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Alternative Splicing Determines the Domain Structure of WWP1, a Nedd4 Family Protein

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Nedd-4-like proteins are E3 ubiquitin-ligase molecules which regulate key trafficking decisions, including targeting of proteins to proteosomes or lysosomes. Here we show that a human Nedd4 family gene, WWP1, is localized on 8q21 and generates at least six isoforms through alternative splicing. We show that alternative splicing affects the domain structure of WWP1, with forms that contain or lack an N-terminal C2 domain. Interestingly, the relative ratio of these forms varies in a tissue-specific manner. Other splice forms were also identified which may disrupt the structure of the C2 domain by removing its predicted C-terminal beta-strands. One splice form generates, through the introduction of a reading frame shift, a C2 domain-only form of WWP1. We discuss the hypothesis that regulation of splice site usage may modulate the activity of WWP1 and possibly other Nedd4 family proteins.

Key Words: **WWP1; Nedd4; Itch; splicing; C2 domain; trafficking; ubiquitin.**

The labeling of specific protein substrates with ubiquitin is a widespread mechanism by which protein function can be posttranslationally regulated. Ubiquitination regulates numerous protein trafficking processes including delivery of targeted proteins to the proteosome, endocytosis and endosomal sorting to the lysosome (1). In the ubiquitination process it is generally E3 class ubiquitin ligases which determine target protein selection. An important group of E3 proteins is the Nedd4 family, which contain a C-terminal HECT class ubiquitin ligase domain (2, 3). In addition, Nedd4 proteins are defined by the presence of an N-terminal C2 domain, which is a membrane lipid interaction motif (4, 5), and two to four WW domains which are thought to mediate target protein selection (6). Nedd4 family proteins have been shown to have diverse functions in different organisms. These roles include down-regulation of membrane proteins by endocytosis (7–13), virus budding, exocytosis of certain membrane proteins (14, 15), transcription factor regulation (16, 17), proteosome dependent degradation (18–21) and endoproteolytic cleavage (22). Given the plethora of roles for Nedd4 family proteins, it is likely that there are numerous regulatory mechanisms for initiating, terminating and targeting of their activity. One way in which a protein's activity can be regulated is through the generation of alternative splice forms. Here we show that WWP1, a human Nedd4 family gene (16–18, 23, 24) generates at least six alternatively spliced isoforms, which affect the domain structure of the protein. We have determined the chromosomal localization of the WWP1 gene and also a closely related processed pseudogene. We show that the identified splice forms of WWP1 are consistent with authentic splice donor and acceptor sites within the WWP1 gene. Interestingly we show that the relative ratio of the different WWP1 splice forms varies in a tissue-specific manner and we discuss the possible functional implications of such variation.

MATERIALS AND METHODS

cDNA clone of WWP1. Sequences of WWP1 have previously been reported (18, 23, 24). The cDNA clone referred to in this study was identified independently in a T47D cell-line (25) cDNA library (gift of Dr. Tania Nolan and Dr. Ged Brady, University of Manchester). The nucleotide numbers used as reference points in this study, refer to clone (2aI) identified in this screen (GenBank AY043361), which consists of 3479 bp and encodes a complete 922-amino-acid ORF. Sequence database searches were performed using BLAST programs at the National Center for Biotechnology Information website.

Identification of splice forms. Tissue mRNA samples were obtained from Clontech. RNA was prepared from the T47D cell line using RNAzol (Gibco-BRL) and a mRNA fraction was obtained following affinity purification using oligo-dT Sepharose (Sigma). RT-PCRs were performed on 50 ng of tissue specific mRNA (Clontech), or 5 ng mRNA template from the T47D cell line, using the SuperScript One-Step RT-PCR system (Gibco-BRL) and a Perkin–Elmer 2400

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cycler. Primer sequences corresponded to nucleotides 2–23 (forward) and 1267–1245 (reverse) of clone 2aI, i.e., 5′-gaaagagggaatcgtgtcttac and 5′-acgatcatcaactcttctttccc, respectively. One cycle of cDNA synthesis was performed at 50°C for 30 min followed by denaturation for 2 min and 35 cycles of PCR amplification (denature at 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min). For identification of splice variants, the purified PCR products were cloned into pGEM T-easy (Promega) and inserts sequenced using an Applied Biosystems automated sequencer and Big Dye chemistry (Perkin–Elmer).

