

# The Intersectin 2 Adaptor Links Wiskott Aldrich Syndrome Protein (WASp)-mediated Actin Polymerization to T Cell Antigen Receptor Endocytosis

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## Abstract

Induction of T cell antigen receptor (TCR) endocytosis has a significant impact on TCR signaling and T cell behavior, but the molecular interactions coordinating internalization of the activated TCR are poorly understood. Previously we have shown that TCR endocytosis is regulated by the Wiskott Aldrich Syndrome protein (WASp), a cytosolic effector which, upon interaction with the cdc42 Rho GTPase, couples TCR engagement to Arp 2/3 complex-mediated actin polymerization. Here we report that WASp associates in T cells with intersectin 2, an endocytic adaptor containing multiple domains including a Dbl homology (DH) domain with the potential to activate Rho GTPases. Intersectin 2 association with WASp increases after TCR engagement, and its overexpression in Cos-7 cells induces WASp translocation to endocytic vesicles within which intersectin 2 colocalizes with both WASp and cdc42. Intersectin 2, but not a DH domain-deleted ( $\Delta$ DH) form of intersectin 2, and stimulation via the TCR also trigger the activation of cdc42. Induction of TCR internalization is also augmented by intersectin 2 and severely impaired by latrunculin B treatment. Thus, intersectin 2 appears to function cooperatively with WASp and cdc42 to link the clathrin endocytic machinery to WASp-mediated actin polymerization and ultimately to occupancy-induced TCR endocytosis.

Key words: TCR endocytosis • actin polymerization • T cell activation • Wiskott Aldrich Syndrome protein • intersectin

## Introduction

Endocytosis of the TCR complex is rapidly induced after receptor engagement and is thought to play an important role in regulation of T cell activation (1–4). Ligand-induced TCR endocytosis has, for example, been implicated in attenuation of T cell responsiveness to antigen and superantigen restimulation (5–8), in the modulation of activated T cell sensitivity to CTL-mediated “fratricidal” lysis (9), and in the regulation of both thymocyte selection and CD4 versus CD8 lineage decisions (10–12). These biologic sequelae of TCR endocytosis have been attributed to the reduction in surface TCR numbers engendered by lysosomal degradation of internalized receptors and a consequent decrease in TCR triggering to a level insufficient for sustaining activation signals (13–15). TCR endocytosis may

also impact on T cell function by either positively or negatively modulating the coupling of TCR components with cytosolic and/or membrane-associated signaling effectors (4, 16) and/or by inducing changes in surface TCR distribution which influence formation or stability of the immunologic synapse (17). Thus, as has also been described for several growth factor receptors (18, 19), TCR internalization appears to play essential and diverse roles in modulating specific cellular responses to receptor stimulation.

The molecular events mediating occupancy-induced TCR endocytosis are not well defined, but include the assembly of clathrin-coated vesicles, a multistep process wherein activated receptors are coupled to clathrin via a heterotetrameric adaptor complex, AP2 (16, 20). AP2 complexes promote clathrin coat assembly and the convergence of multiple enzymes and adaptor proteins which modulate clathrin cage assembly and disassembly and the formation of endocytic vesicles (21). Clathrin-mediated endocytosis of the TCR has been shown to be facilitated by

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a di-leucine and a tyrosine-based AP2-interacting motif within the CD3 $\gamma$  and CD3 $\epsilon$  chains, respectively, and to play integral roles in TCR-triggered proliferation and differentiation, both of which are modulated by a GTPase (Rab5) that links clathrin-coated vesicles to the early endosome (10, 20, 22). However, while clathrin-dependent endocytosis has been studied in many cell systems, little is known about the specific molecular interactions and activities that coordinate clathrin-mediated internalization of the TCR following receptor stimulation.

Recently, our group has identified the Wiskott Aldrich syndrome protein (WASp)\* as an essential component of the TCR endocytic pathway, our data revealing ligand-evoked TCR internalization to be markedly impaired in WASp-deficient T cells (23). WASp contains a number of distinct motifs and domains which imbue this protein with the capacity to participate in a broad range of molecular interactions and to regulate the induction of both transcriptional activation and actin polymerization after TCR stimulation (24). These domains include an NH<sub>2</sub>-terminal EVH1 domain which interacts with the actin-binding protein WIP (25, 26), a cdc42/Rac interactive binding (CRIB) motif, which mediates WASp binding to the activated form of the cdc42 guanosine triphosphatase (27), and a proline-enriched segment through which WASp interacts with Nck, Fyn, and several other SH3 domain-containing proteins (28–30). These latter interactions are not fully characterized in relation to their functional relevance but appear to mediate the coupling of WASp to upstream signaling events evoked by TCR engagement. By contrast, a verprolin homology, cofilin homology, acidic region (VCA) domain located within the WASp COOH terminus interacts with the p21 subunit of the Arp2/3 complex and triggers the nucleation of actin filaments after cdc42-mediated changes in WASp conformation (30–33). The involvement of WASp in both actin polymerization and TCR endocytosis is consistent with an increasing body of evidence suggesting that the actin cytoskeleton plays an integral role in receptor-mediated actin polymerization (34, 35). However, at present the relevance of actin polymerization to TCR internalization is unknown and the molecular mechanisms whereby WASp is coupled to internalization of the TCR have not been established. In this study, we demonstrate the capacity of WASp to interact with intersectin 2, a Dbl homology (DH)-containing endocytic adaptor protein that is known to promote clathrin-mediated endocytosis (36). Intersectin 2 association with WASp is up-regulated after TCR engagement and the coexpression of these proteins in Cos-7 cells induces redistribution of WASp from perinuclear actin bundles to endocytic vesicles within which WASp and intersectin 2 colocalize. Intersectin 2 also colocalizes with the cdc42 GTPase within endocytic vesicles and in the presence, but not absence, of its DH domain, induces cdc42 activation. When overexpressed in Jurkat cells,

intersectin 2 induces a marked increase in ligand-evoked internalization of the TCR. By contrast, induction of TCR endocytosis is severely reduced by latrunculin treatment and by expression of a DH domain-deleted form of intersectin 2. These data reveal that intersectin 2 modulates WASp localization and its capacity to bind cdc42 and thereby initiate actin polymerization. Thus, intersectin 2, cdc42, and WASp function cooperatively to link endocytic pathway components to the actin nucleation machinery and the induction of TCR endocytosis.

## Materials and Methods

**Yeast Two Hybrid Screen.** For the two hybrid screen, the full-length human WASP cDNA was subcloned into the pAS1 vector and the resulting construct transfected into yeast cells harboring the MATCHMAKER activated human T cell DNA library in the pGAD10 vector (CLONTECH Laboratories, Inc.). Yeast colonies demonstrating expression of  $\beta$ -galactosidase and growth in medium supplemented with 25 mM amino-triazole were subcultured for isolation of single colonies from which plasmid DNA was isolated for sequence analysis. All potential positive clones were sequenced at the Samuel Lunenfeld Sequencing Facility, Mount Sinai Hospital (Toronto, Canada) and the sequences deposited in GenBank/EMBL/DBDJ.

