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Cloning and characterization of mPAL, a novel Shc SH2 domain-binding protein expressed in proliferating cells

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Shc adaptor proteins play a role in linking activated cell surface receptors to the Ras signaling pathway in response to receptor mediated tyrosine kinase activation. While the function of Shc in the activation of the Ras pathway via binding to Grb2 has been well characterized, it is becoming increasingly apparent that Shc participates in additional signaling pathways through interactions with other cytoplasmic proteins. Using the yeast two-hybrid system, we have identified a unique Shc binding protein designated PAL (*P*rotein expressed in *A*ctivated *L*ymphocytes) with no similarity to other known proteins. mPAL binds specifically to the Shc SH2 domain and unlike previously described Shc SH2 domain-protein interactions, the association of mPAL and Shc is phosphotyrosine-independent. Both mPAL RNA and protein expression are restricted to tissues containing actively dividing cells and proliferating cells in culture. mPAL expression is induced upon growth factor stimulation and is down-regulated upon growth inhibition. This pattern, and timing of mPAL expression and its association with the Shc adaptor molecule suggests a role for this protein in signaling pathways governing cell cycle progression.

Keywords: SHC; SH2 domain; phosphotyrosine-independent; cell proliferation

Introduction

The ubiquitously expressed Shc adaptor proteins play a role in coupling growth factor receptor activation to intracellular signaling pathways. The mammalian *Shc* gene encodes three overlapping proteins with molecular weights of 46 kDa, 52 kDa and 66 kDa (Pelicci *et al*., 1992; Bonfini *et al*., 1996; Migliaccio *et al*., 1997). All three protein products share a carboxy terminal Src Homology 2 (SH2) domain, a central glycine/proline rich domain with homology to alpha1 collagen (CH1), and an amino terminal phosphotyrosine binding (PTB) domain. The p52 and p46 isoforms differ only by 46 amino acids at the extreme amino terminus of p52 and are generated by the use of alternative translation initiation sites (Pelicci *et al*., 1992). The p66 isoform is produced via alternative splicing of the *Shc* gene and contains an amino terminal extension which encodes a second collagen homology region (CH2) in addition to the common PTB, CH1, and SH2 domains. Interestingly, it has been demonstrated that the expression of p66 is more restricted and some of its biological properties distinct from those of p52 and p46 Shc (Bonfini *et al*., 1996; Migliaccio *et al*., 1997).

Shc proteins are tyrosine-phosphorylated following activation of receptor tyrosine kinases (van der Geer *et al*., 1994), including the epidermal growth factor receptor (EGFR) (Pelicci *et al*., 1992), platelet-derived growth factor receptor (PDGFR) (Yokote *et al*., 1994), nerve growth factor receptor (TrkA) (Obermeier *et al*., 1994), insulin receptor (Pronk *et al*., 1993) and *erb*B-2 (Segatto *et al*., 1993) as well as receptors that lack intrinsic tyrosine kinase activity such as the T-cell receptor (TCR) (Ravichandran *et al*., 1993), B-cell receptor (Saxton *et al*., 1994), receptors for the interleukins (Burns *et al*., 1993; Cutler *et al*., 1993; Ravichandran and Burakoff, 1994) and the erythropoietin receptor (Damen *et al*., 1993). Additionally, tyrosine phosphorylation of Shc has been detected after activation of G-protein coupled receptors (Cazubon *et al*., 1994; Ptaznik *et al*., 1995; Touhara *et al*., 1995; van Biesen *et al*., 1995; Chen *et al*., 1996), ligation of integrins (Maniero *et al*., 1995; Wary *et al*., 1996) and in cells expressing activated Src, Fps, Sea or Lck (McGlade *et al*., 1992a; Crowe *et al*., 1994; Baldari *et al*., 1995; Pelicci *et al*., 1995a), implicating Shc as an important substrate of cytoplasmic tyrosine kinases.

Shc is able to directly bind to tyrosine-phosphorylated proteins including activated receptor tyrosine kinases by virtue of its PTB or SH2 domains (Pawson, 1995; Bonfini *et al*., 1996). The PTB domain of Shc recognizes tyrosine-phosphorylated peptides in which residues amino terminal to the phosphorylated tyrosine determine binding specificity (Songyang *et al*., 1995; van der Geer *et al*., 1995). The Shc PTB domain has been shown to bind directly to N-P-X-pY sequence motifs in the cytoplasmic domains of the EGFR (Blaikie *et al*., 1994), TrkA (Dikic *et al*., 1995), RET (Lorenzo *et al*., 1997) and the IL-2Rβ chain (Ravichandran *et al*., 1996). The Shc SH2 domain preferentially binds to tyrosine phosphorylated peptides in the sequence context pY-E/I-X-I/L/M (where X represents any amino acid) (Songyang *et al*., 1994) and has been proposed to mediate the binding of Shc to the PDGFR (Yokote *et al*., 1994), EGFR (Pelicci *et al*., 1992), RET (Lorenzo *et al*., 1997), and the CD3 zeta chain of the T-cell receptor (Ravichandran *et al*., 1993). While interactions between the Shc SH2 domain and several membrane receptors have been demonstrated, the physiologic importance of these interac-

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tions is less clear, since the PTB domain appears to be predominant in mediating the interaction of SHC with activated growth factor receptors.

Phosphorylated Shc proteins also associate with the Grb2 adaptor protein by binding of the Grb2 SH2 domain to the phosphorylated tyrosine residues within the CH1 domain of Shc (Rozakis-Adcock *et al*., 1992; Salcini *et al*., 1994). Grb2 is stably associated with the Ras guanine nucleotide exchange factor, SOS (Batzer *et al*., 1993; Buday and Downward, 1993; Chardin *et al*., 1993; Egan *et al*., 1993; Rozakis-Adcock *et al*., 1993) and membrane localization of the Grb2-SOS complex results in activation of Ras (Aronheim *et al*., 1994). Therefore it has been proposed that Shc is involved in coupling cell surface receptors to Ras activation. Several studies on the effects of Shc overexpression provide support for this hypothesis. First, coexpression of a dominant-negative mutant of Ras blocks neurite outgrowth in PC12 cells induced by Shc overexpression (Rozakis-Adcock *et al*., 1992). Second, overexpression of Shc in NIH3T3 fibroblasts results in transformation (Pelicci *et al*., 1992) and this can be abrogated by mutation of one of the presumed Grb2 binding sites (Salcini *et al*., 1994). As well, overexpression of Shc enhances EGF-induced activation of MAP kinases (Migliacciao *et al*., 1997) and cell motility and growth in response to hepatocyte growth factor (Pelicci *et al*., 1995b).

The modular structure of Shc proteins permits their interaction with multiple signaling molecules, suggesting that Shc could function to couple activated receptors to pathways other than Ras. Two additional sites of Shc tyrosine phosphorylation have recently been mapped to tyrosine residues 239 and 240 (Y239/240) (Gotoh *et al*., 1996; Harmer and DeFranco, 1997; van der Geer *et al*., 1996). Tyrosine 239 is present within a Grb2 SH2 domain-binding motif, and has been demonstrated to mediate association with Grb2 *in vivo* (Gotoh *et al*., 1997; Harmer and DeFranco, 1997). The Y239/240 phosphorylation sites may also couple Shc to additional downstream SH2 domain-containing proteins, since phosphopeptides corresponding to Y239/240 have been demonstrated to bind to several unidentified proteins and to the newly identified adaptor protein Gads (van der Geer *et al*., 1996; Liu and McGlade, 1998). A novel role for Shc has been suggested in which phosphorylation of Y239/Y240 leads to c-*myc* induction and suppression of apoptosis in Ba/F3 cells, in a Ras-independent manner (Gotoh *et al*., 1996). The Shc PTB domain binds directly to the cytoplasmic enzyme SHIP, an SH2 domain-containing inositol 5-phosphatase, in response to growth factor and cytokine stimulation in hematopoetic cells (Damen *et al*., 1996; Kavanaugh *et al*., 1996; Lioubin *et al*., 1996). Furthermore, proline-rich sequences in the Shc CH1 domain are proposed to mediate the interaction between Shc and the SH3 domain of eps8, a tyrosine-phosphorylated protein involved in EGF receptor-mediated signaling (Matoskova *et al*., 1995; Bonfini *et al*., 1996). Therefore, it is likely that Shc participates in diverse signal transduction pathways by interacting with multiple cytoplasmic signaling molecules.

We have used the two-hybrid system in yeast to screen cDNA libraries for proteins which interact with p52 Shc. Previously, using this technique, we mapped the interaction between the PTB domain of Shc and a specific sequence (NPLH) in the carboxy-terminal region of the tyrosine phosphatase PTP-PEST. These studies were the first to demonstrate tyrosine phosphorylation-independent binding by Shc PTB domain to target sequences (Charest *et al*., 1996). In this study we describe the cloning and characterization of mPAL, a protein that interacts with Shc and whose expression is restricted to proliferating cells. The association of mPAL with Shc occurs via a novel phosphotyrosine-independent interaction with the Shc SH2 domain.

