Mutations in CD2BP1 disrupt binding to PTP PEST and are responsible for PAPA syndrome, an autoinflammatory disorder

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PAPA syndrome (pyogenic sterile arthritis, pyoderma gangrenosum, and acne, OMIM #604416) and familial recurrent arthritis (FRA) are rare inherited disorders of early onset, primarily affecting skin and joint tissues. Recurring inflammatory episodes lead to accumulation of sterile, pyogenic, neutrophil-rich material within the affected joints, ultimately resulting in significant destruction. We recently localized the genes for PAPA syndrome and FRA to chromosome 15q and suggested that they are the same disorder. We have now established this by the identification of co-segregating disease-causing mutations in the CD2-binding protein 1 (CD2BP1; GenBank accession no XM 044569) gene in the two reported families with this disorder. E250Q or A230T amino acid substitutions occur within a domain highly homologous to yeast cleavage furrow-associated protein CDC15. CD2BP1 and its murine ortholog, proline–serine–threonine phosphatase interacting protein (PSTPIP1), are adaptor proteins known to interact with PEST-type protein tyrosine phosphatases (PTP). Yeast two-hybrid assays demonstrate severely reduced binding between PTP PEST and both the E250Q and A230T mutant proteins. Previous evidence supports the integral role of CD2BP1 and its interacting proteins in actin reorganization during cytoskeletal-mediated events. We hypothesize that the disease-causing mutations that we have identified compromise physiologic signaling necessary for the maintenance of proper inflammatory response. Accordingly we suggest classification of PAPA syndrome as an autoinflammatory disease. This CD2BP1-mediated biochemical pathway(s) may function in common inflammatory disorders with apparent etiological overlap, such as rheumatoid arthritis and inflammatory bowel disease.

INTRODUCTION

Autoimmune disorders, estimated to affect 3–5% of the population, pose a significant public health problem (1). Although various criteria have been applied to define classic autoimmunity, in general the destruction of the individual’s own tissues seen in these diseases is thought to result from proliferation of T lymphocytes and/or antibodies directed against self antigens. These antigens may be tissue-specific or ubiquitously expressed, and resulting destructive inflammation may be localized, as in rheumatoid arthritis, or systemic, as in lupus erythematosus. Many of these diseases are associated with particular histocompatibility locus antigens (HLA), suggesting that antigen presentation to T cells is important in the pathogenesis of disease (2–4). A separate but potentially related group of disorders comprises the so-called ‘autoinflammatory’ diseases. Like autoimmune disorders, these conditions involve inflammation that appears to be due to breakdown in self-tolerance; however, autoantibodies or antigen-specific T lymphocytes are not detected (5). There has been considerable recent progress in identifying causative factors in autoimmune/autoinflammatory diseases. However, effective treatments or preventatives are needed for most of these disorders (2).
In humans, clinically distinct autoimmune and autoinflammatory disorders often show familial clustering. Comparison of genome-wide linkage scans has revealed clustering of candidate loci as well, supporting the hypothesis that a common set of susceptibility genes controls the pathogenesis of these diseases (6). The availability of draft or finished human genomic sequence and annotation with single nucleotide polymorphisms (SNPs) will speed the detection of associations between disease and non-HLA susceptibility genes (7). In the meantime, genes for autoimmune/autoinflammatory diseases displaying Mendelian inheritance may be rapidly identified by application of straightforward positional cloning methods, enabling entry into the study of biochemical pathways controlling both rare and common inflammatory diseases with etiological overlap. A recent example is the NOD2 gene, for which distinct mutations have been shown not only to predispose to Crohn’s disease, a common and genetically complex inflammatory disorder, but also to cause Blau syndrome, a rare granulomatous inflammatory disease displaying Mendelian inheritance (8,9).