**Rapid Amplification of cDNA Ends.** A partial intersectin 2 cDNA clone, originally designated SWAP (SH3P18 like WASP associated protein), was completed by rapid amplification of cDNA ends (RACE) from purified Jurkat T cell mRNA using the 5'/3' RACE kit from Roche Molecular Biochemicals and nested gene-specific primers. DNA sequences were assembled and analyzed using the MacVector Program (IBI) and subjected to homology searches using the BLAST algorithm provided by the National Centre for Biotechnology Information.

**Reagents.** Antibodies used for these studies included: polyclonal antibodies specific for WASp (23) and intersectin 2, the latter of which was generated by immunizing New Zealand white rabbits with a glutathione-S-transferase (GST)-fusion protein encompassing the intersectin 2 SH3 domains (amino acids [aa] 757–1,185); rabbit polyclonal anti-human cdc42 and anti-Eps15 antibodies (Santa Cruz Biotechnology, Inc.); and monoclonal antibodies recognizing human CD3 (produced by the UCHT-1 hybridoma), human CD28 (BD PharMingen), the human  $\alpha\beta$  TCR (BD PharMingen), and human c-myc (Santa Cruz Biotechnology, Inc.). Biotinylated anti-human CD3 antibody was purchased from BD PharMingen, biotinylated goat anti-mouse IgG, and Cy3-conjugated goat anti-rabbit Ig from Jackson ImmunoResearch Laboratories, purified rabbit IgG from Bio-Rad Laboratories, phycoerythrin-conjugated streptavidin from BD PharMingen, latrunculin B from Calbiochem-Novabiochem, and 7-amino-actinomycin D (7-AAD) from Sigma-Aldrich. Expression constructs for immunofluorescence studies were derived by subcloning the full-length WASp and intersectin 2 cDNAs into the pEGFP vector-C3 and pDSRED-C1 expression vectors (CLONTECH Laboratories, Inc.). A mutant intersectin 2 cDNA lacking the Dbl homology (DH) domain segment (intersectin 2 $\Delta$ DH) was derived by PCR using primers flanking the sites of the intended deletion (aa 1188–1372) and was then subcloned into the pEGFP vector and sequenced to confirm the presence of the deletion. A cdc42 cDNA cloned into the pRK5 vector was provided by S. Grinstein (Hospital for Sick Children, Toronto, Canada) and a myc-tagged

\*Abbreviations used in this paper: aa, amino acid(s); CRIB, cdc42/Rac interactive binding; DH, Dbl homology; GEF, guanine-nucleotide exchange factor; GST, glutathione-S-transferase; PH, pleckstrin homology; RACE, rapid amplification of cDNA ends; WASp, Wiskott Aldrich Syndrome protein.

Rac1 cDNA cloned into pCDNA3.1 was provided by T. Pawson (Samuel Lunenfeld Research Institute, Toronto, Canada).

**T Cell Stimulation.** For T cell activation, Jurkat E6 cells ( $2 \times 10^7$ ) cultured in RPMI-1640 supplement with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50  $\mu\text{g/ml}$  streptomycin (GIBCO BRL) were stimulated for 2, 5, 10, 30, or 60 min with anti-CD3 (5  $\mu\text{g/ml}$ ) and anti-CD28 (5  $\mu\text{g/ml}$ ) antibodies. Aliquots of all cultures were subjected to anti-phosphotyrosine immunoblotting analysis (see below) to confirm cell activation.

**Immunoblotting and Immunoprecipitation Assays.** To prepare cell lysates, Jurkat or Cos-7 cells ( $2 \times 10^7$ ) were suspended in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM KCl, 150 mM NaCl, 1 mM PMSF, and 1  $\mu\text{g/ml}$  each aprotinin, leupeptin, and pepstatin. After 30 min on ice, unlysed cells were removed by centrifugation and lysates precleared by incubation with Protein A sepharose 6B beads (Amersham Pharmacia Biotech) for 30 min at 4°C followed by an additional 2 h incubation at 4°C with specific antibody or rabbit preimmune serum. The immune complexes were then collected on Protein A sepharose 6B beads by centrifugation, washed three times with lysis buffer, and the bound protein eluted by boiling in Laemmli sample buffer. For immunoblotting analyses, immunoprecipitated or cell lysate proteins were suspended in SDS-PAGE loading buffer, electrophoresed through 10% polyacrylamide gels and transferred to nitrocellulose membrane. After blocking with 3% BSA, filters were incubated for 2 h with appropriate antibody in  $1 \times$  TBST (20 mM Tris, pH 7.5, 0.9% NaCl, 0.05% Tween 20) solution containing 5% skimmed milk. The filters were then incubated for 1 h with a 1/4,000 dilution of Protein-A horseradish peroxidase conjugate (Bio-Rad Laboratories) and for 1 min with ECL substrate (Amersham Pharmacia Biotech), followed by 1–10 min exposure to Eastman Kodak Co. x-ray film.

**Transfection and Immunofluorescence Assays.** Plasmid DNA for expression constructs containing the full-length WASp, cdc42, Rac1, intersectin 2, and intersectin 2  $\Delta\text{DH}$  cDNAs was prepared using the QIAGEN MaxiPrep kit. Cos-7 cells maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50  $\mu\text{g/ml}$  streptomycin (GIBCO BRL) were seeded onto glass slides at a density of  $10^5$  cells/ml and transfected with selected plasmid DNAs using the lipotaxi reagent (Stratagene). At 1 d after transfection the cells were washed and then fixed in 3% cold paraformaldehyde in PBS. Images were obtained using the Olympus 1X-70 inverted microscope equipped with fluorescence optics and Deltavision Deconvolution Microscopy Software (Applied Precision).

**Generation of GST Fusion Proteins.** GST fusion proteins expressing individual or all five of the intersectin 2 SH3 domains were generated by subcloning into the pGEX2T vector PCR-amplified fragments corresponding to the intersectin 2 SH3A (aa 729–790), SH3B (aa 870–928), SH3C (aa 953–1,011), SH3D (aa 1,025–1,089), SH3E (aa 1,099–1,158), or SH3A-E (aa 729–1,158) domains. All constructs were sequenced before their use in pull-down studies. A GST fusion protein containing the Rac and Cdc42-binding region from human PAK1B (GST-PAK CD) was generously provided by John G. Collard (The Netherlands Cancer Institute, Division of Cell Biology, Amsterdam, Netherlands). The GST expression plasmids were transformed into *Escherichia coli* and the fusion proteins then purified from isopropyl-1-thio- $\beta$ -D-galactopyranoside-induced bacteria with glutathione-coupled Sepharose 4B beads (Amersham Pharmacia Biotech). The amount of bound protein was estimated using Coomassie stained SDS gels.

