

# The Ankyrin Repeat-containing Adaptor Protein Tvl-1 Is a Novel Substrate and Regulator of Raf-1\*

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**Tvl-1 is a 269-amino acid ankyrin repeat protein expressed primarily in thymus, lung, and testes that was identified by screening a murine T-cell two-hybrid cDNA library for proteins that associate with the serine-threonine kinase Raf-1. The interaction of Tvl-1 with Raf-1 was confirmed by co-immunoprecipitation of the two proteins from COS-1 cells transiently transfected with Tvl-1 and Raf-1 expression constructs as well as by co-immunoprecipitation of the endogenous proteins from CV-1 and NB2 cells. Tvl-1 interacts with Raf-1 via its carboxyl-terminal ankyrin repeat domain. The same domain also mediates Tvl-1 homodimerization. Tvl-1 was detected by immunofluorescence in both the cytoplasm and the nucleus suggesting that in addition to Raf-1 it may also interact with nuclear proteins. Activated Raf-1 phosphorylates Tvl-1 both *in vitro* and *in vivo*. In baculovirus-infected Sf9 insect cells, Tvl-1 potentiates the activation of Raf-1 by Src and Ras while in COS-1 cells it potentiates the activation of Raf-1 by EGF. These data suggest that Tvl-1 is both a target as well as a regulator of Raf-1. The human homologue of Tvl-1 maps to chromosome 19p12, upstream of MEF2B with the two genes in a head to head arrangement.**

regulate the activity of Raf-1 is phosphorylation. Mitogenic stimulation leads to an increase in Raf-1 phosphorylation on serine, threonine, and in some cases, tyrosine residues (5, 6). Studies using overexpression systems have demonstrated that Raf-1 can be activated both by tyrosine phosphorylation mediated by members of the Src kinase family (7, 8) and by serine phosphorylation, mediated by protein kinase C (9). In contrast, cAMP-dependent protein kinase A (10) and an unidentified G-protein-coupled tyrosine phosphatase down-regulate the activity of Raf-1 (11). The significance of these kinases/phosphatases in regulating Raf-1 activation under physiological conditions is still inconclusive. However, it was recently shown that the Raf-1 activating phosphorylation at Tyr<sup>340</sup>, which is mediated by members of the Src kinase family, is triggered by CD4 cross-linking in T cells (12) and by Fc $\gamma$ RI cross-linking in myeloid cells (13).

Phosphorylation may be the final outcome of the interaction of Raf-1 with other macromolecules. However, the range of the effects of these interactions is quite diverse. Thus, it has been suggested that the interaction of Raf-1 with 14-3-3 (14–16), Hsp90-p50<sup>Cdc37</sup> (17–20), or Ras (21–23) may promote the proper folding of the kinase and may enhance its stability. Moreover, the interaction of Raf-1 with Ras may be responsible for the translocation of Raf-1 to the plasma membrane (24, 25) while the interaction of Raf-1 with Ras or 14-3-3 may promote its oligomerization (26, 27).

The mechanisms by which these interacting macromolecules regulate the activation of Raf-1 are overlapping and interdependent, as exemplified by the interaction of Raf-1 with Ras. The GTP-charged form of Ras binds directly to the amino-terminal conserved region 1 which includes the Ras-binding domain and the cysteine-rich domain of Raf-1 (28–31). This interaction causes translocation of Raf-1 from the cytosol to the plasma membrane where Raf-1 activation takes place (24, 25). The nature of the membrane events and the macromolecules involved in Raf-1 activation have not been well defined to date. One such mechanism, however, may involve binding of Raf-1 to the oligomer forming adaptor protein 14-3-3 (32). Binding to this protein may induce oligomerization and activation of Raf-1 via auto- or trans-phosphorylation.

To study the role of Raf-1 in the transduction of downstream signals we need not only to understand its regulation but also to know its targets. Despite intensive efforts, the only known physiological substrate of Raf-1 to date is MEK (33–35). A recent report has shown that Raf-1 also phosphorylates the phosphatase Cdc25A, a cell cycle regulator, and that phosphorylation enhances the Cdc25A phosphatase activity *in vitro* (36). In addition, Raf-1 was found to phosphorylate Bad, a pro-apoptotic Bcl-2 family member, and may inhibit Bad-in-

Following stimulation of membrane receptors, the Raf-1 kinase is activated and transduces signals to several signaling pathways (1–3). The molecular mechanism of Raf-1 activation is not well understood but appears to be under the control of a host of Raf-1-interacting macromolecules including both proteins and phospholipids (2, 4).

One of the mechanisms by which interacting macromolecules

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duced apoptosis (37). However, whether Cdc25A and Bad can function as physiological downstream effectors of Raf-1 remains to be determined.

The purpose of the work presented here was to identify novel Raf-1 interacting proteins and to determine their role in Raf-1 regulation and signaling. Screening a yeast two-hybrid cDNA library derived from mouse thymus RNA, for such proteins led to the isolation of a novel ankyrin repeat protein, Tvl-1 (from the Greek word *touvlo* which means brick). Tvl-1 interacts with Raf-1 through its carboxyl-terminal ankyrin repeat domain. Similar to 14-3-3, Tvl-1 also forms oligomers. However, in contrast to 14-3-3, Tvl-1 is phosphorylated by Raf-1. Co-expression of Raf-1, Src, and Ras with Tvl-1 in Sf9 cells reproducibly enhanced Raf-1 activity by 3–4-fold. Furthermore, overexpression of Tvl-1 in COS-1 cells enhanced the EGF<sup>1</sup>-induced Raf-1 activation. These findings suggest that Tvl-1 may contribute to the regulation of Raf-1 activation as well as to the transduction of Raf-1 signals.

#### EXPERIMENTAL PROCEDURES

**Yeast Interaction Trap**—The open reading frame of human *Raf-1* (from D. Morrison) was inserted into the pNlex vector so as to be expressed as a fusion with the DNA-binding domain of LexA, a bacterial transcriptional repressor (38). A murine CD4<sup>+</sup> T cell cDNA library (from Stratagene) was subcloned into the expression vector pJG4-5, which utilizes the galactose-inducible *GAL-1* promoter to express library clones as fusions to a nuclear localization sequence, a portable transcriptional activation domain (the “acid blob”, B42), and a hemagglutinin epitope tag (39). Bait and library plasmids were transformed into the yeast strain EGY48/pSH18-34, in which the upstream regulatory elements of the chromosomal *LEU2* gene have been replaced by three copies of the LexA operator and which carries the reporter plasmid SH18-34 containing the *LacZ* gene also under the control of LexA operators. Library clones encoding proteins interacting with Raf-1 were identified based on their ability to activate the LEU2 and LacZ reporters, thus enabling yeast cells to grow and form blue colonies in leucine-free, 5-bromo-4-chloro-3-indoyl  $\beta$ -D-galactoside containing medium.

To determine the specificity of the interaction between Tvl-1 and Raf-1, we carried out yeast interaction mating assays as described (40, 41). Briefly, EGY48 (MAT  $\alpha$ ) haploid cells harboring the plasmid expressing the acid blob-Tvl-1 chimera were mated with a panel of haploid RFY206 (MAT  $a$ ) cells harboring constructs encoding LexA fusion proteins. Besides LexA-Raf-1, the LexA fusions included the constitutively active Raf-1 mutant, Raf-1 Y340D, the amino-terminal 330 amino acids of Raf-1, and 27 other proteins that function in pathways regulating cell proliferation. As in the original screen, the interaction of Tvl-1 with these proteins was determined on the activation of the *LEU2* and *LacZ* reporter genes.

**RNA Isolation, Northern Blotting, and cDNA Libraries**—Poly(A)<sup>+</sup> RNA was isolated using the method of Chomczynski and Sacchi (42). Northern blots were hybridized with the partial *Tvl-1* cDNA probe cloned from the interaction trap library. The same probe was used to screen a murine CD4<sup>+</sup> T cell cDNA library (Stratagene, CA) and a cDNA library constructed by us from rat spleen mRNA. The rat spleen cDNA library was constructed using the Stratagene cDNA cloning kit according to the protocol provided by the manufacturer.