Results

Cloning of Shc binding proteins using the yeast-two-hybrid system

In order to identify binding partners for p52 Shc we used the yeast two-hybrid system. One of the clones isolated when full-length p52 Shc was used as a bait encodes a novel protein sequence designated mPAL which was isolated multiple times from both T-cell (three clones) and 11.5 day mouse embryo libraries (eight clones). This molecule interacts specifically with Shc and not with other non-specific GAL4 fusions tested. Furthermore, mPAL interacted with the isolated SH2 domain of p52 Shc but not the CH1 or PTB domain (data not shown). One of the cDNAs isolated from the yeast two-hybrid screen, encoding nucleotides 42–2130, was used to screen a mouse spleen library (Stratagene). No additional 5′ or 3′ cDNA sequences were identified by this approach so 5′ and 3′ RACE was used to amplify mPAL cDNA from mouse embryo cDNA.

The combined cDNA clone encompasses 2246 bp (Figure 1a). The 2007 bp open reading frame of mPAL includes nucleotides 12–2018. The first in-frame methionine (at nucleotide 12) is a good match for the Kozak consensus sequence (Kozak, 1992) and is used here to designate the initiation codon. Thus the cDNA of mPAL encodes a protein of 668 amino acids with a predicted molecular weight of 75 917 Daltons. A second in-frame methionine occurs 16 amino acids carboxy-terminal to the first, and also appears to be a good match for the Kozak consensus sequence. If this secondary site was used as an initiation codon, a protein of 653 amino acids would be produced with a predicted molecular weight of 74 407 Daltons.

The predicted mPAL protein sequence contains no known protein domains. mPAL contains 23 tyrosine residues, several of which are embedded in good consensus binding motifs for SH2 domains. In addition, two highly acidic regions are encoded in the mPAL sequence. Comparison of both the nucleotide and protein sequences of mPAL with the GenBank databases revealed no significant homology between mPAL and any previously identified proteins. We have isolated a cDNA encoding the human homolog of mPAL which shares 78.2% amino acid identity, with mPAL suggesting that the PAL protein is well conserved between species. In addition, several short murine and human ESTs with approximately 50% amino acid sequence identity to regions of mPAL were identified suggesting that additional mPAL related genes may exist. Interestingly, a partial genomic sequence has also been identified in *Drosophila* which encodes a protein that shares 26.8% identity with

amino acids 115–277 in mPAL, and may represent a mPAL related gene in *Drosophila* (Accession number, AC004364).

Generation of anti-PAL anti-serum and identification of the PAL protein in cell lines

In order to study mPAL protein distribution, polyclonal antibodies were raised in rabbits against a 15 amino acid peptide corresponding to amino acids 17–31 of mPAL. Anti-mPAL antibodies specifically recognized *in vitro* transcribed and translated mPAL (mPAL met2) both by immunoprecipitation and Western blot analysis (Figure 2). Anti-mPAL antibodies were also used for immunoprecipitations from the murine embryonic cell line, P19. Western blot analysis detected an immunoprecipitating protein of 75 kDa which migrated with approximately the same mobility as *in vitro* transcribed and translated mPAL. Immunoprecipitation of *in vitro* transcribed and translated mPAL as well as mPAL immunoprecipitated from undifferentiated P19 cells was blocked by preincubation of the antibody with immunizing peptide, further demonstrating the specificity of this antiserum (Figure 2). The anti-mPAL antibodies are species specific because they do not immunoprecipitate PAL from cell lines derived from species other than mouse. In fact, cloning of human PAL has confirmed that the amino terminal sequence against which our anti-mPAL antibodies were raised is not well conserved in the human protein sequence (RS and JM, unpublished).

The 75 kDa band detected by anti-mPAL antibodies in immunoprecipitates from undifferentiated P19 cells was resolved into two closely migrating bands by SDS–PAGE. *In vitro* transcription and translation of mPAL revealed that this doublet is likely generated by the use of two initiation codons at positions 12 and 56 of the mPAL cDNA (data not shown). This suggests that two isoforms of mPAL are produced *in vivo*, the shorter of which lacks the amino terminal 15 amino acids.

In vivo *association between and mPAL and Shc*

To determine if an *in vivo* association could be detected between mPAL and Shc, a co-immunoprecipitation assay was performed using the human embryonic kidney cell line (293T) co-transfected with pECE-Shc and pcDNA3.1-mPAL expression vectors. The transfected cells were lysed, immunoprecipitated with an irrelevant antibody (rabbit anti-mouse IgG), anti-Shc or anti-mPAL antibodies, followed by immunoblotting with anti-Shc and anti-mPAL antibodies (Figure 3a). The characteristic doublet indicative of p52 and p46 Shc was present in the mPAL immunoprecipitate, and likewise, a 75 kDa protein corresponding to mPAL was detected in the Shc immunoprecipitate, indicating that Shc and mPAL associate *in vivo*. The presence of mPAL immunizing peptide prevented the immunoprecipitation of mPAL and consequently, Shc was no longer observed to co-immunoprecipitate. The p66 isoform of Shc was also observed to co-immunoprecipitate with mPAL (data not shown), indicating that the Shc-mPAL interaction is not limited to the p46 and

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1     GTAAATTTGAAATGGCTGATGATTTGCGGGCTGGTGGAGTTCTGGAACCTATAGCTATGGTTCCACCGAGACCTG
1           M  A  D  D  L  R  A  G  G  V  L  E  P  I  A  M  V  P  P  R  P  D
76    ACTTGGCGCGGAGAAGGAACCGGCGTCCTGGAAGGAAGGTTTATTCTTGGATGCAGATCCATGCAGTGATCAAG
23    L  A  A  E  K  E  P  A  S  W  K  E  G  L  F  L  D  A  D  P  C  S  D  Q  G
151   GCTATCATGCTAATCCAGGTGCTACTGTATAAAACTCTCATACCAGAAGGAAAAACTCCTTTTCCACGAATTATCC
48    Y  H  A  N  P  G  A  T  V  K  T  L  I  P  E  G  K  T  P  F  P  R  I  I  Q
226   AAACAAATGAACTTCTGTTTTATGAACGATTCAGAGCCTATCAAGATTACATTTTAGCTGACTGTAAGGCCTCTG
73    T  N  E  L  L  F  Y  E  R  F  R  A  Y  Q  D  Y  I  L  A  D  C  K  A  S  E
301   AGGTAAAGGAATTCACAGTCAGCTTCTTGGAAAAGGTCCTTGAACCATCTGGATGGTGGGCAGTCTGGCACACTA
98    V  K  E  F  T  V  S  F  L  E  K  V  L  E  P  S  G  W  W  A  V  W  H  T  N
376   ATGTGTTTGAGGTGTTGGTTGAGGTTACAAATGTGGACTTTCCATCCCTGAAGGCGGTCGTAAGGCTTGCAGAGC
123   V  F  E  V  L  V  E  V  T  N  V  D  F  P  S  L  K  A  V  V  R  L  A  E  P
451   CATGCATCTATGAATCTAAATTGAGCACGTTTACCTTGGCCAATGTGAAGGAGCTTTTGGACCTGAAGGAGTTTC
148   C  I  Y  E  S  K  L  S  T  F  T  L  A  N  V  K  E  L  L  D  L  K  E  F  H
526   ATCTGCCTCTGCAGGAGTTGTGGGTGGTATCAGATGACTCACATGAATTCCACCAGATGGCACTTGCAATTGAGC
173   L  P  L  Q  E  L  W  V  V  S  D  D  S  H  E  F  H  Q  M  A  L  A  I  E  H
601   ACGTCAGATTTTTCTACAAACACATCTGGAGGAGTTGGGATGAGGAAGAGGAGGATGAGTATGACTATTTTGTCA
198   V  R  F  F  Y  K  H  I  W  R  S  W  D  E  E  E  E  D  E  Y  D  Y  F  V  R
676   GATGTGTTGAACCTGGACTGAGATTGTATTATGACATACTTGAAGATCGAGTTCCCTCGGGACTTATTGTTGACT
223   C  V  E  P  R  L  R  L  Y  Y  D  I  L  E  D  R  V  P  S  G  L  I  V  D  Y
751   ACCACAATCTGTTGTCTCAATGTGAAGAGAGTTACAGGAAATTTTTAAATCTGAGAAGCAGTTTGTCCAATTGTA
248   H  N  L  L  S  Q  C  E  E  S  Y  R  K  F  L  N  L  R  S  S  L  S  N  C  N
826   ATTCTGATTCTGAGCAGGAAAATATCTCCATGGTGGAAGGGTTAAATTTGTATTCAGAAATTGAACAGTTGAAAC
273   S  D  S  E  Q  E  N  I  S  M  V  E  G  L  N  L  Y  S  E  I  E  Q  L  K  Q
901   AAAAGCTAAAGCTCATTGAGAATCCTTTGTTAAGATATGTTTTTGGTTATCAGAAGAACTCTAATATCCAAGGAA
298   K  L  K  L  I  E  N  P  L  L  R  Y  V  F  G  Y  Q  K  N  S  N  I  Q  G  K
976   AGGGTACTCGTCAAAATGGCCAGAAGGTCATCCATGTGGTTTCCTCCACCATGAAGACAGGTCTACTTCGGTCTC
323   G  T  R  Q  N  G  Q  K  V  I  H  V  V  S  S  T  M  K  T  G  L  L  R  S  L
1051  TATTCAAGGACAGGTTTTGTGAGGAGTCTTGCAAAGAAGAAACAGAAATTAAGTTCCATAGTGATCTGTTGTCTG
348   F  K  D  R  F  C  E  E  S  C  K  E  E  T  E  I  K  F  H  S  D  L  L  S  G
1126  GTATAAATGCCTGCTATGATGGTGACACTGTCATTATTTGTCCTGGCCATTATGTAGTTCATGGCACCTGTTCCA
373   I  N  A  C  Y  D  G  D  T  V  I  I  C  P  G  H  Y  V  V  H  G  T  C  S  I
1201  TAGCTGACTCCATTGAGTTGGAAGGATATGGCCTACCAGATGACATTGTCATAGAAAAGAGGGGCAAAGGAGATA
398   A  D  S  I  E  L  E  G  Y  G  L  P  D  D  I  V  I  E  K  R  G  K  G  D  T
1276  CTTTTGTGGATTGCACGGGTATGGATGTTAAAATTTCAGGCATAAAATTTATTCAGCATGATTCTGTGGAAGGAA
423   F  V  D  C  T  G  M  D  V  K  I  S  G  I  K  F  I  Q  H  D  S  V  E  G  I
1351  TCTTAATCATTCACCATGGCAAGACCACACTGGAAAACTGTGTACTACAATGTGAAACCACAGGAGTCACAGTGC
448   L  I  I  H  H  G  K  T  T  L  E  N  C  V  L  Q  C  E  T  T  G  V  T  V  R
1426  GCACATCAGCAGAACTTTTCATGAAAAACTCAGATGTATATGGTGCCAAGGGTGCTGGTATAGAAATATATCCTG
473   T  S  A  E  L  F  M  K  N  S  D  V  Y  G  A  K  G  A  G  I  E  I  Y  P  G
1501  GAAGTAAATGTACCCTGACTGACAATGGAATCCATCACTGCAAGGAAGGAATTCTCATTAAGGACTTCCTTGATG
498   S  K  C  T  L  T  D  N  G  I  H  H  C  K  E  G  I  L  I  K  D  F  L  D  E
1576  AACATTATGATATTCCCAAAATATCGATGATAAATAACGTCATACACAATAATGAAGGTTATGGTGTTGTTTTGG
523   H  Y  D  I  P  K  I  S  M  I  N  N  V  I  H  N  N  E  G  Y  G  V  V  L  V
1651  TGAAGCCTACAATTTTCTGTGATCTACAGGAAAATACACAAGATGAAATTAATGACAATATGGTTCAGAAAAATA
548   K  P  T  I  F  C  D  L  Q  E  N  T  Q  D  E  I  N  D  N  M  V  Q  K  N  K
1726  AAGAGGCAGATGTCACTGAAGGATTAGATCTGGAAGAAATGCTTCAGTGTGTGGCTAGCAAAATGGAGCCTTATG
573   E  A  D  V  T  E  G  L  D  L  E  E  M  L  Q  C  V  A  S  K  M  E  P  Y  A
1801  CCACAGCTGACTTTAATGAACAAGCTAAGGGAAACTGTGAAATTATAAATGAACTACTTGCTATTTCCATGCAAA
598   T  A  D  F  N  E  Q  A  K  G  N  C  E  I  I  N  E  L  L  A  I  S  M  Q  K
1876  AAGGCCGGATGAAGAAAGACTGAGTGAACTTGGGATTACACAAGCTGATGACAACATAATGTCACAGGAGATGT
623   G  R  M  K  K  R  L  S  E  L  G  I  T  Q  A  D  D  N  I  M  S  Q  E  M  F
1951  TTATTGAAATTATGGGGAACCAGTTTAAGTGGAATGGCAAAGGGAGTTTTGGCACATTTCTTTACTAGCTACAAT
648   I  E  I  M  G  N  Q  F  K  W  N  G  K  G  S  F  G  T  F  L  Y  *
2026  AATATCAATACTCACAAAATACTGTATTTTGAACATGTCTTAAGTATGCTGCTTATATACTTTGCTTCATTTGCT
2101  TCATGGCTGTGTATATATAAAGTGTACTTGACCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
2176  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1 Nucleotide and predicted amino acid sequence of mPAL. The cDNA and predicted amino acid sequence of mPAL is shown. The positions of two potential ATG initiation codons are circled. The peptide against which the anti-PAL antiserum was raised is underlined

