Identification and Relevance of the CD95-binding Domain in the N-terminal Region of Ezrin*

The CD95 (Fas/APO-1) linkage to the actin cytoskeleton through ezrin is an essential requirement for susceptibility to the CD95-mediated apoptosis in CD4+ T cells. We have previously shown that moesin was not involved in the binding to CD95. Here we further support the specificity of the ezrin/CD95 binding, showing that radixin did not bind CD95. The ezrin region specifically and directly involved in the binding to CD95 was located in the middle lobe of the ezrin FERM domain, between amino acids 149 and 168. In this region, ezrin, radixin, and moesin show 60–65% identity, as compared with the 86% identity in the whole FERM domain. Transfection of two different human cell lines with a green fluorescent protein-tagged ezrin mutated in the CD95-binding epitope, induced a marked inhibition of CD95-mediated apoptosis. In these cells, the mutated ezrin did not co-localize or co-immunoprecipitate with CD95. Further analysis showed that the mutated ezrin, while unable to bind CD95, was fully able to bind actin, thus preventing the actin linkage to CD95. Altogether, our results support the specificity of ezrin in the association to CD95 and the importance of the ezrin-to-CD95 linkage in CD95-mediated apoptosis. Moreover, this study suggests that a major role of ezrin is to connect CD95 to actin, thus allowing the CD95 polarization on the cells and the occurrence of the following multiple cascades of the CD95 pathway.

A major requirement for the susceptibility to CD95 (APO-1/Fas)-mediated apoptosis in CD4 + T cells is the CD95 polarization on cell uropods, as a result of CD95 linkage to the actin cytoskeleton through ezrin (1). Ezrin, radixin, and moesin (ERM) are closely related proteins involved in cellular polarization and in various cellular functions (2–4). ERM are found in microvilli, filopodia, membrane ruffles, and cell-to-cell contact sites, where they co-localize and associate with F-actin (5–10). ERM are members of the erythrocyte protein 4.1 superfamily, characterized by a ~300-residue globular N-terminal domain highly conserved in the ERM family (FERM domain) (3, 11–14). ERM interact directly with various membrane proteins, such as CD43, CD44, and intercellular adhesion molecule-1, -2, and 3, through their FERM amino-terminal domain (reviewed in Ref. 3). These proteins can also indirectly bind to H+/Na+ exchanger 3 via the cytoplasmic protein EBP50 (15, 16). ERM are present both in an inactive/closed and in an active/opened form (9, 14), and a direct consequence of ERM activation is their recruitment to the plasma membrane, allowing the ERM linkage to the membrane molecules (17, 18). The ERM/membrane protein interaction is stabilized at the plasma membrane by the ERM association to phosphatidylinositol 4,5-bisphosphate (19, 20) and regulated by tyrosine phosphorylation in Tyr465 and Tyr383 (21–26).

However, whereas the ERM domains involved in actin binding are known (27–30), the specific sites accounting for the binding to membrane proteins are much less defined. Particularly, the specific amino acid sequence included in the N-terminal FERM domain of ERM involved in the binding to the various membrane molecules is not yet known. Recently the three-dimensional structure of ezrin FERM domain has been described (31, 32). The fold consists of three lobes in a trefoil, globally similar to structures reported previously for ERM proteins, with the PIP2 binding domain located on the first and the third lobe (33–35).

In a previous report, we have shown 1) the ezrin/CD95 association in CD95-prone cells (i.e. lymphoblastoid CD4+ T cells and day 6 activated lymphocytes); 2) that moesin is not involved in the association to Fas; and 3) the biological in vivo relevance of this association through treatment with ezrin antisenese oligonucleotides that markedly inhibited susceptibility to CD95-mediated apoptosis of human lymphoid cells (1). Previous data, although providing evidence on the biological relevance of CD95/ezrin interaction, lacked information on 1) the specificity of ezrin in the binding to CD95 (i.e. ezrin versus radixin); 2) the epitopes involved in the ezrin binding to CD95, 3) the evidence for a direct interaction between ezrin and CD95, and 4) the in vivo relevance of this direct interaction. In this study, the specificity of ezrin binding and the sites of ezrin/CD95 association were investigated. The results showed that CD95 and ezrin bind directly, as assessed by both two-hybrid assay and GST pull-down experiments, whereas radixin did not co-immunoprecipitate with CD95 and did not bind to CD95. We also report the CD95 binding sites on ezrin FERM...
domain using deletion series of the ezrin N-terminal domain fused to GST, showing that the ezrin binding site to CD95 is located on the median lobe of ezrin FERM domain. Furthermore, transfection experiments, performed with a GFP-ezrin mutant, obtained substituting the ezrin epitope responsible for the binding to CD95 with the corresponding region of moesin, resulted in the impairment of susceptibility to CD95-mediated apoptosis, consistent with the inability of the mutated ezrin to bind the native CD95.