**Mammalian Cell Lines and Transfections**—COS-1 and CV1 cells were obtained from the American Type Tissue Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin (50 units/ml), streptomycin (50  $\mu$ g/ml), and kanamycin (100  $\mu$ g/ml) (PSK). NIH 3T3 cells cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and PSK. NB2, a prolactin-dependent rat T cell lymphoma line was provided by P. Gout (University of British Columbia). NB2 cells were grown in RPMI 1640 media supplemented with fetal bovine serum (10%) and PSK. COS-1 cells were transiently transfected using the DEAE-dextran/chloroquine method as described previously (43). NIH 3T3 cells were transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's protocol.

**Expression Constructs**—A Tvl-1 expression construct was generated by subcloning the full-length *Tvl-1* cDNA insert into the pCMV5 expression vector (44). FLAG epitope-tagged Tvl-1 (FLAG-Tvl-1) and hemagglutinin (HA) epitope-tagged Tvl-1 (HA-Tvl-1) constructs were generated by inserting the Tvl-1 coding sequence in-frame with the FLAG or the HA epitope tag into the pCMV-5 expression vector. A series of truncated Tvl-1 expression constructs were generated by cloning cDNA fragments of truncated Tvl-1, epitope tagged at their amino terminus with FLAG, into the pcDNA3 expression vector (Invitrogen). CMV-Raf-1 expression constructs were generated by inserting the wild type or constitutively active Raf-1(Y340D) mutant cDNA (7) into the pCMV5 vector. To generate plasmids expressing GST fusion proteins of Raf-1 and its truncated mutants, the cDNA inserts of the full-length Raf-1, its NH<sub>2</sub>-terminal domain (1–323 amino acids) and its COOH-terminal catalytic domain (305–648 amino acids) were, respectively, subcloned into the pEBG expression vector which encodes glutathione *S*-transferase driven by EF1 $\alpha$  promoter (20, 32).

Baculoviruses expressing Tvl-1, the constitutively active Raf-1 mutant Y340D, and the kinase-negative Raf-1 mutant K375M were generated by ligating the appropriate inserts into the transfer vector pVL1393 (PharMingen). The resulting constructs were transfected into Sf9 cells using the Baculogold Transfection Kit (PharMingen). Baculoviruses expressing Raf-1, v-Src, and v-Ha-Ras were provided by Dr. J. Chernoff (Fox Chase Cancer Center).

**Antibodies**—Rabbits were inoculated with (His)<sub>6</sub>-tagged Tvl-1 protein purified from *Escherichia coli* strain M15 (Qiagen) transformed with the construct pQE30-Tvl-1. pQE30-Tvl-1 was constructed by inserting the coding sequence of the full-length Tvl-1 cDNA into the bacterial expression vector pQE30 (Qiagen). *E. coli* transformed with this construct were grown at 37 °C in the presence of ampicillin (50  $\mu$ g/ml) and kanamycin (25  $\mu$ g/ml) to OD<sub>600</sub> = 0.7. Protein expression was induced by growing cells in 0.5 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside at 25 °C for 5 h. Bacteria were collected, resuspended in 10 mM Tris (pH 7.6), 0.5 M NaCl, 0.1% Nonidet P-40, 10% glycerol, 4 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and lysed by sonication on ice. His-tagged Tvl-1 was collected by incubating the clarified lysate with Ni<sup>2+</sup>-NTA resin (Qiagen). The resin beads were washed with lysis buffer containing 25 mM imidazole. The proteins were eluted from the resin by incubation with 100 mM imidazole. His-tagged Tvl-1 isolated by this procedure was shown to be at least 85% pure as determined by Coomassie Brilliant Blue staining of the eluted proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE). Tvl-1 protein was excised from the gel and inoculated into rabbits. A second rabbit polyclonal antibody was raised against a Tvl-1-derived multiple antigen peptide (amino acids 100–113) (synthesized by Research Genetics Inc., Huntsville, AL) according to standard protocols.

Rabbit polyclonal antibody against the carboxyl terminus of Raf-1 (C12) was purchased from Santa Cruz Biotechnologies. An anti-Raf-1 monoclonal antibody (R19120) was purchased from Transduction Labs. The M2 anti-FLAG monoclonal antibody was purchased from Kodak IBI. Anti-HA mouse monoclonal antibody 12CA5 was purchased from Babco.

**Immunoprecipitations and Western Blotting**—Cultured cells were lysed in Nonidet P-40 lysis buffer (20 mM Hepes, pH 7.6, 137 mM NaCl, 0.5% Nonidet P-40, 2 mM EGTA, 10% glycerol, 5  $\mu$ g/ml leupeptin, 5  $\mu$ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium fluoride, 1 mM sodium vanadate). Cell lysates were clarified by centrifugation at 4 °C for 10 min at 12,000  $\times g$  and were precleared by a 30-min incubation with protein A-agarose beads (Life Technologies, Inc.). Immunoprecipitations were carried out by incubating these lysates with the appropriate antibodies for 3 h. The resulting antigen-antibody complexes were collected with protein A- or protein G-agarose beads. The immunoprecipitates were washed three to four times with cold Nonidet P-40 lysis buffer prior to their analysis by SDS-PAGE. Western blots of the immunoprecipitates or of total cell lysates were carried out using Immobilon-P membranes (Millipore).

**Immunofluorescence**—NIH 3T3 cells seeded on glass coverslips were transfected with pCMV5-FLAG-Tvl-1 using LipofectAMINE (Life Technologies, Inc.). Thirty-six hours after transfection, the cells were washed twice with phosphate-buffered saline (PBS), fixed with 3.7% paraformaldehyde (pH 7.4), and permeabilized with 0.1% Triton X-100 in PBS. To block nonspecific antibody binding, the cells were incubated in 10% goat serum in PBS for 60 min at room temperature, prior to being incubated for 60 min with the M2 mouse monoclonal anti-FLAG antibody or the rabbit anti-Tvl-1 antiserum (1:400 dilution in 5% goat serum). Following washing with PBS, the cells were incubated for 30 min with fluorescein isothiocyanate-conjugated goat anti-mouse or anti-rabbit antibodies (Sigma, dilution at 1:400) plus 1  $\mu$ g/ml bisbenzimid

<sup>1</sup> The abbreviations used are: EGF, epidermal growth factor; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

(Hoechst 33258, Sigma). At the end, the coverslips were washed twice in PBS and mounted on glass slides with anti-fade medium (90% glycerol with 1 mg/ml paraphenylene diamine). Fluorescence was recorded by confocal microscopy.

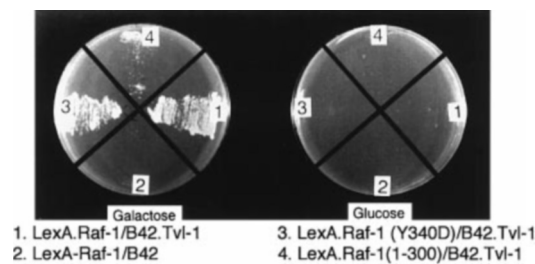
**Protein Kinase Assays in Sf9 Cells**—Raf-1 kinase assays were performed using standard procedures (45). Briefly,  $2 \times 10^6$  Sf9 cells in 60-mm Petri dishes were infected with the desired combination of baculoviruses. After 48 h, cells were washed twice in cold PBS and lysed in the Nonidet P-40 lysis buffer for Raf/Tvl-1 co-immunoprecipitation or in RIPA buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2  $\mu$ g/ml aprotin/leupeptin, 5 mM sodium fluoride, and 5 mM sodium vanadate). Insoluble material was removed by centrifugation at  $12,000 \times g$  for 10 min at 4 °C. Clarified lysates were incubated with rabbit anti-Raf-1 antibody (C12, Santa Cruz Biotechnology) for 3 h at 4 °C, followed by incubation with protein A-agarose beads for 1 h at 4 °C to collect the antigen-antibody complexes. Immunoprecipitates were washed twice with lysis buffers and twice with kinase buffer containing 30 mM Hepes (pH 7.5), 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, and 1 mM dithiothreitol. Kinase reactions were carried out by incubating the immune complexes for 20 min at 25 °C in 40  $\mu$ l of Raf-1 kinase buffer containing 10  $\mu$ M unlabeled ATP, 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (Amersham), and 0.2  $\mu$ g of purified recombinant His-tagged Tvl-1 (His-Tvl-1) or His-tagged kinase inactive MEK (His-MEK (K<sup>-</sup>)). His-MEK (K<sup>-</sup>) was induced and purified as described by Gardner *et al.* (46). Kinase assays using Raf-1 and co-immunoprecipitated Tvl-1 were carried out without added exogenous Tvl-1. Kinase reactions were stopped by adding 3 $\times$  SDS sample buffer (120 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 3% SDS, 10% glycerol, 0.02% bromphenol blue). The products of the kinase reactions were then resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore), and visualized by autoradiography.