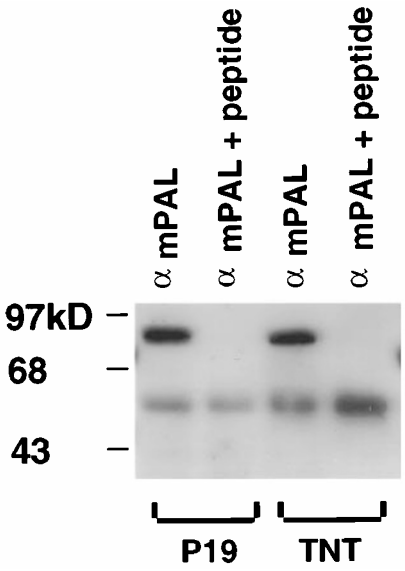


Figure 2 Characterization of specific anti-mPAL antibodies. Undifferentiated P19 cell lysates and *in vitro* translation products (TNT mPAL met2) were immunoprecipitated with an anti-mPAL antibody in the presence or absence of 50 μg immunizing peptide. Western blot analysis was completed with an anti-PAL antibody followed by HRP-Protein A

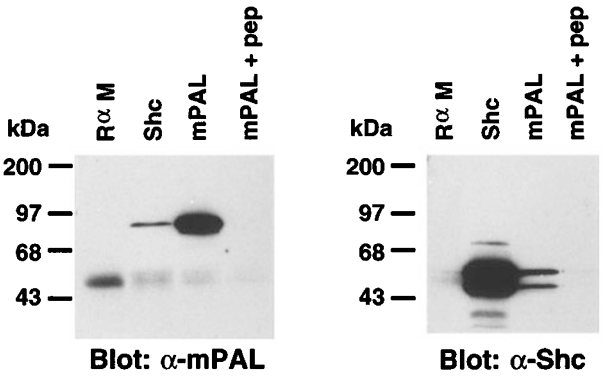


Figure 3 mPAL and Shc associate *in vivo*. The human embryonic kidney cell line (293T) was cotransfected with pECE-Shc and pcDNA3.1-mPAL (amino acids 16–668) expression vectors, lysed and immunoprecipitated with a non-specific antibody (RαM IgG) or with anti-Shc and anti-mPAL antibodies in the presence or absence of 50 μg immunizing peptide. The immunoprecipitates were washed three times, and the bound proteins resolved via SDS–PAGE. Following electrophoretic transfer to PVDF membrane, immunoblotting was performed with anti-Shc and anti-mPAL antibodies

p52 Shc isoforms. Neither Shc nor mPAL were non-specifically immunoprecipitated by the irrelevant antibody.

The SH2 domain of Shc specifically associates with mPAL

To determine which domain of Shc associated with mPAL *in vitro*, precipitation experiments were performed in which immobilized GST and GST-fusion proteins corresponding to the PTB, CH1 or SH2 domain of Shc were incubated with lysate from pcDNA3.1-mPAL-transfected 293T cells, and then subjected to immunoblotting with anti-mPAL antibody (Figure 4a). Only the GST-SH2 domain of Shc (GST-Shc SH2) was able to precipitate mPAL, indicating that this domain is involved in mediating the association of Shc with mPAL. Similarly, a yeast two-hybrid binding study utilizing the Shc PTB, CH1 and SH2 domains in isolation as bait, confirmed that only the Shc SH2 domain is necessary for interaction with mPAL (data not shown).

To examine the specificity of the mPAL-Shc SH2 interaction, a series of GST-SH2 fusion proteins generated from several signal transduction molecules were tested for their ability to precipitate mPAL (Figure 4b). None of the other GST-SH2 domains tested were found to bind to mPAL, indicating that association of the Shc SH2 domain with mPAL was specific.

The interaction of mPAL with the Shc SH2 domain is not dependent on phosphorylation of mPAL

In the previous experiments, mPAL precipitated by the Shc SH2 domain was not detected by immunoblotting with anti-pY antibodies (data not shown), suggesting that tyrosine-phosphorylation of mPAL is not critical for Shc SH2 domain-binding. Additionally, the interaction observed between mPAL and Shc in the yeast-two hybrid system strongly suggests that this interaction is independent of tyrosine-phosphorylation. Furthermore, by virtue of its production in *E. Coli*, recombinant GST-mPAL is not tyrosine-phosphorylated and yet is able to precipitate Shc from NIH3T3 lysate (Figure 5a), supporting the view that tyrosine-phosphorylation of mPAL is not important in the mPAL-Shc SH2 domain interaction.