**In Vitro Binding Assay.** To evaluate WASp binding to GST-intersectin 2 SH3 domain fusion proteins, lysates prepared from

$10^8$  anti-CD3 antibody-stimulated Jurkat cells were incubated at 4°C for 2 h with 5  $\mu\text{g}$  fusion protein immobilized on glutathione-sepharose beads. After several washes in lysis buffer, complexes were resuspended in Laemmli sample buffer, boiled, and analyzed by SDS-PAGE and anti-WASp antibody immunoblotting as described above.

**Cdc42 and Rac Activity Assay.** For detection of cdc42 and Rac activation, Cos-7 cells maintained in 10% fetal bovine serum-supplemented DMEM were transiently cotransfected with either the full-length cdc42 cDNA in pRK5 or with myc-tagged Rac1 cDNA in pCDNA3.1 and with pEGFP-C3 vectors containing either the intersectin 2 or intersectin 2 $\Delta\text{DH}$  cDNAs. Cells were then harvested and suspended in 1 ml lysis buffer. Alternatively, lysates were prepared from Jurkat T cells ( $2 \times 10^7$ ) stimulated with anti-CD3 and anti-CD28 antibodies for varying periods of time (0–60 min). After an adjustment of the Cos-7 cell lysates to ensure approximate equivalence in levels of cdc42 or Rac, both the Cos-7 and Jurkat cell lysates were precleared by incubation with glutathione-sepharose beads for 30 min at 4°C, subjected to centrifugation and the supernatants incubated for 2 h at 4°C with 5  $\mu\text{g}$  of GST alone (as a control) or GST-PAK CD fusion protein immobilized on glutathione-sepharose beads. After several washes in lysis buffer, the complexes were resuspended in sample buffer, and subjected to electrophoresis followed by immunoblotting with anti-cdc42 or anti-myc antibody. To assay total cdc42 and Rac1 expression, aliquots of each cell lysate were also subjected directly to anti-cdc42 or anti-Rac immunoblotting analysis.

**TCR Internalization Assays.** TCR endocytosis was assayed in Jurkat cells ( $2 \times 10^7$ ) treated for 20 min at 37°C with varying amounts (0–3  $\mu\text{M}$ ) latrunculin B or, alternatively, in Jurkat cells transfected with intersectin 2 and intersectin 2 $\Delta\text{DH}$  expression constructs. For these latter studies, plasmid DNA encoding GFP alone, GFP-intersectin 2, or GFP-intersectin  $\Delta\text{DH}$  was introduced into the Jurkat cells by electroporation and the cells incubated for 14 h at 37°C in culture medium. The GFP-expressing populations were then identified by flow cytometric analysis and purified by sorting with the Moflo cell sorter (Cytomation). To assay TCR endocytosis, latrunculin-treated or GFP-positive intersectin 2-transfected cells were incubated for 30 min on ice with either biotinylated anti-CD3 antibody (1  $\mu\text{g/ml}$ ) or anti- $\alpha\beta\text{TCR}$  antibody (1  $\mu\text{g/ml}$ ) followed by biotinylated goat anti-mouse antibody (2  $\mu\text{g/ml}$ ) for a further 30 min on ice. Cells were then washed and treated with 0.1%  $\text{NaN}_3$  on ice or warmed to 37°C for 60 min to allow internalization and treated with 0.1%  $\text{NaN}_3$  on ice. Cells were then stained with PE-conjugated streptavidin, washed, and stained with 7-AAD (to evaluate cell viability) and subjected to flow cytometric analysis using a FACSCalibur™ (Becton Dickinson). The data was expressed as the geometric mean of the population and graphed as the percentage change in the level of surface CD3 or surface TCR expression over the 60 min time course.

## Results and Discussion

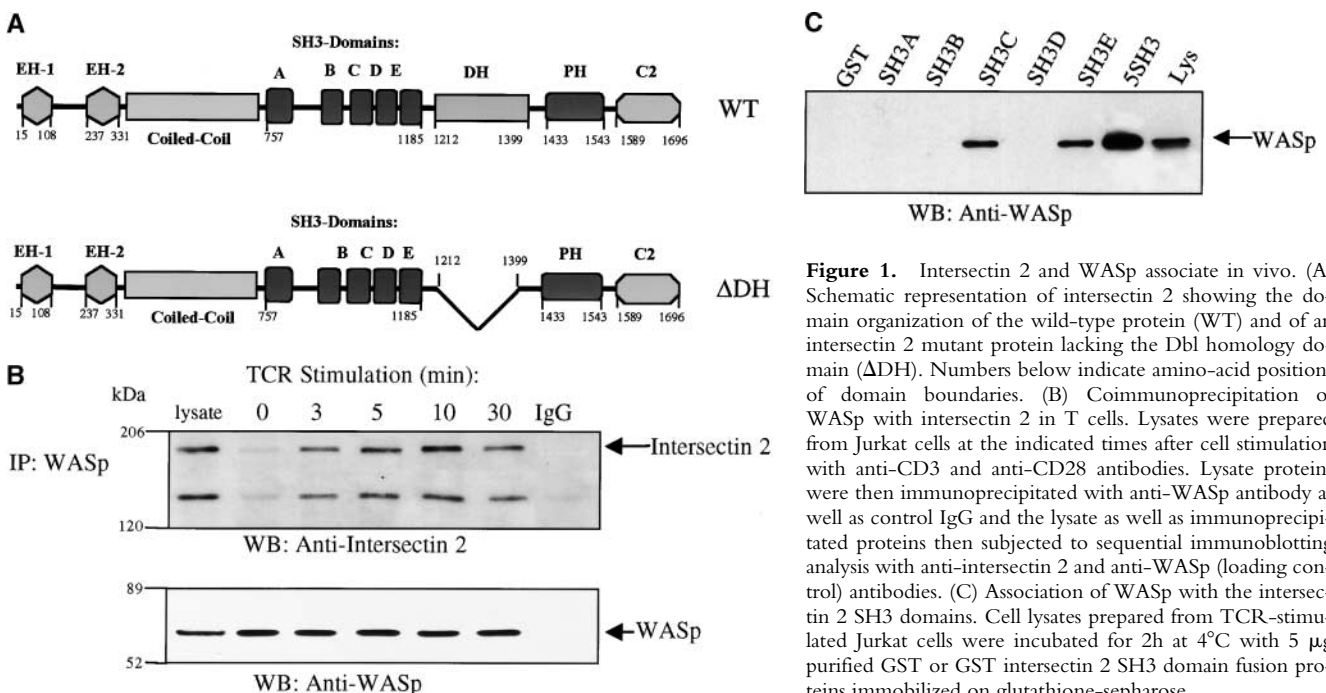
**Identification of Intersectin 2 as a WASp Interacting Protein.** To identify the mechanisms whereby WASp influences endocytosis and other T cell functions, a yeast two hybrid screen for novel WASp interacting proteins was performed using the WASp cDNA as bait and an activated human T cell cDNA library. A number of unique WASp-binding clones were recovered in this system, one of which contained a 1,391 nucleotide cDNA showing 99.8% se-