**Protein Kinase Assays in COS-1 Cells**—Raf-1 kinase assays in COS-1 cells were performed as described previously (20, 26). Briefly, COS-1 cells were transfected with a pEBG-Raf-1 expression construct (20, 26) alone or in combination with the CMV-Tvl-1 expression construct. Twenty-four hours later, cells were serum-starved for 20 h, and then treated with EGF (50 ng/ml, R & D) for 20 min. Cells were lysed using the Triton X-100 lysis buffer (50 mM Tris-Cl, pH 7.6, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 2 mM sodium vanadate, 20 mM  $\beta$ -glycerophosphate, and protease inhibitors). GST-Raf was pulled down by incubating the lysates with glutathione-Sepharose beads (Pharmacia) at 4 °C for 3 h. The beads were washed twice with lysis buffer, twice with lysis buffer containing 0.5 M LiCl, and twice with kinase buffer (40 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol). The Raf kinase reaction was carried out by incubating the pulled down Raf-1 in 60  $\mu$ l of the kinase reaction buffer containing 100  $\mu$ M ATP, 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, and 200 ng of recombinant kinase-dead His-MEK1. Incubations were carried out at 25 °C for 30 min. The products of the kinase reaction were analyzed by SDS-PAGE and autoradiography.

**Metabolic Labeling and Phosphopeptide Analysis**—Two-dimensional phosphopeptide analysis was carried out as described by Boyle *et al.* (47). Briefly, COS-1 cells were transfected with the CMV-Tvl-1 expression construct alone or in combination with the CMV-Raf-1 (Y340D) expression construct. At 24 h after transfection, the cells were cultured in serum-free medium. Sixteen hours later, cells were cultured for 4 h in phosphate-free Dulbecco's modified Eagle's medium containing 1 mCi/ml ortho-[<sup>32</sup>P]phosphate (NEN Life Science Products Inc.). Thirty minutes prior to lysis, half of the cells transfected only with the Tvl-1 expression construct were stimulated with 10% fetal calf serum. <sup>32</sup>P-Tvl-1 was immunoprecipitated from the metabolically labeled cells using rabbit anti-Tvl-1 antiserum and electroblotted onto nitrocellulose membranes. The Tvl-1 protein band excised from the membrane was digested with trypsin. The resulting phosphopeptides were separated using a Hunter thin layer peptide mapping electrophoresis apparatus (CBS Scientific Company, Inc., Del Mar, CA) and visualized by autoradiography.

## RESULTS

**Interaction Trap and Interaction Mating Assays—Identify a Ubiquitously Expressed Ankyrin Repeat Protein, Tvl-1, That Specifically Binds Raf-1**—To identify novel proteins that interact with Raf-1 we screened a CD4<sup>+</sup> murine T cell cDNA library using the interaction trap two-hybrid system in the yeast *Saccharomyces cerevisiae*, as described by Gyuris *et al.* (39). Out of



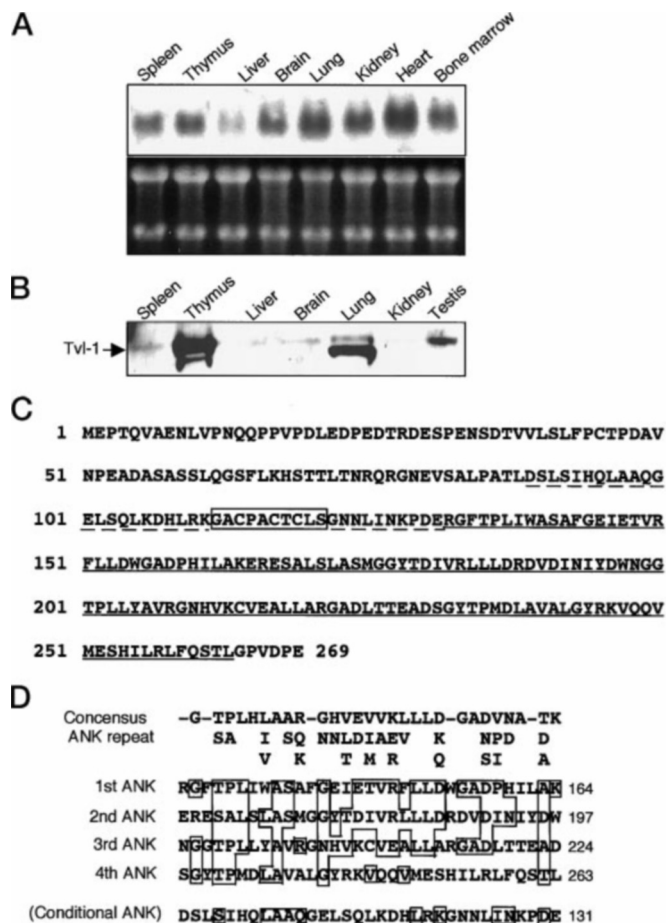
**FIG. 1. Tvl-1 interacts with Raf-1 in the yeast two-hybrid system.** A, B42-Tvl-1 interacts with LexA fusions of Raf-1 (1), Raf-1 Y340D (3) and, less efficiently, with Raf-1(1–330) (4). However, the acid blob B42 alone does not interact with LexA-Raf-1 (2). The observed interactions occurred only in the presence of galactose. The intensity of the interactions of Raf-1 with Tvl-1 and c-Ha-Ras in the yeast two-hybrid system were comparable (data not shown).

50 isolated clones, 15 were sequenced and found to be derived from a single gene, *Tvl-1* (data not shown). The specificity of interaction of the B42-Tvl-1 fusion with Raf-1 was confirmed by the experiment in Fig. 1 which shows that growth is galactose and therefore B42-Tvl-1-dependent and that a full-length Raf-1 interacts with B42-Tvl-1 but not with the B42 domain alone. The same figure shows that Tvl-1 also interacts with the amino-terminal domain of Raf-1 (amino acids 1–330) although with lower efficiency.

To determine whether Tvl-1 interacts with other signaling proteins we carried out interaction mating experiments (40, 41) between EGY48 MAT  $\alpha$  haploid cells harboring the construct encoding the fusion protein B42-Tvl-1 and a panel of 26 RFY206 MAT  $\alpha$  haploid cells harboring constructs encoding LexA fusion proteins. The results showed that Tvl-1 does not interact with *cdc2* (*cdk1*), *cdk2*, c-Ha-Ras, c-Ha-Ras/C186A, Rb, ftz, SSN6, CDI1, daughterless, goosecoid, CDK3, CDI7, CDI11, Dmcdc2, LAR, cyclin C, cyclin E, CLN3, hairy, MYC, MAX, Mxi-1, p53, thyroid hormone receptor, bicoid, and a synthetic polyglutamine peptide (data not shown). These results indicated that the interaction of Tvl-1 with Raf-1 is specific.