EXPERIMENTAL PROCEDURES

Mammalian Two-hybrid Assay—cDNA fragments for human ezrin and CD95 were amplified by reverse transcriptase-PCR, using total RNA extracted from Jurkat cells as template. cDNA for human radixin was obtained from CEM-VBl100 cells as template. The following primers were utilized: CD95(578-586, 149-155), GTCAGCCCATCAATTGTGTTTGGGG and GTGCACTGCGACGACAGGACAG; ezrin(239-245, 586), GTCAGACTCACCAGAAAAGGGA and TCTAGACTCGTCTCGTTTTGTC. Primers for radixin FERM domain were the following: GGATCGAATTCGGCACGAGACAAACCGA and TCTAGACTCTGCCTTCTTTGTC. cDNA for radixin was cloned into the SalI-PstI sites of pM GAL4-BD vector (Promega), and then CD95 fragment was digested with SalI-PstI and cloned into the SalI and PstI sites of pGEM-T vector (Promega), and then CD95 fragment was digested with SalI-PstI and cloned into the SalI and PstI sites of pM GAL4-BD vector (Promega). Ezrin and radixin PCR products were digested with SalI-XhhI and BamHI-HindIII and cloned into the same sites of pVP16-AD cloning vector (Clontech). The resulting plasmids were designed as pM-CD95, pVP16-CD95, pVP16T (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen), pVP16-CP (vector expressing a fusion of the VP 16 AD to a viral coat protein), or pM and pVP16, were utilized. All of the control plasmids were constructed with the aid of the kit manufacturer. Plasmids are detailed in Table I. The correctness of the open reading frame was confirmed in five consenerial GAL4 binding sites. Both the cloning vectors and pG5CAT were designed as pM-CD95, pVP16-CD95, pVP16T (vector expressing a fusion of the GAL4 DNA-BD to the mouse protein pM53), pVP16-CP (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen), pVP16-AD cloning vector (Clontech). The resulting plasmids were designed as pM-CD95, pVP16-CD95, pVP16T (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen), and pVP16-AD, and pVP16-radinx. p5G CAT was the reporter vector containing the CAT gene downstream of five consensus GAL4 binding sites. Both the cloning vectors and pG5CAT were designed as pM-CD95, pVP16-CD95, pVP16T (vector expressing a fusion of the GAL4 DNA-BD to the mouse protein pM53), pVP16-CP (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen), pVP16-AD cloning vector (Clontech). The resulting plasmids were designed as pM-CD95, pVP16-CD95, pVP16T (vector expressing a fusion of the GAL4 DNA-BD to the mouse protein pM53), pVP16-CP (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen), and pVP16-radinx. p5G CAT was the reporter vector containing the CAT gene downstream of five consensus GAL4 binding sites. Both the cloning vectors and pG5CAT were designed as pM-CD95, pVP16-CD95, pVP16T (vector expressing a fusion of the GAL4 DNA-BD to the mouse protein pM53), pVP16-CP (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen), and pVP16-radinx. p5G CAT was the reporter vector containing the CAT gene downstream of five consensus GAL4 binding sites. Both the cloning vectors and pG5CAT were designed as pM-CD95, pVP16-CD95, pVP16T (vector expressing a fusion of the GAL4 DNA-BD to the mouse protein pM53), pVP16-CP (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen), and pVP16-radinx. p5G CAT was the reporter vector containing the CAT gene downstream of five consensus GAL4 binding sites. Both the cloning vectors and pG5CAT were designed as pM-CD95, pVP16-CD95, pVP16T (vector expressing a fusion of the GAL4 DNA-BD to the mouse protein pM53), pVP16-CP (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen), and pVP16-radinx. p5G CAT was the reporter vector containing the CAT gene downstream of five consensus GAL4 binding sites. Both the cloning vectors and pG5CAT were designed as pM-CD95, pVP16-CD95, pVP16T (vector expressing a fusion of the GAL4 DNA-BD to the mouse protein pM53), pVP16-CP (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen), and pVP16-radinx. p5G CAT was the reporter vector containing the CAT gene downstream of five consensus GAL4 binding sites. Both the cloning vectors and pG5CAT were designed as pM-CD95, pVP16-CD95, pVP16T (vector expressing a fusion of the GAL4 DNA-BD to the mouse protein pM53), pVP16-CP (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen), and pVP16-radinx.

Cell Culture, Transfection, and CAT Assay—24 h before transfection, 105 HeLa cells were seeded onto 100-mm plates and cultured in RPMI 1640 10% fetal calf serum. Cells were transfected with 4 µg of each plasmid by calcium phosphate method, as previously described (36). 72 h later, cells were harvested and lysed, and cell extracts were processed to evaluate protein-protein interaction, measured by a CAT ELISA kit (Roche Applied Sciences) according to the manufacturer's instructions. Briefly, antibodies to CAT are prebound to the surface of a microtiter plate, and lysates from transfected cells were added to the wells, allowing expressed CAT binding to the anti-CAT-covered plates, and CAT positivity was revealed by a sandwich ELISA. The absorbance of the sample is determined using an ELISA reader.

Production and Purification of GST Fusion Proteins—A series of deleted N-terminal ezrin fusion proteins were expressed and purified from JM109 bacterial strain (Promega), Ezrin 1–362, ezrin 149–242, ezrin 149–200, ezrin 149–168, and ezrin 508–586 were expressed as GST fusion proteins in pGEX-6P vector (Amersham Biosciences). The obtained protein was about 19 kDa.

Cell Culture, Transfection, and CAT Assay—24 h before transfection, 5 × 105 HeLa cells were seeded onto 100-mm plates and cultured in RPMI 1640 10% fetal calf serum. Cells were transfected with 4 µg of each plasmid by the calcium-phosphate method, as previously described (36). 72 h later, cells were harvested and lysed, and cell extracts were processed to evaluate protein-protein interaction, measured by a CAT ELISA kit (Roche Applied Sciences) according to the manufacturer's instructions. Briefly, antibodies to CAT are prebound to the surface of a microtiter plate, and lysates from transfected cells were added to the wells, allowing expressed CAT binding to the anti-CAT-covered plates, and CAT positivity was revealed by a sandwich ELISA. The absorbance of the sample is determined using an ELISA reader.