sequence identity to a previously reported partial cDNA designated SH3P18 (37). A full-length cDNA of 5,800 bp was subsequently generated by 5' and 3' RACE reactions using RNA from Jurkat cells and was found to contain an open reading frame encoding the recently identified endocytic adaptor protein, intersectin 2. Intersectin 2 and a previously isolated paralogue, intersectin 1, are widely expressed proteins which, as shown in Fig. 1, contain two NH<sub>2</sub>-terminal Eps15 homology (EH) domains, a coiled-coil domain, five SH3 domains, and a COOH-terminal segment that encompasses a Dbl-homology (DH), a pleckstrin homology (PH), and a C2 domain (36, 38). Intersectin orthologs have been identified in *Xenopus* (39), *Drosophila* (40), and mouse (41) and their biochemical and functional characterization has revealed these multimodular proteins to play integral roles in clathrin-mediated endocytosis (38–42). These effects on endocytosis are derived from intersectin interactions with numerous components of the endocytic pathway including, for example, clathrin and AP2 (42), the AP2-binding epsin protein (41, 42), another EH domain-containing endocytic protein, Eps15 (41, 43), and the endocytic modulators, synaptojanin (39, 44) and dynamin (41, 43). Thus, intersectin appears to act as an adaptor or scaffold for assembly of the multiprotein complexes required for clathrin-mediated endocytosis and its interaction with WASp provides a potential mechanism for recruiting WASp into the endocytic pathway.

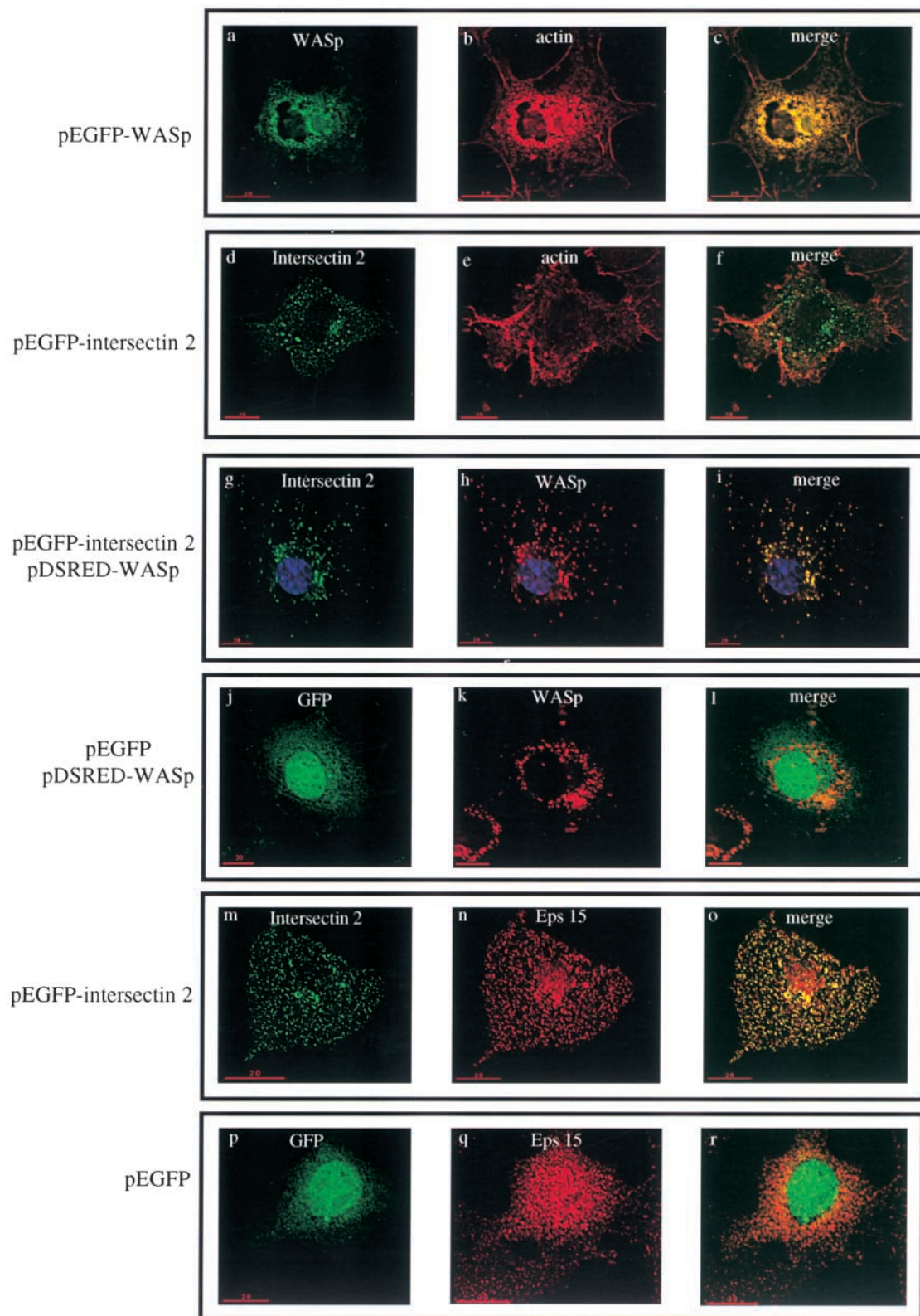
To determine whether WASp association with intersectin 2 occurs in T cells and is modulated by TCR stimulation, the interaction of these proteins was examined in anti-CD3 antibody-stimulated Jurkat cells. As shown in Fig. 1 B, WASp immunoprecipitates from these cells contained both the 194 kD intersectin 2 protein as well as a

142 kD intersectin 2 isoform (intersectin 2s) which has been shown to represent a splice variant lacking the COOH-terminal region encompassing the DH, PH, and C2 domains (36, 41). These isoforms both coimmunoprecipitated with WASp from unstimulated cells, but their association with WASp was markedly upregulated after TCR engagement. Thus, WASp constitutively and inducibly associates *in vivo* with both the long and short isoforms of intersectin 2.