Hybridization of a *Tvl-1*-specific cDNA probe to a Northern blot of poly(A)<sup>+</sup> RNA from normal adult mouse tissues revealed that *Tvl-1* mRNA is widely expressed (Fig. 2A). Western blots of lysates from normal adult mouse tissues probed with a Tvl-1-specific antibody raised against bacterially expressed Tvl-1 protein (Fig. 2B, "Experimental Procedures") revealed that Tvl-1 is expressed primarily in thymus, lung, and testes. The levels of *Tvl-1* mRNA and protein in different tissues correlate only partially, suggesting that protein expression of Tvl-1 may be regulated in part at the post-transcriptional level.

Sequencing of full-length cDNA clones obtained by screening a murine CD4<sup>+</sup> T cell cDNA library revealed that *Tvl-1* encodes a novel 269-amino acid protein containing four ankyrin repeats (48, 49) at its carboxyl terminus (Fig. 2, C and D). Some of the cDNA clones contain a small internal deletion. These clones represent a differentially spliced mRNA species that, when translated, is expected to give rise to a short Tvl-1 protein (deletion marked by a box in Fig. 2C). Deletion of the indicated 10 amino acids generates a potential 5th ankyrin repeat motif that is located upstream of the first one (Fig. 2, C and D). Since both the long and short forms of Tvl-1 interact with Raf-1 (data not shown), the significance of the two forms is currently unknown. Sequencing a full-length cDNA clone isolated from a rat spleen cDNA library showed that the mouse and rat genes exhibit 95% identity at the amino acid level. Screening existing data bases for *Tvl-1*-related sequences identified two overlapping human cosmid clones (R2770 and F14150) which map to human chromosome 19p12 and contain the entire sequence of the human Tvl-1 homologue (accession numbers AC002126 and



**FIG. 2. Tvl-1 is widely expressed and encodes a novel ankyrin repeat protein.** *A*, upper panel: Northern blot analysis of 5  $\mu$ g of poly(A)<sup>+</sup> RNA isolated from the indicated normal adult mouse tissues and probed with <sup>32</sup>P-labeled Tvl-1 cDNA. Lower panel: profile of the ethidium bromide gel of the Northern blot. *B*, Western blot of total cell lysates from normal mouse tissues probed with a polyclonal rabbit antiserum raised against bacterially expressed His-tagged-Tvl-1 protein. While Tvl-1 RNA is expressed at similar levels in all tissues, Tvl-1 protein is expressed at higher levels in thymus, lung, and testis. The observed 33-kDa protein band is specific for Tvl-1 because: (a) it comigrates with the Tvl-1 protein encoded by a CMV-Tvl-1 expression construct transfected into COS-1 cells and (b) detection of this band was competed by preadsorption of the anti-Tvl-1 antiserum with the recombinant Tvl-1 protein (data not shown). *C*, amino acid sequence of the murine Tvl-1 protein deduced from the nucleotide sequence of clone 19 isolated from a murine CD4<sup>+</sup> T cell cDNA library (Stratagene). The underlined sequence defines the ankyrin repeat region. The boxed sequence starting at amino acid 112 is absent from some of the cDNA clones. This is due to differential splicing (data not shown). *D*, alignment of the four ankyrin repeats (ANK) in the COOH-terminal portion of Tvl-1 with the consensus ankyrin repeat sequence (48, 49). The Tvl-1 protein encoded by the differentially spliced mRNA contains a 5th ankyrin repeat motif (conditional ANK and the dashed line in *C*) that is generated following deletion of the boxed 10-amino acid region shown in *C*.

AC003110). The same clone contains a myocyte enhancer-binding factor *MEF2B* (50, 51) upstream of *Tvl-1* in a head to head orientation with *Tvl-1*. Human and mouse Tvl-1 share 85% identity at the amino acid level (data not shown).

The *Tvl-1* clones isolated from the two-hybrid cDNA library encode protein fusions between the COOH-terminal two-thirds of Tvl-1 (amino acids 81–269) and the activation domain B42. Following cloning of the full-length *Tvl-1* gene, we showed that while the full-length protein also interacts with Raf-1, its NH<sub>2</sub>-terminal portion (amino acids 1–96) does not (data not shown). Therefore, the interaction between Raf-1 and Tvl-1 is mediated by the carboxyl-terminal domain of Tvl-1.

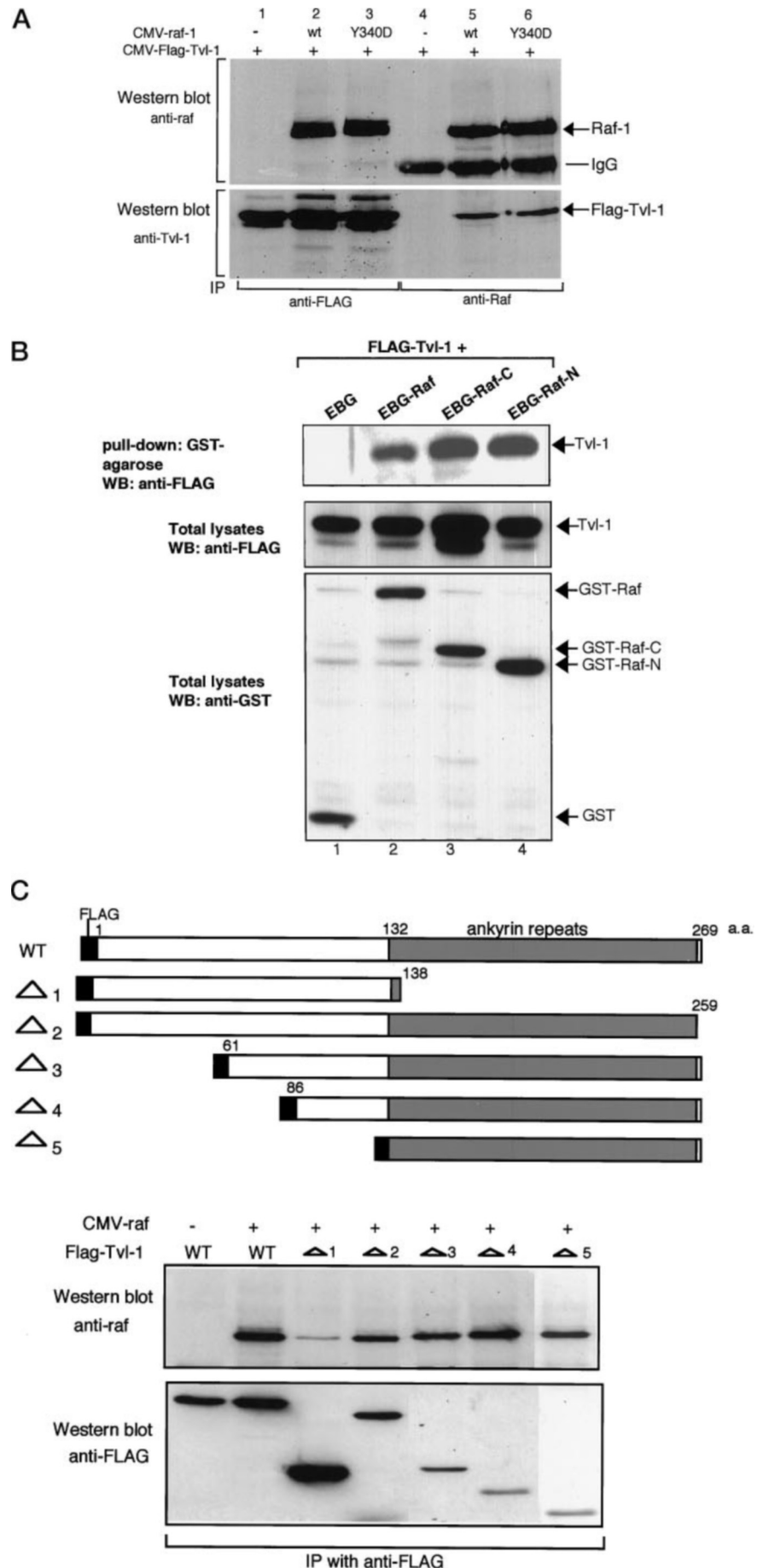
**Tvl-1 Co-immunoprecipitates with Raf-1**—To determine whether Tvl-1 interacts with Raf-1 in mammalian cells, expression constructs of Tvl-1 tagged at their amino terminus with a FLAG epitope tag (FLAG-Tvl-1) and wild type Raf-1 or activated Raf-1 (Y340D) were co-transfected into COS-1 cells in the combinations shown in Fig. 3A. Forty-eight hours later, Tvl-1 or Raf-1 were immunoprecipitated from Nonidet P-40 lysates of the transfected COS-1 cells, with anti-FLAG or anti-Raf antibodies, respectively. Co-precipitating proteins were detected by probing Western blots of the immunoprecipitates with anti-Raf-1 and anti-Tvl-1 antibodies. The results confirmed that Tvl-1 interacts with both the wild type and activated forms of Raf-1 in mammalian cells (Fig. 3A). Similar experiments using NH<sub>2</sub>- or COOH-terminal truncated Raf-1 expression constructs revealed that both truncated Raf-1 proteins interact equally well with Tvl-1 (Fig. 3B). Therefore, Tvl-1 interacts with multiple sites in the NH<sub>2</sub>- and COOH-terminal domains of Raf-1. Moreover, since the kinase-dead Raf-1K375M also binds Tvl-1 (data not shown), the interaction between the two proteins is independent of the Raf-1 kinase activity.