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In Vitro Binding Assay—50 µl of glutathione-Sepharose beads slurry containing 30 µg of GST-ezrin fusion proteins were separately mixed with 15 µg of purified CD95 death domain in NETN buffer for 16 h at 4°C or mixed with 1 mg of Jurkat cell total extract in NETN buffer for 16 h at 4°C. Then these preparations were subjected to Western immunoblot assay with anti-CD95 pAb (C-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-GST pAb (Amersham Biosciences), or anti-mAb (Chemicon). Protein bands were visualized with peroxidase anti-Ig followed by ECL detection (Pierce).

Transfection Assay—The GFP-ezrin/moesin/ezrin (GFP-ez/moe) fusion protein was obtained starting from three separate fragments, ezrin 1–146, moesin 147–173, and ezrin 174–586, and the primers that were

FIG. 1. CD95 association with ERMs. A, detection of ERM on CD95 immunoprecipitates. Western blotting for ezrin or radixin (upper panel) and CD95 (lower panel) on the various immunoprecipitates from CEM-VBl100 whole lysates. Lanes 1, Western blotting for ezrin (upper panel) and CD95 (lower panel) on control immunoprecipitates. Lane 2, Western blotting for radixin (upper panel) and CD95 (lower panel) on control immunoprecipitates. Lane 3, Western blotting for ezrin (upper panel) and CD95 (lower panel) on ezrin immunoprecipitates. Lane 4, Western blotting for radixin (upper panel) and CD95 (lower panel) on radixin immunoprecipitates. Lane 5, Western blotting for ezrin (upper panel) and CD95 (lower panel) on A+G used for immunoprecipitation plus mouse IgGl (as isotype control antibody). B, ezrin, radixin, and CD95 functional domains. The drawing shows ezrin (representative of ERM proteins) tyrosine phosphorylation sites (Tyr145 and Tyr353) for ezrin and Tyr145 and Tyr445 for radixin) placed in the FERM domain (oval) and α-helical region (white bar), respectively, and the carboxy-terminal threonine phosphorylation site (Thr567) in the ezrin molecules (upper panel). Moreover, the CD95 extracellular domain (striped bar), intracytoplasmic domain (black box), and transmembrane region (white box) are shown (lower panel). C, ezrin, radixin, and CD95 fragments used for the two-hybrid assay. The drawing shows the radixin, ezrin, and CD95 fragments used to perform the two-hybrid assay. Particularly, the whole radixin FERM domain and ezrin 1–362 including the whole ezrin FERM domain plus the α-helical fragment with the second tyrosine phosphorylation site (Tyr267) lacking in radixin (upper panel) and the intracytoplasmic region of CD95 (residues 185–335) (lower panel) are shown.
CD95 Binding Epitope on Ezrin FERM Domain

The mammalian two-hybrid assay for the interaction between ezrin or radixin and CD95 was analyzed by measurement of expression of the reporter gene CAT through an ELISA colorimetric analysis. The positivity of ezrin CD95 interaction was evidenced by comparison of absorbance values of the various conditions with the positive controls. Condition A was as follows. Positive controls: 1) CAT (activation domain cloning vector)/pVP16-ezrin (vector expressing a fusion of the GAL4 DNA-BD to the mouse protein p53/pVP16-T (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen)/pG5CAT (mammalian reporter vector). Condition B was as follows. Negative controls: 1) pM53/pVP16-CP/pG5CAT: pM-53 (vector expressing a fusion of the GAL4 DNA-BD to the mouse protein p53/pVP16-CP (vector expressing a fusion of the VP16 AD to a viral coat protein, which does not interact with p53)/pG5CAT (mammalian reporter vector): 2) pM/pVP16-ezrin/pG5CAT: pM (GAL4 DNA-binding domain cloning vector)/ pVP16 (activation domain cloning vector)/pG5CAT (mammalian reporter vector): 3) pM-CD95/pVP16/pG5CAT: pM-CD95 (vector expressing a fusion of the GALA DNA-BD to the CD95 cytoplasmic domain)/pVP16 (activation domain cloning vector)/pG5CAT (mammalian reporter vector): 4) pM/pVP16-ezrin/pG5CAT: pM (GAL4 DNA-binding domain cloning vector)/pVP16-ezrin (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen)/pG5CAT (mammalian reporter vector). Condition C was as follows. Positive controls: 1) CAT (CAT enzyme 0.25-0.125 ng/ml) 0.262-0.176 0.186

Table I

| Transfected plasmids | Assorbance |
|----------------------|------------|
| Condition A          | 1 CAT (0.25-0.125 ng/ml) 0.262-0.176 |
| Condition B          | 1 pM53/pVP16-CP/pG5CAT: pM-53 (vector expressing a fusion of the GAL4 DNA-BD to the mouse protein p53/pVP16-CP (vector expressing a fusion of the VP16 AD to a viral coat protein, which does not interact with p53)/pG5CAT (mammalian reporter vector): 2) pM/pVP16-ezrin/pG5CAT: pM (GAL4 DNA-binding domain cloning vector)/ pVP16 (activation domain cloning vector)/pG5CAT (mammalian reporter vector): 3) pM-CD95/pVP16/pG5CAT: pM-CD95 (vector expressing a fusion of the GALA DNA-BD to the CD95 cytoplasmic domain)/pVP16 (activation domain cloning vector)/pG5CAT (mammalian reporter vector): 4) pM/pVP16-ezrin/pG5CAT: pM (GAL4 DNA-binding domain cloning vector)/pVP16-ezrin (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen)/pG5CAT (mammalian reporter vector). |
| Condition C          | 1 PM/ezrin149/pG5CAT: PM-CD95 (vector expressing a fusion of the GALA DNA-BD to the CD95 cytoplasmic domain)/pVP16 (activation domain cloning vector)/pG5CAT (mammalian reporter vector): 2) pM-CD95/pVP16-CP/pG5CAT: pM-CD95 (vector expressing a fusion of the GALA DNA-BD to the CD95 cytoplasmic domain)/pVP16 (activation domain cloning vector)/pG5CAT (mammalian reporter vector): 3) pM-CD95/pVP16-CP/pG5CAT: pM-CD95 (vector expressing a fusion of the GALA DNA-BD to the CD95 cytoplasmic domain)/pVP16 (activation domain cloning vector)/pG5CAT (mammalian reporter vector): 4) pM/pVP16-ezrin/pG5CAT: pM (GAL4 DNA-binding domain cloning vector)/pVP16-ezrin (vector expressing a fusion of the VP16 AD to the SV40 large T-antigen)/pG5CAT (mammalian reporter vector). |