Two families have been described with similar inflammatory disorders, called PAPA syndrome and familial recurrent arthritis (FRA) (10,11). Both PAPA syndrome and FRA are characterized by autosomal dominant inheritance of early-onset, destructive, recurrent inflammation of joints, skin and muscle. Synovial tissue biopsy reveals massive polymorphonuclear infiltrate without the presence of immunoglobulin or complement deposits. Pyoderma gangrenosum-like ulcerative lesions occur in the skin of some affected individuals, as does severe cystic acne. Episodic inflammatory arthritis typically does not resolve spontaneously, and must be treated with intra-articular steroids or surgical drainage of the infiltrate. An associated infectious or environmental agent responsible for triggering these flares has yet to be identified. We have hypothesized that inflammatory episodes in both disorders may arise via a mechanism having relevance to other diseases with apparent etiological overlap, such as cystic acne, rheumatoid arthritis and inflammatory bowel disease. We therefore initiated positional cloning of the gene(s) responsible for PAPA syndrome and FRA. By linkage analysis of genome-wide scans, we mapped both PAPA syndrome and FRA in two extended kindreds to overlapping intervals of chromosome 15q.

We now report positional cloning of a single gene responsible for both PAPA syndrome and FRA. Genes encoded in the 15q critical region were assembled from available databases, and exons were determined in silico and screened for heterozygous mutations. In one gene encoding CD2-binding protein 1, two different missense mutations were identified that co-segregated with the clinical presentation of PAPA syndrome and FRA, respectively, in the two originally-reported extended kindreds. Yeast two-hybrid interaction trap assays revealed that the known binding between the PEST-type protein tyrosine phosphatase (PTP PEST) and CD2BP1 is almost completely abolished in the presence of either disease-causing missense mutation. These results support our original suggestion that PAPA syndrome and FRA (now referred to collectively as ‘PAPA syndrome’) are the same disease and demonstrate that alteration of CD2BP1 is responsible for this severe inflammatory disease.

RESULTS

Construction of a transcription map of the PAPA syndrome critical region

We constructed a partial physical and transcription map of the 15q interval linked to PAPA syndrome by assembling bacterial artificial chromosome (BAC) clones and known genes in silico from public databases. A high-throughput genomic sequence of BAC clones found in GenBank was searched for coding regions by a combination of BLAST analysis (13) against several protein and nucleotide databases and application of exon prediction algorithms. The resulting physical map confirmed genes identified from the G3 and GB4 radiation hybrid maps (14,15) (data not shown). A schematic representation

Figure 1. Partial physical and transcription map of the PAPA syndrome critical region. PAPA syndrome was previously localized to a 10 cM interval of chromosome 15q between D15S1023 and D15S979 (right vertical bar) in the originally described family. FRA was localized to a larger region between D15S5D and D15S127 (left vertical bar) encompassing the PAPA syndrome locus. Candidate genes encoded in this interval are shown: cellular retinoic acid-binding protein 1 (CRABP1); CD2BP1; reticulocalbin 2 (RCN2); cathepsin H (CTSH); BCL2-related protein A1 (BCL2A1); interleukin-16 (IL16); SH3-domain GRB2-like 3 (SH3GL3). Those shown in bold were screened for mutations in affected individuals.
of the PAPA syndrome critical region and selected candidate genes is shown in Figure 1.

Selection of candidate genes and mutation screening

PAPA syndrome is characterized by dominant inheritance of severe and unusual episodic arthritis without discernible infection. Erythematous ulcerative skin lesions (pyoderma gangrenosum) and cystic acne also occur in some but not all affected family members (Fig. 2A and B). These findings prompted us to examine genes having a possible role in the inflammatory response. Affected individuals from family FRA1 were screened for mutations in selected genes, including interleukin-16 (IL16), cellular retinoic acid-binding protein 1 (CRABP1), and BCL2-related protein A1 (BCL2A1) by direct DNA sequencing or denaturing high performance liquid chromatography (DHPLC) (16). The CD2-binding protein 1 (CD2BP1) gene was considered a plausible candidate because it encodes a phosphoprotein highly expressed in hematopoietic tissues, and evidence suggests that the CD2BP1 protein may regulate cytoskeletal organization in hematopoietic cells (17).