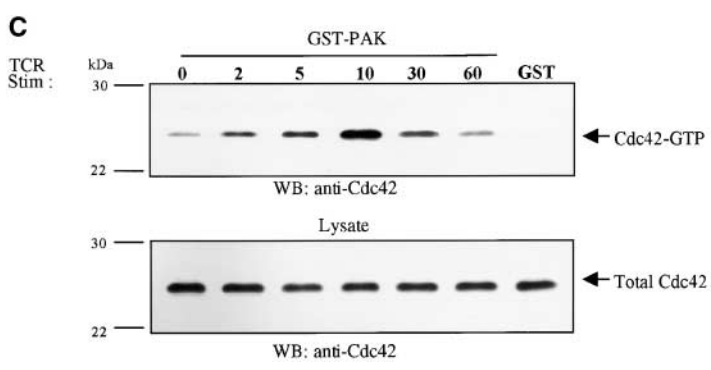
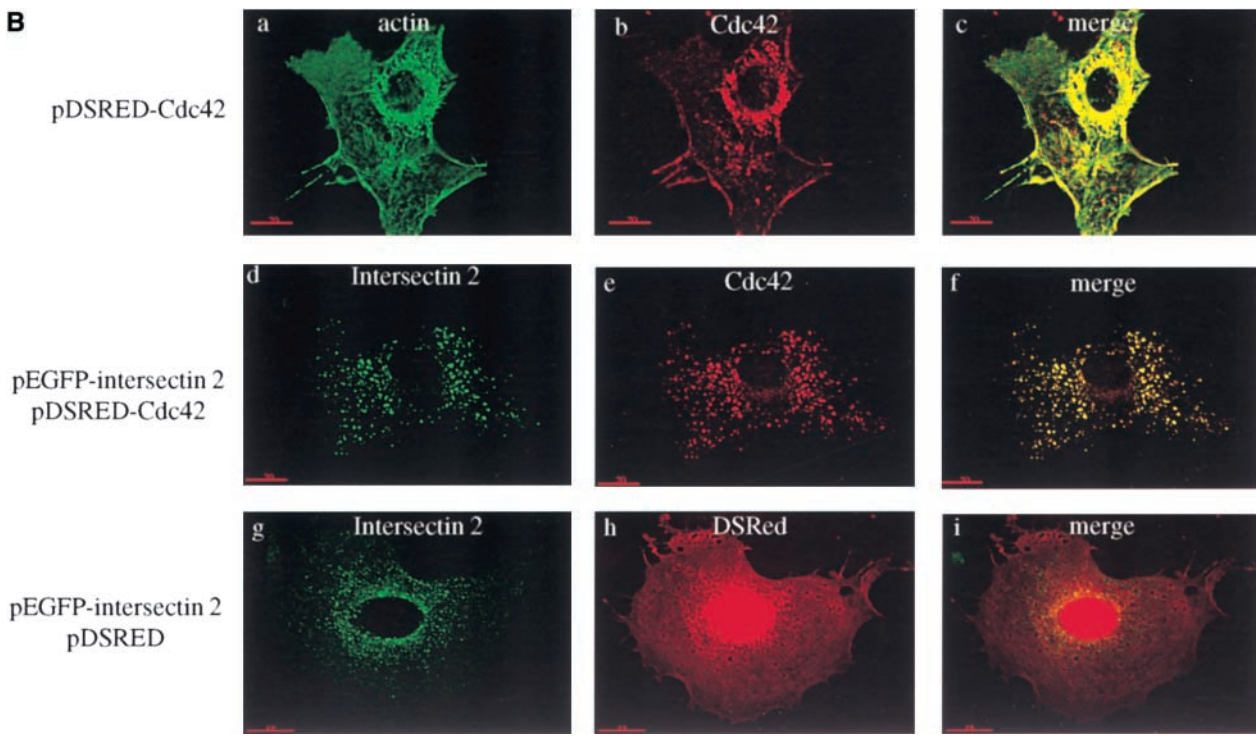
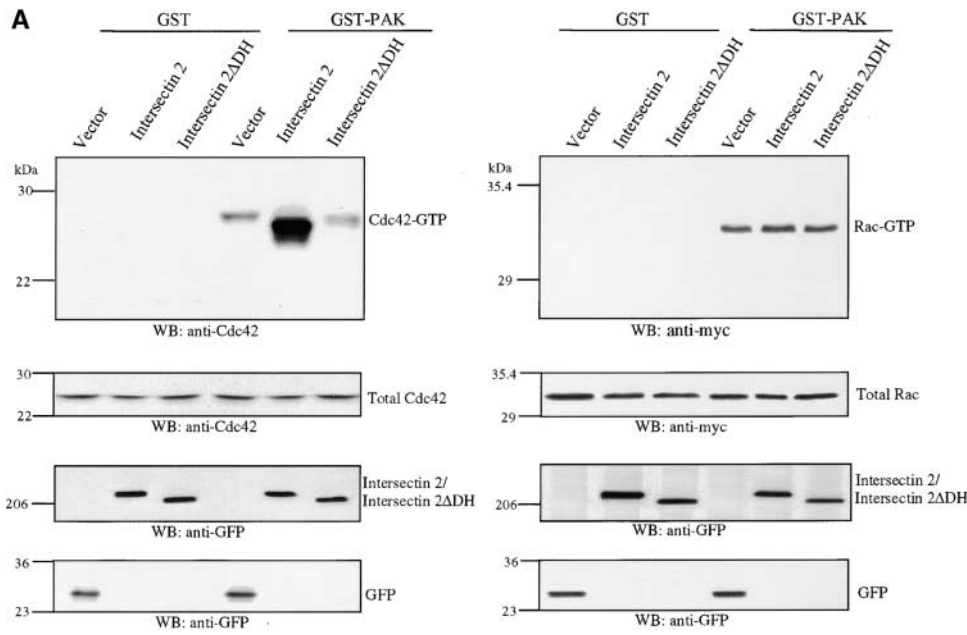
Because WASp contains a proline-rich region with multiple PXXP and two PPPPXXRG SH3 domain binding motifs, the role of the intersectin 2 SH3 domains in mediating association of these proteins was also explored. To this end, the five intersectin SH3 domains were expressed together or individually as GST fusion proteins and examined for their capacity to precipitate WASp from lysates of stimulated Jurkat cells. As shown in Fig. 1 C, WASp was able to bind either the five SH3 domains together or the middle (SH3C) and the most COOH-terminal (SH3E) SH3 domains individually. By contrast, neither GST alone, nor the individual SH3A, SH3B, or SH3D domains showed any association with WASp. While these data reveal that the intersectin 2 SH3C and SH3E domains mediate its interaction with WASp, the higher signal intensity elicited using the full complement of intersectin 2 SH3 domains, suggests that these modules act cooperatively to augment WASp binding. Similarly, the SH3 domains of another WASp binding partner, Nck, are thought to coordinately mediate the association of these proteins (28). In view of data revealing the intersectin 1 SH3 domains to interact with the dynamin GTPase (39), it is likely that the intersectin 2 SH3 domains also mediate association with endocytic molecules, but whether such interactions occur concurrently via SH3



