 3) and its association with the actin cytoskeleton (Fig. 6B, lane 3). Preincubation of the cells with 100 nM wortmannin for 20 minutes before cell stimulation results in an inhibition of cofilin dephosphorylation under both conditions (Fig. 6A, lanes 4 and 6). In addition, wortmannin significantly blocks the association of cofilin with the actin cytoskeleton, irrespective of the kind of receptor stimulation (CD2 or CD3 x CD28) (Fig. 6B, lanes 4 and 6).

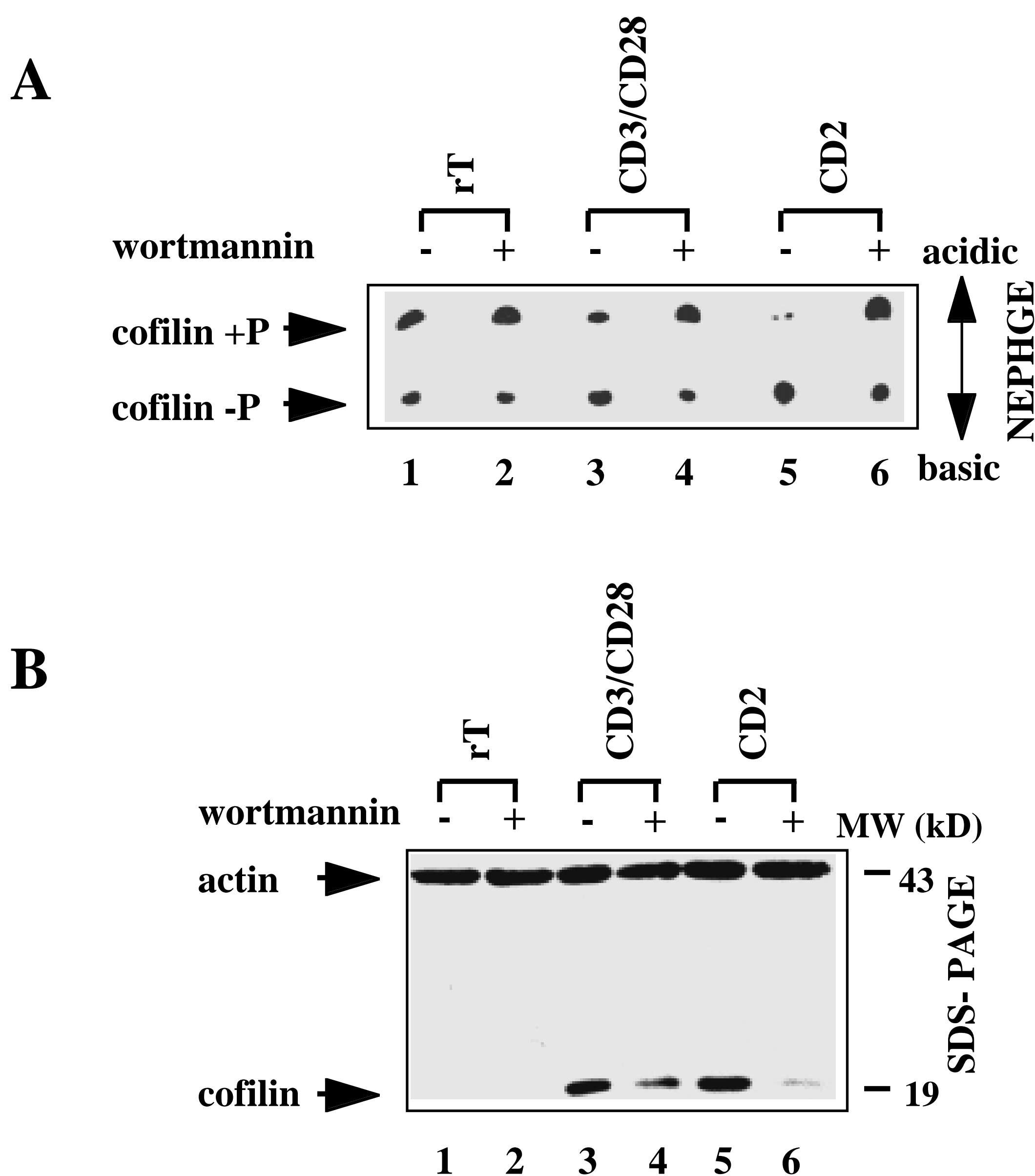


Figure 6

Dephosphorylation of cofilin and its association with the actin cytoskeleton are inhibited by wortmannin.
PBTs were activated by CD3 x CD28 cross-linking (lanes 3 and 4) or CD2 stimulation (lanes 5 and 6) for 1 hour in the presence (lanes 2, 4 and 6) or absence (lanes 1, 3 and 5) of 100 nM wortmannin. rT indicates unstimulated resting PBTs (lanes 1 and 2).
(A) The phosphorylation state of cytoplasmic cofilin was revealed by NEPHGE-Western blot analysis.
(B) The amounts of cofilin and actin in the actin cytoskeletal fractions were determined by SDS-PAGE/Western blot analysis.

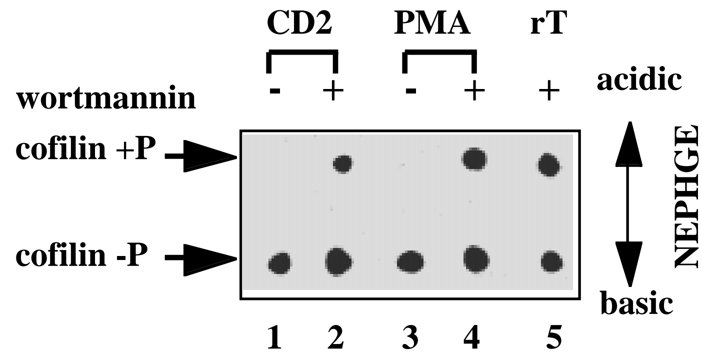
3.5 Stimulation with PMA does not influence the association of cofilin with the actin cytoskeleton

Phorbolesters, such as phorbol myristate acetate (PMA) are considered to represent a stimulus which mimicks receptor mediated costimulation of T lymphocytes (Weiss et al., 1986). As shown in Fig. 7A (lanes 3 and 4) this mode of T cell activation indeed leads to a wortmannin sensitive dephosphorylation of cofilin. However, in marked contrast to antibody initiated surface receptor stimulation (Fig. 6), the association of cofilin with the actin cytoskeleton can not be induced by stimulation with PMA (Fig. 7B, lane 3) or PMA plus the calcium ionophore (A23187) (Fig. 7C, lanes 3 and 4).

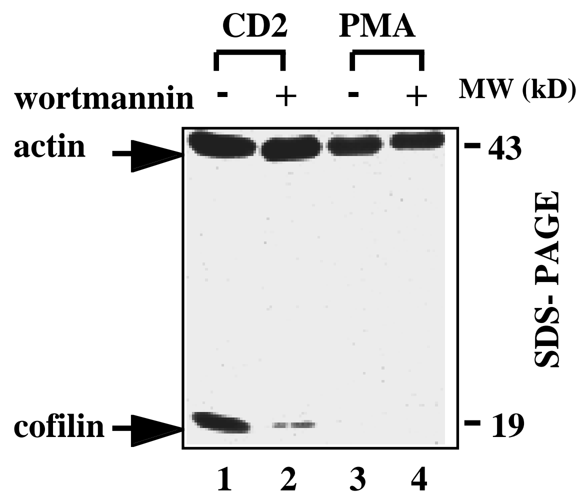
Figure 7

The association of cofilin with the actin cytoskeleton is not inducible through PMA.
(A) PBTs were activated by CD2 receptor triggering (lanes 1 and 2) or PMA (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 100 nM wortmannin. rT indicates unstimulated resting PBTs (lane 5). The phosphorylation state of cytoplasmic cofilin was analysed by NEPHGE-Western blot analysis.
(B) PBTs were activated by CD2 receptor triggering (lanes 1 and 2) or PMA (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 100 nM wortmannin. The amounts of cofilin and actin in the actin cytoskeletal fractions were determined by SDS-PAGE/Western blot analysis.
(C) PBTs were activated with 10 nM PMA (lanes 2, 4, and 5) plus different concentrations of the ionophore A23187 (lanes 4 and 5). As a positive control, cells were stimulated with PMA plus anti-CD3 (lane 3). rT indicates unstimulated resting PBTs (lane 1). The amounts of cofilin and actin in the actin cytoskeletal fractions were determined by SDS-PAGE/Western blot analysis.

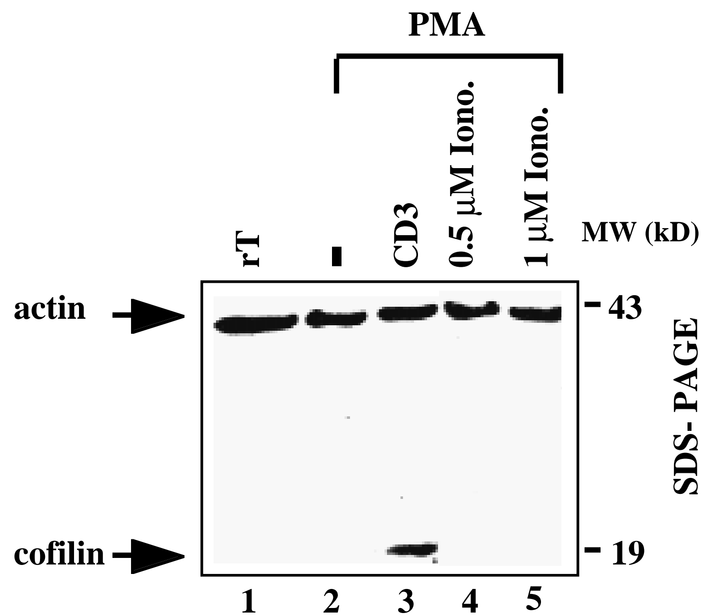
A



B



C



3.6 Wortmannin does not inhibit the constitutive association of cofilin with the actin cytoskeleton in Jurkat T lymphoma cells

In Jurkat T lymphoma cells cofilin exists mainly in its dephosphorylated form (due to permanent activity of serine phosphatases) (Samstag et al., 1996). As expected, in these transformed cells cofilin associates with F-actin in the absence of external stimuli (Fig. 8, lane 1). Interestingly, here, in contrast to the situation described in untransformed T lymphocytes, wortmannin neither inhibits the spontaneous cofilin dephosphorylation (not shown) nor its constitutive association with the actin cytoskeleton (Fig. 8, lanes 2-5). These findings imply that in Jurkat cells a transforming event downstream of PI3-kinase has occured which leads to continuous cofilin activation through dephosphorylation.

