A protein related to a proteasomal subunit binds to the intracellular domain of the p55 TNF receptor upstream to its 'death domain'

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Abstract **A novel protein that binds specifically to the intracellular domain of the p55 tumor necrosis factor (TNF) receptor was cloned by two-hybrid screening of a HeLa cell cDNA library. Data bank searches revealed high sequence similarity of the protein (55.11) to yeast, nematode and plant proteins, whose functions are yet unknown. Significant similarity was also found between 55.11 and *SEN3*, the yeast equivalent of the p112 subunit of the 26S proteasome. Deletion analysis showed that the protein binds to the p55 receptor upstream to the region involved in induction of cell death.**

Key words: Death domain; Receptor; Proteasome; Signaling; Tumor necrosis factor; Two-hybrid system

1. Introduction

Triggering of the two TNF receptors, the p55 receptor (p55-R; CD120a) and the p75 receptor (p75-R; CD120b), initiates a wide range of effects, including modulation of differentiation patterns, activation of various immune mechanisms, induction of cell death, and stimulation of cell growth [1–3]. As with other receptors, these varied activities and their coordinated induction are likely to be effected by heterogeneity of functional motifs in the TNF receptors and of effector proteins with which the receptors interact. Currently, the majority of information available on the mechanisms of action of the TNF receptors concerns the C-terminal regions in the receptors' intracellular domains. In the p75-R, a region of about 78 amino acids in the C terminus of the receptor is involved in the enhancement of T cell growth and NFκB activation by TNF. Two proteins that bind to this C terminal region (TRAF1 and TRAF2) probably take part in the involved signaling [4]. Studies on the mechanisms of action of p55-R have focused on a conserved sequence motif of about 80 amino acids at its C terminus, which signals for cell death, and therefore, has been called the 'death domain' [5,6]. This motif, which is also present in some other proteins [7,8], can self-associate and bind to analogous sequences. The death domain of p55-R binds to itself and to a death domain motif present in Fas/APO1 (CD95), a structurally-related receptor that also signals for cell-death. Also the death domain of Fas/APO1 self-associates and binds to an analogous sequence in a recently cloned cytoplasmic protein, MORT1, which appears to participate in induction of cell death by Fas/APO1 [8,9]. Besides inducing cell death, the death domain of p55-R contributes to the induction of noncytocidal TNF effects, including anti-viral state and activation of the acid sphingomyelinase [6,10]. However, signaling for some TNF effects involves regions of p55-R other than its death domain. For example, the TNF-induced activation of the neutral sphingomyelinase involves a region located upstream to the death domain, and the induction of nitric oxide synthase involves both the death domain and a region located upstream to it [6,10]. Besides being involved in signaling, the membrane proximal part of p55-IC participates in the regulation of uptake of the receptor [11]. We report here the cloning of a protein that binds to the intracellular domain of the p55-R (p55-IC) at a region upstream to its death domain.

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The nucleotide sequence of 55.11 will appear in the GenBank/EMBL Data Bank under Accession Number X86446.

Abbreviations: GST, glutathione S-transferase; Fas-IC, intracellular domain of Fas/APO1; FLAG-55.11, the region extending between residues 309 and 900 in the 55.11 protein and which was N-linked to the FLAG octapeptide; IC, intracellular domain; p55-R, the p55 receptor for TNF; p75-R, the p75 receptor for TNF; p55-IC, intracellular domain of the p55-R; TNF, tumor necrosis factor.

2. Materials and methods

2.1. Cloning of the cDNA of 55.11

A partial cDNA of 55.11 (nts 925–2863, which encode for amino acids 309–900; see Fig. 4) was cloned by a two hybrid screen [12] of a Gal4 activation domain-tagged HeLa cell cDNA library (Clontech, Palo Alto, CA, USA), as previously described [9]. The rest of the 55.11 cDNA (nts 1–924, which encode for amino acids 1–308) was cloned by PCR from a human fetal liver cDNA library. The nucleotide sequence of 55.11 was determined in both directions by the dideoxy chain termination method.

2.2. Two hybrid β-galactosidase expression tests

β-galactosidase expression tests were performed as previously described [8], except that in part of the tests, the pVP16 vector, which contains the activation domain of VP16, was used instead of pGAD-GH, the Gal4 activation domain vector. Numbering of residues in the proteins encoded by the cDNA inserts are as in the Swiss-Prot data bank. Deletion mutants were produced by PCR, and point mutants by oligonucleotide-directed mutagenesis [13].