used to direct their synthesis were the following: ezrin, C15, CTGCAGACTCCACCAAGAAAAACA and GATCCAGACTGCTTGCTGACTCTC; moesin, 149, GTACTCCTGGCCGGAGACAG and GATCCGCCTGCCTTGAGC; ezrin, 149, GTTCATGCTGGCATGCGGG and GAATTTCTACGGGCTCTAAGAC. PCR products were cloned into pTopo-TA vector (Invitrogen) and then excised with the appropriate restriction enzymes (XhoI and KpnI, KpnI and BamHI, and BamHI and EcoRI, respectively) and ligated to acquire a single fragment that subsequently was ligated in the pEGFPN1 vector (Clontech) at the EcoRI or XhoI and EcoRI sites to produce the GFP-ez/moe fusion protein. Primers utilized to produce the full-length ezrin were CTGCAGACTCCACTGCAAGAAAAACA and GATCCAGACTGCTTGCTGACTCTC, and the GFP-ez/moe fusion protein was obtained as described above. Plasmids encoding the GFP-ez/moe or GFP-ez/moe fusion proteins were transfected into HeLa cells growing on coverslips using the calcium phosphate protocol (36), and analyzed by Western blot. CD95, ezrin, radixin, and GFP-tagged proteins were detected with an anti-CD95 pAb (C20; Santa Cruz Biotechnology), an anti-ezrin mAb (clone 1E4; MBL, Japan), an anti-radixin mAb (Transduction Laboratories), an anti-GFP tag mAb (clone 1E4; MBL, Japan), and visualized with peroxidase anti-IgG followed by ECL detection (Pierce). CD95, radixin, and ezrin GFP-tagged proteins were immunoprecipitated overnight at 4°C in the presence of protein A + G-Sepharose beads (protein G plus; Sigma) from precleared cell lysates, respectively, with an anti-CD95 antibody (clone DX2; Calbiochem), an anti-CD95 antibody (clone 3C12; Sigma), an anti-radixin pAb (C15; Santa Cruz Biotechnology), and GFP mAb (clone 1E4; MBL, Japan). Mouse IgG1, (Santa Cruz Biotechnology), was used as control isotype. Immunoprecipitated beads were washed four times in lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 10% glycerol, 10 μg/ml supplemented with mixture of protease inhibitors and phosphatase inhibitors), resuspended in SDS sample buffer, and resolved by 10% SDS-PAGE. Then proteins were transferred to nitrocellulose membrane and analyzed by Western blotting.

Western Blotting and Immunoprecipitation—Whole extracts from HeLa, Hut78, Jurkat or CEM-VBL100 cells, and GFP-ezrin and GFP-ez/moe HeLa or Hut78 transfected cells were resuspended in SDS sample buffer, denatured by boiling, separated by 12% SDS-PAGE, and analyzed by Western blot. CD95, ezrin, radixin, and GFP-tagged proteins were detected with an anti-CD95 pAb (C20; Santa Cruz Biotechnology), an anti-ezrin mAb (Chemicon), and anti-GFP tag mAb (clone 1E4; MBL, Japan), and visualized with peroxidase anti-IgG followed by ECL detection (Pierce). CD95, radixin, and ezrin GFP-tagged proteins were immunoprecipitated overnight at 4°C in the presence of protein A + G-Sepharose beads (protein G plus; Pierce) from precleared cell lysates, respectively, with an anti-CD95 antibody (clone DX2; Calbiochem), an anti-CD95 antibody (clone 3C12; Sigma), an anti-radixin pAb (C15; Santa Cruz Biotechnology), and GFP mAb (clone 1E4; MBL, Japan). Mouse IgG1, (Santa Cruz Biotechnology), was used as control isotype. Immunoprecipitated beads were washed four times in lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 10% glycerol, 10 μg/ml supplemented with mixture of protease inhibitors and phosphatase inhibitors), resuspended in SDS sample buffer, and resolved by 10% SDS-PAGE. Then proteins were transferred to nitrocellulose membrane and analyzed by Western blotting.