We designed PCR primers from the CD2BP1 sequence to amplify the cDNA in six overlapping fragments (Fig. 3). These amplimers were generated by reverse transcription–polymerase chain reaction (RT–PCR) of total RNA from affected family members FRA1-1 and FRA1-10 Epstein–Barr virus (EBV) lymphoblastoid cell lines. Analysis by denaturing high-performance liquid chromatography (DHPLC) detected heteroduplex formation in the fragment amplified by primers 4F and 4R, suggesting the presence of a sequence variant in one allele. Interestingly, alternative splicing of this region in activated T cells has been reported to generate two transcripts differing by 57 nucleotides (17). However, we detected only the longer version in amplified patient lymphoblastoid cDNA (data not shown). The exon/intron boundaries of CD2BP1 were derived by alignment of the cDNA sequence with a human genomic sequence assembled from available databases. This analysis indicated that the CD2BP1 gene contains 15 exons, with the alternatively spliced region encompassing exon 12. Primers were designed to amplify each exon (Fig. 3 and Table 1).

Sequence analysis of a portion of exon 11 identified a G-to-C transversion at nucleotide 964 of the CD2BP1 cDNA creating an E250Q variant that co-segregates with disease in the FRA family (Fig. 4A). Similar analyses in members of the PAPA family detected a G-to-A transition at nucleotide 904 of CD2BP1 predicted to create an A230T variant in affected individuals (Fig. 4B). Sequencing of the cloned 5′ exon 11 or

Figure 2. Clinical features of PAPA syndrome. PAPA syndrome is characterized by autoimmune destruction of joints and skin. (A) Arthritis in the proband FRA1-1 characteristically involves primarily the elbows, knees and ankles. Shown here is severe swelling of the knee during a flare. (B) Ulcerative skin lesion (pyoderma gangrenosum) is shown for the FRA1-1 proband.

Figure 3. Structure of the CD2BP1 gene. Predicted exons are shown by alternating gray and black shading and are numbered below each line. Primer sequences used for PCR amplification of cDNA are underlined; primer names are given in bold print above the sequence for forward primers and below for reverse. The G904A and G964C mutations are shown.
Table 1. Exons of the CD2BP1 gene

<table>
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<tr>
<th>Exon</th>
<th>Position in cDNA</th>
<th>Forward PCR primer 5'-3'</th>
<th>Position</th>
<th>Reverse PCR primer 5'-3'</th>
<th>Position</th>
<th>Product bp</th>
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<tr>
<td>1</td>
<td>5' UTR –252</td>
<td>AGACTGTGTCCCTCCATCACC</td>
<td>–95</td>
<td>TGGAGATTCACGCTAGCTC</td>
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<td>AGAACAGAAGGAGCTCCCTT</td>
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The position of each exon in the published CD2BP1L sequence is given. Positions of oligonucleotide primers are given as distances in nucleotides from the 5' or 3' end of each exon; for example, '–95' is 95 nucleotides away from the beginning or end of the exon, and '+16' is 16 nucleotides within the exon.