2.3. Northern analysis

Total RNA was isolated using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH), denatured in formaldehyde/formamide buffer, electrophoresed through an agarose/formaldehyde gel, and blotted to a GeneScreen Plus membrane (Dupont, Wilmington, DE, USA) in 10 × SSPE buffer, using standard techniques. The blots were hybridized with the cDNA of 55.11 (nts 925–2,863), radiolabeled with the random-prime kit (Boehringer Mannheim Biochemica, Mannheim, Germany), and washed stringently. Autoradiography was performed for 1 week.

2.4. Expression of 55.11 cDNA in HeLa cells and binding of the 55.11 protein to glutathione S-transferase fusion proteins of p55-IC

Glutathione S-transferase (GST) fusions with p55-IC (GST-p55IC) and with p55-IC truncated below amino acid 345 (GST-p55IC345) were produced and adsorbed to glutathione-agarose beads as previously

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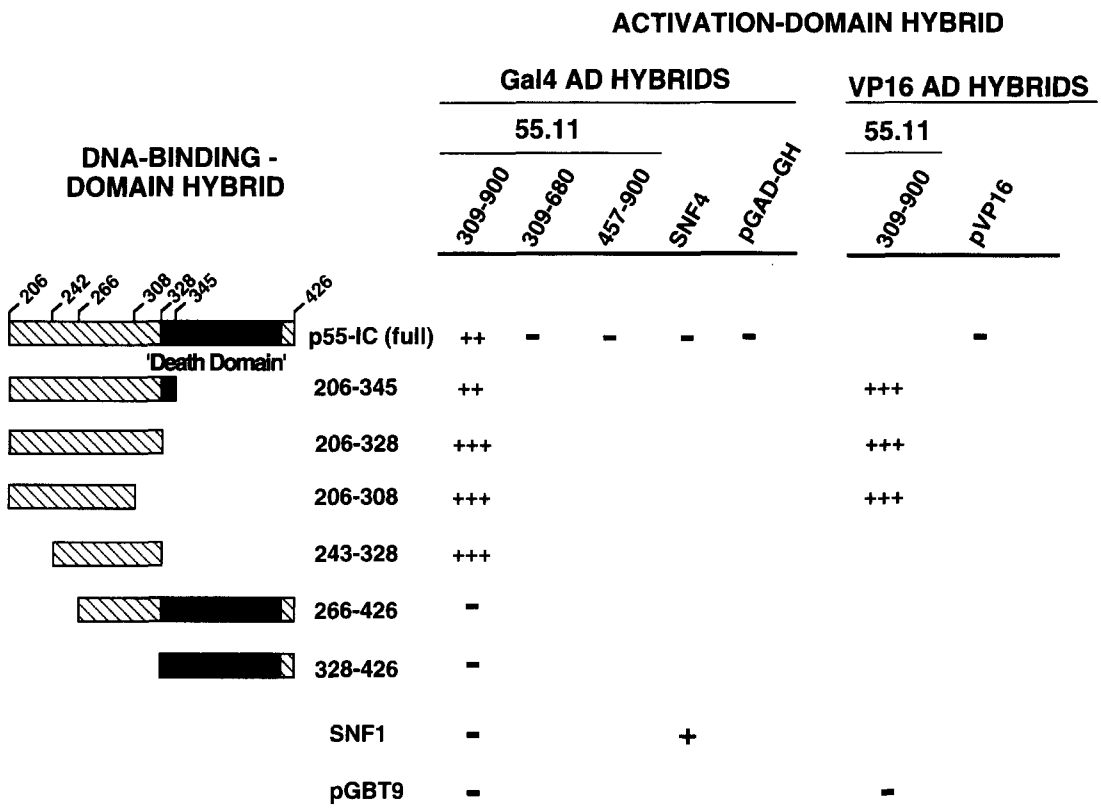


