Brief Definitive Report

A Potential Mechanism of "Cross-Talk" between the p55 Tumor Necrosis Factor Receptor and Fas/APO1: Proteins Binding to the Death Domains of the Two Receptors Also Bind to Each Other

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Summary

The p55 tumor necrosis factor (TNF) receptor and Fas/APO1 induce cell death via distinct regions in their intracellular domains. Three cytoplasmic proteins that bind to these receptor regions have been identified recently. One, MORT1 (also called FADD), binds to Fas/APO1 but not to p55-R; another, TRADD, binds to the p55 TNF receptor but not to Fas/APO1; and the third, RIP, binds weakly to both receptors. The regions within these proteins that are involved in binding to the receptors and the receptor regions to which they bind share a common sequence motif, that of the "death domain." This study shows that the death domain motifs in MORT1, TRADD, and RIP bind effectively to each other, a mode of binding that may allow "cross-talk" between the functional expression of the p55 TNF receptor and Fas/APO1.

The p55 TNF receptor (p55-R) and Fas/APO1 are structurally related and activated by distinct yet structurally related ligands, which share the ability to induce death in cells. The physiological roles of these receptors are different. The ligand molecules activating the p55-R are mainly formed by mononuclear phagocytes, and cell death induction by the receptor occurs as part of a wide repertoire of other induced functional changes, most of which are involved in the inflammatory process (1, 2). Fas/APO1 activation, on the other hand, occurs in response to a lymphocyte-produced ligand and apparently leads almost solely to cell death (3). There seem also to be differences in the mechanisms of cell death induction by p55-R and Fas/APO1. They are manifested in different morphological features of the death processes. Also, the death processes occur synergistically, induced differentially in cells of different lines and modulated differently by drugs and biological regulators (4–6). Yet there is also evidence that cell death induction by p55-R and Fas/APO1 involves similar, or even common, mechanisms, both in the early and late events of the death induction processes. The structural requirements for the induction of cell death by the two receptors reside in intracellular domain regions that have sequence homology; in both receptors, these regions (called the "death domains") tend to self-associate, which apparently contributes to the initiation of their signaling (7–10). Cell death induction by both receptors involves a critical function of protease(s) related to the IL-1 β-converting enzyme (ICE) (11–14), and both can be suppressed in a cell type–specific manner by BCL2 (15, 16). The mechanistic level at which the interaction between the signaling by p55-R and Fas/APO1 occurs, and how this interaction results in the observed similarities and differences in their effects, have not been documented.

Three cytoplasmic proteins—MORT1 (17) (or FADD [18]), RIP (19), and TRADD (20)—that bind to the intracellular domains of p55-R or Fas/APO1 and apparently take part in the induction of their cytocidal effect have been cloned recently. All these proteins were found to contain the sequence motif shared between the death domains (DD) of the intracellular domains of p55-R and Fas/APO1. As in the receptors, the DD motifs in the three cytoplasmic proteins seem to be sites of protein–protein interaction. The three proteins interact with the p55-R and Fas/APO1 intracellular domains by the binding of their DDs to those in the receptors, and in both TRADD and RIP (though not in MORT1) the DDs self-associate. In this study, we explored further the associations among the three cytoplasmic proteins and the two receptors resulting from their DD interactions. MORT1 and TRADD were found to bind differentially to Fas/APO1 and p55-R, but also to each other, and both bound effectively to RIP. This mode of binding may allow for functional "cross-talk" between p55-R and Fas/APO1 early in their signaling cascades.

E.E. Varfolomeev and M.P. Boldin contributed equally to this work.