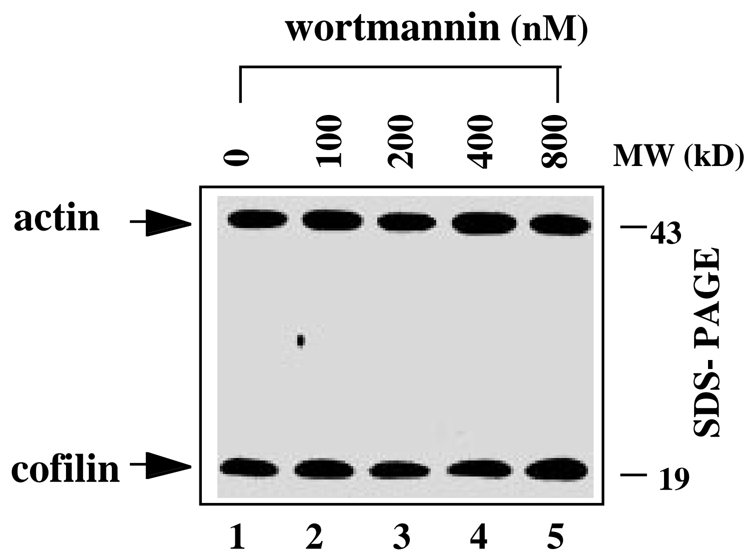


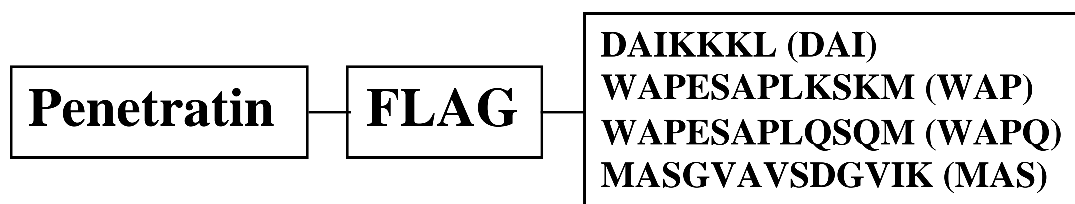
Figure 8

In Jurkat T lymphoma cells cofilin constitutively associates with the actin cytoskeleton independent of PI3-Kinase activity.
Unstimulated Jurkat T cells were treated with 100-800 nM wortmannin as indicated lanes 2-5 for 5 hours and compared to untreated cells (lane 1). The amounts of cofilin and actin in the actin cytoskeletal fractions were determined by SDS-PAGE Western blot analysis.

3.7 The activation induced association of cofilin with the actin cytoskeleton is blocked by cofilin derived peptides

With the aim of blocking the interaction of cofilin with the actin cytoskeleton *in vivo*, peptides corresponding to the residues 1-12 (MAS), 104-115 (WAP), and 122-128 (DAI) of the human cofilin sequence were synthesized. As a control, a non-actin binding control peptide (WAPQ) was designed by substitution of lysines 112 and 114 to glutamines in peptide WAP (Fig. 9A). A FLAG epitope (Hopp et al., 1988) was added to the N-terminus of each peptide to allow their detection by a monoclonal FLAG-antibody. To enable peptide entry into cells, these oligo-peptides were individually coupled to Penetratin, a 16 amino acid region of the Antennapedia homeodomain which has been shown to translocate through biological membranes in a rapid and energy-independent fashion without endosomal degradation (Perez et al., 1992; Derossi et al., 1994).

Direct binding of these synthetic peptides to cytoskeletal actin was analysed in peptide binding assays *in vitro*. To this end, actin was immunoprecipitated from actin cytoskeletal fractions of resting T cells, in which no association of the endogenous cofilin with F-actin exists. As shown in Fig. 9B, the peptides MAS and WAP, corresponding to residues 1-12 and 104-115, significantly bound to the immunoprecipitated actin, whereas the control peptide WAPQ clearly did not. The peptide DAI, corresponding to the residues 122-128, showed only low binding to isolated cytoskeletal actin. Therefore, only the peptides MAS and WAP, as well as the control peptide WAPQ, were selected for subsequent *in vivo* competition experiments.



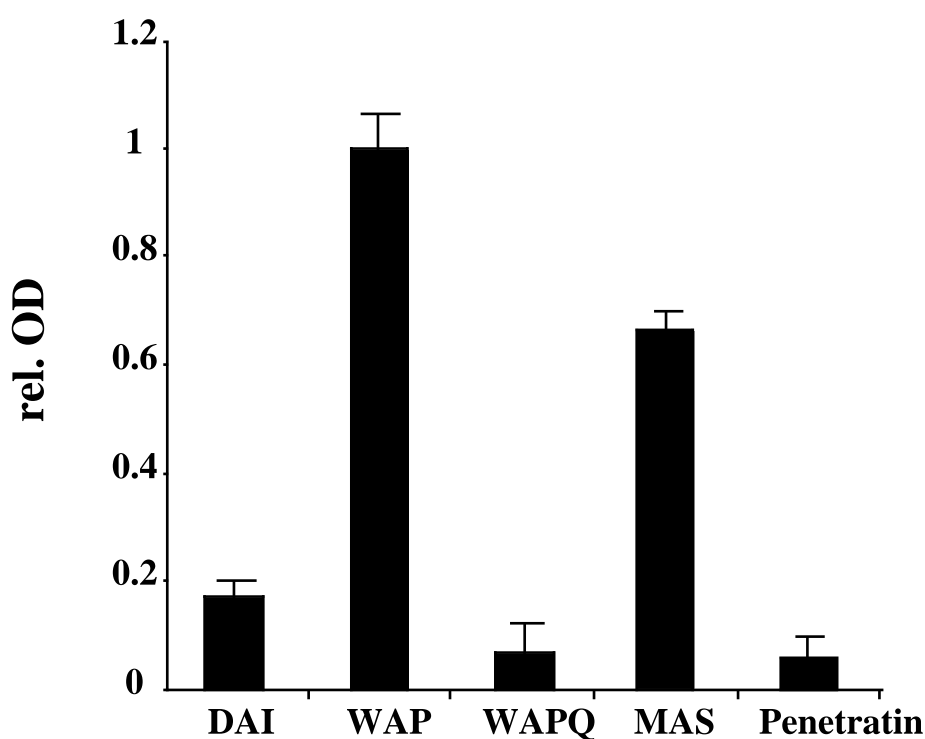


Figure 9

(A) Generation of Penetratin-cofilin peptides. The N-terminus of each peptide, corresponding to residues 1-12 (MAS), 104-115 (WAP), and 122-128 (DAI) of the human cofilin sequence was tagged by a FLAG epitope. A negative control peptide (WAPQ) was synthesized by substitution of two lysines (Lys 112 and Lys 114) to glutamines of peptide 104-115. All peptides were coupled to the peptide carrier molecule Penetratin (Appligene). The Penetratin coupled peptides were purified by HPLC and mass spectrometry.

(B) In vitro binding assays of Penetratin coupled peptides to cytoskeletal actin.
Actin was immunoprecipitated from isolated actin cytoskeletal fractions of resting PBT cells. Peptides which bound to cytoskeletal actin were detected by a FLAG specific mAb in an ELISA system.

3.8 Penetratin coupled peptides efficiently internalize into living cells

To confirm that Penetratin coupled peptides are able to enter living primary T lymphocytes, PBTs were incubated with the different peptides for 2 hours at 37¡C. Then, uptake of the peptides was determined by intracellular fluorescence staining employing a FLAG-specific mAb and subsequent flow cytometric analysis. Fig. 10 shows that all penetratin coupled peptides (MAS, WAP, WAPQ) indeed efficiently entered T lymphocytes. Importantly, all tested peptides, including the negative control peptide (WAPQ) were taken up with comparable efficiency. No negative effects on cellular viability were exerted by any of these peptides.

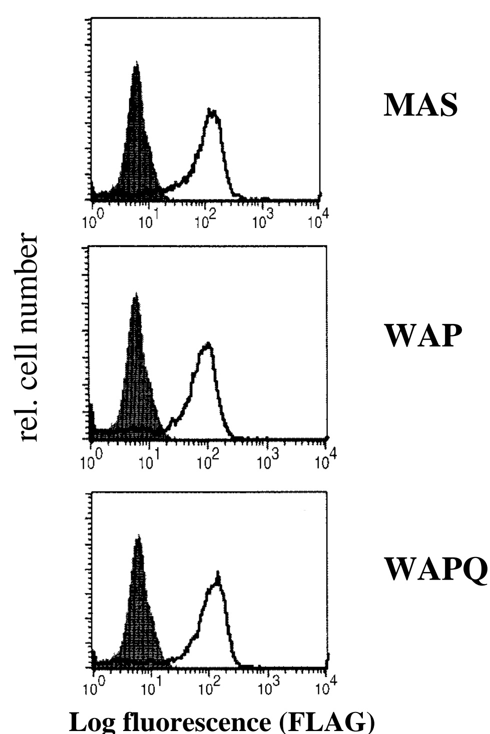


Figure 10

Internalization of penetratin coupled peptides into living PBT cells.
PBTs were incubated with the Penetratin coupled peptides for 2 hours at 37¡C, and the uptake of these peptides was determined by intracellular flow cytometric analysis employing a FLAG specific mAb. The fluorescence intensities of cells without peptide treatment as a negative control (black area) and of the peptide treated cells (white area) are shown as an overlay.