Fig. 1. Binding of the 55.11 protein to p55-IC, within transformed yeast. Binding of human p55-IC (residues 206–426) and various deletion mutants to activation domain hybrids containing 55.11 was examined in transfected SFY526 yeast. Gal4 DNA binding domain constructs (pGBT9) and either Gal4 (pGAD-GH) or VP16 (pVP16) activation domain constructs were used. The binding was assessed by a two-hybrid β-galactosidase expression filter assay. SNF1 and SNF4 served as positive controls and the empty Gal4 and VP16 vectors as negative controls. +++ and ++ indicate the development of strong color within 20 and 60 min of initiation of the assay, respectively. – indicates no development of color within 24 h. Blanks indicate not tested. The 55.11 protein did not bind to lamin, cyclin D, and the intracellular domains of human Fas/APO1 (residues 175–319), CD40 (residues 216–277), and p75-R (residues 287–461) (data not shown).

described [9,14,15]. The cDNAs of 55.11 (nts 1–2863), of FLAG-55.11, and of luciferase were expressed in HeLa cells. FLAG-55.11 is the region extending between residues 309 and 900 in the 55.11 protein (the partial cDNA of 55.11 (nts 925–2863), originally cloned by the two hybrid screen), N-linked to the FLAG octapeptide (Eastman Kodak, New Haven, CT). Expression of the fusion proteins was accomplished using a tetracycline-controlled expression vector (HtTA-1) in a HeLa cell clone that expresses a tetracycline-controlled transactivator [9,16]. Metabolic labeling of the expressed proteins with [^{35}S] Met and [^{35}S]Cys (Dupont, Wilmington, DE, and Amersham, Buckinghamshire, UK), lysis of the HeLa cells, immunoprecipitation, and binding of the labeled proteins to the GST fusion proteins were performed as described before [8], except that 0.5% rather than 0.1% Nonidet P-40 was present in the cell lysis buffer. The immunoprecipitations of 55.11 and FLAG-55.11 were achieved using a rabbit antiserum (diluted 1:500) raised against a GST fusion protein containing the region of 55.11 that extends between amino acids 309 and 900 and a mouse monoclonal antibody against the FLAG octapeptide (M2; Eastman Kodak; 5 μg/ml of cell lysate).

3. Results and discussion

Since the death domain of p55-IC tends to self-associate [9], two-hybrid screens [12] for proteins that bind to p55-IC yield primarily the cDNA of p55-IC itself. However, when screening a HeLa cell library with p55-IC we isolated a cDNA clone (55.11) whose binding site to p55-IC appeared distinct from the death domain. The protein bound to a truncated p55-IC from which the death domain had been deleted (construct 206–328 in Fig. 1), more effectively than to nontruncated p55-IC. It also bound to an even further C terminally truncated construct (construct 206–308) and to a construct from which both the death domain and a membrane proximal part were deleted (construct 243–328). However, the 55.11 protein did not bind to a construct that was N-terminally truncated down to amino

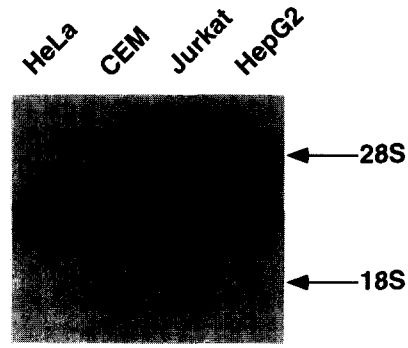


Fig. 2. Northern analysis of the RNA from several cells lines, using the 55.11 cDNA as a probe. The cell lines examined were HeLa, CEM, Jurkat, and Hep G2 cells derived from human epithelioid carcinoma, an acute lymphoblastic T cell leukemia, an acute T cell leukemia, and a hepatocellular carcinoma, respectively. The 55.11 cDNA (nts 925–2863) was used as a probe. Samples consisted of 10 μg of RNA/lane.