Serine/theronine-phosphorylation-dependent SH2 binding has been reported (Cleghon and Morrison, 1994; Malek and Desiderio, 1994; Pendergast *et al*.,

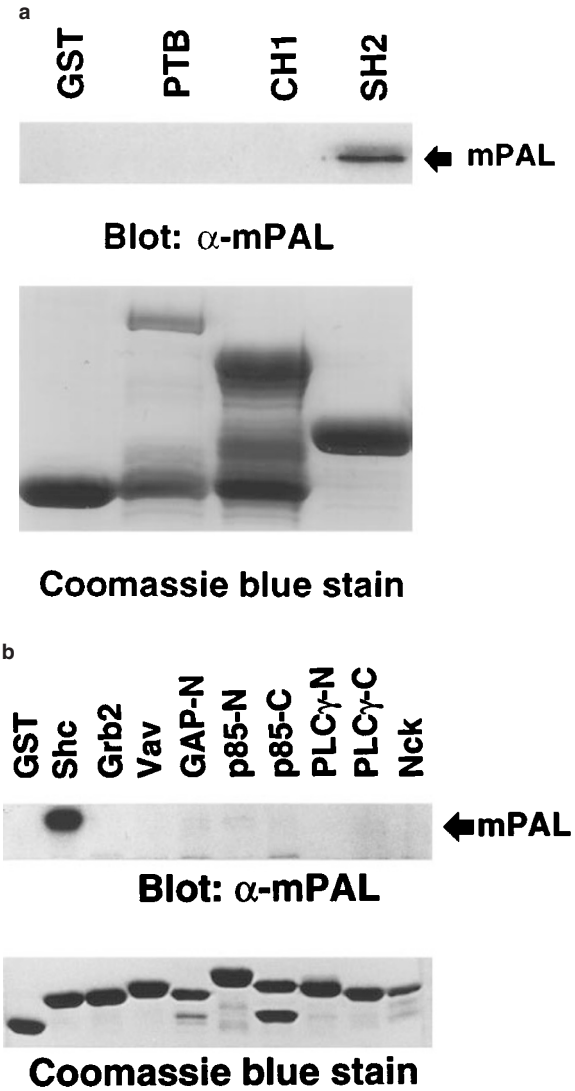


Figure 4 mPAL binds specifically to the SH2 domain of Shc. (**a**) The PTB, CH1 and SH2 domains of Shc were expressed as glutathione-S-transferase (GST) fusion proteins and immobilized onto glutathione-Sepharose beads. Five μg of GST or GST-fusion proteins were individually incubated with lysate (0.5 mg total protein) from 293T cells transfected with pcDNA3.1-mPAL. Coprecipitating proteins were resolved via SDS–PAGE, transferred to PVDF membrane, and immunoblotted with anti-mPAL antibody. The GST fusion proteins were visualized by Coomassie blue staining. (**b**) mPAL binds specifically to the SH2 domain of Shc. A panel of GST-SH2 fusion proteins (Shc SH2 domain, Grb2 SH2 domain, Vav SH2 domain, N-terminal SH2 domain of ras-GAP, N-terminal SH2 domain (p85-N) and C-terminal SH2 domain (p85-C) of p85 subunit of PI-3 kinase, N-terminal SH2 domain (PLCγ-N), and C-terminal SH2 domain (PLCγ-C) of phospholipase C-γ1, and Nck SH2 domain) were immobilized on glutathione-Sepharose beads. Five μg of immobilized GST or GST-fusion protein were individually incubated with 293T-mPAL lysate, the beads washed several times, and the remaining bound proteins resolved via SDS–PAGE, transferred to PVDF membrane, and immunoblotted with anti-mPAL antibody

1991) and thus it was of interest to determine if the mPAL-Shc SH2 interaction was phosphorylation-dependent. However, no phosphoproteins are immunoprecipitated with anti-mPAL antibodies from *in vivo* ^{32}P-orthophosphate labeled undifferentiated P19 cells or proliferating NIH3T3 cells, indicating that mPAL is not a phosphoprotein (data not shown). However, free phosphotyrosine competed for binding of mPAL to the Shc SH2 domain, whereas free phosphoserine or phosphothreonine did not (Figure 5b), implying that excess free phosphotyrosine, which is able to occupy the phosphotyrosine-binding pocket of the Shc SH2 domain, prevented interaction with mPAL. In addition, an arginine-to-alanine mutation in the conserved FLVRES motif (R397A) in the βB region of the Shc SH2 domain, which disrupts its interaction with phosphorylated EGF receptors also abrogated binding of GST-Shc SH2 to mPAL *in vitro* (Figure 5c). Similarly, the R397A mutation in full length Shc abolished the co-immunoprecipitation of mPAL and Shc *in vivo* (Figure 5d). This suggests that the mPAL-Shc SH2 interaction requires structural elements similar to those involved in SH2 phosphotyrosine-peptide interactions.

Expression of mPAL in murine tissues

A single mPAL RNA transcript of less than 2.4 kb in size was identified by Northern blot analysis of RNA isolated from a variety of murine tissues as well as from murine embryos at various stages of development (Figure 6). This size is in agreement with the cDNA

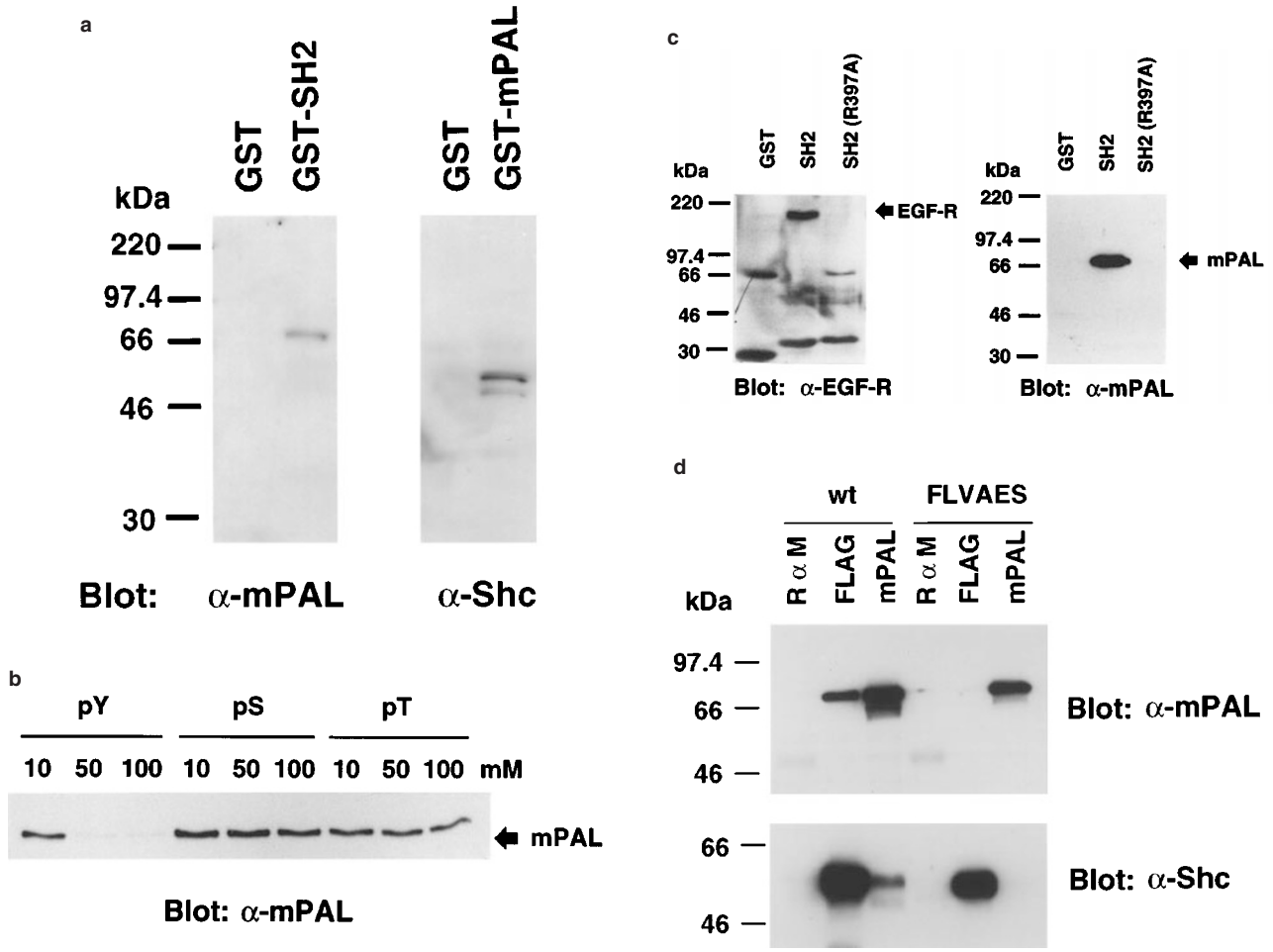


Figure 5 Binding of mPAL to Shc is phosphotyrosine-independent. (**a**) Association of GST-mPAL and Shc in NIH3T3 cells. Ten μg of immobilized recombinant GST (GST), GST-Shc SH2 (GST-SH2) domain and GST-mPAL (GST-mPAL) fusion protein were individually incubated with approximately 1 mg of NIH3T3 lysate. The beads were washed several times, and the remaining bound proteins resolved via SDS–PAGE, transferred to PVDF membrane, and immunoblotted with either anti-Shc or anti-mPAL antibodies. (**b**) Free phosphotyrosine disrupts the mPAL-Shc SH2 interaction. 2.5 μg of immobilized GST-Shc SH2 domain fusion protein was incubated with 293T-mPAL lysate in the presence of increasing concentrations of free phosphotyrosine, phosphoserine or phosphothreonine as indicated. The beads were washed several times, and the remaining bound proteins resolved via SDS–PAGE, transferred to PVDF membrane, and immunoblotted with anti-mPAL antibody. (**c**) Shc SH2 R397A does not interact with mPAL. A Shc SH2 domain mutant (SH2(R397A)) which is unable to bind to phosphotyrosine-containing substrates (R397 mutated to A), and the wild type Shc SH2 domain (SH2) were expressed as GST-fusion proteins, and immobilized onto glutathione-Sepharose beads. Five μg of GST or GST-fusion protein were individually incubated with EGF stimulated HER14 cells (left panel) or 293T-mPAL lysate (right panel), the beads washed several times, the remaining bound proteins resolved via SDS–PAGE, transferred to PVDF membrane, and immunoblotted with anti-mPAL antibody. (**d**) Shc containing an inactivated SH2 domain does not coimmunoprecipitate with mPAL 293T cells were co-transfected with Flag-tagged wild type (wt) p52 Shc or R397A Shc (FLVAES) and pcDNA3.1-mPAL. Immunoprecipitations were peformed from cell lysates using a non-specific antibody (RαM IgG) or with anti-FLAG and anti-mPAL antibodies. Immunoprecipitating proteins were resolved via SDS–PAGE. Following electrophoretic transfer to PVDF membrane, immunoblotting was performed with anti-Shc and anti-mPAL antibodies

size of 2.2 kb. mPAL RNA was strongly expressed in RNA isolated from testis, and was present at a much lower level in spleen, lung and heart, but was absent from brain, liver, skeletal muscle. mPAL RNA was observed at all stages of embryonic development (data not shown). This pattern of expression suggests that mPAL expression is restricted to tissues containing a proliferating cell population, but is absent from quiescent tissues. The low levels of mPAL RNA detected in murine lung and heart may result from tissue contamination with activated lymphocytes which express high levels of mPAL (Figure 9).