Figure 4. Segregation of the G964C or G904A mutations with FRA or PAPA syndrome, respectively. (A) The proband FRA1-1 is denoted by an arrow. Reverse sequence and DHPLC traces are shown for each individual, except FRA1-2 and FRA1-13 for which DHPLC was not performed.
exon 10 amplifiers from affected individual FRA1-1 or PAPA1-114, respectively, confirmed the presence of both mutant and wild-type sequences. DHPLC analysis of 228 unrelated control chromosomes revealed no heterozygous sequence variants in PCR-amplified CD2BP1 exons 10 or 11; DNA sequencing revealed no differences from the published sequence in 72 of these (data not shown). By our analysis, neither the E250Q or A230T alteration has been described in the predicted untranslated region (UTR) of the protein tyrosine phosphatase PTP PEST. The failure of clone 81 to grow in the absence of histidine when in combination with the mutant construct, versus its strong growth with the wild-type CD2BP1, suggested that the E250Q change disrupts CD2BP1:PTP PEST binding. However, this interaction was not completely abrogated. Plating clone 81:CD2BP1 E250Q–GAL4BD cotransformants on media that does not select for interactions (−Leu, −Trp, +His) resulted in colonies with weak but reproducible lacZ activity, indicating low-level interaction with the mutant. We quantitatively assayed the interactions between clone 81 and either wild-type or E250Q mutant CD2BP1 by measuring β-galactosidase (β-GAL) activity. Triplicate measurements resulted in an average of 1.2 β-GAL units for wild-type CD2BP1:clone 81 interaction, compared with an average of 1.2 β-GAL units for the E250Q mutant CD2BP1:clone 81 interaction. It therefore appears that a residual interaction of approximately 1.2% of wild-type levels still occurs between PTP PEST and the E250Q mutant, but is insufficient to result in histidine prototrophy in the two-hybrid screen. In repeated experiments, when the A230T mutant was transformed with clone 81, the level of β-GAL activity was reduced to less than 10% of wild-type levels, confirming that this residue is also important for PTP PEST interaction with CD2BP1. In parallel quantitative two-hybrid experiments we found no detectable differences in the interactions between CD2 and wild-type, E250Q or A230T CD2BP1 mutants; however, deletion of the CD2BP1 SH3 domain completely abolished this interaction, confirming the previous observation that this domain is essential for CD2 binding.

**DISCUSSION**

We conclude that the heterozygous mutations in the CD2BP1 gene cause PAPA syndrome in the two families we have studied. Given what is currently known about CD2BP1 function combined with the clinical features of this disorder, we have formulated two alternative models of disease pathogenesis. The first derives from the resemblance of PAPA syndrome to classic autoimmune disease. By definition, autoimmune disorders are mediated by the adaptive immune system. The presence of the E250Q change disrupts CD2BP1:PTP PEST binding.
response, in which T and/or B cells respond to specific self antigens and ultimately produce tissue damage and destruction (4,23). According to this model, flares seen in PAPA syndrome would be initiated by reaction of a subset of T or B cells to a specific component of skin, muscle and joints (10). CD2BP1 is expressed in T-cells and has been proposed to be important in regulating T-cell behavior by modulation of CD2 activity (17). CD2 is a cell surface molecule that binds to the glycoprotein CD58 on opposing antigen-presenting cells (APCs), thereby improving the efficiency with which T-cell receptors find a major histocompatibility complex (MHC) molecule together with an antigenic peptide (24,25). Increasing levels of CD2BP1 in transfected cells reduce CD2-mediated sheep red blood cell (RBC) rosetting, implying that CD2BP1 downregulates CD2-mediated T-cell adhesion activity (17). Although we have not detected infiltration by T or B cells in affected tissues (11), we cannot rule out the possibility that disease-causing mutations in CD2BP1 may alter T-cell activity in PAPA syndrome patients and precipitate the observed influx of polymorphonuclear cells (neutrophils) into inflammatory sites.

The severe neutrophil infiltration in affected tissues suggests a second model of disease pathogenesis in which inflammation results directly from dysfunction of the innate immune response. In this scenario, disease-causing mutations in CD2BP1 somehow exaggerate the signal for proliferation and infiltration of inflammatory initiator cells, alter apoptotic pathways and/or inhibit their clearance, leading to the observed abnormal accumulations of neutrophil-rich material. The recent report that the CD2BP1 protein binds pyrin, the protein encoded by the MEVF gene responsible for familial mediterranean fever (FMF), supports this model (26). FMF is a rare, recessively inherited autoinflammatory disease characterized by self-limited episodes of fever with arthritis, and sterile inflammation of peritoneal and pleural membranes (27). Although clinically distinct from PAPA syndrome, FMF is also characterized by neutrophil infiltration into target tissues, and pyrin itself is expressed in neutrophils but not T or B cells (28,29). It is interesting that of five proteins (including pyrin) responsible for inherited autoinflammatory diseases, four contain apoptosis-related domains, and two are well-recognized regulators of apoptosis. This has led to the hypothesis that mutations in these genes may inhibit apoptosis, leading to prolonged accumulation of neutrophils (30). Perhaps disease-causing mutations in CD2BP1 alter a pyrin-mediated apoptotic pathway in neutrophils.