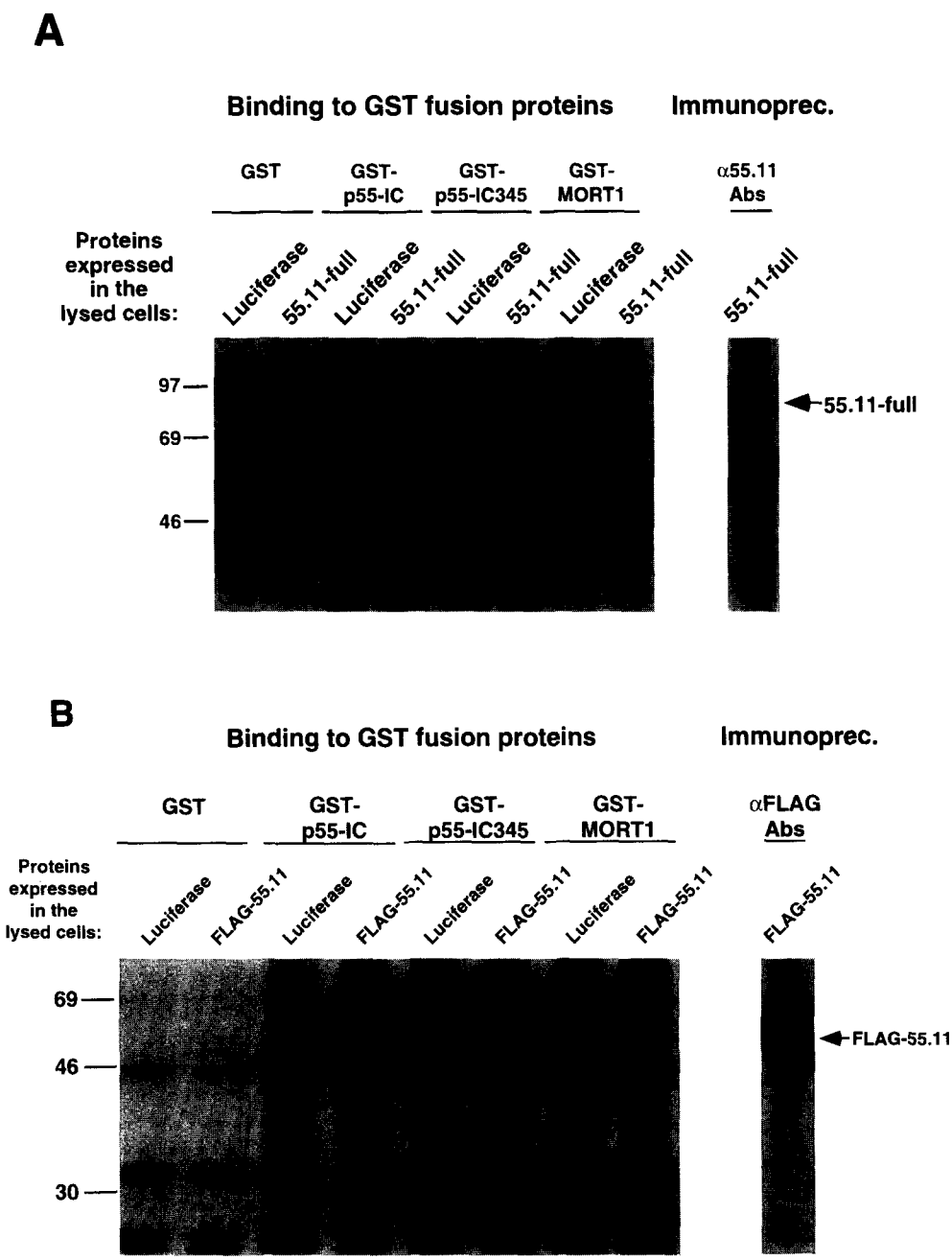