In agreement with data obtained by Northern blot analysis, mPAL protein was expressed in mouse spleen, testis and thymus all of which contain proliferating cells, but was absent from normal quiescent tissues (data not shown). In addition, mPAL protein levels are elevated in all murine cell lines tested to date, provided that the cells are actively proliferating. Furthermore, when murine PAL cDNA was used to probe a human tumour cell line Northern blot, elevated levels of human PAL RNA were detected in all lines (RS and JM, unpublished data). Overall, the pattern of expression of mPAL RNA and protein support a role for mPAL in signaling pathways governing cellular proliferation.

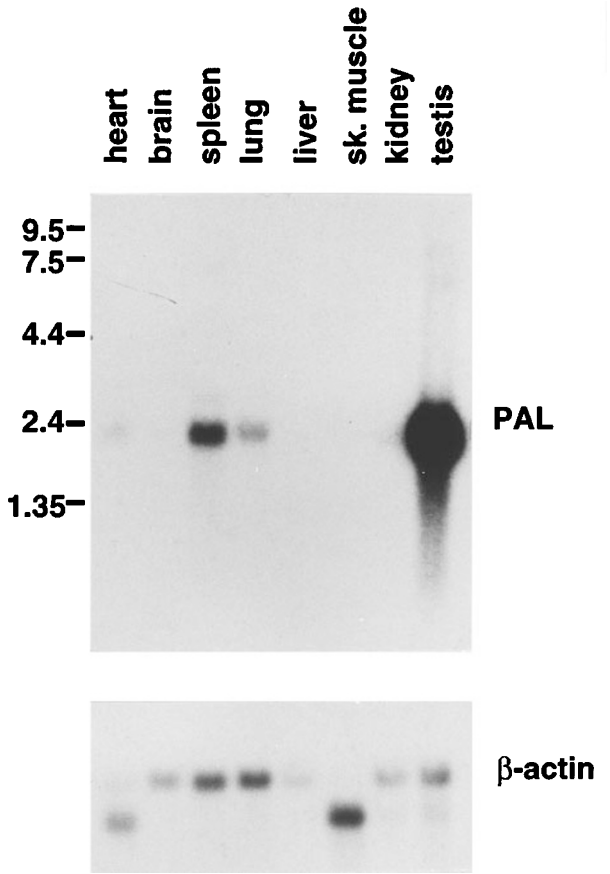


Figure 6 Distribution of mPAL RNA in murine adult tissues. A multiple tissue Northern blot of poly $(A)^+$ RNA extracted from adult murine tissues was purchased from Clontech. A ^{32}P-labeled 1.5 kb *Aff*III/*Pst*I fragment of mPAL cDNA was utilized as probe. Molecular weight standards are indicated. The membrane was exposed to X-ray film overnight at $-80°C$. Equal loading of mRNA was confirmed by reprobing the membrane with β-actin

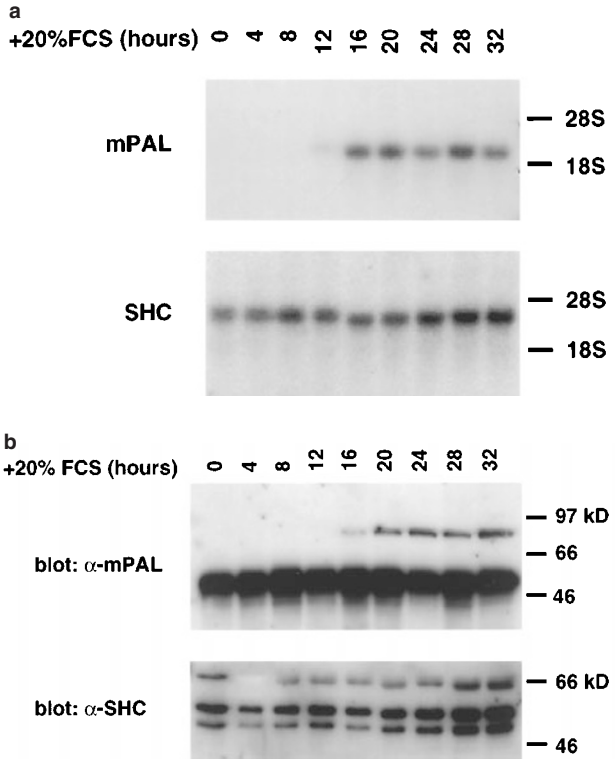
Induction of mPAL by addition of exogenous growth factors

To determine whether mPAL expression could be induced by exogenous growth factors and to study mPAL levels during the cell cycle, serum starved NIH3T3 cells were treated with fresh media containing 20% fetal bovine serum and total RNA was extracted at various time points. Quiescent, serum starved NIH3T3 cells do not express detectable levels of mPAL RNA (Figure 7). mPAL RNA levels remained undetectable for 8 h following addition of serum, however expression increased detectably by 12–32 h when RNA levels were high, while Shc RNA levels remain constant throughout the cell cycle (Figure 7a). Detectable mPAL protein expression appeared to lag behind RNA levels, as mPAL protein is only detectable at the 16 h time point (Figure 7b). mPAL protein levels increase from 16–20 h and remain constant thereafter. By comparison, Shc protein levels are unchanged in both quiescent and cycling cells. The moderate increase in Shc protein levels observed following 20 h is most likely related to an increase in cell number or protein content at these late time points. Cell cycle analysis demonstrated a well synchronized passage of these cells through the cell cycle following addition of fetal calf serum (Figure 7c). Cells remained primarily in G0/G1 through 8 h of stimulation, and began to enter S phase by 12 h. The majority of cells had passed through G2/M by 20–24 h and had re-entered G0/G1. Despite re-entry into G0/G1, levels of mPAL RNA and protein remained constant in actively cycling cells. Taken together, these data indicate that mPAL expression is low or absent in cells in G0/G1, but is elevated in cells committed to cell cycle progression, or actively cycling cells.

Expression of mPAL is down-regulated in cell lines by contact inhibition and terminal differentiation

Since mPAL mRNA and protein levels appeared to correlate with cell proliferation in tissues, levels of RNA were analysed in proliferating versus contact inhibited NIH3T3 cells in culture. NIH3T3 cells were plated at approximately 50% confluence in DMEM supplemented with 10% fetal bovine serum, and allowed to proliferate over several days. Levels of mPAL RNA were measured by Northern blot analysis and evaluated relative to cell confluence (Figure 8a). Actively growing, subconfluent NIH3T3 cells expressed relatively high levels of mPAL RNA, however, within 24 h after confluence was reached, levels of mPAL RNA decreased to undetectable levels. In contrast, Shc RNA levels remained constant throughout the time course, irrespective of cell density.

The embryonic carcinoma cell line, P19, can be induced to differentiate to a neural phenotype by incubation with retinoic acid and to a muscle phenotype with DMSO (McBurney, 1993). Following 6–7 days of treatment with retinoic acid, as many as 85% of P19 cells have been demonstrated to express neuronal markers and become post-mitotic (McBurney *et al*., 1988; McBurney, 1993). DMSO-treated P19 cells differentiate towards mesodermal and endodermal lineages. Approximately 25% cardiac muscle cells are



c

	% cells in phase								
hours in 20% fcs	0	4	8	12	16	20	24	28	32
G0/G1	92.0	92.5	89.0	73.0	21.5	32.0	54.0	29.0	51.5
S	5.0	5.0	9.5	26.0	67.5	26.0	37.0	50.0	25.0
G2/M	3.0	2.5	1.5	1.0	11.0	42.0	9.0	21.0	23.5

Figure 7 mPAL induction in serum-stimulated NIH3T3 cells. NIH3T3 cells were cultured in media containing 0.5% fetal calf serum for 48 h, then stimulated by addition of media containing 20% fetal calf serum. Following stimulation mRNA and protein was harvested from cells at the times indicated. (**a**) Northern blot analysis. Ten μg of total RNA from each sample were separated on formaldehyde-agarose gels and transferred to Genescreen nylon membrane. Blots were probed for mPAL RNA as described above. This blot was exposed to X-ray film for 72 h at -80°C. The membrane was stripped and reprobed for Shc (lower panel). (**b**) Western blot analysis. At each timepoint, mPAL was immunoprecipitated from 1.0 mg of lysate and immunoblotted with anti-mPAL antibodies. In the lower panel, 20 μg of whole cell lysate was immunoblotted for Shc. (**c**) Cell cycle analysis. Cells were harvested at each time point, stained with propidium iodide and subjected to cell cycle analysis by flow cytometry

observed following 6–7 days of DMSO treatment (Rudnicki *et al*., 1990; McBurney, 1993), while the remainder of cells in culture continue to grow and differentiate into skeletal muscle as well as other less well defined cell types (McBurney, 1993). Levels of mPAL protein were measured by immunoprecipitation and Western blot analysis in P19 cells induced to differentiate in the presence of retinoic acid or DMSO (Figure 8b). While the parental, rapidly proliferating cell line expressed mPAL protein, DMSO-differentiated P19 cells expressed significantly lower levels of mPAL. Furthermore, in P19 cells treated for 7 days in the presence of retinoic acid, mPAL is virtually undetectable. Levels of β-tubulin remain constant throughout differentiation. These data further support the hypothesis that mPAL expression is restricted to proliferating cells and is down-regulated upon growth inhibition.