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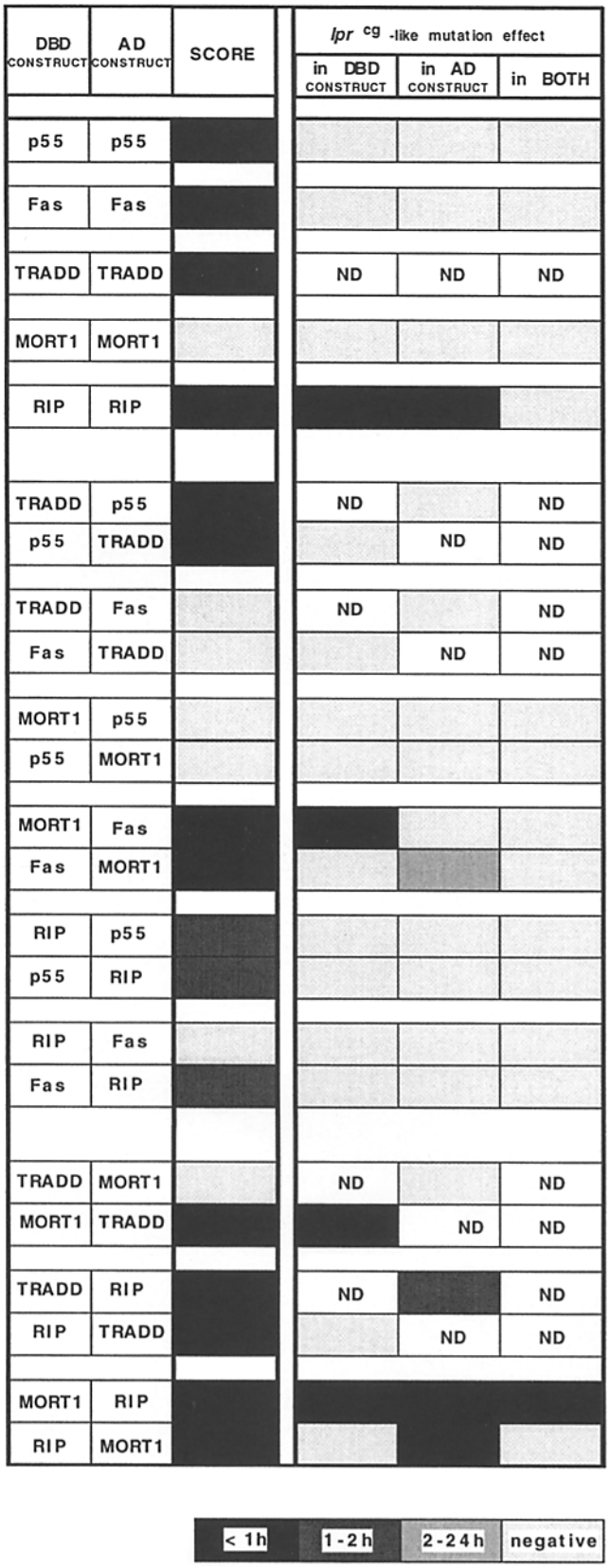

Figure 1. Interactions of the DDs of p55-R, Fas/APO1, MORT1, TRADD, and RIP in a yeast two-hybrid test, and the effect of *lpr*^{cg}-like mutations in these proteins on their interactions. Assessment of the interaction of Ga14 hybrid constructs encompasses the following human proteins truncated upstream to their DD motifs: p55-R (residues 326–426), Fas/APO1 (residues 210–319), MORT1 (residues 92–208), TRADD (residues 195–312), and RIP (residues 261–372), as well as of the following point mutants of these proteins: p55-R L351N, Fas/APO1 V238N, MORT1 V121N, and RIP F308N, whose mutation sites within the DDs correspond to that found in the Fas/APO1 of the *lpr*^{cg} mice. Each cDNA insert was introduced both into the Ga14 DBD and the Ga14 AD constructs (pGBT9 and pGAD-GH), and the binding of inserts in both constructs to all other inserts within transfected SFY526 yeasts was assessed by a β-galactosidase expression filter assay. The results are presented in terms of the time required for development of strong color. *ND*, not done.

Materials and Methods

Two-hybrid β-Galactosidase Expression Tests. cDNA inserts were cloned by PCR, either from the full-length cDNAs cloned previously in our laboratory or from purchased cDNA libraries. Residue numbering in the proteins encoded by the cDNA inserts are as in the Swiss-Prot Data Bank. Point mutants were produced by oligonucleotide-directed mutagenesis (21). β-galactosidase expression in yeasts (SFY526 reporter strain [22]) transformed with these cDNAs in the pGBT-9 and pGAD-GH vectors (DNA binding domain [DBD] and activation domain [AD] constructs, respectively) was assessed by a filter assay (10) When expressed in the pGAD-GH vector, RIP and its DD had some cytotoxic effect on the yeasts, manifested in a low yield of yeast colonies. They did not have any such cytotoxic effect when expressed (to a lower extent) in the pGBT-9 vector.