For either model of disease pathogenesis, we hypothesize that low-level inflammation is constitutively present in PAPA syndrome, and clinical flares may result from mild physical trauma that induces accumulation of hyperresponsive inflammatory cells. Consistent with this, members of both families described here report ulcerative lesions arising at sites of parenteral injections (10, our unpublished observations). Whether PAPA syndrome is mediated by lesions in adaptive or innate immunity, or some alternative pathway, will require detailed functional analyses in cells of affected patients.

Various experiments have supported the hypothesis that CD2BP1 and PTP PEST function in cytoskeletal regulation. It was originally shown by confocal microscopy that endogenous murine CD2BP1 associated with the cortical actin cytoskeleton, lamellipodia and membrane-bound F-actin in the cytokinetic cleavage furrow in dividing NIH 3T3 cells. Overexpression of the protein caused formation of distinct morphological changes in NIH 3T3 cells, with formation of extended filopodia (18). In the human Jurkat J77 T cell line, stable CD2BP1 transfectants showed normal motility and filopodial formation in response to CD2 triggering, whereas cells lacking CD2BP1 were non-responsive, suggesting that CD2BP1 regulates CD2-activated integrin adhesion pathways in T cells. PTP PEST is expressed in hematopoietic tissues, and has been proposed to negatively regulate lymphocyte activation (17). In fibroblasts homozygously deleted for PTP PEST, murine CD2BP1, as well as other known PTP PEST substrates p130Cas, paxillin and focal adhesion kinase (FAK), are hyperphosphorylated, and this correlates with a significant increase in the number of cells apparently blocked in a late stage of cytokinesis (31). Further studies of morphology and function in cells of patients affected with PAPA syndrome, as well as cell transfection studies, will determine whether disease-causing mutations in CD2BP1 confer cytoskeletal dysregulation.

The interaction of CD2BP1 with PTP PEST is well described (18). This interaction is mediated by the coiled-coil domain of murine CD2BP1 and the terminal 20 amino acids of hematopoietic stem cell fraction PTP, or related phosphatases (18). Introduction of a W232A mutation within the same coiled-coil domain abolished the interaction with the hematopoietic stem cell fraction PTP in vitro and in vivo. The mutant protein was also hyperphosphorylated, implying that CD2BP1 is a substrate of PTPs (22). Our data confirm the strong interaction between PTP PEST and CD2BP1 and demonstrate loss of binding between the two mutants A230T and E250Q that lie within the predicted coiled-coil domain. Whether the pathophysiology of PAPA syndrome is due to loss of function from reduction in PTP PEST binding, a dominant negative effect due to alteration of CD2BP1 interaction with other proteins or an alternative mechanism will require further study. In any case, it is interesting that the changes produced by CD2BP1 missense mutations are sufficient to confer susceptibility to inflammatory episodes, suggesting that CD2BP1 is critical in the homeostasis of inflammation. This also suggests to us that more drastic nonsense or deletion mutations in CD2BP1 could confer chronic, severe inflammation or lethality.