T-cell activation results in mPAL expression and the formation of a mPAL-Shc complex

The restricted expression pattern of mPAL suggested that the association of Shc and mPAL *in vivo* might be limited to specific cell types or to cells which have been induced to proliferate and therefore express high levels of mPAL protein. Since the proliferation rate of primary T-cells is readily regulated *in vitro*, the levels of mPAL expression and its association with Shc were investigated in resting versus activated T-cells. Primary mouse lymphocytes, activated by cross-linking CD3, expressed high levels of mPAL while unstimulated cells did not (Figure 9a). The timing of the expression of mPAL was coincident with T-cell activation and proliferation. Shc immunoblots of anti-mPAL immunoprecipitates revealed the coprecipitation of Shc with mPAL following T-cell activation and concurrent with mPAL expression. Furthermore, constitutively activated primary T-cells isolated from a CTLA4 deficient mouse (Waterhouse *et al*., 1995; Marengere *et al*., 1996) expressed mPAL protein while mPAL was undetectable in T-cells isolated from a wild type mouse (Figure 9b). Immunoprecipitation of mPAL and Shc, followed by immunoblotting with anti-mPAL and anti-Shc clearly demonstrated the coprecipitation of mPAL with Shc from the CTLA4 deficient T-cell lysates but not from unstimulated wild type cells.

Likewise Shc was present only in anti-mPAL precipitates from CTLA4 deficient lymphocytes (Figure 9b).

Discussion

This paper reports the cloning and characterization of a novel Shc SH2 binding protein which we have designated mPAL (murine *P*rotein expressed in *A*ctivated *L*ymphocytes). The mPAL protein sequence contains no known protein domains and appears to be unrelated to previously identified proteins. The mPAL protein is conserved because we have also identified a human cDNA encoding a protein 78.2% identical to mPAL (RS and JM, unpublished data). In addition, a

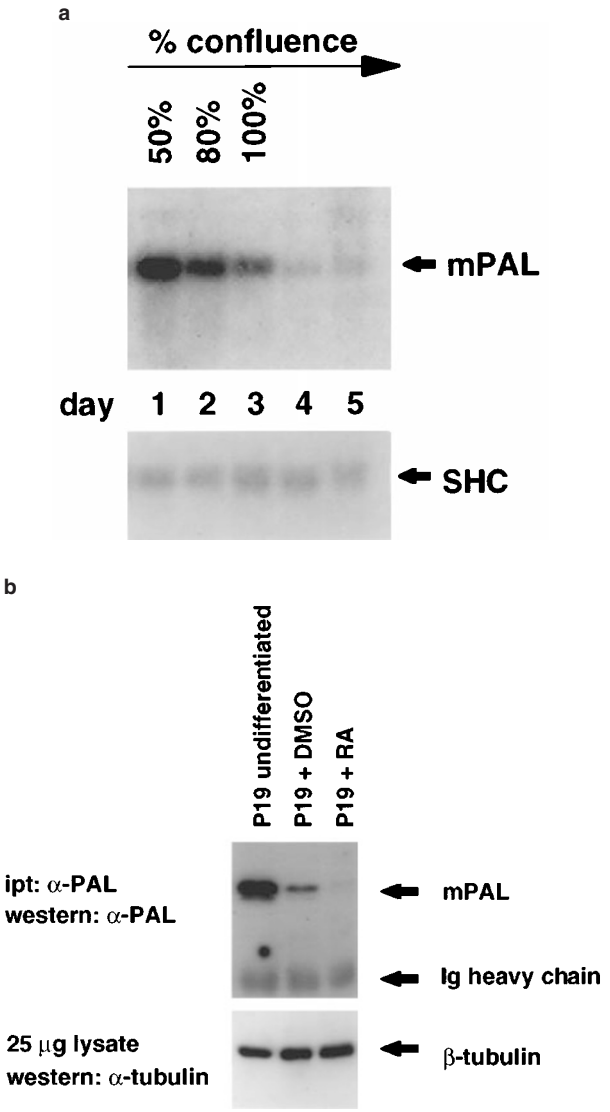


Figure 8 mPAL expression is down-regulated following inhibition of cell growth. (**a**) mPAL levels are down-regulated upon contact inhibition. RNA was harvested from NIH3T3 cells over five consecutive days and the percentage confluence of each plate was noted at the time of harvest. mRNA samples (10 μg/lane) were separated on formaldehyde-agarose gels and transferred onto Genescreen nylon membrane. Blots were probed for mPAL and exposed to X-ray film at -80°C for 2 days. Blots were stripped and reprobed for Shc expression. (**b**) Expression of mPAL decreases in differentiating P19 cells. The pluripotent murine embryonic carcinoma cell line, P19, was differentiated in the presence of retinoic acid and DMSO. mPAL immunoprecipitates from each sample were immunoblotted with anti-mPAL. This result was reproducibly obtained in three separate experiments. Twenty-five μg of whole cell lysate was subjected to SDS–PAGE and Western blot analysis with anti-β tubulin as probe to control for equal loading

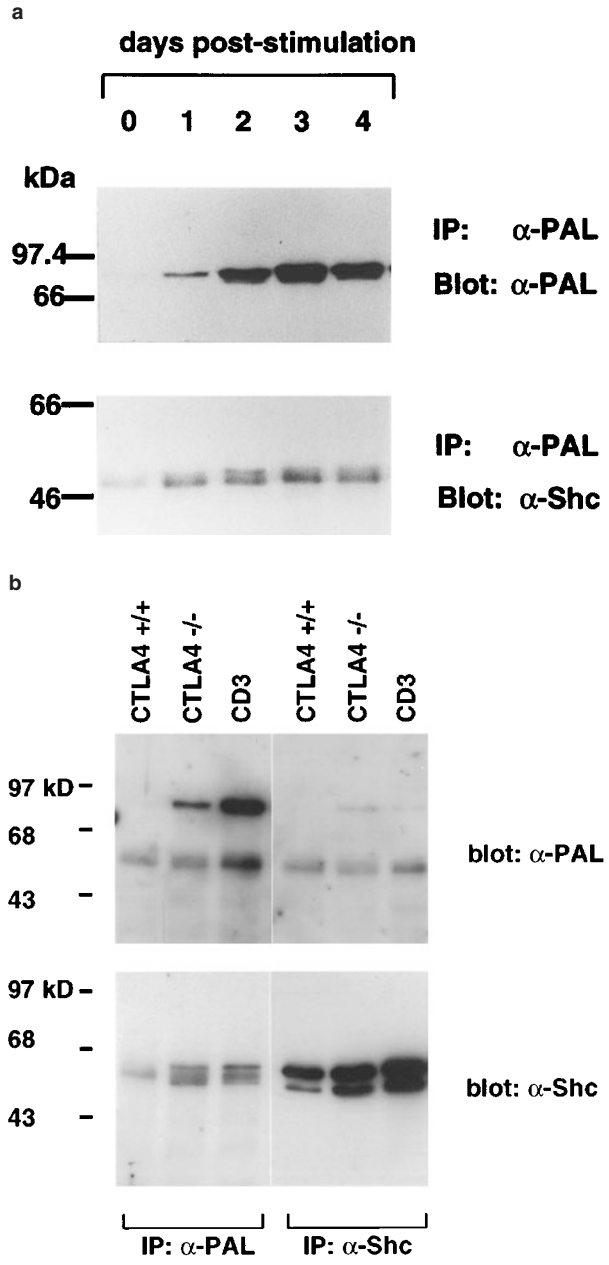


Figure 9 mPAL expression is upregulated and associates with Shc following T-cell activation. (**a**) mPAL is expressed in anti-CD3 activated murine T-cells. T-cells isolated from wild type mice were stimulated by cross-linking the T-cell receptor with anti-CD3 antibodies. Samples were collected over 4 days and lysed in NP40 lysis buffer. mPAL was immunoprecipitated from 1.0 mg of lysate for each sample and Western blot analysis was performed with anti-mPAL antibodies. The blot was then stripped and immunoblotted with anti-Shc antibodies. (**b**) mPAL and Shc co-immunoprecipitate in activated T cells. T-cells isolated from wild type and CTLA4 deficient mice, as well as CD3 activated T cells, were lysed in NP40 lysis buffer. Shc and mPAL were each immunoprecipitated from 1 mg of lysate and immunoprecipitating proteins were resolved via SDS–PAGE. Duplicate gels were run and transferred to PVDF membrane. Membranes were immunoblotted for both Shc and mPAL. In order to clearly visualize Shc co-precipiation, the mPAL immunoprecipitations Western blotted with α-Shc were overexposed relative to the corresponding Shc immunoprecipitations

partial cDNA encoding a rat homolog of mPAL has been isolated from undifferentiated PC-12 cells which shares 92.5% identity with mPAL (Lee *et al*., 1995). The partial sequence of a *Drosophila* gene also has been reported in GenBank (accession number, AC004364) which encodes a protein 26.8% identical to mPAL and may represent a mPAL-related protein.

mPAL was isolated in a yeast-two-hybrid screen from murine T-cell and embryo libraries in which the p52 isoform of Shc was used as 'bait'. In addition we have observed an *in vivo* interaction between Shc and mPAL in mammalian cells by co-immunoprecipitation. mPAL binds to Shc via its SH2 domain and this interaction is specific because SH2 domains from other proteins did not bind mPAL. Furthermore, the mechanism of SH2 binding is novel. Unlike other SH2 domain-phosphopeptide interactions, the mPAL-Shc SH2 domain interaction appears to be independent of tyrosine-phosphorylation. Similar phosphotyrosine-independent interactions with SH2 domains have been described between Lck and the ubiquitin binding protein p62 (Joung *et al*., 1996; Vadlamudi *et al*., 1996), between v-*abl* and Shc (Raffel *et al*., 1996) as well as between Grb10 and the Raf1 and MEK1 kinases (Nantel *et al*., 1998).