Fluorescence in situ hybridization (FISH). FISH was performed using cosmids LA0835L9, LA0848B17, and LA0849F10 (Los Alamos National Laboratory, supplied by UK HGMP Resource Centre) which were identified by screening of the chromosome 8-specific genomic clone filter, LA08NCO1 (Los Alamos National Laboratory, supplied by UK HGMP Resource Centre), using clone 2aI as a probe. For FISH, the genomic clones were labeled with biotin using a Bionick kit (Gibco-BRL). 80 ng of labeled probe was precipitated in the presence of 3–5 μg Cot1 DNA and then dissolved in hybridization mixture (50% formamide, 10% dextran sulfate and 2× SSC). Probes were denatured at 80°C for 5 min, left to preanneal for 30 min, and then applied to denatured metaphase spreads prepared from normal PHA-stimulated human lymphocytes. The metaphase spreads were denatured in 70% formamide in 2× SSC at 73°C and dehydrated in an ethanol series. Probes were left to hybridize for 12–16 h at 37°C then washed at 42°C in 50% formamide/2× SSC and then in 2× SSC. The biotinylated probes were detected with FITC-conjugated avidin (Vector Laboratories) and the metaphase spreads counterstained with 4,6-diamino-2-phenylindole (DAPI). Images were captured with a cooled charge-coupled device (CCD) camera attached to a Zeiss axioskop microscope, and then analyzed using Quips (Vysis, Inc., Downers Grove, IL) software. At least 10 metaphases were examined for each probe.

RESULTS

Identification of WWP1 Splice Forms

To analyze the expression of the WWP1 gene a RT-PCR protocol was adopted. Two primers were designed which were located near the 5′ end of the WWP1 open-reading frame and which flanked the C2 domain containing region. Primers were first tested on a RNA template derived from the T47D cell line (25), since we had already established that WWP1 was expressed in these cells. Surprisingly, six RT-PCR products were detected (A to F), which were cloned and sequenced (Figs. 1a and 1b). Form A corresponded to the expected sequence of WWP1, while forms E and F corresponded to forms lacking the C2 domain. Other forms were also identified, which corresponded to deletions of the C-terminal end of the C2 domain (form C) and truncations of the protein ORF within or before the C2 domain (B and D, respectively). These truncations arose due to the introduction of a frame shift leading to an in frame stop codon. The barely detectable splice form D could be translated to give only a short 32 amino acid peptide from the existing initiating methionine. We cannot, however, rule out that a downstream methionine could be used to initiate another C2 minus form of the protein from this transcript. Splice form C, as well as deleting part of the C2 domain and some sequence C-terminal to it, also introduced a single amino acid change (Leu to Phe) at the new exon–exon boundary.