CD2BP1 or its murine ortholog was previously shown to interact with two additional proteins, c-Abl and WASP. The c-Abl proto-oncoprotein involved in various human leukemias is a tyrosine kinase that appears to be important in regulation of the cytoskeleton and binds actin filaments directly through a C-terminal domain (32). The protein also binds and phosphorylates murine CD2BP1. A model has been proposed suggesting that CD2BP1 functions to couple PTP PEST to c-Abl, allowing dephosphorylation and hence regulation of activity of the c-Abl protein (20). WASP is linked to actin polymerization via interaction with the Rho family GT Pase CDC42Hs (33). Mutations in WASP are responsible for the X-linked hematopoietic deficiencies and platelet abnormalities of WAS as well as X-linked congenital neutropenia (34–36). WASP binding to murine CD2BP1 appears to be phosphorylation—dependent; mutation of tyrosine 367 within the SH3 binding domain to aspartate or glutamate to mimic the negative charge
of a phosphorylated tyrosine severely reduced co-immunoprecipitation of the two proteins (21). Although binding between WASP (or CD2) and both E250Q and A230T mutant forms of CD2BP1 was unaltered in yeast two-hybrid experiments (our unpublished results), in mammalian cells, these interactions may be affected as a result of CD2BP1 hyperphosphorylation in the absence of PTP PEST. In particular, WASP association with CD2BP1 is predicted to decrease in the presence of the disease-causing mutations. In addition, the subsequent activities each of these substrates might be altered by an inability to couple to PTP PEST.

Pyoderma gangrenosum (PG) and cystic acne are conditions sometimes associated with common inflammatory autoimmune diseases. PG is a painful disease of the skin in which erythematous sterile pustules develop into ulcerative necrotic lesions, typically on the feet and extremities. Although successful treatments have been described, PG can be fatal (37–39). Kindreds segregating PG have been reported (40); however, a genetic etiology is not generally attributed to this disease. The disease occurs most often in association with rheumatoid arthritis or inflammatory bowel disease (Crohn’s disease or ulcerative colitis). In children, PG is most often associated with ulcerative colitis (41). In this regard, it is interesting that the human PTP PEST is encoded on chromosome 7q in a region exhibiting evidence of linkage to inflammatory bowel disease (42). Cystic acne is common, estimated to occur in 10–20% of adolescents and young adults. More rarely it is associated with arthritis or PG (43–45). These observations suggest to us some etiological overlap between PAPA syndrome and more common and genetically complex disorders such as rheumatoid arthritis and inflammatory bowel disease. Functional studies of CD2BP1, PTP PEST and other CD2BP1-binding partners will be important in elucidating pathways involved in PAPA syndrome and related inflammatory diseases.

MATERIALS AND METHODS

Electronic transcript mapping

A high-throughput genomic sequence of BACs identified from public databases (primarily at the National Center for Biotechnology Information) was obtained from GenBank. The NIX platform (http://menu.hgmp.mrc.ac.uk/menu-bin/Nix/Nix.pl) was used for both exon prediction and BLAST analyses.

CD2BP1 exon prediction

The genomic structure of CD2BP1 was derived by aligning the CD2BP1 cDNA sequence to a human genomic sequence obtained from available databases by the Seqman program (DNASTAR, Inc., Madison, WI).

PCR amplification

Whole-cell RNA was isolated from EBV-transformed lymphoblastoid cell lines established from patients FRA1-1 and FRA1-10 by a modified guanidine thiocyanate lysis procedure (Roche Boehringer Mannheim, Indianapolis, IN). RNA was converted to cDNA by both random priming and dT priming with reverse transcriptase (Roche Boehringer Mannheim). CD2BP1 was produced in six fragments by amplification of cDNA with the following primers: CD2BP1-1F: ATG ATG CCC CAG CTC GCA; CD2BP1-1R: CCT TCT GCC TCT CAC GAA AC; CD2BP1-2F: CAA ATG GAG AAT GTG GGC AG; CD2BP1-2R: TGG CTT TGT GTC TCT TCT; CD2BP1-3F: CCA AGA AGA CAT ACC AGC AGA AG; CD2BP1-3R: CCG CAC TTC CTC GTA GAG; CD2BP1-4F: GCA GTG TGT CAA GGA TGA TG; CD2BP1-4R: GTC TCT GTC GAC GCA GCA G; CD2BP1-5F: CGC CCT ACC AGA ACT ATT ACG; CD2BP1-5R: CCA CAG TCC ACC AGC CAT; CD2BP1-6F: CAG CCG AGA ACC CAG ATG A; CD2BP1-6R: AAG ACA GAA CGC CCT CCT TT. Amplification conditions for all cDNA fragments were 35 cycles, 94°C 30 s, 56°C 30 s, 72°C 60 s. CD2BP1 exons were amplified from genomic DNA by oligonucleotide primers described in Table 1.