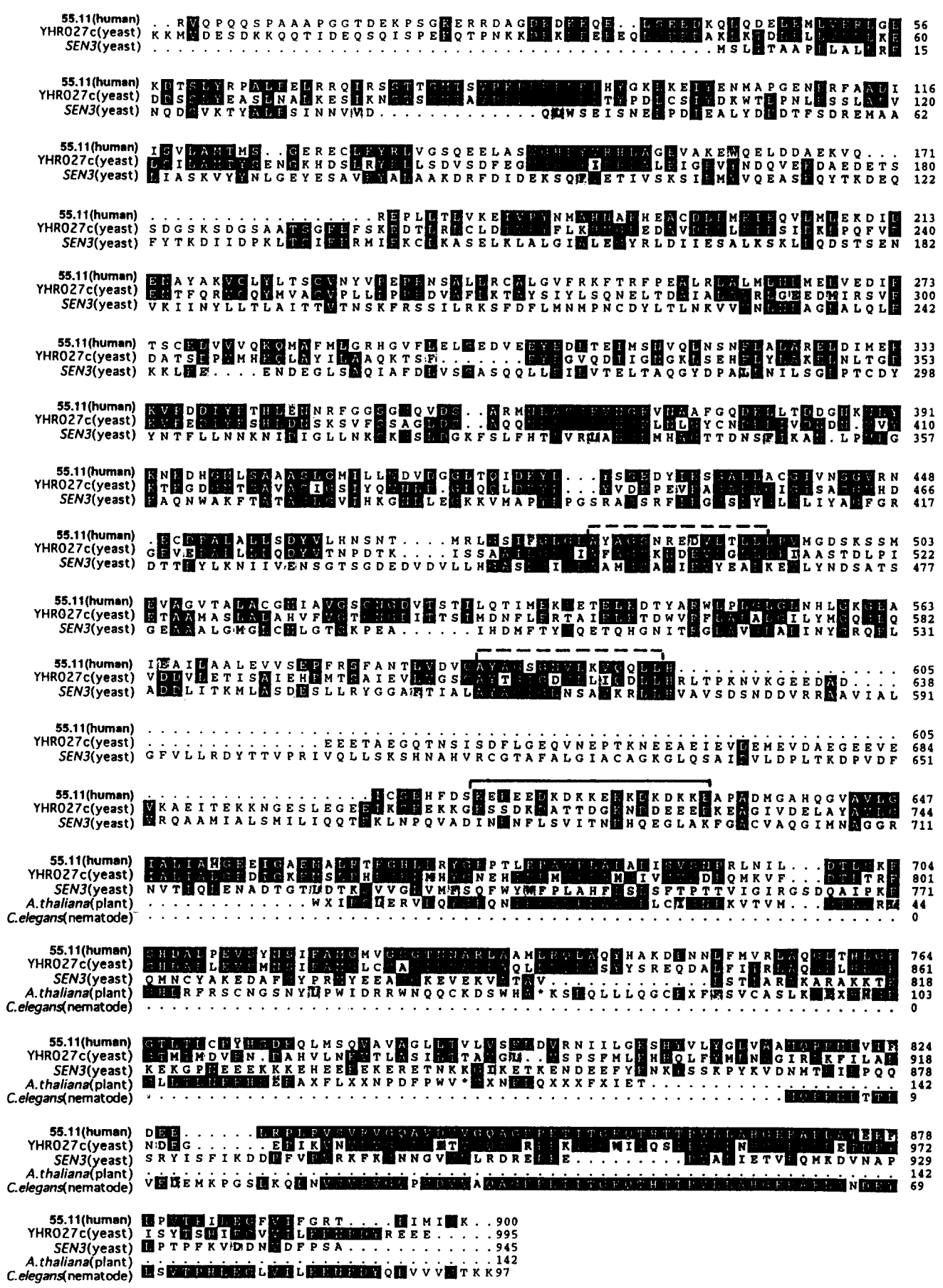