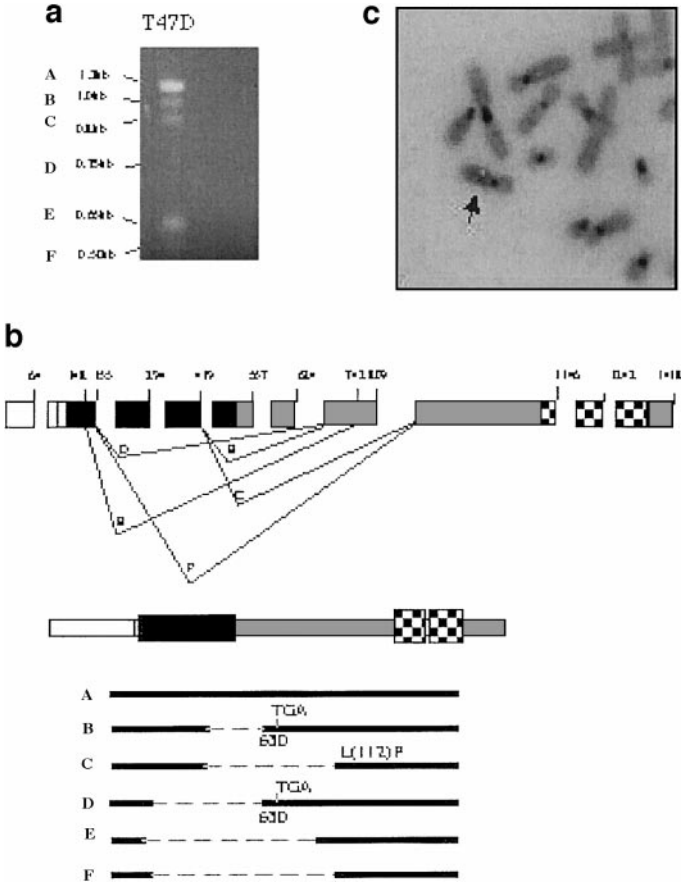

FIG. 1. Genomic organization and splice forms of the 5′ end of the WWP1 reading frame. (a) Ethidium bromide-stained agarose gel showing RT-PCR products obtained. Six bands were identified, labeled A to F, and subcloned. (b) Exon structure of the 5′ end of WWP1 gene (shown not to scale). Boxes denote exons identified in full-length form A. Unshaded region represents 5′ untranslated sequence, black boxes represent C2 domain region, and checkered boxes represent WW domain coding sequence. Numbers indicate nucleotide positions of splice sites, compared to clone 2aI (GenBank Accession AY043361). Alternative usage of splice junctions in forms B–F is indicated below. Note that form E uses alternative splice donor and acceptor sites that lie within the A form exons; all other splice variants are derived from exon skipping. The six alternative splice products are depicted schematically below. Horizontal thick black lines indicate the extent of the RT-PCR products derived from the splice forms A to F, with dashed lines indicating the portions removed in different splice forms. Positions of in frame stop codons (TGA) introduced in splice forms B and D are indicated. In splice form C the new splice junction introduces an amino acid change of Leu 112 to Phe as indicated, but the remainder of the open reading frame is intact. (c) FISH mapping of WWP1 genomic clones LA083519, LA0848b17, and LA0849f10 to chromosome 8q21. Positive signal represented by double light spots is indicated with an arrow. Only a partial metaphase spread is shown.

Analysis of a sequence alignment of the C2 domain of WWP1 with other C2 domains, together with consideration of the known C2 domain fold (4, 26), allowed prediction of the secondary structure elements within the WWP1 C2 domain sequence (Fig. 2). From this analysis it could be concluded that the B and C splice

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PLC-d1            ...LRVRIISGQQLPKVNKNKNSIVDPKVIVEIHGVGR-
hNedd4            ...VRVKVIAGIGLAKKDI--LGASDPYVRVTLYDPMN
mNedd4            ...VRVKVIAGIGLAKKDI--LGASDPYVRVTLYDPMS-
mNedd4-2             *TSLPLPRSLLEEVFL--PGICDPYVKLSLYVADEN
hNedd4-2          ...LDLPLSPLPTSDELFL--PGICDPYVKLSLYVADEN
WWP1-A            ...LQVTVSSAKLKRKKN---WFGTAIYTEVVVDG----
WWP1-B            ...LQVTVSSAKLKRKKN---WFGTAIYTEVVVDG----
WWP1-C            ...LQVTVSSAKLKRKKN---WFGTAIYTEVVVDG----
WWP2              ...LTLKVVSAKPKVHNR---QPRINSYVEVAVDGLPS-
Itch              ...LQITVISAKLKENKKN--WFGPSPYVEVTVDG----
AIP4-b           ...LQITVISAKLKENKKN--GWGPSPYVEVTVDG----

PLC-d1    DTGS-RQTAVITN-NGFNPKWDMEFEPEVTVPDLALVRFMVEDYDSSSKN
hNedd4    GVLTSVQTKTIKK--SLNPKWNEEILFRVLP-QRHRILFEVFDENRLTRD
mNedd4    GILTSVQTKTIKK--SLNPKWNEEILFRVLP-QRHRILHEVFDENRLTRD
mNedd4-2  RELALVQTKTIKK--TLNPKWNEEFYFRVNP-SNHRLLFEVFDENRLTRD
HNedd4-2  RELALVQTKTIKK--TLNPKWNEEFYFRVNP-SNHRLLFEVFDENRLTRD
WWP1-A    -----EITKTAKSSSSSNPKWDEQLTVNVTP--QTTLEFQVWSHRTLKAD
WWP1-B    -----EITKTAKSSSSSNPKWDEQLTVNVTP--QTTLEFQVWSHRTLKAD
WWP1-C    -----EITKTAKSSSSSNPKWDEQLTVNVTP--QTTLEFQVWSHRTLKAD
WWP2      -----ETKKTGKRIGSSELLWNEIIILNVTA--QSHLDLKVWSCHTLR-N
Itch      -----QSKKTEKCNNTNSPKWKQPLTVIVTP--TSKLCFRVWSHQTLKSD
AIP4-b    -----QSKKTEKCNNTNSPKWKQPLTVIVTP--VSKLHFRVWSHQTLKSD