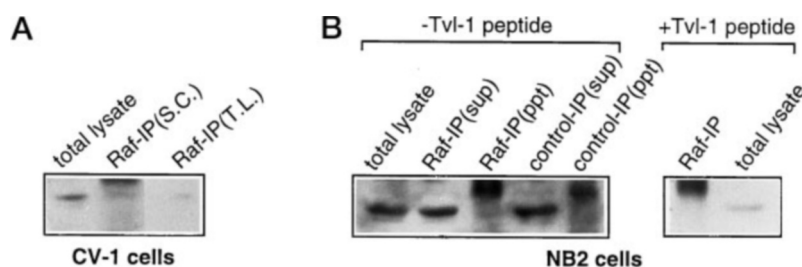
To map the Tvl-1 domain that interacts with Raf-1, a series of Tvl-1 deletion mutants in-frame with an amino-terminal FLAG tag were constructed and cloned in the pCMV5 expression vector. Co-transfection of these constructs with Raf-1 in COS-1 cells (Fig. 3C) confirmed the results of the two-hybrid experiments in yeast by showing that it is indeed the carboxyl-terminal ankyrin repeat region of Tvl-1 that interacts with Raf-1.

The preceding experiments were carried out using lysates of transiently transfected COS-1 cells that express high levels of both Tvl-1 and Raf-1. To determine whether the two proteins interact also when expressed at physiological levels, we immunoprecipitated Raf-1 from Nonidet P-40 lysates of CV-1 and NB2 cells, two cell lines that express both proteins. Immunoprecipitations were carried out using an anti-Raf-1 antiserum raised against the Raf-1 peptide C12 (Santa Cruz) or the monoclonal anti-Raf-1 antibody (R19120, Transduction Labs) (Fig. 4). Control immunoprecipitations of the NB2 cell lysates were also carried out using a control antibody against the interleukin-9 receptor. The immunoprecipitates were subjected to SDS-PAGE, and following Western blotting, they were probed with a rabbit antiserum raised against the full-length Tvl-1 protein (Fig. 4A). In the NB2 cell experiment Western blots were probed with the anti-Tvl-1 antibody raised against the Tvl-1 peptide (amino acids 100–113) in the presence or absence of excess peptide (Fig. 4B). The results (Fig. 4, A and B) showed that endogenous Tvl-1 co-immunoprecipitates with endogenously expressed Raf-1 from lysates of both CV-1 and NB2 cells.

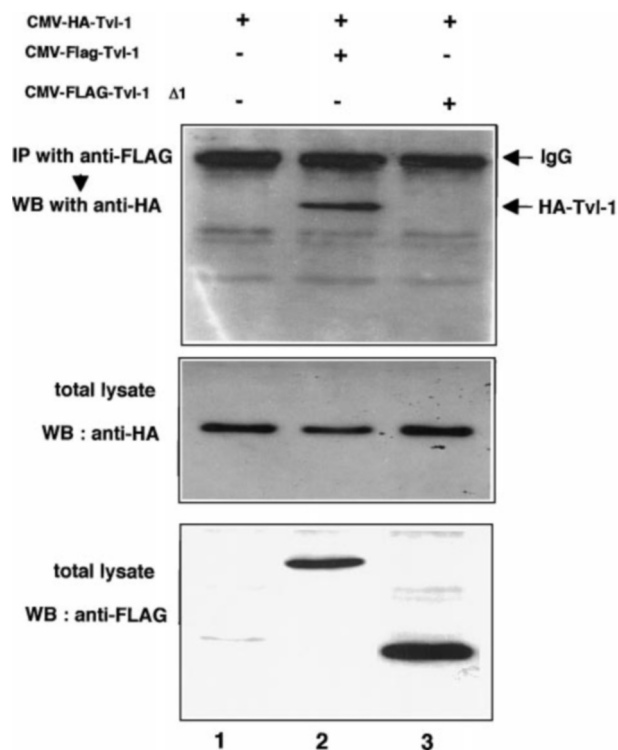
**Tvl-1 Expressed in Transiently Transfected COS-1 Cells Forms Homodimers**—Notch, an ankyrin repeat-containing protein has been shown to oligomerize via homotypic intermolecular interactions between ankyrin repeats (52). To determine whether Tvl-1, also an ankyrin repeat protein, homodimerizes, expression constructs of HA-tagged and FLAG-tagged Tvl-1 were co-transfected into COS-1 cells. Tvl-1 was immunoprecipitated using the anti-FLAG antibody from Nonidet P-40 lysates of the transfected cells harvested 48 h later. Following SDS-PAGE and Western blotting, the resulting immunoprecipitates were probed with the anti-HA antibody. The results (Fig. 5) revealed that Tvl-1 molecules indeed homodimerize. Since HA-Tvl-1 and a FLAG-tagged Tvl-1 deletion lacking the ankyrin repeat domain do not dimerize (Fig. 5, lane 3), we conclude that homodimerization is likely to be mediated by the ankyrin repeat region.

**FIG. 3. Tvl-1 interacts with Raf-1.** *A*, COS-1 cells were transfected with FLAG-tagged Tvl-1, wild type Raf-1 (*wt*) and constitutively active Raf-1 (*Y340D*) in the vector pCMV5. Cells were lysed 48 h later, and FLAG-Tvl-1 was immunoprecipitated (*IP*) with the anti-FLAG M2 monoclonal antibody (*lanes 1–3*). Raf-1 and Flag-Tvl-1 were detected in the immunoprecipitates by Western blotting using rabbit anti-raf antiserum (*C12*, *upper part*) and rabbit anti-Tvl-1 antiserum (*lower part*). COS-1 cell lysates were also immunoprecipitated with the anti-raf-1 C12 antibody which detects very weakly the endogenous Raf-1 protein of COS-1 cells. Western blots of these immunoprecipitates were probed with the same anti-raf-1 and anti-Tvl-1 antibodies (*lanes 4–6*). *B*, both NH<sub>2</sub>-terminal and COOH-terminal domains of Raf-1 interact with Tvl-1. The EBG-based expression constructs of full-length (*lane 2*), NH<sub>2</sub>-terminal (*lane 4*) and COOH-terminal (*lane 3*) domains of Raf-1 were co-transfected with the FLAG-tagged Tvl-1 expression construct into COS-1 cells. GST-fusion proteins from transfected cell lysates were pulled down and Western blotted with the anti-FLAG monoclonal antibody (*top panel*). Total cell lysates were also immunoblotted with anti-FLAG (*middle panel*) and anti-GST antibodies (*bottom panel*). *C*, Mapping the Raf-1 interacting Tvl-1 domains. *Upper panel*, schematic representation of FLAG-tagged wild-type Tvl-1 (*WT*) and five Tvl-1 deletion mutants. *Lower panel*, co-immunoprecipitation of Raf-1 and the FLAG-tagged Tvl-1 deletion mutants (shown in the *upper panel*) from COS-1 cells transfected with the indicated expression constructs. Cell lysates were immunoprecipitated with anti-FLAG M2 monoclonal antibody, Western blotted either with the rabbit anti-Raf-1 C12 antibody (*upper panel*), or with the rabbit anti-FLAG antibody (*lower panel*). Note that the interaction between Raf-1 and the Tvl-1Δ1 mutant representing the NH<sub>2</sub>-terminal domain of Tvl-1 was very weak.