Structural elements involved in Shc SH2 domain-phosphotyrosine peptide interactions may mediate the mPAL-Shc SH2 domain interaction, since occupancy of the phosphotyrosine-binding pocket with free phosphotyrosine, or mutation of the conserved R397 residue involved in phosphotyrosine binding disrupts the mPAL-Shc SH2 domain interaction. Alternatively, mPAL could be binding a region near the Shc SH2 phosphotyrosine binding pocket and phosphotyrosine binding or the R397A mutation may alter the conformation of the SH2 domain, or local charge distribution on the domain surface and disrupt the mPAL binding site. Further characterization of the exact sequences of mPAL required for Shc-SH2 domain binding will be required to resolve the molecular basis of the mPAL and Shc interaction. It is possible that binding of mPAL to the Shc SH2 domain is mediated by the acidic regions in mPAL because phosphotyrosine-independent interactions between SH2 domains of other proteins such as Bcr-Abl, Lck and Blk and sequences rich in serine and glutamic acid have been previously reported (Pendergast *et al*., 1991; Malek and Desiderio, 1994; Joung *et al*., 1996). In some cases, serine phosphorylation has been shown to be important for SH2 domain binding (Pendergast *et al*., 1991; Malek and Desiderio, 1994). This does not appear to be the case for the mPAL-Shc interaction since the interaction cannot be competed for by free phosphoserine or phosphothreonine, and we have been unable to detect any phosphorylation of the mPAL protein.

The binding of the Shc SH2 domain to mPAL represents an alternative role for the Shc SH2 domain which had previously been reported to couple Shc to activated transmembrane receptors rather than downstream cytoplasmic signaling molecules. In fact, there is growing evidence that Shc binds to proteins distal to receptor tyrosine kinases via its SH2 domain. The isolated Shc SH2 domain has been demonstrated to function as a dominant-negative mutant and block signaling from the PDGF and EGF receptors (Gotoh *et al*., 1995; Ricketts *et al*., 1996; Roche *et al*., 1996). Furthermore, the Shc SH2 domain can effectively block EGF signaling in cells, even if introduced as much as 10 min following EGF stimulation (Ricketts *et al*., 1996). Consistent with these observations, expression the SH2 domain of the neural specific ShcC adaptor protein has been shown to block Erk1 and Erk2 activation by the EGF receptor and to inhibit EGF receptor mediated transformation of cells, however association of the isolated Shc SH2 domain and the activated receptor is undetectable (O'Bryan *et al*., 1998). These data have led both Ricketts *et al*. (1996) and O'Bryan *et al*. (1998) to hypothesize that the Shc SH2 domain serves an important function at a point distal to EGF receptor activation and that additional proteins are required for EGF receptor signaling. We have demonstrated by subcellular fractionation and by expression of Green Fluorescent Protein-labeled mPAL in mammalian cell lines that mPAL is primarily a cytoplasmic protein (data not shown) and may therefore represent a downstream target of the Shc SH2 domain following receptor activation.

mPAL mRNA and protein are elevated in tissues containing proliferating cells and in cell lines, but are absent in normal, quiescent tissues and growth-arrested cells. Factors which stimulate cell cycle progression and proliferation also stimulate mPAL expression, whereas factors which inhibit cellular proliferation including serum withdrawal, contact inhibition, and terminal differentiation, inhibit the expression of mPAL. Interestingly, a partial cDNA encoding mPAL was identified as a gene expressed in fertilized single cell mouse embryo (accession C87425). These data support a role for mPAL in cell growth and differentiation. mPAL may be involved in progression through the cell cycle rather than the immediate early response because mPAL mRNA is expressed during entry into S phase and passage through G2/M. Preliminary data from cyclohexamide treated cells also supports the hypothesis that mPAL is not an immediate early response gene (data not shown). Microinjection of the Shc SH2 domain or anti-Shc antibodies has been shown to inhibit both S phase entry and *Fos* induction in PDGF stimulated NIH3T3 cells (Roche *et al*., 1996). Since mPAL is a target of the Shc SH2 domain, it is possible that mPAL represents a component of the growth regulatory pathway blocked by Shc dominant-negative mutants.

Given that mPAL was isolated from a T-cell library and T-cells represent a normal cell lineage whose proliferative rate is readily regulated in culture, we also studied mPAL expression and its association with Shc in response to T-cell activation. While unstimulated T-cells lack mPAL protein, T-cells activated by cross-linking the T-cell receptor and constitutively activated T-cells isolated from CTLA4$^{-/-}$ mice express high levels of mPAL. Furthermore, Shc and mPAL complexes were found to co-immunoprecipitate from activated T-cells, albeit at low levels. The percentage of mPAL which can be detected in complex with Shc probably under-represents the actual amount Shc-mPAL in the cell. This may be due in part to the antibodies used to detect Shc, since they are directed against the SH2 domain and therefore may not efficiently precipitate Shc proteins in which the SH2 domain is complexed with another molecule. Since

mPAL protein is detectable only in proliferating T-cells, the formation of a mPAL-Shc complex is regulated, at least in part, by mPAL expression levels.

The function of mPAL in cell cycle progression and the physiologic outcome of the mPAL-Shc interaction remains to be determined. The association of mPAL with the Shc SH2 domain could imply that mPAL is a component of a novel signaling pathway downstream of Shc. Alternatively, mPAL could regulate Shc function by modifying complex formation between the Shc SH2 domain and tyrosine phosphorylated targets. Taken together, the data presented here indicate that mPAL may function in signaling pathways governing cell cycle progression.

Materials and methods

Unless otherwise indicated, reagents were purchased from Sigma chemicals.

Yeast two-hybrid

Full length human p52 Shc was fused to the DNA binding domain of GAL4 (GAL4-DB) by cloning into pAS-1. The yeast strain used (Y153) contained both HIS3 and *lacZ* reporter genes driven by promoters containing GAL4 binding sites, and was deleted for endogenous GAL4 (Bai and Elledge, 1996). Yeast transformed with pAS-1 GAL4-Shc were screened for expression of the GAL4-Shc fusion protein by Western blot analysis of yeast lysates using anti-Shc antibodies (data not shown). The GAL4 Shc expressing strain was assayed for transcriptional activation of HIS3 and *lacZ*, and was negative for auto-activation of GAL4 driven promoters. A plasmid cDNA-GAL4 transactivation domain (GAL4-TA) fusion library, derived from total T-cell cDNA (a generous gift from S Elledge) and, a mouse embryo GAL4-TA fusion library (Clontech) were used to transform yeast carrying the GAL4-Shc DB fusion constructs and interacting clones were selected on his^- media supplemented with 20 mM aminotriazole. After 72 h nitrocellulose replicas were made and assayed for β-galactosidase activity directly. Over 3×10^6 clones were screened from both libraries and 42 and 100 His and β-gal positive clones were picked up from the T-cell and embryo libraries, respectively. The isolated cDNAs were checked for insert size and introduced into a second strain of yeast (Y187) and tested for β-galactosidase activity alone, or when mated with yeast carrying either GAL-Shc or GAL4 fused to other unrelated proteins to eliminate false positives.

cDNA screening/constructs

A mouse spleen library was screened with the mPAL cDNA isolated from the yeast-two-hybrid screen. cDNAs corresponding to nucleotides 42–2130 encoding mPAL amino acids 16–668, were subcloned into Bluescript SK^- (Stratagene) and utilized for *in vitro* transcription and translation (mPAL met2).

The remaining 5′ and 3′ cDNA ends of mPAL were identified using 5′ and 3′ RACE of Marathon Ready cDNAs (Clontech) from murine embryo and spleen as per manufacturer's instructions. A full length construct encoding mPAL amino acids 1–668 was cloned by PCR from murine embryo Marathon Ready cDNA and cloned directly into pCR2.1 (Invitrogen). This construct was sequenced for accuracy and was used for *in vitro* transcription and translation (mPAL met1).

Cell culture

P19 and 293T cells were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Sigma), 200 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol, 5 U/ml penicillin C and 5 μg/ml streptomycin sulfate. NIH3T3 cells were cultured in 10% calf serum (Sigma), 200 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol, 5 U/ml penicillin C and 5 μg/ml streptomycin sulfate.