**Figure 1.** Intersectin 2 and WASp associate *in vivo*. (A) Schematic representation of intersectin 2 showing the domain organization of the wild-type protein (WT) and of an intersectin 2 mutant protein lacking the Dbl homology domain ( $\Delta$ DH). Numbers below indicate amino-acid positions of domain boundaries. (B) Coimmunoprecipitation of WASp with intersectin 2 in T cells. Lysates were prepared from Jurkat cells at the indicated times after cell stimulation with anti-CD3 and anti-CD28 antibodies. Lysate proteins were then immunoprecipitated with anti-WASp antibody as well as control IgG and the lysate as well as immunoprecipitated proteins then subjected to sequential immunoblotting analysis with anti-intersectin 2 and anti-WASp (loading control) antibodies. (C) Association of WASp with the intersectin 2 SH3 domains. Cell lysates prepared from TCR-stimulated Jurkat cells were incubated for 2h at 4°C with 5  $\mu$ g purified GST or GST intersectin 2 SH3 domain fusion proteins immobilized on glutathione-sepharose.



**Figure 2.** Colocalization of ectopically expressed intersectin 2 and WASp. Subcellular distribution of WASp and intersectin 2 was analyzed by confocal immunofluorescent microscopy of Cos-7 cells transiently transfected with pEGFP-WASp alone (a–c), pEGFP-intersectin 2 alone (d–f and m–o), pEGFP-intersectin 2, and pDSRED-WASp (g–i), or, as controls, pDSRED-WASp and the pEGFP vector (j–l) and pEGFP alone (p–r); actin was visualized with rhodamine-phalloidin (b, c, e, and f) and Eps15 was visualized using anti-Eps15 and Cy3-conjugated secondary antibody (n, o, q, and r). Panels c, f, i, l, o, and r represent merged images from a and b, d and e, g and h, j and k, m and n, and p and q, respectively, with overlap indicated in yellow. The images shown are representative of three independent experiments.



**Figure 3.** Effects of intersectin 2 and T cell stimulation on cdc42. (A) Intersectin 2 induces cdc42 activation. Cell lysates were prepared from Cos-7 cells expressing intersectin 2, intersectin 2ΔDH, or empty vector-(pEGFP) and either the cdc42 (left panel) or myc-tagged Rac1 cDNAs (right panel). Equal amounts of lysate proteins were either loaded directly on the gel or incubated with 5μg GST or GST-PAK CD fusion proteins immobilized on glutathione-sepharose. The lysate and GST-PAK CD-bound proteins were then separated by SDS-PAGE and analyzed by immunoblotting with anti-cdc42 (left panel) or anti-myc (right panel) antibody to detect total cdc42/Rac1 and cdc42-GTP/Rac1-GTP, respectively. To control for loading, the lysate filters were washed and reprobbed with anti-GFP antibody. (B) Intersectin 2 colocalizes with cdc42. Intersectin 2 effects on the subcellular

domains distinct from those used for WASp binding or at overlapping sites remains to be determined.

**Intersectin 2 Colocalizes with WASp and Redirects Its Subcellular Localization.** To determine whether intersectin 2 binding to WASp impacts on the subcellular distribution of these proteins, DSRED or GFP tagged versions of these proteins were expressed individually and then together in Cos-7 cells and their localization relative to each other and to the actin cytoskeleton was examined by immunofluorescence microscopy. As shown in Fig. 2, a–c, an evaluation of WASp and actin staining patterns in cells expressing WASp alone revealed WASp to be located in actin-containing bundles or aggregates clustered in a perinuclear distribution. By contrast, intersectin 2 overexpression was associated with a punctuate staining pattern that showed negligible overlap with the actin meshwork (Fig. 2, d–f) and which closely resembles the staining patterns reported for Eps15 and several other endocytic pathway components (41, 45). Importantly, coexpression of WASp and intersectin 2 did not affect the localization of intersectin 2 within the cell, but markedly altered the distribution of WASp such that the latter colocalized with intersectin 2 within the small vesicular structures (Fig. 2, g–i). To confirm that these latter structures represent endocytic vesicles, intersectin 2–transfected cells were also used to compare the subcellular distribution of intersectin 2 with that of Eps15, an AP2-binding protein previously localized to clathrin-coated pits and vesicles (45). As shown in Fig. 2, m–o, cells stained with anti-Eps15 antibody showed a punctuate pattern of fluorescence very similar to that observed for intersectin 2 and superimposition of these images revealed these proteins to be almost completely colocalized. Together, these findings indicate that intersectin 2 not only associates with WASp, but also regulates the localization of WASp within the cytosol so as to target WASp to sites of endocytic activity. These observations are reminiscent of data showing that the murine intersectin 1 homologue, Eps1, regulates the subcellular localization of both dynamin and Eps15 (41, 43, 46). Similarly, intersectin 2 may provide a physical vehicle for positioning WASp in proximity to multiple endocytic proteins and thereby recruiting WASp into the clathrin-mediated endocytic pathway.

(continued)

distribution of cdc42 were examined by confocal immunofluorescence analysis of Cos-7 cells transfected with pDSRED-cdc42 alone (a–c), pDSRED-cdc42 and pEGFP-intersectin 2 (d–f) or, as a control, pEGFP-intersectin 2 with the pDSRED vector (g–i); actin was visualized with rhodamine phalloidin (b and c). Panels c and f and i represent merged images from a and b, d and e, and g and h, respectively, with overlap indicated in yellow. (C) TCR engagement induces cdc42 activation. Lysates were prepared from Jurkat cells at the indicated times after TCR stimulation and equal amounts of lysate proteins were either loaded directly on the gel or incubated with 5  $\mu$ g GST-PAK CD fusion proteins immobilized on glutathione-sepharose. The lysate and GST-PAK CD-bound proteins were then separated by SDS-PAGE and analyzed by immunoblotting with anti-cdc42 antibody to detect total cdc42 and cdc42-GTP, respectively.