Fig. 3. In vitro binding of the protein encoded for by 55.11 to GST fusion proteins containing portions of p55-IC. Binding of the full length 55.11 protein (55.11-full) (A) and of FLAG-55.11 (amino acids 309–900 of 55.11, encoded by the initially cloned partial cDNA, fused at the N terminus with the FLAG octapeptide) (B). The cDNAs for the full-length 55.11, FLAG-55.11, and luciferase (control) were expressed in transfected HeLa cells and metabolically labeled with [^{35}S]Met and [^{35}S]Cys. The following proteins were fused with GST: full-length p55-IC (GST-p55-IC), p55-IC C-terminally truncated up to amino acid 345 (GST-p55-IC345), and the MORT1 protein (GST-MORT1). GST-MORT1 and GST alone served as controls. Lysates of the transfected cells were immunoprecipitated with antibodies against the 55.11 protein or the FLAG octapeptide. The proteins were analyzed by SDS-polyacrylamide gel electrophoresis (10% acrylamide), followed by autoradiography.

acid 266 (Fig. 1). These findings indicate that the binding site for 55.11 is located in the region that extends between residues 243 and 308 of p55-IC and that the N terminus of this binding site is between residues 243 and 266.

Transfer of the cDNA for 55.11 from the originally cloned 'prey' construct, which contained the Gal4 activation domain, to a prey construct containing the VP16 activation domain did not decrease the binding efficiency of the 55.11 protein to p55-IC (Fig. 1). Thus, the structure(s) involved in this binding appear to reside within the 55.11 molecule and not to involve the site of fusion of 55.11 with the activation domain. However, binding of 55.11 to p55-IC was abolished by even limited truncations of the 55.11 protein at either its C (construct 309–680) or N terminus (construct 457–900. Residue 309 is the first residue in the protein encoded by the partial cDNA clone originally isolated in the two hybrid screen). The observed binding


55.11(human)
YHR027c(yeast)
SEN3(yeast)
A.thaliana(plant)
C.elegans(nematode)

appeared to be specific since 55.11 did not bind to other proteins, including three receptors of the TNF/NGF receptor family (p75-R, Fas/APO1 and CD40; data not shown).

Northern analysis using the 55.11 cDNA as a probe (Fig. 2) revealed, in several cell lines, a single hybridizing transcript of about 3 kb, which is larger than the cDNA (2 kb). Using oligonucleotide primers that correspond to the 55.11 sequence, we cloned by PCR a 5′ extending sequence whose length was about 1 kb. The sum of the length of this 5′ extending sequence with that of the originally cloned cDNA approximates the length of the 55.11 transcript. The 3 kb cDNA that encompassed both these portions was effectively expressed in transfected HeLa cells yielding a protein of about 84 kDa, which suggests that the 3 kb cDNA contains a translational start site.

To ascertain that 55.11 can indeed bind to p55-IC and to exclude involvement of yeast proteins in this binding, the in vitro interaction of GST p55-IC fusion proteins, produced by bacteria, with the protein encoded by the 3 kb 55.11 cDNA (55.11-full), produced by transfected HeLa cells, was examined. The protein encoded by 55.11 bound to fusion proteins that contained the full p55-IC (GST-p55IC) or a truncated p55-IC that lacked most of the death domain (GST-p55IC345) (Fig. 3A). The protein did not bind to GST alone or to GST fused to the MORT1 protein (control). Similarly, the HeLa cell-expressed protein encoded by the initially cloned partial cDNA of 55.11 in fusion with the FLAG octapeptide (FLAG-55.11) bound in vitro to GST-p55IC and GST-p55IC345, but not to GST or GST-MORT1 (Fig. 3B).

Data bank searches revealed that parts of the sequence of the 55.11 cDNA (Accession Numbers T03659, Z19559, and F09128) and its mouse homologue (Accession Numbers X80422 and Z31147) have already been determined during arbitrary sequencing of cDNA libraries. A cDNA sequence (Accession Number U18247) that encodes for a human protein of 596 amino acids present in cultures of human hepatoma HC10 cells is almost identical to that of 55.11. This hepatoma protein lacks an N terminal portion (amino acids 1–297) corresponding to that of 55.11 and also differs from 55.11 at the regions that correspond to residues 297–377 and residues 648–668 in 55.11. The searches of the data bank also revealed that proteins with very high sequence homology to 55.11 exist in *Saccharomyces cerevisiae* (yeasts), *Arabidopsis thaliana* (plants) and *Caenorhabditis elegans* (worms). In yeast, there are two known proteins whose DNA sequences resemble that of 55.11 (the open reading frame YHR027c and *SEN3*; Fig. 4). The sizes of both are close to that of 55.11. YHR027c is known only by the sequencing of a genomic clone while *SEN3* has been cloned as a cDNA. The sites within 55.11 that are similar to those in *SEN3* correlate to the sites of its similarity to YHR027c, although much more similarity is evident between 55.11 and YHR027c than between 55.11 and *SEN3*. The DNA sequence information available for the *Arabidopsis thaliana* and *Caenorhabditis elegans* proteins, although only partial, clearly shows that these proteins are as similar to 55.11 as the YHR027c protein of yeast. The only one of these four proteins whose nature has been elucidated so far is the yeast *SEN3*, whose homology to 55.11 is limited. *SEN3* has been identified as the yeast equivalent of the p112 subunit of an activator of the 20S proteasome (the proteolytic core of the 26S proteasome [17,18]) (M. R. Culbertson and M. Hockstrasser, personal communication).