In order to characterize the serum mediated induction of mPAL, NIH3T3 cells were grown to 70–80% confluence, washed twice in phosphate buffered saline (PBS) and then transferred to media containing 0.5% fetal bovine serum for 48 h to achieve quiescence. Serum starved cells were then transferred to media containing 20% fetal bovine serum. Samples were harvested for protein or RNA isolation at the times indicated. Cells isolated at each time point were stained with propidium iodide and subjected to cell cycle analysis by flow cytometry.

P19 cells were differentiated to a neural phenotype following incubation for 6–7 days in the presence of 5×10^{-7} M retinoic acid or a muscle phenotype by incubation with 0.75% DMSO for 5 days. Techniques used were as described by Jones-Villeneuve *et al.* (1982) and McBurney *et al.* (1982).

Primary murine T-cells were isolated from wild type and CTLA4 deficient mice (a kind gift of Luc Marengere). Wild type murine T-cells were activated by cross-linking the T-cell receptor on anti-CD3 coated tissue culture plates as described by Marengere *et al.* (1997).

Preparation of GST-fusion proteins

The Shc SH2 mutant (R397A) was engineered using PCR-based site-directed mutagenesis. The PCR product containing the R397A mutation was cloned using *Bam*HI and *Eco*RI restriction sites into the pGEX-2T vector (Pharmacia). The PAL cDNA encoding amino acids 11–648 was cloned using *Xho*I restriction sites into the pGEX-4T3 vector (Pharmacia). Additional GST-fusion proteins; Shc PTB (Blaikie *et al.*, 1994); Shc SH2 (Pelicci *et al.*, 1992); Grb2 SH2 (Rozakis-Adcock *et al.*, 1992); Vav SH2 (Margolis *et al.*, 1992); GAP-N SH2, PLCγ-N SH2, PLCγ-C SH2 (Anderson *et al.*, 1990); and p85-N SH2, p85-C SH2 (McGlade *et al.*, 1992b) have been previously described. Nck SH2 contains amino acids 281–377 of human Nck (Lehman *et al.*, 1990), and the GST Shc-CH1 consists of amino acids 212–376 of human Shc. GST-SH2 fusion proteins were prepared as follows: log-phase *Escherichia coli (E. Coli)* DH5-α cells were grown in the presence of 100 μg/ml amphicillin, and induced with 1 mM isopropythiogalactopyranoside (IPTG) for 3 h at 37°C. The cells were pelleted, lysed in 1 ml of ice-cold NP-40 lysis buffer (50 mM HEPES, pH 7.25, 150 mM NaCl, 2 mM EDTA, 100 μM $ZnCl_2$, 1% (v/v) Nonidet-P40, 100 μM sodium pervanadate, 10% (v/v) glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM Pefa-Bloc), sonicated on ice three times for 30 s, and the lysates clarified by centrifugation for 10 min at 11 000 r.p.m., 4°C. The lysates were incubated with glutathione-Sepharose beads (Pharmacia) for 30 min at 4°C, the beads washed several times with NP-40 lysis buffer, and resuspended in an equal volume of phosphate-buffered saline (PBS) containing 1 mM dithiothreitol (DTT). Each of the purified GST-fusion proteins was quantitated by SDS–PAGE followed by Coomassie staining and comparison with known BSA standards.

Transient transfections

Human p52 Shc in pECE has been previously described (Pelicci *et al.*, 1992). The mouse p66 Shc cDNA was

subcloned into pcDNA1 (unpublished). Mouse p52 Shc with an activated SH2 domain was generated through PCR-based mutagenesis (R397 mutated to A). The PAL cDNA encoding amino acids 11–648 was cloned into pcDNA3.1 (Invitrogen). 293T cells in 10 cm culture dishes were transfected with 10 μg of pECE-Shc and/or pcDNA3.1-mPAL expression vectors using Lipofectin (Gibco-BRL) according to manufacturer's instructions.

Antibodies

Anti-mPAL antibodies were raised in rabbits against the peptide sequence VPPRPDLAAEKEPAS, corresponding to amino acids 17–31 in the predicted protein sequence of mPAL. An carboxy-terminal cysteine was added to facilitate conjunction to keyhold limpet haemocyanin (KLH) and to couple the peptide to a Sulfolink column (Pierce) used for affinity purification of anti-mPAL antibodies. Crude anti-mPAL was used at a concentration of 1:500 for Western blot analysis and 20 μl per immunoprecipitation. Affinity purified antibody was used at a final concentration of 1 μg/ml for Western blotting and at 2 μg per immunoprecipitation.

Affinity purified anti-Shc antibodies (Pelicci *et al*., 1992) were used at a 1:500 dilution for Western blot analysis and 2 μg per immunoprecipitation. In some experiments, a monoclonal anti-Shc antibody (Transduction Laboratories) was used at a dilution of 1:250.

The anti-phosphotyrosine antibody RC20H (Transduction Laboratories) was used at a dilution of 1:2500 for Western blot analysis. The anti-phosphotyrosine antibody 4G10 (UBI) was used for Western blot analysis at a dilution of 1:1000, anti-EGFR antibody (Upstate Biotechnology) was used at a dilution of 1:500 for Western blot analysis, and anti-β-tubulin antibody (Amersham) was used at a concentration of 1 μg/ml for Western blot analysis.

Immunoprecipitation and Western blot analysis

Each 10 cm plate of adherent cells was lysed in 1 ml of NP-40 lysis buffer for 15 min at 4°C. Tissue lysates were also homogenized in NP-40 lysis buffer using a polytron homogenizer (Kinematica AG). Lysates were transferred to a 1.5 ml microfuge tube and centrifuged at 10 000 *g* (maximum speed) for 10 min at 4°C. Typically, a volume of lysate containing 1 mg of total protein was incubated with 20 μl crude anti-mPAL antiserum or 2 μg affinity purified anti-mPAL antiserum and 100 μl of 10% Protein A Sepharose (Sigma) and the volume increased to 1 ml with NP-40 lysis buffer. Samples were incubated at 4°C with gentle rotation for 1 h. Immune complexes were then washed three times in 1 ml NP-40 lysis buffer. Samples were boiled for 5 min in 40 μl reducing SDS–Laemmli sample buffer prior to loading onto a 10% polyacrylamide gel and separation by SDS–PAGE. Proteins were electrophoretically transferred to Immobilon-P (PVDF) membrane (Millipore), and incubated in a blocking solution of 5% skim milk powder in TBST (20 mM Tris-HCl, pH 8.3, 150 mM NaCl, 0.05% Tween 20) for a minimum of 1 h prior to addition of antibody overnight at 4°C. Membranes were washed three times in TBST and incubated for 1 h at 4°C with a 1:3000 dilution of HRP-coupled protein A (Biorad). Following incubation with secondary antibodies, membranes were washed three times in TBST and developed using ECL (Amersham) as per manufacturer's instructions.

GST-Precipitation experiments

The cell lysates of transiently-transfected 293T cells were prepared as described above, and incubated with approximately 5 μg of GST-fusion protein coupled to glutathione-Sepharose 4B beads for 2 h at 4°C. The beads were washed three times with NP-40 lysis buffer, resuspended in 25 μL of SDS-sample buffer, the proteins resolved on a 10% SDS-polyacrylamide gel and transferred to Immobilon-P membrane. Immunoblotting and detection of immunoreactive bands was carried out as previously described.

Competition studies assessing the phosphotyrosine dependence of GST-Shc SH2 and mPAL association were carried out in the presence of 10, 50 and 100 mM O-phospho-L-threonine, O-phospho-L-serine, or O-phospho-L-tyrosine which were added to the cell lysates prior to incubation with immobilized GST-Shc SH2 fusion protein.

In vitro *transcription and translation*

T7 TNT-coupled Reticulocyte Lysate systems (Promega) was used to transcribe and translate cDNA constructs encoding full length mPAL amino acids 1–668 (met1) in PCR2.1 (Invitrogen) and amino acids 16–668 (met2) in Bluescript SK(−) (Stratagene) Bluescript SK, according to the manufacturer's instructions.

Northern blot analysis

Northern blots containing 2 μg of poly(A)$^+$ mRNA isolated from a variety of murine tissues and embryos were purchased from Clontech.

Where specified, total RNA was prepared from cultured cells using the TRIzol Reagent (Gibco/BRL) as per manufacturer's instructions. RNA samples (10 μg/lane) were separated on formaldehyde-agarose gels and transferred onto Genescreen nylon membrane (NEN-Dupont) as described by Sambrook *et al*., 1989.

Blots were probed with a 1522 bp *Afl*III/*Pst*I fragment of the mPAL cDNA which was radiolabeled by random hexamer priming (Pharmacia). Blots were initially prehybridized for 4 h at 42°C in a solution of 50% formamide, 4 × SSPE, 1% SDS, 0.5% skim milk powder, 10% dextran sulfate and 10 mg/ml sheared salmon sperm DNA. ^{32}P-Radiolabeled probe was then added at 10^6 c.p.m./ml and the blot further incubated for 16 h at 42°C. Blots were washed twice for 10 min at room temperature in 2 × SSC, 0.1% SDS, then twice at 65°C in 0.1 × SSC, 0.1% SDS and then exposed to film. The blots were also probed with radiolabeled β-actin cDNA (Clontech) or Shc cDNA as an indicator of RNA loading.

Accession number

The cDNA sequence of murine PAL has been submitted to GenBank (accession number: AF017152).

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