Cell Death Assay—HeLa and Hut78 cells, either untransfected or GFP-transfected, were cultivated in 6-cm plates. Forty-eight hours after transfection, two-color flow cytometric analysis was performed after the following treatments: 1) 24 h after CD95 triggering (500 ng/ml anti-CD95 antibody, clone CH11; Upstate Biotechnology, Inc., Lake Placid, NY); 2) 6-h TNF-α exposure (50 IU/ml Sigma); or 3) 6-h of staurosporin (STS, 1 μM; Sigma). Untreated cells were considered as control. At the end of treatments, cells were washed and stained by using annexin V-alexa 568 (Molecular Probes, Inc., Eugene, OR). By using this technique, transfected cells showed green fluorescence emis-
FIG. 4. Susceptibility to apoptosis of the transfected cells. A, schematic representation of GFP-ez/moe chimeric fusion protein. Ezrin aa 147–173 (i.e. containing CD95 binding epitope) and moesin aa 147–173, replacing the same ezrin amino acids in the chimeric protein, are compared. Differences are framed. B, biparametric analysis of GFP-transfected HeLa cells after staining with annexin V-alexa 568 performed in living cells. First column, untransfected cells; second column, GFP alone-transfected cells; third column, wild type GFP-ezrin-transfected cells; fourth column, GFP-ez/moe-transfected cells. First row, control untreated cells; second row, α-Fas-treated cells; third and fourth rows, cells treated with TNF-α or STS, respectively. In quadrants I and IV nonapoptotic cells are shown: transfected cells (single GFP-positive, quadrant I) or nontransfected cells (GFP/annexin V double negative, quadrant IV). In quadrants II and III, apoptotic cells are shown; events in quadrant II of the dot plots correspond to transfected cells undergoing apoptosis (double GFP/annexin V-positive). In quadrant III, nontransfected apoptotic cells (GFP-negative/annexin V-positive) are included. The numbers in the quadrants represent the percentage obtained in one experiment representative of four. C,
sion due to GFP, and then they were easily distinguishable from non-transfected cells. Apoptotic cells showed red fluorescence emission due to the annexin V binding. Green/red double positive events thus corresponded to apoptosis of transfected cells. The samples were analyzed with a FACScan cytometer (Becton Dickinson) equipped with a 488-nm argon laser. At least 50,000 events have been acquired. Data were recorded and statistically analyzed by a Macintosh computer using CellQuest Software. Statistical analysis of apoptosis data was performed by using Student’s t test. Data reported are the mean of four separate experiments ± S.D. Only p values of less than 0.01 were considered as significant.

**Confocal Microscopy and Intensified Video Microscopy Analyses—** For microscopy analyses, HeLa and Hut78 cells were seeded on coverglasses placed in 60-mm Petri dishes. 48 h after transfection with the various GFP plasmids described above, cells were fixed (paraformaldehyde (3%), 30 min, + 4°C) and permeabilized (Triton X-100 (0.5%), 10 min, room temperature). For localization of CD95 and the various GFP-tagged ezrin fusion proteins, samples were incubated at 37°C for 30 min with polyclonal antibodies to CD95 (Santa Cruz Biotechnology) and then incubated with anti-rabbit IgG TRITC conjugate (Sigma). For detection of the naive ezrin on untransfected cells, monoclonal antibody to ezrin (Biogenesis) was used. Then samples were washed in PBS and treated with fluorescent secondary antibody Rhodamine Red (R-6393, goat anti mouse IgG (H+L) Molecular Probes Europe BV, Poort-Gebouw, Leiden, The Netherlands). Cells were observed and analyzed by a confocal laser-scanning microscope (FV500; Olympus Optical Co. Europe GmbH, Hamburg, Germany) or by intensified video microscopy (IVM) (1). For IVM analyses, images were captured by a color-chilled 3CCD camera (DELT A Systems), and figures were obtained by adding CD95-TRITC (red) and ezrin (FITC or GFP; green) images through the OPTILAB (Grafe Tek, France) software for image analysis.

**RESULTS**

**CD95 ERM Association**

We first performed experiments aimed at investigating a possible direct interaction between CD95 and ERM proteins. Our previous results in human lymphocytes suggested that only ezrin interacted with CD95, whereas moesin was not implicated in the binding to CD95 (1). However, radixin was not expressed in human lymphocytes (1, 37). Therefore, we preliminarily evaluated the radixin binding to CD95 in cellular systems expressing radixin. To this purpose, we carried out co-immunoprecipitation experiments using a clone of CEM cells (VBL-100), known to fully express radixin (38). As shown in Fig. 1, consistently with a previous report (1), ezrin and CD95 co-immunoprecipitated, whereas radixin was undetectable in CD95 immunoprecipitates (Fig. 1A). Notably, in the same cell extracts, P-glycoprotein (p170) proved to co-immunoprecipitate with radixin (data not shown), further supporting our previous findings (38). This first set of results showed that radixin/CD95 interaction did not occur in cells expressing radixin, whereas in the same cells, CD95 interacted with ezrin, further supporting a specific role of ezrin in connecting CD95 to actin.