**FIG. 4. Interaction of endogenous Raf-1 and Tvl-1.** *Panel A*, Raf-1 was immunoprecipitated from lysates of CV-1 cells using the anti-Raf peptide antiserum (C12) from Santa Cruz (S.C., lane 2) or the anti-Raf-1 monoclonal antibody from Transduction Labs (T.L., lane 3). A Western blot of the resulting immunoprecipitates was probed with the anti-Tvl-1 antiserum. Co-immunoprecipitating protein band comigrates with Tvl-1 in total cell lysates. *Panel B*, Raf-1 was immunoprecipitated from NB2 Cell lysates using anti-Raf C12 antibody (Raf-IP, lanes 2 and 3). Anti-interleukin 9 receptor antibody was used as a negative control (control-IP, lanes 4 and 5). Both the immunoprecipitates (ppt) and the supernatants (sup) of the immunoprecipitation reactions were Western blotted with an anti-Tvl-1 peptide antiserum in the absence (-) as well as in the presence (+) of excess peptide. Note that the protein band comigrating with Tvl-1 (Raf-IP, ppt; lane 3) from anti-Raf-1 immunoprecipitates was absent from the control anti-IL-9 receptor immunoprecipitate (control-IP, ppt; lane 5). Moreover, this Tvl-1 band was detected only in the absence of peptide competition (compare lanes 3 and 6).



**FIG. 5. Tvl-1 expressed in transiently transfected COS-1 cells homodimerizes.** COS-1 cells were transfected with the indicated combinations of HA-tagged wild type Tvl-1 and FLAG-tagged wild type or COOH-terminal truncated Tvl-1 (FLAG-Tvl-1 $\Delta 1$ , Fig. 3C) expression constructs. Anti-FLAG immunoprecipitates of transfected cell lysates were Western blotted with the anti-HA monoclonal antibody (*upper panel*). The *lower two panels* show the Western blots of total lysates derived from the same cells and probed with anti-HA or anti-FLAG monoclonal antibodies.

**Subcellular Localization of Tvl-1**—To define the subcellular localization of Tvl-1, we transfected NIH 3T3 cells with a FLAG-tagged Tvl-1 construct, either transiently or stably, and we stained the transfected cells with the anti-FLAG antibody M2. The results (Fig. 6) revealed that Tvl-1 is present in both the cytoplasm and the nucleus. Similar results were obtained using affinity purified anti-Tvl-1 antibody instead of the FLAG antibody (data not shown). The detection of Tvl-1 in the cytoplasm is compatible with its interaction with Raf-1, a cytoplasmic protein. However, since Tvl-1 is also present in the nucleus, it is likely that it may interact with nuclear proteins and may contribute to the regulation of nuclear functions.

**Raf-1 Phosphorylates Tvl-1 in Vitro and in Vivo**—To determine whether Tvl-1 serves as a Raf-1 substrate we first carried

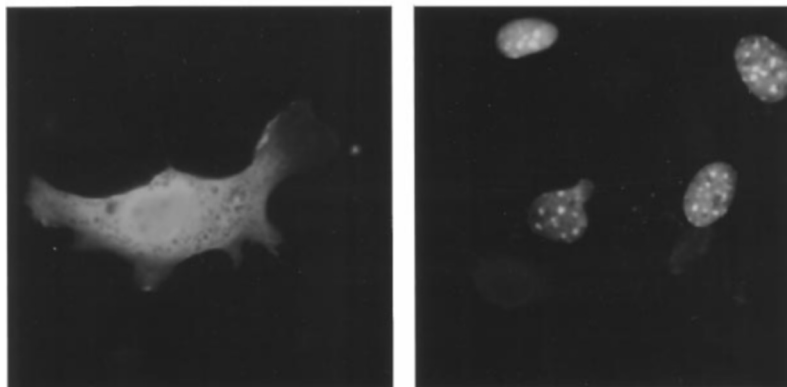
out *in vitro* kinase assays using Raf-1 immunoprecipitated from baculovirus-infected Sf9 cells co-expressing Raf-1 and Tvl-1. The kinase inactive mutant of Raf-1, K375M, and the activated mutant of Raf-1, Y340D, were used as controls. Wild type Raf-1 was activated by co-expression of v-Ha-Ras and v-Src (Fig. 7A). In a separate experiment, bacterially expressed (His)<sub>6</sub>-Tvl-1 was added as the exogenous substrate in Raf-1 *in vitro* kinase assays. The results in Fig. 7, A and B, revealed that Tvl-1 co-immunoprecipitates with, and is phosphorylated only by catalytically active Raf-1. By comparing the degree of phosphorylation of Tvl-1 and MEK-1 by Raf-1 *in vitro*, we found that both proteins served equally well as Raf-1 substrates (data not shown). Inspection of the Tvl-1 sequence revealed a motif SALSLASMGG starting at amino acid 168, which is similar to the MEK-1 motif (L/A)-S-(T/S)-G phosphorylated by Raf-1 and represents a candidate phosphorylation site (53).

To determine whether Raf-1 induces phosphorylation of Tvl-1 in mammalian cells, <sup>32</sup>P-Tvl-1 was immunoprecipitated from ortho[<sup>32</sup>P]phosphate-labeled COS-1 cells expressing Tvl-1 alone or in combination with constitutively active Raf-1 (Y340D). Tvl-1 immunoprecipitated from singly transfected <sup>32</sup>P-labeled cells stimulated with serum for 30 min was used as a control. Immunoprecipitated Tvl-1 was electroblotted onto nitrocellulose membranes, digested with trypsin. The resulting products were separated using a Hunter thin layer peptide mapping electrophoresis apparatus. As shown in Fig. 7C, the tryptic phosphopeptide map of Tvl-1 from COS-1 cells stimulated with serum is very similar, if not identical, to the one from serum-starved COS-1 cells co-expressing Tvl-1 and active Raf-1. These data indicate that Raf-1 indeed phosphorylates Tvl-1 *in vivo* and is the primary kinase to phosphorylate Tvl-1 in the serum-stimulated cells.

**Tvl-1 Potentiates the Activation of Raf-1**—To determine whether Tvl-1 modulates the activity of the Raf-1 kinase we examined the kinase activity of Raf-1 in Sf9 cells co-expressing Tvl-1 and Raf-1 in the presence or absence of v-Src plus v-Ha-Ras. To this end, Sf9 cells were infected with baculoviruses expressing Tvl-1, Raf-1, v-Src, and v-Ha-Ras in the combinations shown in Fig. 8A. Forty-eight hours later the infected cells were lysed in a RIPA lysis buffer. An *in vitro* kinase reaction carried out using Raf-1 immunoprecipitated from these lysates, and histidine-tagged kinase-dead MEK-1 as the exogenous substrate revealed that Tvl-1 potentiates the activation of Raf-1 by Src and Ras. The potentiation of the activation of Raf-1 by Tvl-1 is highly reproducible, with very similar results obtained in three independent experiments. To determine whether Tvl-1 has a similar effect on the activity of the Raf-1 kinase in mammalian cells, we overexpressed Tvl-1 in COS-1 cells and examined the kinase activity of Raf-1 following

anti-FLAG

Hoechst 33258



**FIG. 6. Subcellular localization of Tvl-1.** Immunofluorescence of NIH 3T3 cells transfected transiently with a FLAG-Tvl-1 expression construct and costained with the anti-FLAG monoclonal antibody (*left panel*) and the nucleus staining dye Hoechst 33258 (*right panel*).

overnight serum starvation and EGF stimulation for 20 min. As shown in Fig. 8B, while overexpression of Tvl-1 had no effect on the kinase activity of Raf-1 in unstimulated cells, it significantly enhanced the activation of Raf-1 by EGF. Similar results were obtained in three independent experiments.