PLC-d1    DFIGQSTIPWNS-------LKQGYRHVHLLSKNGDQHPSATLFVKISIQD*
hNedd4    DFLGQVDVPLYPLPTENPRLERPYTFKDFVLHPRSHKSRVKGYLRLKMTYLPKTSGSEDDNAEQAEELEPG...
mNedd4    DFLGQVDVPLYPLPTENPRMERPYTFKDFVLHPRSHKSRVKGYLRLKMTYLPKNGSEDENADQAEELEPGW...
MNedd4-2  DFLGQVDVPLSHLPTEDPTMERPYTFKDFLLRPRSHKSRVKGFLRLKMGYMPKNGGQDEENSEQRDDMEHG...
HNedd4-2  DFLGQVDVPLSHLPTEDPTMERPYTFKDFLLRPRSHKSRVKGFLRLKMAYMPKNGGQDEENSDQRDDMEHG...
WWP1-A    ALLGKATIDLKQALLIHNRKLERVKEQLKLSLENKNGIAQTGELTVVLDGLVIEQENITNCSSSPTIEIQE...
WWP1-B    ALLGKATIDLKQALLIHNRKCWLLKARME*
WWP1-C    ALLGKATIDLKQALLIHNRKFNGESSSFAPTDNASVTGTPVVSEENALSPNCTSTTVEDPPVQEILTSSEN...
WWP2      ELLGTASVNLSNVLKNNGGKMENMQLTLNLQTENKGSVVSGGKLTIFLDGPTVDLGNVPNGSALTDGSQLP...
Itch      VLLGTAGLDIYETLKSNNMKLEEVVMTLQLVGDKEPTET-MGDLSVCLDGLQVEAEVVTNGETSCSESTTQ...
AIP4-b    VLLGTAALDIYETLKSNNMKLEEVVVTLQLGGDKEPTET-IGDLSICLDGLQLESEVVTNGETTCSESASQ...
AIP4-a                                                MKTTLLKLIFCLNFDVKGASQ...
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FIG. 2. Alignment of the C2 domain of WWP1 with the C2 domain from phospholipase C-delta1 (PLC-d1), whose structure is known (26) and with C2 domains from Nedd4 family proteins. Bold lettering indicates the conserved clusters of hydrophobic amino acids that allow the positions of the eight beta-strands (underlined sequence of PLC-d1) to be deduced (4, 26). Asterisks mark positions of in-frame stop codons. The sequences of three splice forms of WWP1 (A, B, C corresponding to those depicted in Fig. 1) are indicated. Italic lettering in WWP1-B and C indicates sequence replacing the normal C2 domain sequence in these splice forms. AIP4-a and -b represent two alternative forms of the human AIP4/Itch gene identified from EST sequences (see text). AIP4-a contains an alternative N-terminus and initiating methionine (italic lettering) compared to the AIP4-b splice form. The mouse Nedd4-2 (mNedd4-2) ORF is predicted to start at the double underlined methionine (28), due to an earlier in-frame stop codon (asterisk). However, analysis of the predicted 5′ untranslated region reveals a C2 domain signature with very high homology to human Nedd4-2 (hNedd4-2). Homology between mNedd4-2 and hNedd4-2 only breaks down near the beginning of the C2 domain (italic lettering), in the vicinity of the mNedd4-2 in frame stop codon. The accession numbers of the sequences used were PLC-d1 (B28821) human Nedd4 (D42055), mouse Nedd4 (P46935), mouse Nedd4-2 (AF277232), human Nedd4-2 (KIAA0439), human WWP1 (AY043361), human WWP2 (NM007014), mouse Itch (NM008395), human AIP4 (CACO9387, BF203990, AU129193).

forms led to deletions of parts of the C2 domain which encompassed the terminal two beta strands.