**Binding of Ezrin FERM Domain to CD95**

Two-hybrid Assay—To evaluate a possible direct interaction between ezrin and CD95, two-hybrid assay experiments were performed. The mammalian two-hybrid system was used in order both to allow the occurrence of post-transductional changes, such as tyrosine phosphorylation, and to avoid possible false positive results. By analogy with other ERM-binding membrane proteins, the two-hybrid system was performed using either the ezrin or the radixin FERM domain and the whole CD95 cytoplasmic domain. Particularly, the ezrin N-terminal 392 aa, including the FERM domain and both tyrosine phosphorylation sites (i.e. Tyr440 and Tyr490) (39) or the whole radixin FERM domain (known to lack Tyr490) were fused to the GAL4 transactivation activation domain of the pVP16-AD, and the CD95 cytoplasmic domain (aa 185–335) was fused to the GAL4 DNA binding domain of the pGAL4-BD cloning vector (Fig. 1, B and C). pG5CAT containing the CAT gene was used as reporter plasmid. Plasmids containing the CD95 and ezrin or radixin fragments and the plasmid containing CAT were transfected in HeLa cells. As control experiments, the interaction of mouse p53 with either SV40 large T antigen (as positive control) or with polyoma virus coat protein (as negative control) was evaluated (Table I). The two-hybrid interaction between CD95 and ezrin constructs was detected by expression of CAT assessed by a CAT ELISA colorimetric assay (detailed under “Experimental Procedures”). In seven of seven repeated experiments, the results clearly showed that the ezrin N-terminal domain interacted directly with CD95 cytoplasmic domain, whereas no detectable interaction was observed between CD95 and radixin. The values of absorbance obtained from CD95/ezrin interaction were comparable with positive controls, whereas no CAT expression was detected in all negative controls (Table I). This set of experiments showed that CD95 and ezrin interact directly and that this interaction is specific for the ezrin FERM domain.

**GST in Vitro Binding Assay—**In order to identify the specific ezrin epitopes involved in the ezrin/CD95 interaction highlighted by the two-hybrid experiments, a series of GST pull-down assays were performed. With the purpose of mapping the region responsible for CD95/ezrin association, a series of recombinant truncations of the ezrin FERM domain were constructed (Fig. 2). Thus, we investigated the CD95 binding to a series of ezrin fragments produced as GST fusion proteins in a prokaryotic expression system. In order to confirm and extend the two-hybrid assay results, we first explored the ability of the GST-ezrin variants to interact with the recombinant CD95. To this purpose, we utilized the whole CD95 cytoplasmic domain purified protein, in GST pull-down experiments. The results first showed that GST-ezrin 1–362 bound to CD95, confirming the results of the two-hybrid assay (not shown). We thus performed experiments using a series of recombinant truncations of the ezrin FERM domain in order to individuate the epitope accounting for the CD95 binding. The chosen regions were ezrin 149–242, ezrin 149–200, ezrin 149–168, and ezrin 169–242. We preliminarily evaluated the specificity of our GST-ezrin fusion proteins, blotting with an anti-GST antibody the bacterial lysates containing the various fusion proteins (not shown). Thus, we evaluated the binding of the various GST-ezrin fusion proteins to CD95. The results showed a CD95-binding activity for GST-ezrin 149–242, GST-ezrin 149–200, and GST-ezrin 149–168, as assessed by Western blot for CD95 (Fig. 3A). However, GST-ezrin 169–242 did not show any detectable CD95 binding activity, together with the carboxyl-terminal truncation (ezrin 508–586), including the consensus sequence motif for the binding to actin (27) (Fig. 3A).

In order to verify the possible occurrence of CD95/ezrin fragments binding in a more physiological condition, we performed additional experiments using a constitutively expressed CD95.
CD95 Binding Epitope on Ezrin FERM Domain

To this purpose, we performed the GST assay, pulling down the total extracts of human T cells expressing high level of constitutive CD95 (i.e. Jurkat cells) on the same ezrin fragments used in the experiments with the recombinant CD95. The results were highly consistent with those obtained with the recombinant CD95, in showing the binding of GST-ezrin 149–242, GST-ezrin 149–200, and GST-ezrin 149–168 to the Jurkat cells CD95, as detected by Western blot with an anti-CD95 antibody (Fig. 3B). Again, the GST-ezrin 169–242 and the GST-ezrin 508–586 did not show any detectable CD95-binding activity (Fig. 3B). As a further control, we also evaluated the interaction of the native actin with the ezrin fragments. The results showed that actin was exclusively detected in Jurkat total extracts and in the GST-ezrin 508–586 pulled down Jurkat total extracts (Fig. 3C), confirming the specificity of our ezrin fragments for the binding to either CD95 or actin, as appropriate.

In Vivo Characterization of CD95 Binding Domain

In order to investigate the relevance of the identified ezrin region in susceptibility to CD95-mediated apoptosis, two human cell lines of different histotype and prone to CD95-mediated apoptosis (Hut78 and HeLa) were used. These cell lines were transiently transfected with different expression vectors encoding GFP alone, GFP-tagged wild type (GFP-ezrin), and mutated ezrin (GFP-ez/moe). In order to avoid the occurrence of ezrin 4.1 domain mutations interfering with its globular folding, a chimeric ezrin/moesin/ezrin fusion protein (GFP-ez/moe) was obtained, where ezrin aa 148–168 were replaced with the corresponding amino acid sequence of moesin, that displays a similar folding to ezrin but does not bind CD95 (1, 32, 35) (Fig. 4A; see “Experimental Procedures”). Seventy-two hours after transfection, cells were lysed and analyzed to verify the presence of the tagged protein. Western blot analysis showed the expression GFP-ezrin fusion proteins in total lysates of transfected cells (not shown). Thus, the functional role of this ezrin epitope in CD95-mediated apoptosis in intact cells was verified. HeLa and Hut78 cells were transfected with the above described plasmids, and transfected cells were triggered with α-Fas, TNF-α (whose receptors do not interact with ezrin (1)), or STS (a stimulus of intrinsic/mitochondrial pathway (40)). The transfection efficiency in our experimental system ranged from 18.6 to 47.9%. Biperametric fluorescence-activated cell sorting analysis of GFP/annexin V double-positive cells revealed that 1) only GFP-ez/moe cells were significantly (p < 0.01) protected from the CD95-induced apoptosis (Fig. 4, B and C, second row, quadrant II), 2) GFP-ezrin did not confer any significant protective effect, 3) in the same experimental conditions, no protective effect against TNF-α- or STS-induced apoptosis was observed (Fig. 4, B and C, third and fourth rows, respectively), and 4) comparable percentages of apoptotic cells were detected in GFP-ezrin cells, untransfected cells, and cells transfected with GFP-vector alone (Fig. 4, B and C, first and second columns).