Induced Expression, Metabolic Labeling, and Immunoprecipitation of Proteins. Since the size similarity of the DDs makes it difficult to distinguish between them in gel electrophoresis, we chose to examine the interaction of MORT1, TRADD and RIP by coexpressing the full-length MORT1 protein with the DDs of TRADD and RIP. The proteins, N-linked to the FLAG octapeptide (Eastman Kodak Co., New Haven, CT), or to an influenza hemagglutinin (HA) epitope (YPYDVPDYA [23]) were expressed in HeLa cells using a tetracycline-controlled expression vector and labeled metabolically with [^{35}S]Met (55 μCi/ml) and [^{35}S]Cys (10 μCi/ml) (EXPRE^{35}S^{35}S protein labeling mix; DuPont, Wilmington, DE), as described before (17). The cells were then lysed in RIPA buffer (1 ml/5 × 10^5 cells), and the lysates were precleared by incubation with irrelevant rabbit antiserum (3 μl/ml) and protein G–Sepharose beads (Pharmacia, Uppsala, Sweden; 60 μl/ml). Immunoprecipitation was performed by 1-h incubation at 4°C of 0.3-ml aliquots of lysate with mouse mAbs (5 μg per aliquot) against the FLAG octapeptide (M2; Eastman Kodak Co.), HA epitope (12CA5 [23]), or the p75 TNF receptor (#9; [24]) as a control, followed by an additional 1-h incubation with protein G–Sepharose beads (30 μl per aliquot). The immunoprecipitates were washed three times with REPA buffer and analyzed by SDS-PAGE.

Results

The interactions of the DDs of human p55-R, Fas/APO1, TRADD, MORT1, and RIP were evaluated first by a yeast two-hybrid test. The CDNAs encoding these domains were expressed as fusion proteins with the Ga14 DNA binding and activation domains (DBD and AD constructs) in the yeast SFY526 reporter strain, and the binding of these fusion proteins to each other was assessed by deter-

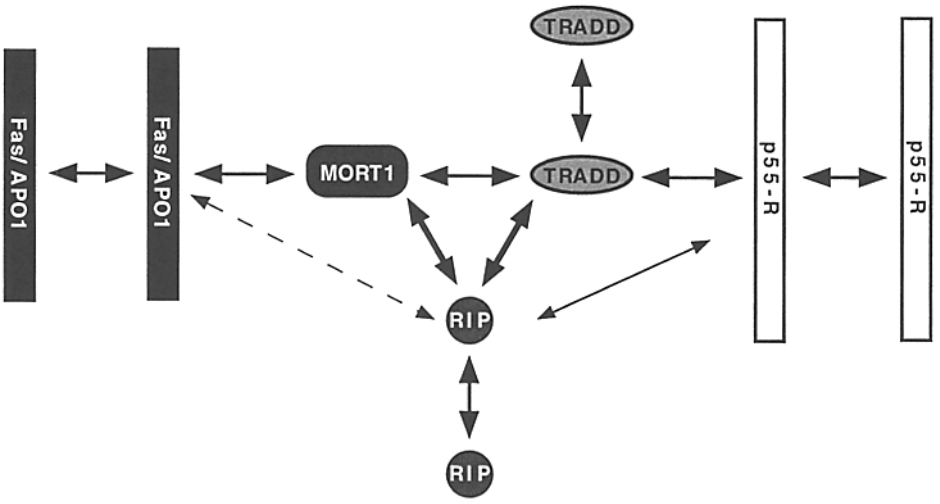

Figure 2. Diagrammatic illustration of the DD interactions observed in the yeast two-hybrid tests. The lengths and thicknesses of the arrows connecting the DD icons correspond to the intensity of the interactions as observed in the experiment described in Fig. 1.

mining β-galactosidase expression by the yeasts. The results of these tests are summarized in Fig. 1 and illustrated diagrammatically in Fig. 2.