#### DISCUSSION

In this report we presented the cloning and initial characterization of a gene (*Tvl-1*) that encodes a novel ankyrin repeat protein that interacts with Raf-1 and is phosphorylated by the Raf-1 kinase. Through its interaction with Raf-1, Tvl-1 also promotes the activation of the Raf-1 kinase by EGF or Src/Ras. The interaction between Tvl-1 and Raf-1 is specific since Tvl-1 failed to interact with other kinases such as cdc2, CDK2, ERK1, Akt2, Akt3, and ILK-1 as well as a series of additional signaling molecules (data not shown). Moreover, Raf-1 failed to co-immunoprecipitate with other ankyrin repeats containing proteins including Cdk inhibitor p16 and ILK-1. Finally, Tvl-1 was shown to interact with Raf-1 not only in cells overexpressing the two proteins, but also in untransfected cells expressing physiological levels of the endogenous proteins. The phosphorylation of Tvl-1 by Raf-1 is also specific in that it is due to Raf-1 and not a contaminating co-immunoprecipitated kinase. Thus, Tvl-1 is not phosphorylated by Raf-1 expressed in the absence of Src and/or v-Ha-Ras, but is phosphorylated by the constitutively active Raf-1 mutant Y340D. Moreover, the kinase-dead mutant Raf-1K375M did not phosphorylate Tvl-1 when immunoprecipitated from lysates of Sf9 cells co-expressing this mutant and v-Ha-Ras plus v-Src. Tvl-1 was detected in both the cytosol and the nucleus of cells transiently transfected with Tvl-1 expression constructs. These findings suggest that the interaction of Tvl-1 with Raf-1 and its consequences are physiologically possible.

Although studied extensively, the molecular mechanism(s) of Raf-1 activation by growth factors remain relatively poorly understood. The process of activation is regulated by the interaction of Raf-1 with a host of cellular proteins, one of which is the Tvl-1 protein described in this report. Tvl-1, similar to other interacting proteins such as 14-3-3, does not activate Raf-1 by itself (32). Instead, it potentiates the activation of Raf-1 by signals transduced by other signaling molecules such as Src/Ras or induced by growth factors such as EGF. Earlier studies had shown that Raf-1 can be activated by both Ras-dependent and independent mechanisms (54–56). The findings presented in this report suggest that Tvl-1, similar to 14-3-3, is involved in the activation of Raf-1 by signals that are Ras-dependent.

Tvl-1 may potentiate the activation of Raf-1 by affecting its conformation. This in turn may influence the association of Raf-1 with other activating molecules. Alternatively, Tvl-1 may

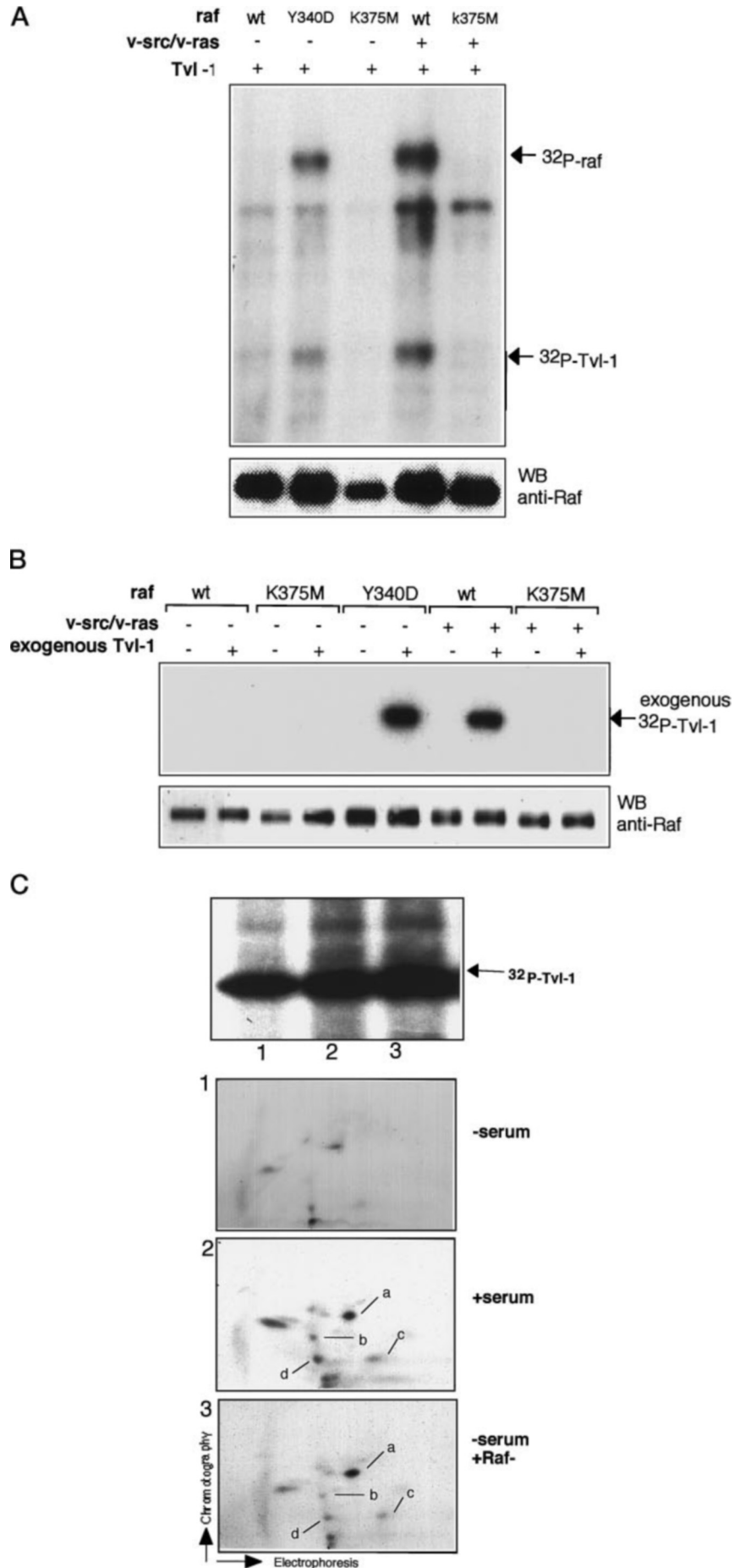
function as a scaffold protein that contributes to the assembly of Raf-1-activating multimolecular complexes, or may contribute to the association of Raf-1 with the plasma membrane. A scaffolding role for Tvl-1 is supported by the fact that it contains multiple ankyrin repeat motifs that function as domains of protein-protein interaction, and oligomerize spontaneously. The potential involvement of Tvl-1 in promoting the association of Raf-1 with the plasma membrane was suggested by findings that other Raf-1-interacting proteins, such as Ras, may enhance Raf-1 activity by this mechanism (24, 25, 57). In the case of Tvl-1 this does not seem to be the case, however, based on two lines of evidence. First, a myristylated form of Tvl-1, which is membrane-associated, failed to enhance the activation of Raf-1 (data not shown). Second, following serum or EGF stimulation, we failed to convincingly detect the membrane translocation of Tvl-1 by immunofluorescence staining (data not shown). We conclude that Tvl-1 either forms a bridge between Raf-1 and other Raf-1 activating proteins or directly affects Raf-1 protein conformation facilitating its activation by other interacting proteins.