3.9 The activation induced association of cofilin with the actin cytoskeleton *in vivo* is blocked by cell permeable cofilin derived peptides

To investigate whether the cofilin peptides can block the association of cofilin with the actin cytoskeleton *in vivo*, freshly isolated human peripheral blood T cells (PBT) were preincubated with the Penetratin coupled peptides MAS and WAP (Fig. 11A, lanes 2-4), the control peptide WAPQ (Fig. 11A, lanes 5-7), or without peptides (Fig. 11A, lane 1), respectively for 2 hours. Subsequently, the T lymphocytes were stimulated via CD2 receptors in order to induce the association of cofilin with the actin cytoskeleton. The amounts of actin and cofilin in the actin cytoskeletal fractions were determined by Western blot analysis. As shown in Fig. 11A and 11B, this activation induced interaction was indeed blocked in a dose dependent manner by preincubation of cells with MAS and WAP peptides. The combination of the MAS and WAP peptides acts synergistically in inhibiting the association of cofilin with the actin cytoskeleton *in vivo*, as determined by densitometric quantification of cofilin band intensities (Fig. 11B). In contrast, presence of the control peptide WAPQ did not alter the amounts of cofilin in the actin cytoskeletal fractions of CD2-activated T cells (Fig. 11A lanes 5-7, and Fig. 11B). This synergistic inhibitory effect of the cofilin peptides MAS and WAP *in vivo* indicates that the residues 1-12 and 104-115 of human cofilin function cooperatively in the interaction between cofilin and F-actin in activated T lymphocytes.

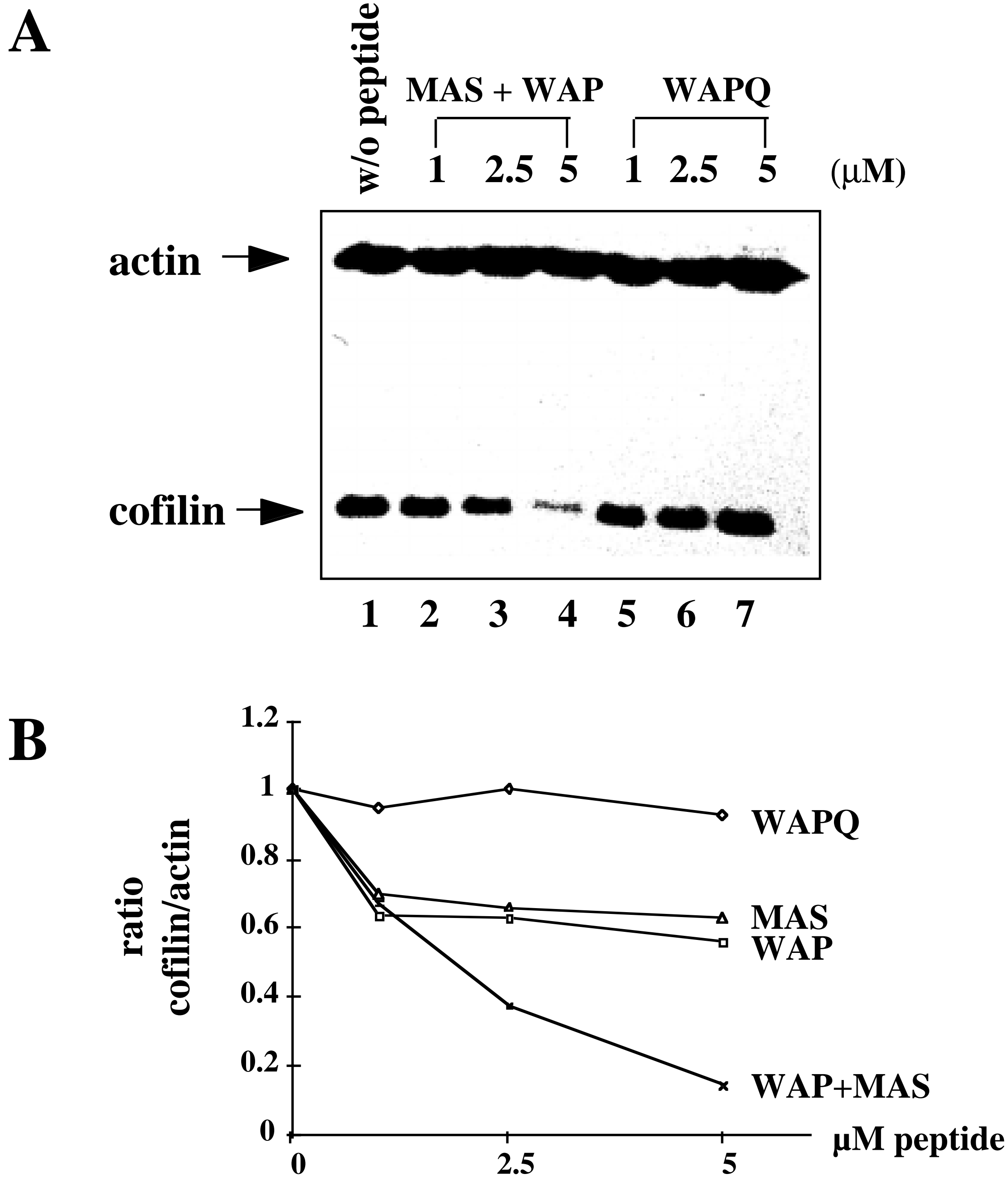


Figure 11

The interaction of cofilin with the actin cytoskeleton is blocked by MAS and WAP peptides in vivo.

PBT cells were preincubated with peptides MAS, WAP, WAPQ, the combination of MAS and WAP peptides or without peptide, respectively. Subsequently, PBT cells were stimulated via CD2 for 2 hours. The amounts of actin and cofilin in the actin cytoskeletal fractions were determined by Western blot analysis (A). In (B), a densitometric quantification of such Western blots is shown (expressed as the ratio of cofilin to actin band intensities).

3.10 Cofilin is involved in the process of the receptor cap formation

The next series of experiments was aimed at investigating the functional consequences of the interaction of cofilin with the actin cytoskeleton during T cell activation processes.

The clustering of T cell surface receptors upon receptor cross-linking (capping) plays an important role in the recruitment of intracellular signaling molecules to membrane receptors and the subsequent induction of cell proliferation (Fisher et al., 1998; Holsiger et al., 1998; Snapper et al., 1998). Receptor cap formation of T lymphocytes is known to depend on a functional actin cytoskeleton (De Petis, 1974).

To investigate the potential functional roles of cofilin in regulating the T cell actin cytoskeleton, it was analyzed whether cofilin participates in the process of receptor cap formation in human peripheral blood T lymphocytes. Following receptor cap formation by mAb mediated cross-linking of CD2 receptors in human PBTs, a co-localization of cofilin with CD2 caps is observed (>90% of the capped cells) (Fig. 12A). Employing an actin specific antiserum co-accumulation of actin at the CD2-caps was detected as well (Fig. 12B). Note that here F-actin can not be visualized by staining with phalloidin, since binding of phalloidin to F-actin is blocked by cofilin once it has bound to actin (Carlier and Pantaloni, 1997; Moon et al.,1993).

Many studies have shown that PI3-kinase and the Rho family of small GTPases, including Rho, Rac and Cdc 42, regulate the reorganization of the actin cytoskeleton. Therefore, the potential co-localization of these proteins with CD2 receptor caps was also examined. As shown in Fig. 12C, the p85 subunit of PI3-kinase is detectable in CD2 caps. Moreover, one of the major PI3-kinase substrates, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), also co-localizes in CD2 caps (Fig. 12D). In contrast, no co-localization of the Rho GTPases Rac1 (Fig. 12E), RhoA or Cdc42 (data not shown) was found.