Mutation analysis

DHPLC was performed by WAVE analysis (Transgenicom, San Jose, CA) utilizing conditions recommended by WaveMaker v3.3.3 software. CD2BP1 amplified fragments 1, 2, 3 and 5 were analyzed at an oven temperature of 65°C, fragments 4 and 5’ exon 11 at 64°C, and fragment 6 at 66°C. PCR amplimers were purified from 1.2% agarose gels with the QIAquick gel purification kit (Qiagen, Hilden, Germany) and sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) or Tetrads (MJ Research, Waltham, MA) thermocycler. Reactions were performed in 25 μl containing 1.5 mM MgCl2, 500 μM KCl, 0.25 mM each dNTP, 1.0 pmol each primer, 0.5 U Taq polymerase (PE Corp., Norwalk, CT), and 100 ng patient DNA. Amplification conditions were 35 cycles of 94°C 30 s, 56°C 30 s, 72°C 60 s, except 5’ exon 11, for which an annealing temperature of 55°C was used.

Yeast two-hybrid analysis

The wild-type CD2BP1 cDNA was PCR amplified from a human spleen cDNA library using primers 1 (5’ATTGGATC-CAGCTGAGTTCAAAAGATG 3’) and 2 (5’AAATTGCAG-TAGCAAGGTGGGACAGTGC 3’). The CD2BP1 SH3 domain deletion construct was built using primer 1 and primer 3 (5’ AATGTGACGGGTCTCCGTTATCTCTCGT 3’). Amplification products were purified, restriction-digested with BamHI and SalI and cloned into the corresponding sites of pAS2-1 (Clontech, Palo Alto, CA). The E250Q and A230T mutant cDNA clones were derived by PCR site-directed mutagenesis of wild-type CD2BP1 and subcloned into pAS2-1. The cytoplasmic domain of CD2 was PCR-amplified
from a human spleen cDNA library using primers 4 (5’ ATTGGATCCGACCCACACGAGATCTAGTA 3’) and 5 (5’ AATGGTCAGTACGCAAAGTTGGGACAGTG 3’). Amplification products were purified, restriction-digested with BamHI and SacI and cloned into the corresponding sites of ACT2AD (Clontech). Wild-type and mutant constructs were purified, and the DNA sequence of the fusion proteins was confirmed by automated fluorescence sequencing as described above. Yeast two-hybrid screening was performed using the strains, media, vectors and protocols from the Matchmaker two-hybrid system (Clontech). Cells containing the wild-type CD2BP1-GAL4BD construct were transformed with 10 μg of a human thymus cDNA library in pACT2 (Clontech). Approximately 2 × 10^6 transformants were spread on SD/-Leu/-Trp/-His/+3-AT plates. An aliquot was also plated on SD/-Leu/-Trp to test co-transformation efficiencies. After incubation at 30°C, positive clones were patched on SD/-Leu/-Trp/-His/+3-AT (25 mm) plates, and assayed for β-galactosidase (β-GAL) activity using a colony-lift filter assay. Quantitative β-gal assays were performed in triplicate using CPRG (Roche Molecular Biochemicals) as a substrate. Binding domain constructs were cured from yeast clones by growth in -Leu media followed by duplicate plating to -Trp media to identify clones that had lost the GAL4BD construct. These cured clones were then re-transformed with the E25Q mutant-GAL4BD construct or with the wild-type construct, and were restested for histidine prototrophy.

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