The DDs of p55-R, Fas/APO1, TRADD, and RIP were able to self-associate. The DD of MORT1 lacked this ability, even though the full-length MORT1 protein does self-associate (17), apparently through an interaction that involves the region upstream of its DD.

The DD of TRADD bound to the DD of p55-R, but not to the DD of Fas/APO1, whereas the DD of MORT1 behaved in the converse fashion.

The DD of RIP, like the full-length RIP protein (19, was able to bind both to the DDs of Fas/APO1 and p55-R. Binding was significantly weaker, though, than that of the DDs of TRADD or MORT1 to these receptors. Although RIP was initially identified by virtue of its binding in a two-hybrid screen to Fas/APO1 (19), this binding was quite weak, and could be observed only when the RIP DD was highly expressed in the yeasts, by introducing it into the AD construct. There was no measurable binding when the DD of RIP was introduced into the DBD construct, which has a lower expression effectivity. A longer RIP insert, corresponding to amino acids 161–372 in the protein, did not bind more effectively to Fas/APO1 (not shown).

Apart from their observed binding to the DDs of p55-R or Fas/APO1, the DDs of each of the three cytoplasmic proteins tested bound also to each other. These interactions were all effective. Notably, the effectivity of binding of the DD of RIP to the DDs of MORT1 and TRADD was significantly greater than that of its binding to the DDs of p55-R and Fas/APO1.

A similar pattern of interaction was observed in the *HF7c* yeast reporter strain, regularly used in our laboratory for two-hybrid screens. Indeed, in a recent attempt to clone proteins that bind to MORT1 by a two-hybrid screen, we found that a significant proportion of the cloned cDNAs encode TRADD or RIP (not shown).

In specificity tests for the two-hybrid assay, we did not observe binding of the DD motifs to any of a number of irrelevant proteins, including SNF1, the intracellular domain

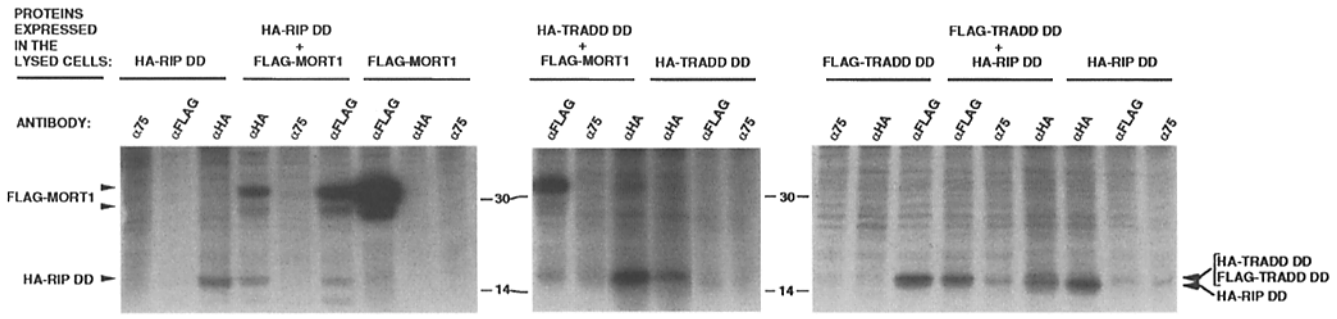

Figure 3. Interactions of MORT1, TRADD, and RIP within transfected HeLa cells. MORT-1 (nucleotides 19–753 in GenBank accession number U24231), fused at its NH_2 terminus with the FLAG octapeptide, and the DDs of TRADD (amino acids 195–312) and of RIP (amino acids 261–372), fused at their NH_2 termini either with the FLAG octapeptide or the HA epitope (23), were expressed, either alone or in mixtures of two in HeLa cells, and metabolically labeled with [^{35}S]Cys and [^{35}S]Met. Cross-immunoprecipitation of the coexpressed proteins was performed using the indicated antibodies. The proteins were analyzed by SDS-PAGE (15% acrylamide) followed by autoradiography. In cell lysates containing MORT1 and RIP, coimmunoprecipitation of both proteins could be obtained using antibodies against either one of them. However, in lysates containing TRADD and RIP, coimmunoprecipitation of the two proteins was observed only when using antibody against RIP, and in lysates containing TRADD and MORT1, it was observed only with an antibody against TRADD, apparently because of steric hindrance.