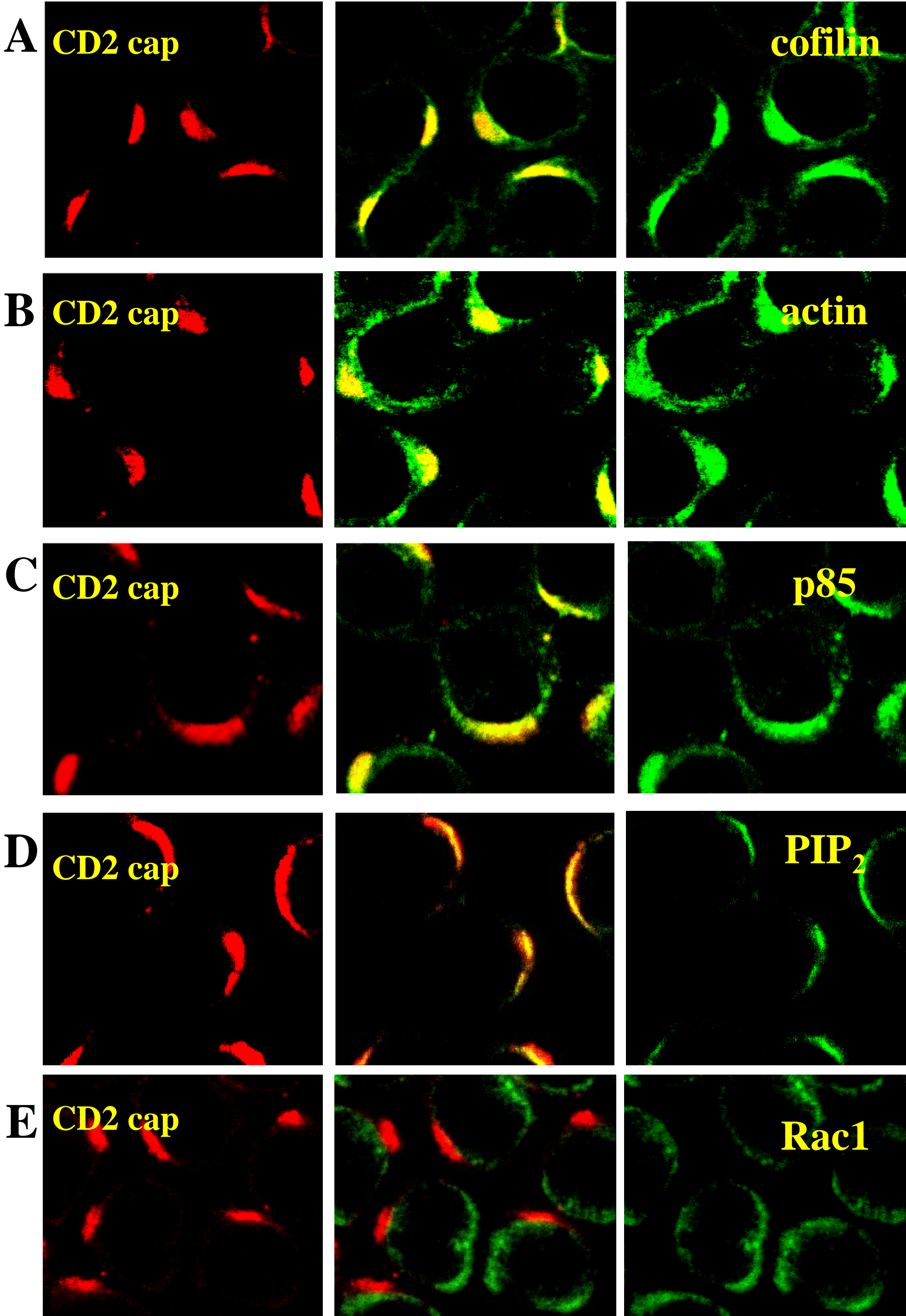

A
CD2 cap
cofilin
B
CD2 cap
actin
C
CD2 cap
p85
D
CD2 cap
PIP2
E
CD2 cap
Rac1

Figure 12

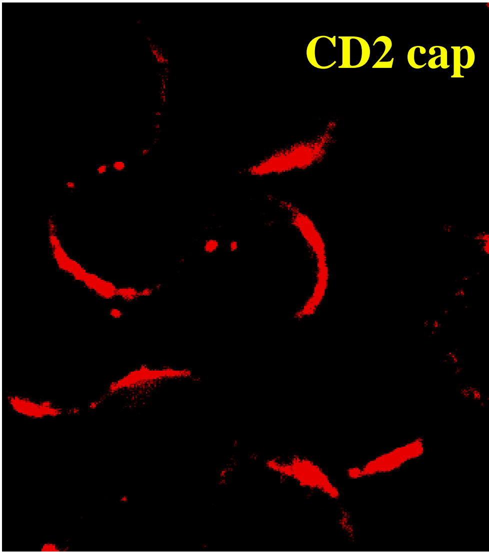
Cofilin, actin, PI3-kinase and PtdIns(4,5)P_2 (PIP_2), but not Rac1, co-localize with CD2 receptor caps.
Receptor caps were induced in PBTs by incubation for 45 min with CD2 antibodies which were cross-linked by Texas-red labeled secondary antibodies. Cofilin (A), actin (B), the p85 subunit of PI3-kinase (C), PtdIns(4,5)P_2 (PIP_2) (D) and Rac1 (E) were visualized by indirect immunofluorescence staining employing Cy-2 labeled secondary antibodies. CD2 caps (red, left panels) and the counterstained proteins (green, right panels) were analyzed with a confocal laser scanning microscope. In the digital overlays of red and green fluorescence (middle panels) co-localizing proteins appear yellow.

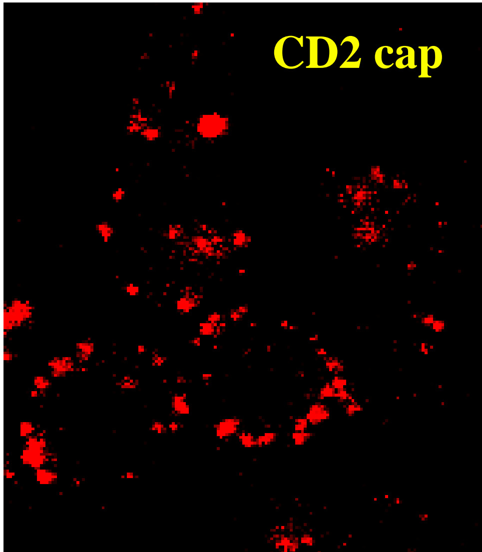
Since cofilin strongly co-localizes with CD2 receptor caps, the next experiments were aimed at investigating whether cofilin derived peptides could influence the formation of CD2 receptor caps. To this end, purified human resting T lymphocytes were preincubated with cofilin derived peptides MAS and WAP (Fig. 13B) or control peptide WAPQ (Fig. 13A), respectively. Then caps were formed by cross-linkage of CD2 receptors. In the presence of the control peptide WAPQ, CD2 receptor caps are induced and cofilin clearly co-localizes within these receptor caps (Fig. 13A). In contrast, pretreatment with the cofilin derived peptides MAS and WAP completely prevented CD2 receptor cap formation and co-accumulation of cofilin (Fig. 13B). Thus, these data provide evidence that the interaction of cofilin with the actin cytoskeleton is a critical event during the process of receptor cap formation.

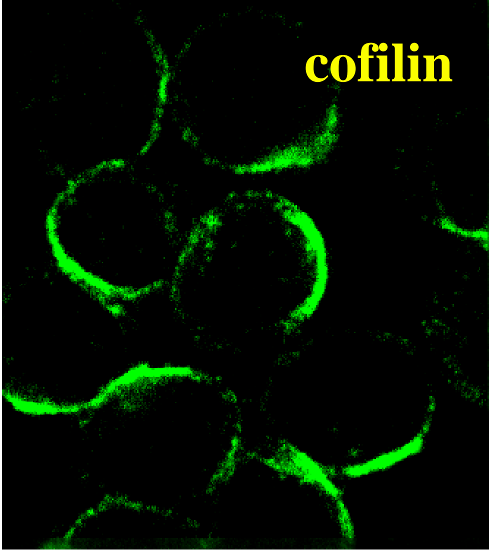
Figure 13

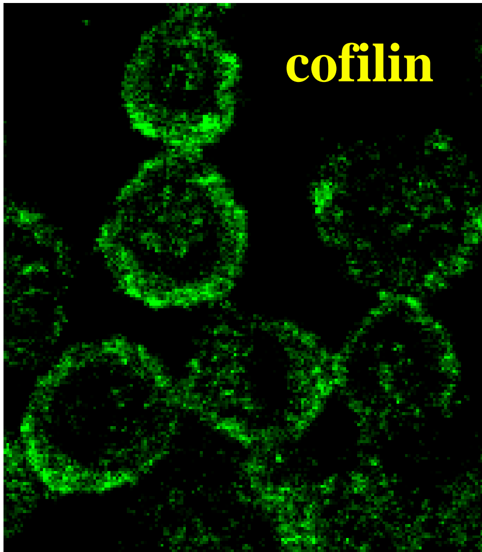
CD2 receptor cap formation is prevented by cofilin derived peptides.
PBT cells were preincubated with the combination of MAS and WAP peptides (each 2.5 µM) (B) or WAPQ (5 µM) (A), respectively. Subsequently, CD2 receptor caps were induced by incubation with CD2 mAbs which were cross-linked by Cy-3 labeled secondary antibodies. Cells were fixed and permeabilized, and cofilin was counterstained, employing Cy-2 labeled secondary antibodies. The CD2 caps (red, upper panels) and cofilin (green, middle panels) were visualized by indirect immunofluorescence staining and analyzed with a confocal laser scanning microscope. In the digital overlays of red and green fluorescence (lower panels) co-localizing proteins appears yellow.