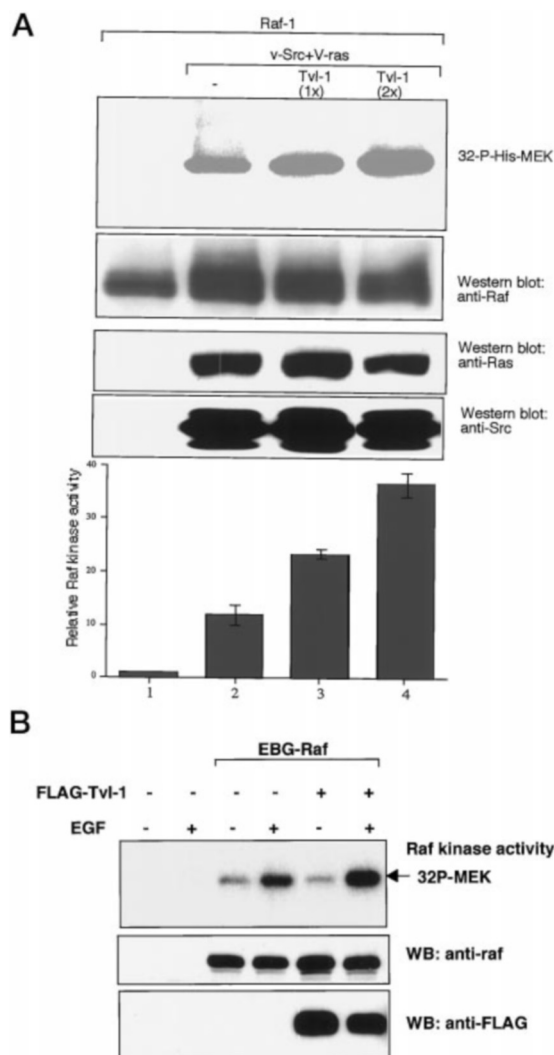
The only other known Raf-1 substrates to date are MEK-1, as well as perhaps Cdc25A and Bad. Phosphorylation of MEK-1 activates the kinase and contributes to the propagation of the signal from Raf-1 to the mitogen-activated protein kinase (33–35, 58). Phosphorylation of Cdc25A may contribute to the activation of this phosphatase (36). Finally, phosphorylation of Bad may prevent its interaction with anti-apoptotic Bcl-2 family members in the mitochondria, thus inhibiting apoptosis (37). The outcome of Tvl-1 phosphorylation is presently unknown. Given that Tvl-1 enhances Raf-1 activation, phosphorylation of Tvl-1 by Raf-1 may represent a feedback inhibitory signal that may alter the binding affinity between the two proteins and promote their dissociation. Alternatively, phosphorylated Tvl-1 may contribute to the transduction of Raf-1 signals. To date we know that Tvl-1 does not activate the mitogen-activated protein kinase in NIH 3T3 cells.<sup>2</sup> It remains to be determined whether Tvl-1 affects the phosphorylation of Cdc25A and Bad by Raf-1, or whether it transduces signals to other growth factor-activated pathways.

A recent report (59) that appeared after the original submission of this manuscript, identified Tvl-1 as a protein that contributes to the assembly of the transcriptional complex regulating the expression of the major histocompatibility complex class II genes. Tvl-1 (named RFXANK) enhances the stability of binding of the transcription factors RFX5 and RFXAP to the sequence in the major histocompatibility complex class II promoter. These findings combined with the data presented here

<sup>2</sup> J-H. Lin and P. N. Tschlis, unpublished results.

**FIG. 7. Tvl-1 is phosphorylated by Raf-1 *in vitro* and *in vivo*.** *A, upper panel: in vitro* kinase assays of Raf-1 immunoprecipitated from Sf9 cells infected with baculoviruses directing the expression of wild type (*wt*) Raf-1, kinase-dead (K375M) Raf-1 or constitutively active (Y340D) Raf-1, v-Src, and v-Ras as well as Tvl-1 as indicated. Tvl-1 co-immunoprecipitating with Raf-1 was highly phosphorylated only in the lysates derived from cells expressing both active Raf-1 and Tvl-1. *Lower panel*, Raf-1 was expressed approximately equally in all the baculovirus-infected cultures of Sf9 cells. *B, upper panel: in vitro* kinase assays of Raf-1 immunoprecipitated from lysates of Sf9 cells, infected with the indicated baculoviruses. Recombinant (His)<sub>6</sub>-Tvl-1 protein purified from *E. coli* was used as the exogenous kinase substrate. *Lower panel*, Western blot of the same Sf9 cell lysates probed with the anti-Raf-1 antibody. *C*, Raf-1 phosphorylates Tvl-1 *in vivo*. In the *top panel*, COS-1 cells transfected with Tvl-1 alone (*lanes 1 and 2*) or in combination with a constitutively active Raf-1-Y340D construct (*lane 3*) were serum-starved overnight and labeled with ortho[<sup>32</sup>P]phosphate for 4 h. The cells in *lane 2* were stimulated with 10% fetal calf serum for 30 min prior to harvesting. Cells were lysed, and the <sup>32</sup>P-labeled Tvl-1 was immunoprecipitated, fractionated by SDS-PAGE, electroblotted onto a nitrocellulose membrane, and visualized by autoradiography. In the *lower three panels*, the <sup>32</sup>P-labeled Tvl-1 bands were cut out from the membrane and digested with trypsin. The resulting tryptic phosphopeptides were separated electrophoretically on a thin-layer cellulose plate (pH 1.9), followed by ascending chromatography in isobutyric acid buffer. The phosphopeptides were visualized by autoradiography. Note that the tryptic peptide map of Tvl-1 from serum-stimulated cells is similar to that from the cells co-expressing active Raf-1(Y340D).





**FIG. 8. Tvl-1 enhances the activation of Raf-1 induced by v-Ras/v-Src in Sf9 cells and by EGF in COS-1 cells.** *A, top panel:* *in vitro* kinase reactions were carried out using Raf-1 immunoprecipitated from lysates of Sf9 cells infected with the indicated baculovirus combinations. Bacterially expressed kinase-dead His<sub>6</sub>-MEK1 was used as the exogenous kinase substrate. *Middle panel:* Western blot of the total infected cell lysates probed with anti-Raf, anti-Ras, and anti-Src antibodies. *Bottom panel:* quantitation of the Raf-1 kinase activity from the top panel by PhosphorImager. *B, COS-1 cells* were transfected with pEBG (lanes 1 and 2) or pEBG-Raf-1 expression construct (lanes 3–6) alone or in combination with the pCMV-FLAG-Tvl-1 expression construct (lanes 4 and 5). Twenty-four hours after transfection, cells were serum-starved overnight, and stimulated with EGF (50 ng/ml) for 20 min. Cells were lysed and GST-Raf-1 was pulled down for kinase assays using recombinant kinase-dead His<sub>6</sub>-MEK1 as the exogenous substrate (*top panel*). Total cell lysates were Western blotted and probed with anti-Raf-1 or anti-FLAG antibody (*lower two panels*). The experiments in both *A* and *B* were repeated three times with similar results.

suggest that indeed Tvl-1 may define a novel class of scaffold proteins that promote the assembly of a variety of macromolecular complexes. In addition, they raise the question whether Raf-1 regulates gene expression by directly phosphorylating the transcriptional adaptor Tvl-1.

Overall, the data presented in this report show that Tvl-1 interacts with Raf-1 in mammalian cells even when it is expressed at natural levels. Although the precise role of this interaction in mammalian cells has not been fully explored, our data from overexpression systems clearly show that Tvl-1 has the potential to both regulate Raf-1 and to function as a Raf-1 downstream target.

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