To explain the mechanism by which transfected cells were protected from CD95-mediated apoptosis, we carried out reciprocal co-immunoprecipitation experiments blotting with anti-GFP tag mAb on CD95 immunoprecipitates and with anti-CD95 mAb on GFP tag immunoprecipitates, in untransfected Hut78 cells, or in Hut78 cells transfected with the GST-ezrin or with the GST-ez/moe (Fig. 5). The results showed that the GFP-ez/moe was undetectable in the CD95 immunoprecipitates, suggesting that the mutated ezrin did not interact with CD95 (Fig. 5A). As expected, the anti-GFP antibody recognized the GFP-tagged ezrin exclusively in the CD95 immunoprecipitates from GFP-ez/moe cells, without cross-reacting with the ezrin of untransfected cells (Fig. 5A). Furthermore, a specific CD95 corresponding band was detected in GFP immunoprecipitates from GFP-ez/moe-transfected cells, whereas CD95 was undetectable in the GFP immunoprecipitates from GFP-ez/moe-transfected cells (Fig. 5B). In Fig. 5, A and B, the presence of GFP-tagged ezrin in GFP immunoprecipitates and CD95 in CD95 immunoprecipitates is shown, respectively. Comparable results were obtained with HeLa cells (data not shown). Finally, we analyzed by laser-scanning confocal microscopy and
intensified video microscopy (IVM) (1) the relative distribution of ezrin, CD95, and GFP-ezrin or GFP-ez/moe in HeLa (Fig. 6A) and Hut78 (Fig. 6B) transfected or untransfected cells. The results showed that GFP-ez/moe and CD95 did not co-localize, while localizing in similar subcellular compartments (Fig. 6, A and B, first panel). On the other hand, ezrin and CD95 proved to polarize and co-localize in both GFP-ezrin-transfected and -untransfected cells (Fig. 6, A and B, second and third panel, respectively). This set of results showed that GFP-ez/moe and CD95 did not co-immunoprecipitate or co-localize in GFP-ez/moe-transfected cells, consistent with the resistance of these cells to CD95-mediated apoptosis. However, these data did not provide direct evidence for the mechanism through which in transfected cells the mutated ezrin might interfere with the CD95-mediated apoptosis in the presence of wild-type ezrin.

**Mutated Ezrin Prevents the Binding of the Wild-type Ezrin to Actin**

In order to provide a mechanism responsible for the mutated ezrin-induced inhibition of susceptibility to CD95-mediated apoptosis, we hypothesized that the mutated ezrin might behave as dominant negative. To this purpose, GFP-ez/moe-transfected Hut78 cells were analyzed by IVM to assess the presence and behavior of endogenous ezrin (stained in red), as compared with the GFP-tagged mutated ezrin. The results clearly showed that endogenous and mutated ezrin were co-expressed (Fig. 7), while differently localized, in transfected cells, the red stain being mostly detectable at the plasma membrane and the GFP staining being more diffused into the cytoplasm (Fig. 7). On the basis of previous reports (1, 41), it seemed clear that the most important function of ezrin in the CD95 apoptotic pathway was to connect CD95 to actin, thus allowing CD95 to be polarized on a cell protrusion (42). Therefore, we reasoned that the mutated and the wild-type ezrin did not differ in their actin-binding domain. This suggested us that the mechanism responsible for our results might be due to a competition between the mutated and the endogenous ezrin in their binding to actin. In this case, mutated ezrin could be favored in its binding to actin, due to its transfection-induced
overexpression, thus preventing the CD95 binding to actin and, as a consequence, its polarization on the cell. Hence, we performed reciprocal co-immunoprecipitation experiments aimed at evaluating the CD95 and GFP-tagged ezrin binding to actin, in both GFP-ez/moe and GFP-ezrin transfected cells. The results demonstrated that actin was detectable in GFP immunoprecipitates from whole lysates of both GFP-ez/moe and GFP-ezrin-transfected Hut78 cells (Fig. 8A). Consistently wild type and mutant GFP-tagged ezrin were detectable in actin immunoprecipitates from whole lysates of the same cells (Fig. 8B). Moreover, CD95 was only barely detectable in the actin immunoprecipitates from GFP-ez/moe-transfected cells, while fully detectable in GFP-ezrin-transfected and untransfected cells (Fig. 8C). These results, together with the data showing the inability of CD95 to co-immunoprecipitate with the mutated ezrin, highly supported our hypothesis that in cells transfected with the GFP-ez/moe, the mutated ezrin, while being unable to bind CD95, was fully able to bind actin, conceivably leading to a competition with the wild-type ezrin in the linkage to actin. As positive controls, GFP and actin were detected on GFP and actin immunoprecipitates, respectively (Fig. 8, A–C).