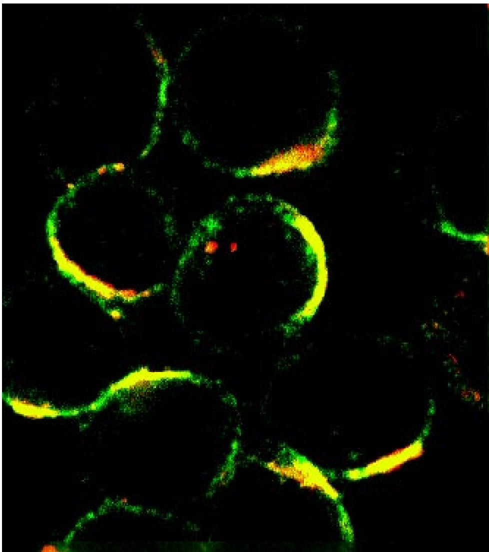
A: WAPQ **B: MAS+WAP**

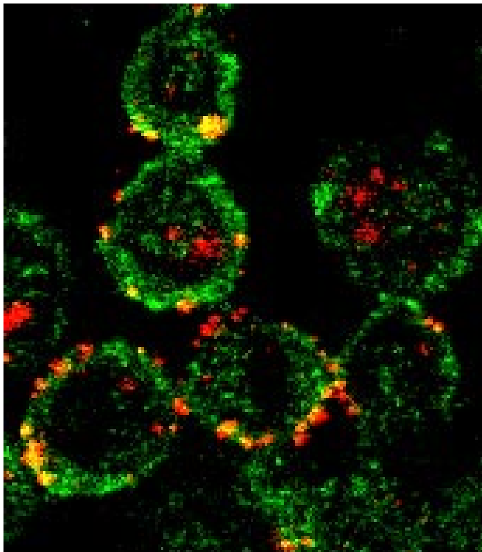












3.11 The interaction of cofilin with the actin cytoskeleton is required for T cell activation

To investigate which functional T cell responses are dependent on the interaction between F-actin and cofilin, the effects of the cofilin derived peptides on cytokine production, expression of T cell surface activation markers and T cell proliferation were analyzed.
In a first set of experiments, proliferation of the human peripheral blood T lymphocytes was examined. To this end, PBT cells were preincubated with the cofilin derived peptides MAS and WAP, the control peptide WAPQ, or without peptides, respectively. Subsequently, cells were stimulated via CD2 receptors (Fig. 14A) or, alternatively, by the combination of PMA plus calcium ionophore (A23187) (Fig. 14B), which bypasses proximal receptor mediated signaling events leading to direct activation of protein kinase C (PKC) and an increase of the intracellular free calcium concentration. In both modes of stimulation a strong proliferative response was observed. However, as shown in Fig. 14A, the CD2 induced PBT cell proliferation was significantly inhibited by preincubation with the peptides MAS and WAP. In contrast, upon stimulation of T lymphocytes with PMA plus ionophore, the cofilin derived pepetides MAS and WAP failed to inhibit cell proliferation (Fig. 14B). The control peptide WAPQ had no effect on T cell proliferation. These findings suggest that the cofilin/F-actin interaction is essential for early receptor mediated activation steps that are located upstream of PKC activation and Ca^{2+} mobilization.

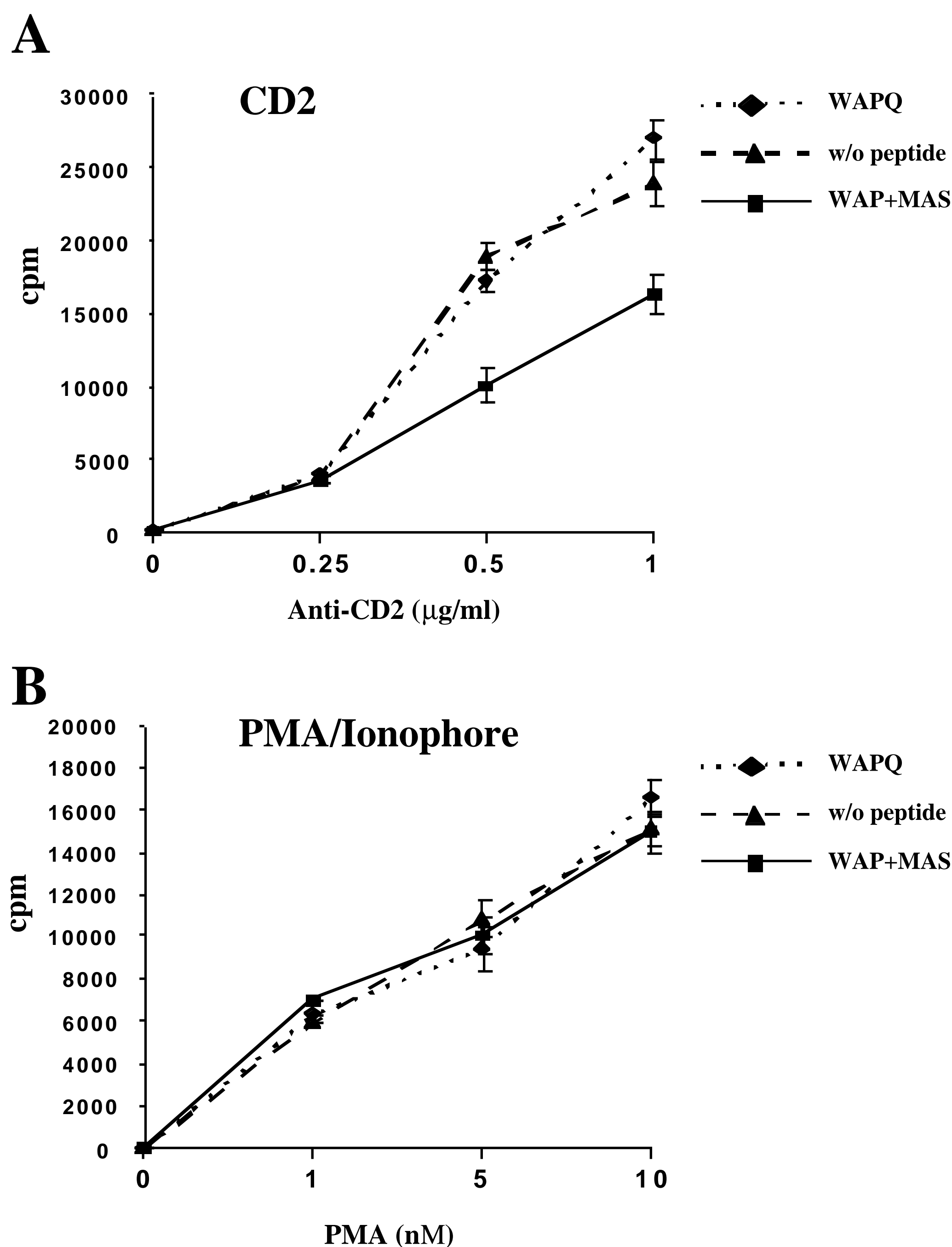


Figure 14

***CD2-induced T cell proliferation is inhibited by cofilin derived peptides*.**
Proliferative responses of resting PBT cells, preincubated with the combination of MAS and WAP peptides (2.5 µM each ■), WAPQ (5 µM, ◆), or without peptide treatment (▲), respectively. (A) PBT cells were stimulated with various concentrations of anti-CD2. (B) Alternatively, T cells were stimulated with various concentration of PMA plus ionophore (A23187). All cultures were maintained for 48 hours adding ^{3}H-thymidine during the last 16 hours of culture. The experiments were repeated at least 3 times providing comparable results.

To further explore the role of cofilin in T cell activation processes, the effects of cofilin derived peptides on cytokine production was analyzed. Following CD2 stimulation of resting human T lymphocytes, the amounts of the T cell growth factor Interleukin-2 (IL-2) as well as the proinflammatory cytokine γIFN in the T cell culture supernatants were determined by ELISA.

As shown in Fig. 15A, preincubation with the WAP and MAS peptides strongly inhibited the CD2 inducible IL-2 production. In marked contrast, the CD2 induced production of γIFN was dramatically enhanced by preincubation with the peptides WAP and MAS (Fig. 15B). The control peptide WAPQ was ineffective in both cases. These results indicate that the interaction of cofilin with F-actin is differentically involved in the production of cytokines.

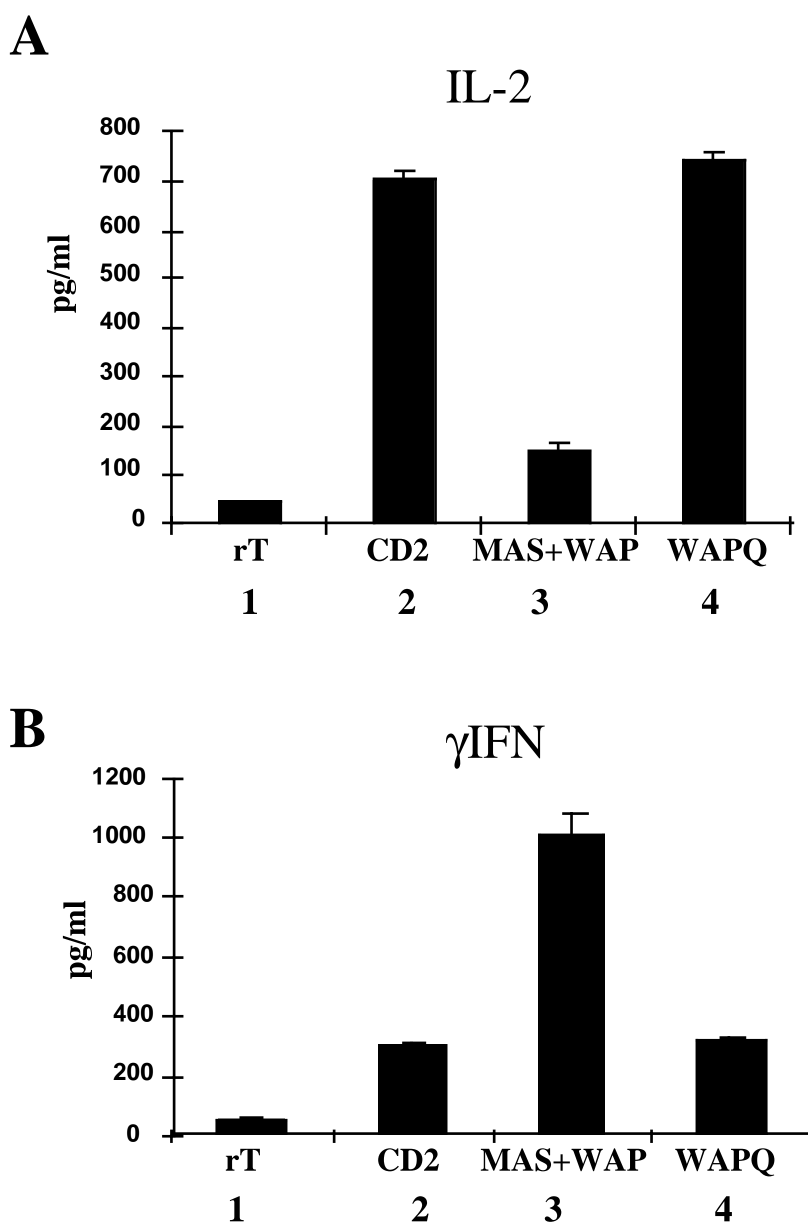


Figure 15

IL-2 production is inhibited by cofilin derived peptides, whereas γIFN production is enhanced. PBT cells were preincubated with the combination of MAS and WAP peptides (2.5 µM each) (A and B, bars 3) or WAPQ (5 µM) (A and B, bars 4), subsequently, T cells were activated via CD2 receptor triggering. Bar 2 in A and B shows the CD2 activated cells without peptide treatment. rT indicates unstimulated resting PBTs (A and B, bars 1). The production of IL-2 (A) and γIFN (B) was determined in cell culture supernatants harvested after 48 h of T cell culture. A representative experiment of three independent experiments is shown.