Conserved amino acid sequence motifs were not discerned within the protein encoded for by 55.11, except for a repetitive 'KEKE' sequence that extends between Lys^{614} and Glu^{632}. Such 'KEKE' sequences, which are present in many proteins, including proteasomal subunits and chaperonins, may promote association of protein complexes [19]. A sequence $AYAGS(x)_8LL$ appears twice in the 55.11 protein (at sites 479 and 590, see Fig. 4); no functional significance for this sequence has yet been described.

Findings in this study suggest several possible routes that could be explored to elucidate the function served by the 55.11 protein and how its binding to the p55-R contributes to TNF activity. The finding that the 55.11 protein is bound to a region in the p55-R distinct from the death domain indicates that it affects noncytocidal TNF activiti(es). There are indications that the region to which the 55.11 protein binds contains sequence motifs involved in TNF induced expression of nitric oxide synthase [6] and activation of neutral sphingomyelinase [10]. However, these motifs have not yet been fully defined. The region is particularly rich in proline, serine, and threonine residues; yet it does not contain the RPM1 and RPM2 proline-rich motifs, present in several other cytokine receptors [20]. Proline residues follow two serines and two threonines in the N terminal part of the binding region of the 55.11 protein, making them potential sites for phosphorylation by MAP kinase, CDC2, and other proline-dependent kinases [21], which may affect receptor binding by the 55.11 protein. More accurate definition of the sequence elements within the p55-R that are involved in 55.11 binding, and those involved in the effects of TNF on nitric oxide synthase and neutral sphingomyelinase, will help clarify the role of the 55.11 protein in signaling.

The marked sequence similarity of the 55.11 protein with *SEN3*, the yeast equivalent of the p112 protein, provides additional clues to the function of the 55.11 protein. If, as does the p112 protein, the 55.11 protein occurs in the regulatory complex of the 26S proteasome, than its binding to p55-R may modulate the proteolytic function of the proteasome, or conversely, contribute to the degradation of the p55-R itself within the proteasome. Of note, interferon γ, whose activities are closely related to those of TNF, has pronounced effects on the composition and function of the proteasomes [24,25]. Furthermore, although there is no information regarding how the TNF receptors are degraded, the rapidity with which TNF binding decreases after inhibition of protein synthesis, suggests that these molecules have a very short half-life, consistent with the

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Fig. 4. Comparison of the deduced amino acid sequence of human 55.11 to that of related proteins present in lower organisms. The sequences of amino acids predicted for: the 55.11 cDNA; an open reading frame (YHR027c) within a cosmid derived from the 8th chromosome of *Saccharomyces cerevisiae* (nts 21253–24234, Accession Number U10399); *SEN3*, the cDNA of a *Saccharomyces cerevisiae* protein (Accession Number L06321); a partial cDNA of a protein of the plant *Arabidopsis thaliana* (Accession Number T21500); and a partial cDNA of a protein of the nematode *Caenorhabditis elegans* (Accession Number D27396). The 'KEKE' sequence in 55.11 is marked with a solid line and the sequence $AYAGS(x)_8LL$ with broken lines. The sequences were aligned using the PILEUP and PRETTYBOX programs of the GCG package. Gaps introduced to maximize alignments are denoted by dashes.

possibility that their degradation occurs in the proteasomes (unpublished data). Additional studies of the significance of the sequence similarity between the 55.11 protein and the p112 protein, and of the particular function of the p112 unit within the proteasome, will help clarify the functional significance of the 55.11 protein interaction with the p55-R.

Although TNF itself has only been found in multicellular organisms, the occurrence of proteins closely related to 55.11 in yeast, as well as in plants and nematodes, indicates that this TNF-receptor associated protein serves an evolutionary conserved function. This is not surprising since a number of known molecules and mechanisms regulated by TNF seem to have appeared rather early in the evolutionary process. For example, several protein kinases known to signal TNF effects, as well as transcriptional factors affected by these kinases, also occur in yeast where they have important roles in signaling [21–23]. The technical advantage of the genetic set up in yeast for studying the function of an unknown protein, coupled with the advantage of the cellular composition of nematodes for studying the role of an unknown protein in a multicellular organism, will facilitate elucidation of the function of the 55.11 protein.

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