**DISCUSSION**

The membrane protein connection to the actin filaments in conferring a polarized behavior to the cells is an important clue of cell biology (39, 43). ERM appears the most involved protein family in exerting this function (3, 44–46). We have previously demonstrated that the association between CD95 and ezrin is an important feature of susceptibility to CD95-mediated apoptosis and that this association has relevant biological significance inasmuch as treatment with ezrin antisense oligonucleotides strongly inhibited the susceptibility to CD95-mediated apoptosis of live human lymphoid cells (1). In this study, we provided evidence that the ezrin/CD95 association occurs through a direct binding between the ezrin FERM domain and the CD95 cytoplasmic domain. Notably, although a number of ezrin partners have been identified (reviewed in Ref. 3), the mechanisms by which ezrin exerts its functions are still mostly unclear. In fact, whereas ezrin binding domains to actin are well characterized (27–30), the ezrin epitopes involved in the specific linkage to membrane proteins are unknown. The results of our study not only showed that the intracellular domain of CD95 bound directly to the ezrin FERM domain, but, using the GST pull-down assay, we were able to show that a small epitope contained in the ezrin FERM domain is involved in the binding to CD95. In *in vitro* analysis, using various deletion mutants, revealed that determinants of the ezrin binding to CD95 were located within amino acids 149–168 in the middle lobe of ezrin FERM domain. Moreover, we have newly shown that radixin, while not expressed in many cell types, including lymphocytes (1, 37), did not co-immunoprecipitate with CD95 in radixin-expressing cells and did not interact with CD95, as assayed with the two-hybrid assay. These results, together with previous evidence that moesin is not involved in the binding to CD95 (1), suggested that ezrin has a specific role in linking CD95 to actin. To further evaluate the biological relevance of these results, we performed transfection experiments in two different human cells lines using a GFP-ezrin construct mutated in the epitope responsible for CD95 binding. In order to avoid the occurrence of ezrin 4.1 domain mutations interfering with its globular folding, we obtained a chimeric ezrin/moesin/ezrin fusion protein, where ezrin aa 148–166 were replaced with the corresponding amino acid sequence of moesin, which displays a similar folding to ezrin but does not bind CD95 (1, 32, 35) (GFP-ez/moe). The results of these experiments have shown that GFP-ez/moe did not co-immunoprecipitate or co-localize with CD95 in transfected cells. Consistent with these data, cells expressing the mutated ezrin were strongly and specifically protected from CD95-mediated apoptosis, whereas no protection was observed after treatment of transfected cells with other apoptotic stimuli, such as TNF or staurosporin. With these results, we provided *in vitro* evidence that the ezrin 148–166 was specifically involved in the binding to CD95, and the ezrin-to-CD95 linkage was key in rendering a cell prone to CD95-mediated apoptosis. However, we did not
provide data supporting a possible mechanism through which the mutated ezrin inhibited susceptibility to CD95 apoptosis in cells simultaneously expressing wild-type ezrin. Hence, we performed experiments aimed at verifying the hypothesis that the mutated ezrin might behave as dominant negative in transfected cells. The background of these experiments was that the most conceivable function of ezrin in the CD95 apoptotic pathway is to connect CD95 to actin and that GFP-εz/moe and wild-type ezrin, while differing in their membrane-binding domains, maintained the same actin-binding domain. In fact, CD95-to-actin connection has proven to be key both in driving CD95 polarization on the cells and susceptibility to CD95 triggering (47). Moreover, dramatic effects on ezrin and actin connection to actin has been shown to be key both in rendering CD95-to-actin connection has proven to be key both in driving CD95 signaling (41). The results showed a marked decrease in the detection of actin in the CD95 immunoprecipitates and a high level of actin in the GFP-εz/moe-immunoprecipitates in GFP-εz/moe-transfected cells. These data gave support to our hypothesis, suggesting that a competition between the mutated ezrin and the endogenous ezrin in the binding to actin occurred in transfected cells. The overexpression of the mutated ezrin in transfected cells favored its binding to actin, in turn preventing the CD95 binding to actin and, as a consequence, the CD95 polarization on the cell. CD95 connection to actin has been shown to be key both in rendering cells susceptible to CD95-mediated apoptosis (1) and in allowing DISC formation, CD95 internalization (41), and GD3 engagement (47). Moreover, dramatic effects on ezrin and actin cytoskeleton occur following CD95 triggering (48), and disruption of cytoskeleton may induce apoptosis via CD95 activation (49). Hence, our data add new support to the key role of ezrin in allowing the CD95 linkage to actin, in turn suggesting that the mutated ezrin dominantly interfered with CD95 signaling by preventing CD95 interaction with actin. Altogether, our results show that 1) the ezin/CD95 linkage is ezrin-specific, whereas both radixin and moesin seem not to be involved in the linkage to CD95 in CD95-susceptible cells; 2) the ezrin region responsible of the CD95 binding consists of 20 amino acids included in the median lobe of the ezrin FERM domain, where ezrin shows relevant differences from both moesin and radixin, supporting ezrin specificity for CD95 binding; 3) ezrin mutations on the mapped CD95 binding epitope result in the loss of CD95-to-εzrin linkage, consistently with the inhibition of sensitivity to CD95-mediated apoptosis. On the basis of our data, we mapped the CD95 binding site on ezrin, between amino acids 149 and 168. Notably, in this sequence ezrin, radixin and moesin show only a 60–65% identity (Fig. 9A), whereas previous analyses have shown that ERM proteins have ~86% of identity in the whole FERM domain (14). This epitope is located within the ezrin FERM domain, in a region that is included between amino acids 12–115 and 233–310 (Fig. 9B), the two determinants of the Pap2 binding domain (20). Last, our results showed that mutated ezrin may lead to a cell refractoriness to CD95-mediated apoptosis in binding actin but be unable to bind CD95, thus interfering with the CD95 to actin linkage. This is consistent with the hypothesis that ezrin-mediated CD95 linkage to actin may have a role not only in conferring cell susceptibility to CD95 triggering but also in driving the actin-dependent DISC formation and CD95 internalization (41).

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