of the human p75 TNF receptor, lamin, cyclin D, and the DD of the rat low-affinity nerve growth factor (NGF) receptor (not shown). To further assess the binding specificity, we introduced point mutations to the p55-R, Fas/APO1, MORT1, and RIP DDs at sites corresponding to that of I-225 in the mouse Fas/APO1 sequence. A naturally occurring replacement mutation of this residue, found in *lpr*^{cg} mice, abolishes signaling by Fas/APO1 (9, 25) as well as its interaction with MORT1 (17, 18). Mutation of the corresponding residues in the DDs of human p55-R (L351N) and Fas/APO1 (V238N) had a similar effect. The mutated proteins were not able to self-associate, nor to bind to TRADD, MORT1, or RIP. Also, introduction of a replacement mutation to the DD of RIP at the site corresponding to that of the *lpr*^{cg} mutation (F308N) resulted in loss of its ability to bind to Fas/APO1, MORT1, and TRADD, as well as to self-associate, although the mutated protein still bound to the normal RIP DD. On the other hand, in MORT1, the *lpr*^{cg}-like mutation (V121N) had only a limited effect. It resulted in less effective binding to Fas/APO1, which, for some reason, was observed only when the mutated protein was introduced into the AD construct but not in the DBD construct.

To test whether the interactions observed between TRADD, MORT1, and RIP in the yeast two-hybrid tests occur also in eukaryotic cells, we coexpressed MORT1 and the DDs of TRADD and RIP within transfected HeLa cells and attempted to immunoprecipitate them from the cell lysates. Immunoprecipitation resulted in precipitation of the coexpressed proteins, indicating that they bind to each other within the HeLa cells (Fig. 3).

Discussion

Although the evidence is still largely indirect, TRADD, MORT1, and RIP appear to play important roles in the initiation of the cytocidal effect of p55-R and Fas/APO1 (26). The binding of these proteins to the receptors, which occurs through their DDs, apparently is required for their contribution to the signaling. A recent study showing that stimulation of Fas/APO1 in cells evokes binding of MORT1 to this receptor suggests that the DD interactions observed within transfected yeasts occur also within the mammalian cell, and take part in the process of signaling induction (27). Although the DDs of all the proteins examined in this study have the ability to bind to other DDs, there is clear specificity in this interaction. The DD of TRADD binds to that of p55-R, but not to the DD of Fas/APO1. The DD of MORT1 binds to the DD of Fas/APO1, but does not bind to the DD of p55-R. This specificity in the action of proteins that take part in the signaling activity of p55-R and Fas/APO1 may well contribute to the differences in function of the two receptors.

In addition to their differential binding to the DDs of p55-R and Fas/APO1, the DDs of TRADD and MORT1 also are able to bind effectively to each other, and both are capable of binding to the DD of RIP more effectively than do the DDs of Fas/APO1 or p55-R. Thus, even though distinct, the signaling cascades affected by TRADD and MORT1 may well be coordinated through their mutual interactions. The nature of this coordination may vary, depending on the way in which the different interactions of the DD in a given protein affect each other. These interactions may occur together or be exclusive; they may also modulate each other. One possible way for such modulation is indicated by the occurrence in RIP of sequence motifs characteristic of protein kinases. If this protein indeed possesses protein kinase activity, it may be capable of phosphorylating MORT1 and TRADD upon binding to them, thereby modulating their function. One plausible consequence of the association of TRADD and MORT1, and of the binding of RIP to both proteins, is integration of their effects, at least in part. This integration may account for the fact that cell death induction by p55-R and Fas/APO1 exhibit certain similarities as well as distinct features; this could also result in sharing of other activities of the two receptors.

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