To determine whether other Nedd4 family genes generated similar alternative transcripts we examined ESTs in the GenBank database corresponding to Nedd4 genes previously thought to lack a C2 domain. The mouse Nedd4 family protein Itch was originally reported to lack C2 domain sequence (27); however, reanalysis of the sequence showed a C2 domain was indeed present (24). The sequence of AIP4 (GenBank Accession CACO9387), the human orthologue of mouse Itch (18), does not contain a C2 domain sequence. Through searching of the GenBank database we identified two human ESTs (BF203990, AU129193) which encoded an alternative N-terminus of AIP4 with a recognizable C2 domain. This finding suggests that alternative splicing of Itch/AIP4 occurs similarly to WWP1. In the case of Itch/AIP4 however, the ORF of the alternate splice product was found to include a novel translation start site fused to sequences downstream of the C2 domain (Fig. 2). In contrast all the alternative forms of WWP1 that we have detected have the same predicted translation start site. It has also been reported that the ORF of mouse Nedd4-2 lacks a C2 domain (28). However, analysis of the translated mouse Nedd4-2 mRNA sequence (GenBank accession AF277232) upstream of the putative initiation methionine, revealed the presence of a C2 domain signature (Fig. 2). We compared the predicted mouse Nedd4-2 C2 domain sequence with the incomplete sequence of hu-

man Nedd4-2 which also contains a recognizable C2 domain sequence and found that the two amino acid sequences were virtually identical apart from at the N-terminal end of the C2 domain. The human and mouse sequences differ around the location where an in frame stop codon is found in the mouse sequence (Fig. 2) but not in the human. This prompts us to speculate that these human and mouse forms represent two different splice forms of the Nedd4-2 gene which initiate translation from different sites and that further splice forms of mouse Nedd4-2 may exist which allow its C2 domain translation.

Chromosomal Localization of the WWP1 Gene

To confirm that the identified RT-PCR products were genuine splice variants of WWP1, we investigated the structure of the genomic region around the alternatively spliced region. Initial FISH mapping to human metaphase chromosomes, using the 2aI cDNA as a probe, gave positive signals on both chromosome 3 and 8 (data not shown). Screening of a chromosome 8 specific genomic clone filter LA08NCO1 (Los Alamos National Laboratory, supplied by UK HGMP Resource Centre) allowed the identification of three positively hybridizing cosmids (LA0835l9, LA0848b17 and LA0849f10). The latter were used as probes for FISH mapping to human metaphase chromosomes. All three cosmids mapped to 8q21 (Fig. 1c). No positively hybridizing clones were obtained from a chromosome 3 specific filter. At this time publicly available DNA sequence from the Human Genome Project became available (GenBank AC083845) which, in conjunction with the cDNA sequence, allowed the intron–exon boundaries at the 5′ end of the WWP1 gene to be determined. Appropriate splice junction sequences were identified corresponding to those required to generate all of the six splice forms (Fig. 1b). This analysis confirms that the observed RT-PCR products reflect genuine alternative splice site usage and are not experimental artifacts. All the splice forms, apart from E, arose by skipping of intervening exons which were used in splice form A. Splice form E, however, arose from use of alternative donor and acceptor sites lying within two of the A form exons (Fig. 1b).

Interestingly, during this analysis a very closely related genomic DNA sequence was identified which was located on chromosome 3 (GenBank AC016962). Translation of the sequence revealed high amino acid similarity to WWP1 but the amino acid reading frame was interrupted in several places with stop codons (Fig. 3). This result together with the fact that the genomic sequence was not interrupted by introns suggested that the chromosome 3 sequence was a processed pseudogene. This finding may explain the original positive signal obtained by FISH using the WWP1 cDNA probe. Analysis of the sequence of the identified alternative forms of WWP1 showed that none of these forms could have arisen from transcription of its related pseudogene.

Expression of WWP1 Splice Variants Is Widespread and Regulated in a Tissue-Specific Manner

To determine whether alternative splicing of WWP1 was a general phenomenon or a specific feature of splicing misregulation within the T47D tumour cell line, we compared expression of WWP1 splice forms across a number of different human tissues. Figure 4 shows that WWP1 was expressed in all tissues investigated. While alternative splicing occurred in all these samples, the relative ratios of different splice products appeared to be regulated in a tissue-specific fashion. In all samples the C2 domain plus and minus forms (A and E) were most highly represented and the relative proportion of other forms was significantly lower compared to the tumor cell line T47D. Splice forms B and D were not detected in tissue derived RNA samples. This was true even in tissues that gave a relatively strong signal for the A and E forms, such as skeletal muscle. In testis and bone marrow RNA, the ratio of the E to A forms of WWP1 was higher compared to that of adult liver tissue RNA. Other samples, including fetal and adult brain, skeletal muscle and fetal liver tissue, showed an intermediate ratio of the E to A forms. It was interesting to note that the RNA from fetal liver contained a higher ratio of E to A forms compared with adult liver, suggesting that this ratio may be developmentally regulated in some circumstances.