Finally, the effects of cofilin derived peptides on the expression levels of different activation markers on the surface of activated T lymphocytes was investigated. To this end, human resting T lymphocytes were preincubated with the cofilin derived peptides MAS plus WAP, the control peptide WAPQ, or without peptides. Subsequently, PBT cells were activated via CD2 receptors and the expression of different activation markers on the surface of activated T cells was measured by flow cytometric analysis. Interestingly, different influences of the cofilin derived peptides on the CD2-induced expression of various activation markers were observed. Notably, the upregulation of CD69, an early T cell activation marker, was almost completely inhibited by preincubating the cells with cofilin derived peptides (WAP+MAS) (Fig. 16A). In marked contrast, a strong increase in the surface expression of MHC class II molecules in the presence of WAP and MAS peptides was observed (Fig. 16B). This might be a secondary effect due to the observed increase in γIFN production, which has been described to induce upregulation of MHC expression. The CD2 induced upregulation of CD25 expression (IL-2 receptor α chain) was not significantly altered by preincubation with cofilin derived peptides (Fig. 16C). In all cases, the control peptide WAPQ did not show any effect.

Taken together, the WAP and MAS peptides exert strong inhibitory effects on T cell proliferation, IL-2 production and the expression of CD69, whereas the production of γIFN and the expression of MHC class II molecules are significantly enhanced.

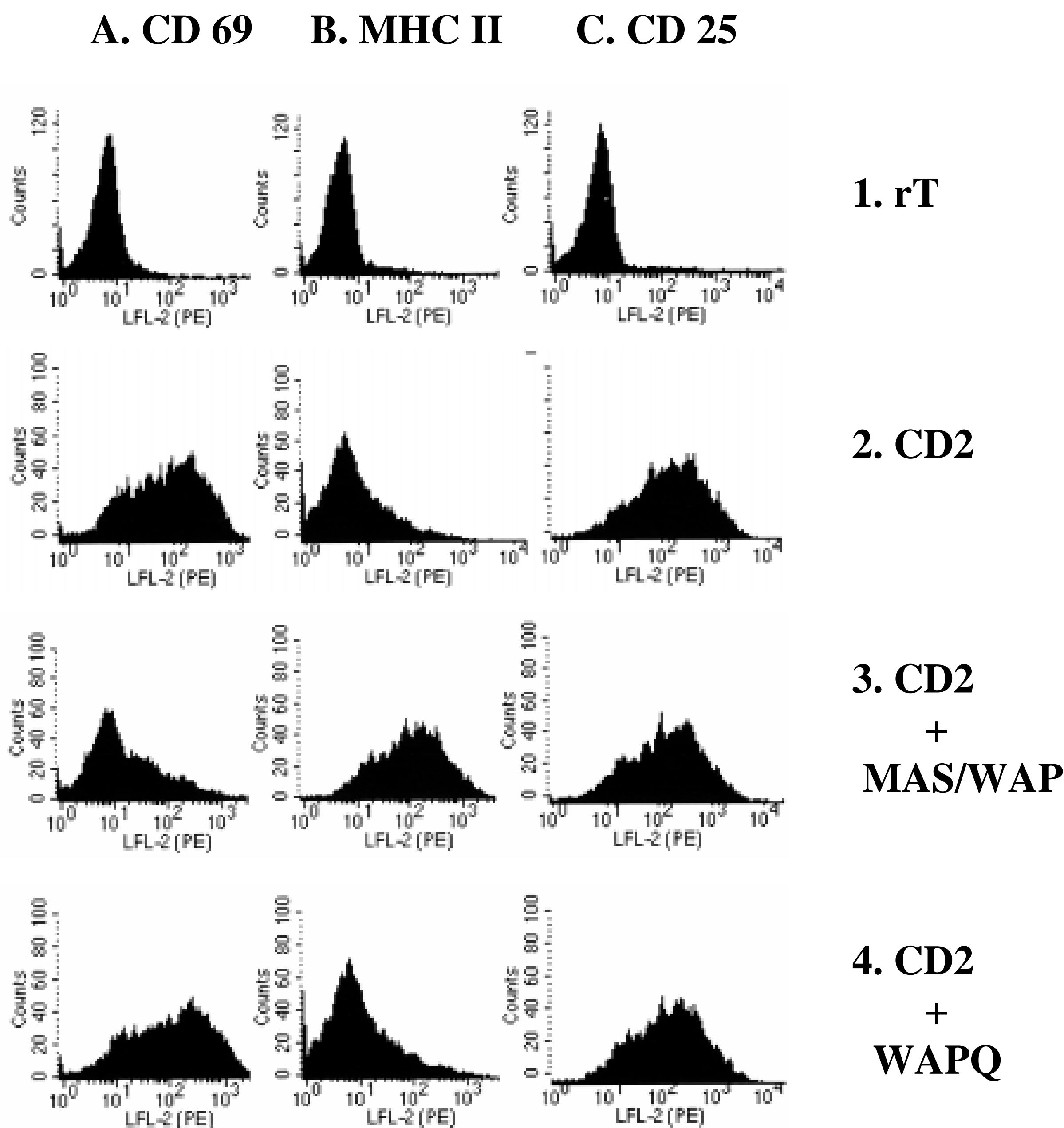


Figure 16

Diverse effects of the cofilin derived peptides on the expression of different activation markers. PBT cells were preincubated with the combination of MAS and WAP peptides (2.5 µM each 3), WAPQ (5 µM. 4) or without peptide (2), respectively. Subsequently, PBT cells were activated by CD2 receptor triggering (2, 3 and 4). Unstimulated resting PBTs (rT) are shown in (1). Expression of CD69 (A) was monitored after 16 hours, MHC II (HLA-DR) (B) and CD25 (C) after 48 hours of culture.

3.12 The cofilin derived peptides inhibit CD2-mediated activation induced cell death (ACID)

As shown above, the interaction of cofilin with the actin cytoskeleton plays an important role for the activation of primary T lymphocytes. In previously activated T cells or hybridoma cell lines stimulation induces apoptosis, a process which has been termed activation induced cell death (AICD) (Kabelitz et al., 1993). To address the question whether cofilin also affects AICD, the influence of cofilin derived peptides on CD2-induced apoptosis of the IL-2 dependent human T cell clone D798.18 cells was investigated (Wesselborg et al., 1993). Following preincubation with cofilin derived peptides MAS and WAP, control peptide WAPQ or without peptide treatment, D798.18 cells were stimulated with three mitogenic CD2 mAbs (M1, M2 and 3PT) directed against different CD2 epitopes. After 6 hours of incubation, apoptosis was determined by two different experimental methods: (1) by Annexin V and PI double staining (Fig. 17A) or, alternatively, (2) by terminal transferase (TdT)-mediated nick end labeling (TUNEL) staining (Fig. 17B).

Fig. 17 demonstrates that CD2 stimulation efficiently induced apoptosis in this T cell clone. This induction of apoptosis by anti-CD2 mAbs was significantly reduced by pre-incubation with MAS and WAP peptides (Fig 17A-3, 17B-3). As expected, no effect was observed by a treatment with the control peptide WAPQ (Fig. 17A-4, 17B-4). These data clearly demonstrate that the cofilin derived peptides MAS and WAP prevent activation induced programmed cell death in D798.18 cells.