**Intersectin 2 Regulates cdc42 Localization and Activation.** In addition to its numerous protein binding molecules, intersectin 2 contains a DH domain, a motif characteristically positioned in tandem with a PH domain and shown to confer catalytic function to guanine-nucleotide exchange factors (GEFs) for Rho family GTP-binding proteins (47). These latter proteins include cdc42, which upon activation, can associate with WASp and induce a change in its structural conformation that is required for induction of WASp VCA domain-Arp 2/3 complex-mediated actin polymerization (31–33). These observations raise the possibility that intersectin 2 modulates WASp function by virtue of catalyzing the conversion of GDP-bound cdc42 to its activated, GTP-bound form. To address this possibility, intersectin 2 effects on cdc42 activation were evaluated using a GST fusion protein containing the CRIB domain of the p21 activated kinase, PAK. Like WASp, PAK is a downstream target of cdc42 that interacts via its CRIB region with the GTP, but not GDP-bound form of this GTPase (48, 49). Accordingly, to determine whether intersectin 2 triggers activation of cdc42, the amount of cdc42 precipitated by GST-PAK CD was evaluated in Cos-7 cells transfected with cdc42 alone or in conjunction with intersectin 2. As shown by anti-cdc42 immunoblotting analysis (Fig. 3 A), the amount of cdc42 precipitated by the GST-PAK CD protein was very substantially increased in the context of intersectin 2 expression, although levels of total cdc42 were comparable in the control and the intersectin 2–transfected cells. To confirm that this effect of intersectin 2 reflected its DH domain activity, cdc42 activation was also evaluated in Cos-7 cells transfected with cdc42 and a mutant form of intersectin 2 lacking the DH domain (intersectin 2 $\Delta$ DH). Although anti-GFP immunoblotting analysis revealed pEGFP-intersectin 2 $\Delta$ DH to be expressed at levels comparable to pEGFP-intersectin 2 in these cells, intersectin 2 $\Delta$ DH evoked negligible binding of cdc42 to the GST-PAK CD fusion protein (Fig. 3 A). These data indicate that intersectin 2 has GEF activity for cdc42 and also link this activity to the Dbl homology domain.

Because the PAK CRIB domain associates with GTP-bound Rac1 as well as cdc42 (48), the potential for intersectin 2 to regulate other Rho family GTPases was also evaluated by assaying the capacity of the GST-PAK CD fusion protein to precipitate Rac1 from Rac1 and intersectin 2/intersectin 2 $\Delta$ DH cotransfected Cos cells. The results of this analysis revealed no effect of either intersectin 2 or intersectin 2 $\Delta$ DH on the activation of Rac1 (Fig. 3 A). This latter finding suggests that intersectin 2 may specifically target cdc42, a possibility consistent with data indicating at least some DH domain-containing proteins to show restricted specificity for particular Rho family members (50).

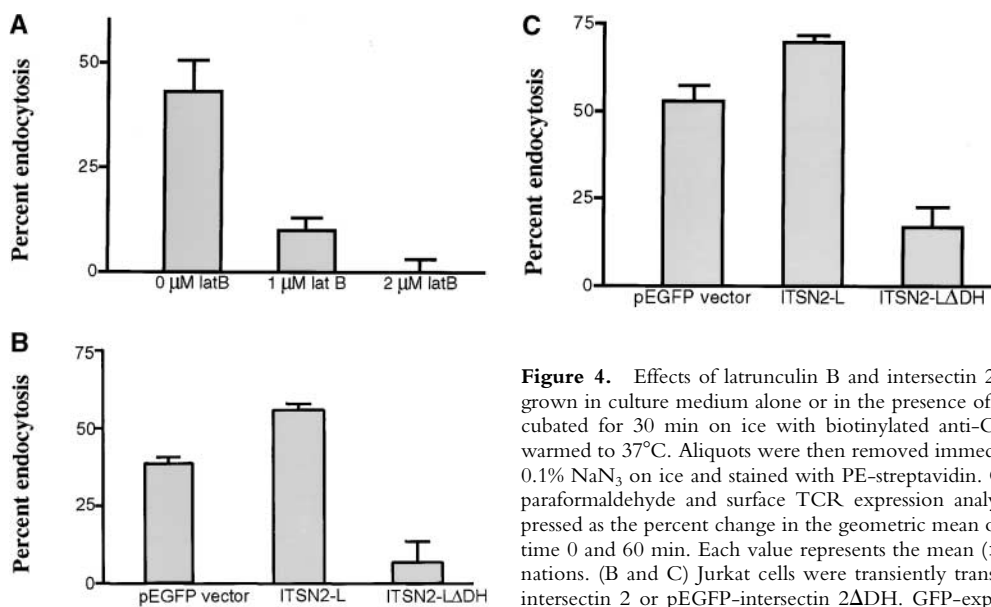
To determine whether intersectin 2 also modulates the subcellular distribution of cdc42, the localization of both these proteins was also examined in Cos-7 cells. As shown in Fig. 3 B (a–c), immunofluorescent analysis of cells ectopically expressing cdc42 revealed this GTPase to be located in perinuclear, actin-containing bundles, a distribution similar to that observed for WASp. However, when

cotransfected with intersectin 2, cdc42, exhibited a punctuate staining pattern which overlapped almost entirely with that of intersectin 2 (Fig. 3 B, d–f). Thus, like WASp, cdc42 localization within the cell is regulated by intersectin 2, the latter of which appears to direct cdc42 to endocytic vesicles and thereby place cdc42 in a favorable position to be targeted by intersectin 2 DH-mediated GEF activity. These data suggest that in addition to its critical role in the regulation of actin rearrangement (51), cdc42 may also participate in clathrin-mediated endocytosis. Similarly, the Rac and Rho GTPases have both been implicated in the clathrin-coated vesicle formation required for receptor-mediated endocytosis (52).

The capacity for intersectin 2 to stimulate cdc42 activation suggests that intersectin 2 not only associates with WASp, but may modulate WASp downstream functions through the regulation of cdc42 activity. However, while cdc42 is thought to play a role in T cell polarization following APC contact (53), the extent to which TCR engagement induces activation of this GTPase is unknown. To address this issue and thereby elucidate whether cdc42 activation represents a potential mechanism for functionally coupling intersectin 2 with WASp, the amounts of active cdc42 present in Jurkat cells at varying times following TCR stimulation were evaluated again using the GST-PAK CD protein to precipitate GTP-bound cdc42. As shown in Fig. 3 C, the results of this analysis revealed amounts of active cdc42 to progressively increase after cell stimulation while levels of total cdc42 in the cells remained unchanged. Thus, cdc42 activation, which can trigger cdc42 binding to WASp, is elicited by TCR engagement and may therefore represent an intermediary step in the coupling of intersectin 2 to the induction of WASp VCA domain-Arp 2/3 complex-mediated actin

polymerization. These data suggest that intersectin 2 drives not only the translocation of WASp to endocytic vesicles, but also the expression of WASp effector functions required for TCR endocytosis.