DISCUSSION

An accumulation of data from different organisms has shown that Nedd4 family proteins, through targeted protein ubiquitination, mediate a variety of protein trafficking decisions on the secretory, endocytic and proteosomal pathways. Some members of the family have been shown to mediate several functions. Little is known, however, about how the balance between these roles is achieved and regulated. Here we have demonstrated that the human Nedd4 family protein, WWP1, undergoes alternative splicing which generates forms of the protein that have or lack the C2 domain. Analysis of the relative abundance of the different WWP1 splice forms across a range of different tissues found evidence that the ratio of alternative forms can be regulated in a tissue-specific manner. We also presented evidence that a similar splicing in or out of the C2 domain occurs in at least one other Nedd4 family gene, namely human AIP4/Itch and possibly also in Nedd4-2. The splicing in or out of the C2 domain in Nedd4 family proteins is likely to have important consequences on their activity. One possible consequence is that alternative forms are targeted to differ-

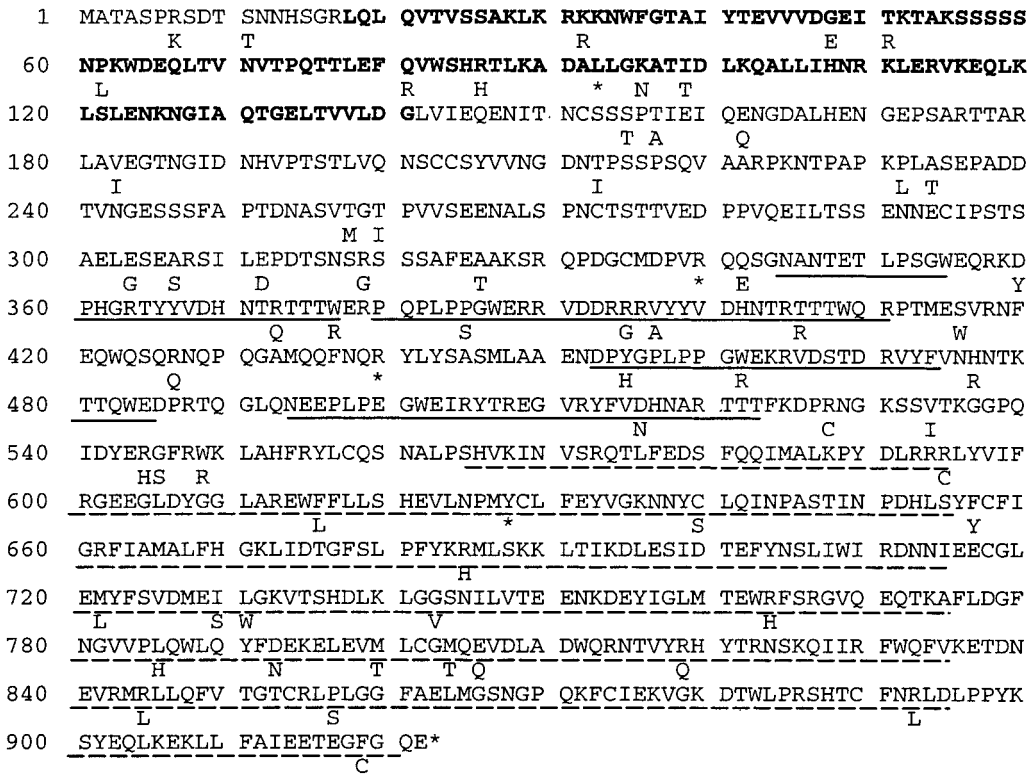

FIG. 3. A pseudogene sequence related to WWP1 on chromosome 3. The amino acid sequence is shown of the complete open-reading frame of WWP1, with amino acid differences in the interrupted reading frame of the pseudogene shown below. Asterisks indicate positions of in-frame stop codons in the pseudogene sequence. The C2 domain of WWP1 is indicated with bold lettering, the four WW domains are underlined with a solid line and the HECT domain is underlined with a broken line. Amino acid substitutions in the pseudogene are distributed evenly throughout the sequence in both conserved and nonconserved regions.