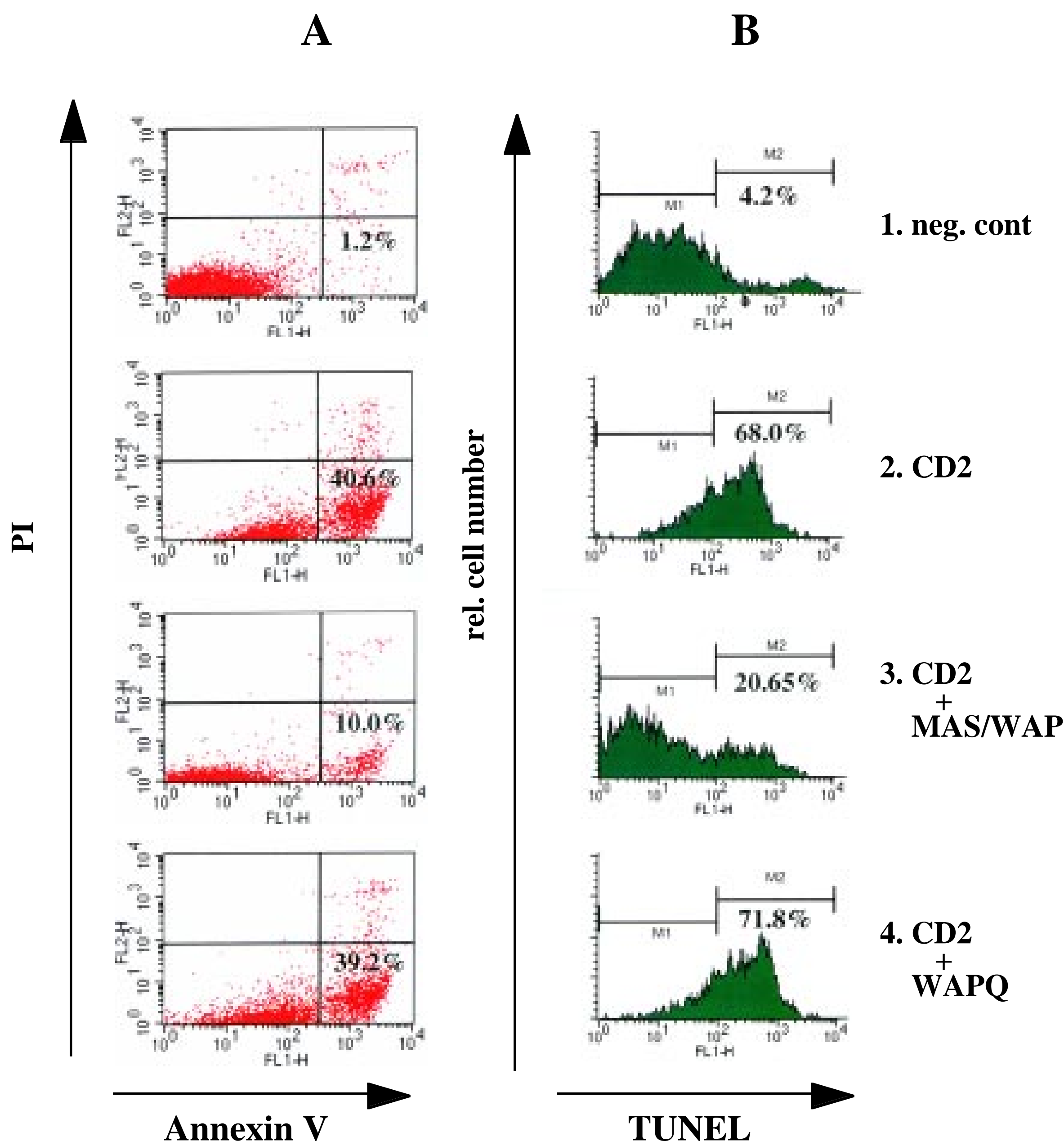


Figure 17

CD-mediated apoptosis (ACID) is inhibited by cofilin derived peptides.
The T cell clone D798.18 was preincubated for 2 hours with the combination of MAS and WAP peptides (2.5 µM each) (3), WAPQ (5 µM) (4) or without peptide (1 and 2), respectively. Subsequently, cells were activated for 8 hours with anti-CD2 mAb. (1) shows, as a negative control the unstimulated T cell clone. (A) Uptake of PI (Y-axis) and staining with annexin V-FITC (X-axis) of D798.18 cells cultured for 6 hours. (B) DNA fragmentation was revealed Tdt-mediated nick end labeling (TUNEL assay) (incorporation of FITC labled dUTP) and intracellular flow cytometric analysis.

3.13 Cofilin derived peptides do not influence CD95-induced apoptosis

D798.18 cells express CD95 (Fas) receptors and are sensitive to the CD95 mediated-apoptotic signal when directly triggered via CD95 receptors. To determine whether the cofilin derived peptides MAS and WAP also affect CD95 mediated programmed cell death, apoptosis the D798.18 T cell clone was triggered with anti-CD95 mAb in the presence of MAS and WAP peptides, WAPQ peptide or in the absence of peptides. Anti-CD95 induced cell death (apoptosis) was detected by Annexin V and TUNEL assays.

Interestingly, as shown in Fig.18 both apoptosis assays demonstrated that in marked contrast to the CD2-induced ACID, the cofilin derived pepetides MAS and WAP did not affect apoptosis in response to CD95 triggering. This result provides evidence for the involvement of cofilin in the CD2-mediated signaling pathway of activation induced apoptosis, but not on CD95 (Fas)-mediated signaling.

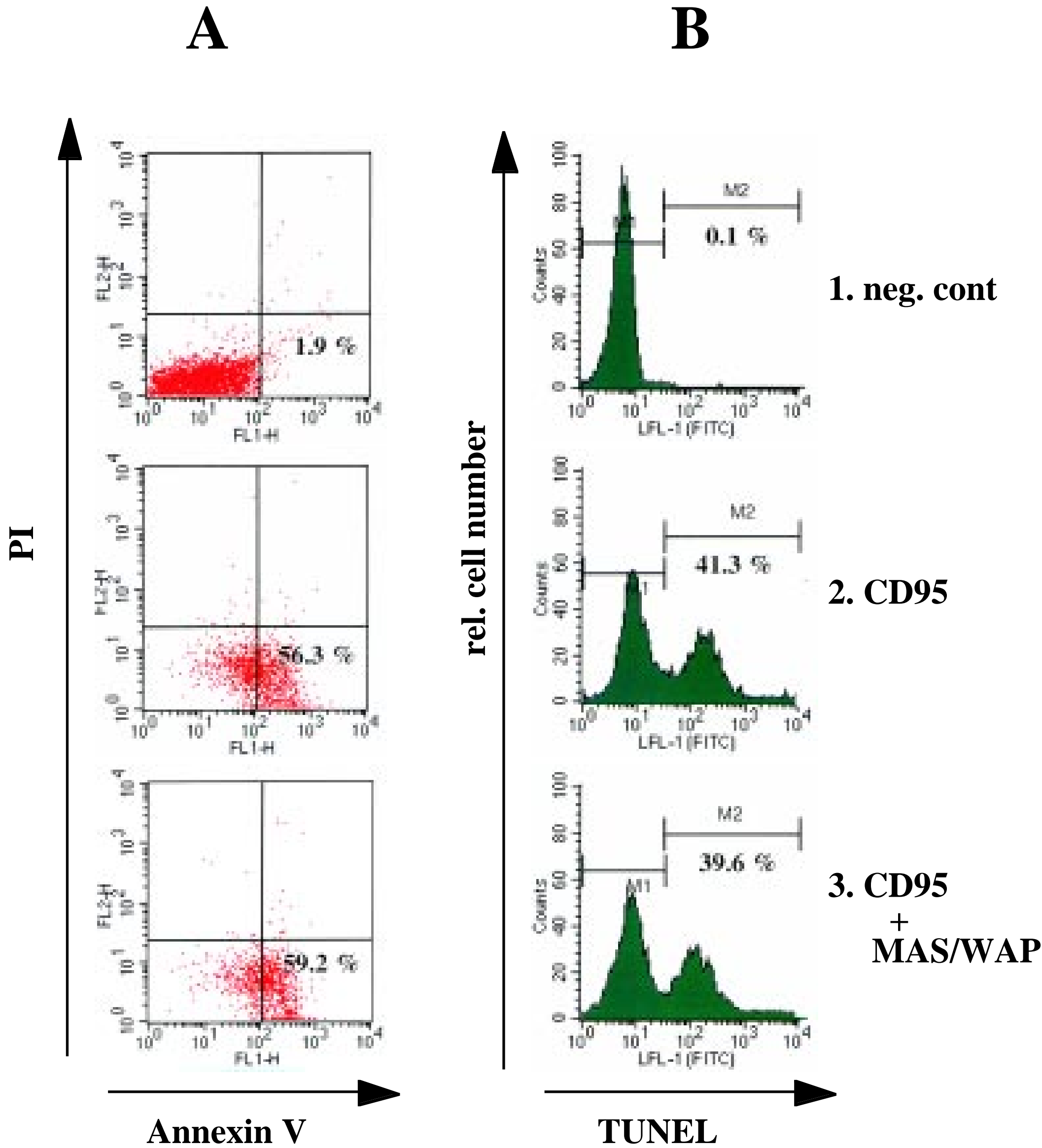


Figure 18

CD95 induced apoptosis is not inhibited by cofilin derived peptides.

The T cell clone D798.18 was preincubated for 2 hours with the combination of MAS and WAP peptides (2.5 µM each) (3) or without peptide (1 and 2), respectively. Subsequently, cells were activated by CD95 receptor triggering. (1) Unstimulated T cells. (A) Uptake of PI (Y-axis) and staining with annexin V-FITC (X-axis) of D798.18 cells cultured for 5 hours. (B) TUNEL assay of fixed cells after 6 hours of stimulation with CD95 mAb. DNA fragmentation was revealed by Tdt mediated nick end labeling (incorporation of FITC labeled dUTP) and subsequent intracellular flow cytometric analysis.

3.14 Apoptosis induced via CD2 is inhibited by soluble anti-CD95 mAb

Fas mediated cell death has been suggested to be the major pathway of activation induced apoptosis of T cell hybridomas (Brunner et al., 1995; Dhein et al., 1995; Ju-S et al., 1995; Yang et al., 1995).

To determine whether CD2 induced apoptosis in the D798.18 T cell clone is dependent on CD95/CD95 ligand (Fas/FasL) interaction, cells were stimulated via CD2 in the presence or absence of a blocking anti-CD95 mAb (soluble anti-CD95 mAb F(ab) fragments, which prevent the interaction between CD95 and its ligand without inducing apoptotic signals). Apoptotic cells were determined by the Annexin V assays. Fig. 19 shows the results of a representative experiment, demonstrating that the treatment with the blocking anti-CD95 mAb inhibited CD2 induced apoptosis in a dose dependent manner. This result demonstrates that the CD95/CD95 ligand (Fas/FasL) system is involved in CD2 induced apoptosis in the D798.18 T cell clone.