*TCR Internalization Requires Actin Polymerization and Is Regulated by Intersectin 2.* The potential role for intersectin 2 in triggering cdc42 binding to WASp and WASp-mediated actin polymerization suggests that WASp effects on TCR endocytosis reflect, at least in part, its capacity to promote Arp 2/3 complex-driven actin nucleation. However, while actin cytoskeletal organization has now emerged as a critical component of receptor-mediated endocytosis (34, 35), the relevance of actin polymerization to TCR internalization is not well established. Accordingly, we investigated the requirement for actin polymerization in ligand-induced TCR endocytosis by assaying the effects of an actin monomer-sequestering reagent, latrunculin B (54), on the levels of biotinylated TCR remaining on Jurkat cells after cell stimulation. For these studies, cells were stimulated in the presence of concentrations of latrunculin B shown to abrogate (2  $\mu$ M) or markedly inhibit (1  $\mu$ M) TCR-evoked actin polymerization, but to leave cell viability intact (data not shown). As shown in Fig. 4 A and previously reported for other receptor/cell systems (34, 35), latrunculin B inhibited TCR endocytosis in a concentration-dependent manner and exposure to 2  $\mu$ M latrunculin essentially abrogated TCR internalization in these cells. These observations indicate an integral role for actin polymerization in TCR endocytosis and therefore concur with recent data implicating the actin cytoskeleton in T cell internalization of peptide ligands and in the trafficking of the B cell antigen receptor to both endosomes and lysosomes (55, 56). Thus induction of actin polymerization represents a likely mechanism whereby WASp and



**Figure 4.** Effects of latrunculin B and intersectin 2 on TCR endocytosis. (A) Jurkat cells grown in culture medium alone or in the presence of 1  $\mu$ M or 2  $\mu$ M latrunculin B were incubated for 30 min on ice with biotinylated anti-CD3 antibody (1  $\mu$ g/ml), washed, and warmed to 37°C. Aliquots were then removed immediately or 60 min thereafter, mixed with 0.1% NaN<sub>3</sub> on ice and stained with PE-streptavidin. Cells were then fixed for 15 min in 4% paraformaldehyde and surface TCR expression analyzed by flow cytometry. Data are expressed as the percent change in the geometric mean of PE-streptavidin fluorescence between time 0 and 60 min. Each value represents the mean ( $\pm$  SEM) of three independent determinations. (B and C) Jurkat cells were transiently transfected with pEGFP (vector), pEGFP-intersectin 2 or pEGFP-intersectin 2 $\Delta$ DH. GFP-expressing cells were then isolated by cell sorting and incubated for 30 min on ice with either 1  $\mu$ g/ml biotinylated anti-CD3 antibody (B) or with 1  $\mu$ g/ml anti- $\alpha$  $\beta$  TCR antibody followed by 2  $\mu$ g/ml biotinylated goat anti-mouse antibody (C). The cells were then assayed for TCR internalization as described above. Each value represents the mean ( $\pm$  SEM) of three independent determinations.



potentially intersectin 2-mediated regulation of WASp influence TCR endocytosis.

In view of the potential importance of the intersectin 2/cdc42/WASp axis to TCR internalization, the role of intersectin 2 in TCR internalization was also directly investigated utilizing Jurkat cells transfected with plasmids encoding GFP-tagged intersectin 2, intersectin 2 $\Delta$ DH, or vector control. GFP-expressing cells were isolated by cell sorting and the effects of both anti-CD3 and anti-TCR antibody stimulation on TCR internalization then assayed using streptavidin to detect biotinylated surface TCRs. As is consistent with an important role for intersectin 2 in promoting TCR endocytosis, intersectin 2 overexpression in these cells resulted in a substantive increase in anti-CD3 as well as anti-TCR-evoked TCR internalization (Fig. 4, B and C). By contrast, in both the anti-CD3 and anti-TCR antibody-treated cells, intersectin 2 $\Delta$ DH expression was associated with a marked reduction in antigen receptor internalization, a result which suggests that this protein acts dominant negatively to disrupt the endocytic function of endogenous intersectin 2. This latter possibility is also supported by the finding that induction of cdc42 activation appears lower in Cos-7 cells expressing intersectin 2 $\Delta$ DH than in cells expressing empty vector (Fig. 3 A). These data therefore confirm that occupancy evoked TCR endocytosis is regulated by intersectin 2 and imply that this regulation is realized via the modulation of cdc42 and, by extension, WASp function.

In summary, the current data identify intersectin 2 as a WASp-binding protein which associates inducibly with WASp after TCR stimulation and promotes the induction of TCR endocytosis. The data also reveal an interplay between intersectin 2, cdc42, and WASp, wherein intersectin 2 directs the translocation of WASp and cdc42 to clathrin-coated endocytic vesicles and utilizes its DH domain to induce both cdc42 activation and internalization of the TCR. Because the activation of cdc42 and its consequent interaction with WASp is required for the WASp VCA domain to activate the Arp 2/3 complex (33), these data suggest that intersectin 2 modulates not only the subcellular localization of WASp, but also its capacity to evoke actin polymerization. This link between intersectin 2, an endocytic adaptor, and the actin cytoskeleton is consistent with the current data revealing TCR internalization to be dependent on actin polymerization and with recent data identifying a role for another endocytic protein, Pan1p, in induction of actin polymerization in yeast (57). Thus intersectin 2-mediated activation of cdc42 provides a potential point of convergence for the clathrin-mediated endocytic and Arp 2/3-initiated actin nucleation pathways that mediate internalization of the TCR after its engagement.

Although actin polymerization appears to be required for TCR endocytosis, the mechanisms whereby actin cytoskeletal arrangements impact on clathrin-mediated endocytosis are not clear. Actin cytoskeletal rearrangement may allow for the appropriate subcellular compartmentalization of endocytic components and may be needed for such endocytic events as vesicular budding, scission, and movement (52).

This latter possibility is supported by recent data revealing the WASp-related N-WASp molecule to be recruited to the sites of actin comet tails in endosomes and lysosomes where it may provide a nidus for the actin nucleation required for vesicular trafficking (58). In addition to these issues, it is also unclear whether the functional coupling of actin-nucleating to endocytic proteins is relevant to biological processes other than receptor internalization. Intersectin 1, for example, has recently been implicated in the activation of mitogenic signaling pathways (59, 60) and should intersectin 2 subserve a comparable function, its interaction with WASp may impact on transcriptional activation as well as receptor endocytosis. Finally, in view of the reported interaction of N-WASp with several endocytic proteins in neurons (61, 62), it is also possible that WASp is recruited to the TCR endocytic pathway by more than one endocytic effector. While all of these issues require further investigation, the capacity of intersectin 2 to promote TCR internalization, WASp targeting to endocytic vesicles, cdc42 activation and, by extension, WASp-mediated actin polymerization, indicates a critical role for the linkage of WASp with intersectin 2 specifically in connecting endocytic and cytoskeletal dynamics. Thus, while intersectin 2 association with WASp may subserve several biological functions, the current data indicate that intersectin 2, cdc42, and WASp function cooperatively to regulate TCR endocytosis and thereby provide a mechanistic framework for coupling the clathrin-mediated endocytic pathway and the actin nucleation machinery in T cells.

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