ent locations and thus may possibly regulate target protein selection. This hypothesis is supported by data, which show that the C2 domain of Nedd4 is important for its localization to lipid rafts (29). An alternative and intriguing possibility is that the outcome of target selection and ubiquitination could depend on the presence or absence of its C2 domain. In this regard, it is important to note that C2 domains in other proteins mediate interactions with proteins as well as membrane lipid. For example, the C2 domain of Nedd4 binds to annexin XIII (29) and C2 domains of synaptotagmin mediate interactions with syntaxin, a SNARE protein involved in membrane fusion (30) and AP-2, a protein involved in formation of clathrin coated pits during endocytosis (31). It is possible therefore that the C2 domains of Nedd4 family proteins play a direct role in mediating their trafficking functions. Interestingly, in yeast, the C2 domain of Rsp5 was found not to be required for ubiquitination of its target permease proteins, but the C2 domain was required for this ubiquitination to lead to their endocytosis (32). Thus the consequence of WWP1 or Itch activity could depend on whether their target proteins are ubiquitinated by a C2 positive or negative form. Interestingly a C2 minus form of Nedd4 is generated posttranslationally by caspase-dependent cleavage during apoptosis, although the biological significance of this is not known (33).

A number of less abundant alternative forms of WWP1 were also identified. Splice forms B and C removed the predicted C-terminal two beta-stands from the C2 domain. In addition form B introduced a premature stop codon, allowing a predicted open reading frame encompassing only the truncated C2 domain. It is not clear whether the removal of these terminal beta-strands would allow a functional C2 domain to fold, since we can find no precedent in the literature for such a variant C2 domain. It is possible that the new amino acid sequences, which replace the two beta-strands in these splice variants, could play a part in stabilizing the fold. Expression and structural analysis of the isolated truncated domain would reveal the stability of such a fold. It is tempting to speculate that the presence of such products could have a consequence on the function of WWP1. It is possible that a single C2 domain, or a WWP1 molecule with a defective C2 domain, could have a dominant negative effect on WWP1 activity. It will be important to establish in cells where there is an increase in relative proportion of these forms, such as in the T47D tumour cell line,

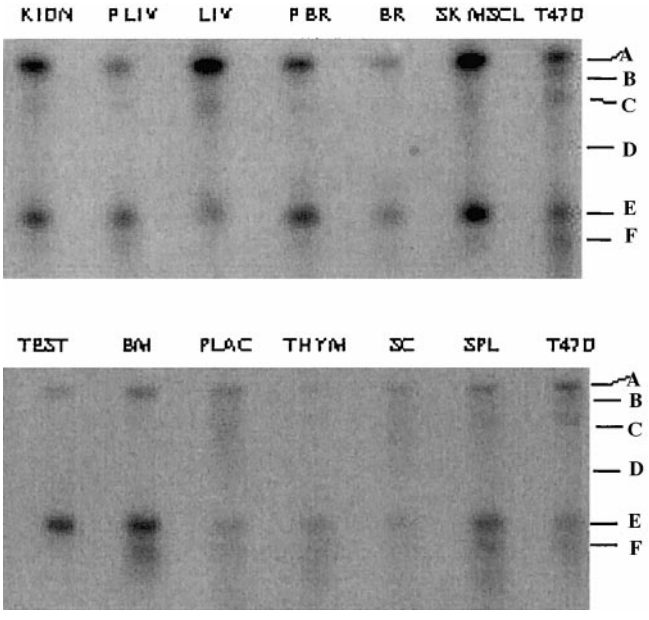

FIG. 4. Expression of splice variants of WWP1 in normal tissues and cell lines. Southern blots of RT-PCR products obtained from 50 ng of mRNA derived from human kidney (KIDN), fetal liver (FLIV), adult liver (LIV), fetal brain (FBR), adult brain (BR), skeletal muscle (SKMSCL), testis (TEST), bone marrow (BM), placenta (PLAC), thymus (THYM), spinal chord (SC), spleen (SPL). The RT-PCR product from 5 ng of T47D cell line mRNA is at the end of each panel as a comparison, with the six identified splice products labeled A to F. Southern blots were probed with a ^{32}P-labeled, nested fragment lying within the region flanked by, but not including, the primer sequences.

whether their expression can be linked to altered cellular behavior.

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