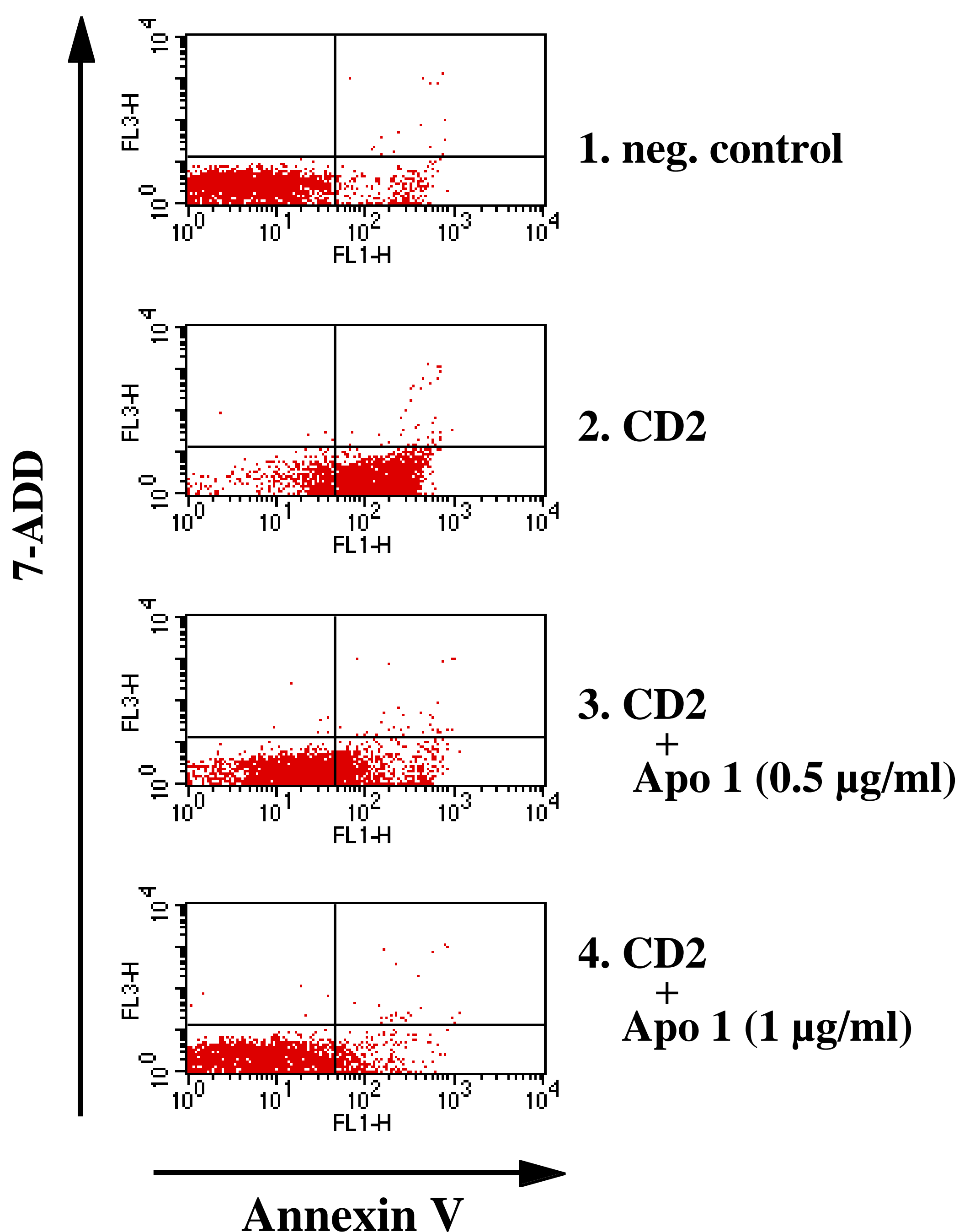


Figure 19

Inhibiton of activation induced apoptosis in anti-CD2 stimulated D798.18 cells by soluble anti-CD95 mAb

Anti-CD2 stimulated D798.18 cells were cultured in the presence of F(ab) fragments of CD95 mAb in different concentrations (3, 4), or in the absence of anti-CD95 (1 and 2). The unstimulated T cell clone is shown in (1). Apoptosis was evaluated by flow cytometric analysis of Annexin V-FITC (X-axis)/7-AAD (Y-axis) stained cells after 6 hours of culture.

3.15 Cofilin derived peptides inhibit the expression of CD95 ligand

As described above, CD95 signals are involved in CD2-induced apoptosis of D798.18 cells, therefore, the ability of cofilin derived peptides to affect the expression of CD95 ligand during the CD2-induced apoptotic process was examined. The CD95 ligand is a 40 kD type II membrane protein. Human CD95 ligand is released as a 26 kD protein from the T cell membrane by metalloproteinases (Kayagaki et al., 1995; Oyaizu et al., 1997).
D798.18 cells were treated with CD2 mAbs in the presence of cofilin derived peptides (MAS+WAP), the control peptide (WAPQ) or in the absence of peptide. The expression of CD95 ligand on the cell surface after 6 hours of culture was determined by flow cytometric analysis. Fig. 20A (2) demonstrates that CD2 stimulation induces surface expression of CD95 ligand on D798.18 cells. Detection of CD95 ligand expression following CD2 stimulation could be improved by preincubation of D798.18 cells with an inhibitor of matrix metalloproteinases, KB 8301, which blocks the cleavage of membrane bound forms of human CD95 ligand (Fig. 20B (2)). The expression of CD95 ligand was strongly inhibited by MAS and WAP peptides, both in the presence (Fig. 20B, (3)) and absence (Fig. 20A, (3)) of the matrix metalloproteinases inhibitor KB 8301. The control peptide WAPQ showed no effect on CD95 ligand expression. These results indicate that cofilin is involved in the signaling pathway downstream of CD2 receptor stimulation leading to CD95L expression but upstream of the CD95/CD95L interaction during activation induced apoptosis. Moreover, the cofilin derived peptides likely have no influence on cleavage of human membrane bound CD95 ligand.

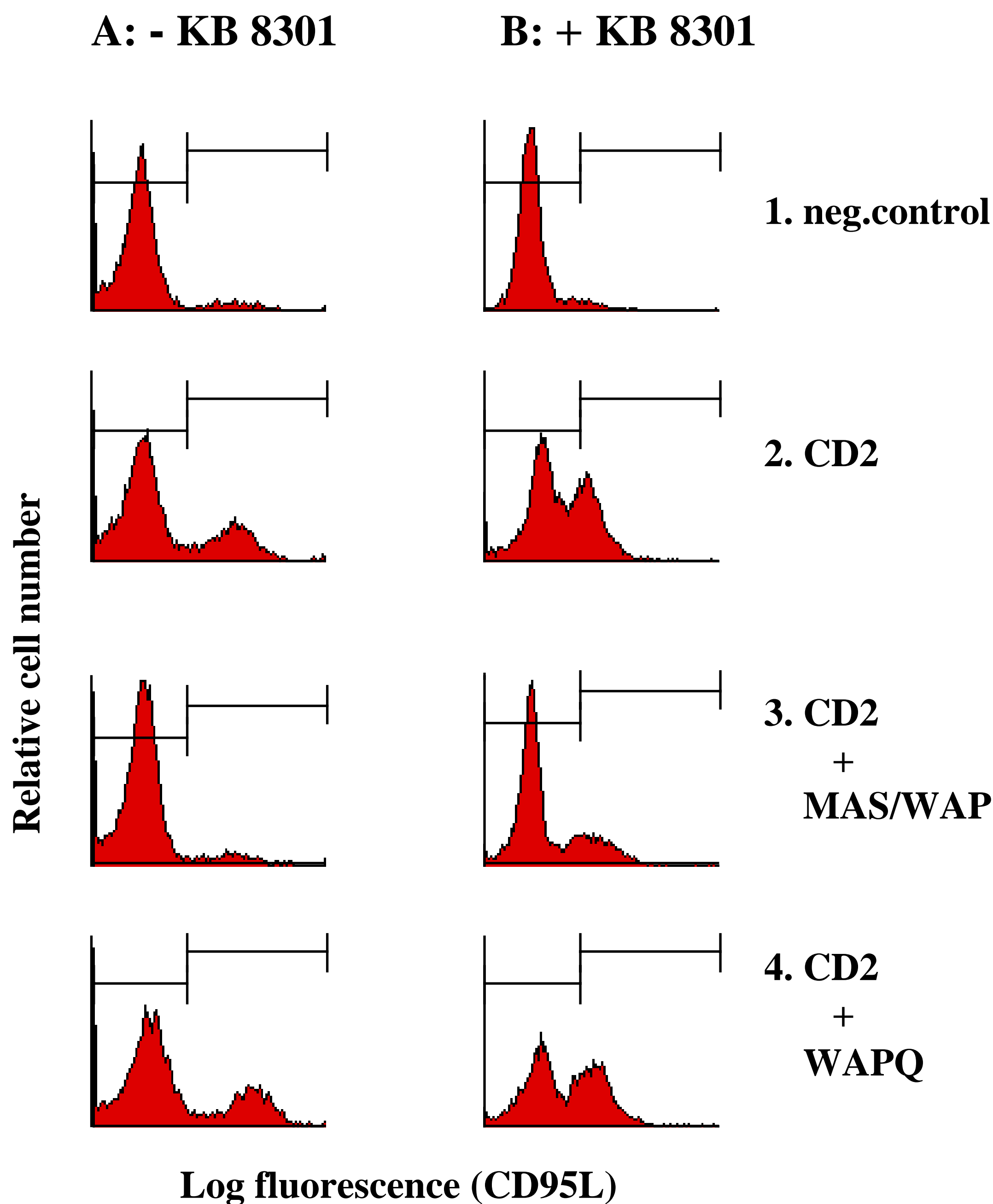


Figure 20

Expression of human CD95 ligand on the surface of the T cell clone D798.18 is prevented by cofilin derived peptides.
The expression of CD95 ligand on the human T cell clone D798.18 was analysed by flow cytometry in the presence (B), or absence of a metalloproteinase inhibitor, KB8301 (A). D798.18 cells were pretreated with a combination of MAS and WAP peptides (3), control peptide WAPQ (4) or without peptide (1 and 2). (1) shows, as a negative control, unstimulated cells. In (2) (3) and (4) the cells were stimulated via CD2 triggering for 6 hours to induce CD95 ligand expression. Fluorescence intensity (Y-axis) versus cell number (X-axis) is shown.