Cell, Vol. 87, 845–855, November 29, 1996, Copyright ©1996 by Cell Press

CAR1, a TNFR–Related Protein, Is a Cellular Receptor for Cytopathic Avian Leukosis–Sarcoma Viruses and Mediates Apoptosis

Jürgen Brojatsch,* John Naughton,*
Melissa M. Rolls,* Kurt Zingler,†
and John A. T. Young*
*Department of Microbiology and Molecular Genetics
Harvard Medical School
Boston, Massachusetts 02115
†Department of Microbiology and Immunology
University of California School of Medicine
San Francisco, California 94143

Summary

Viral envelope (Env)–receptor interactions have been implicated in the cell death associated with infection by subgroups B and D avian leukosis–sarcoma viruses (ALVs). A chicken protein, CAR1, was identified that permitted infection of mammalian cells by these viral subgroups. CAR1 bound to a viral Env fusion protein, comprising an ALV-B surface Env protein and the Fc region of an immunoglobulin, indicating that it is a specific viral receptor. CAR1 contains two extracellular cysteine-rich domains characteristic of the TNFR family and a cytoplasmic region strikingly similar to the death domain of TNFR1 and Fas, implicating this receptor in cell killing. Chicken embryo fibroblasts susceptible to ALV-B infection and transfected quail QT6 cells expressing CAR1 underwent apoptosis in response to the Env–Ig fusion protein, demonstrating that this cytopathic ALV receptor can mediate cell death.

Introduction

Avian leukosis–sarcoma viruses (ALVs) are divided into cytopathic and noncytopathic subgroups. In contrast to infection by noncytopathic viral subgroups (A, C, and E), which does not lead to cell killing, infection by cytopathic viral subgroups (B, D, and F) can lead to the death of as much as 30%–40% of target cells during the acute phase of infection (Weller et al., 1980; Weller and Temin, 1981). Cells killed by cytopathic ALVs contain approximately 300–400 copies of unintegrated viral DNA per cell, whereas noncytopathic ALV infections do not lead to accumulation of unintegrated viral DNA (Weller et al., 1980; Weller and Temin, 1981). Also, addition of antisera to prevent viral reinfection abrogates the viral cytopathic effect (CPE; Weller et al., 1980). These observations have led to a proposal that the cell killing associated with cytopathic ALV infections might be due to massive viral superinfection (Weller et al., 1980; Weller and Temin, 1981). Indeed, infected cells that survive this transient period of cell killing are resistant to viral superinfection because the cognate receptors are functionally down-regulated (reviewed by Weiss, 1993). Multiple rounds of viral infection have also been implicated in the CPE associated with other retroviruses, including HIV-1 (Somasundaran and Robinson, 1987; Stevenson et al., 1988; Pauza et al., 1990; Robinson and Zinkus, 1990), spleen necrosis virus (Keshet and Temin, 1979), and the feline leukemia virus FeLV-FAIDS (Donahue et al., 1991). In the case of HIV-1, however, superinfection is not required for cell killing (Bergeron and Sodroski, 1992; Laurent-Crawford and Hovanessian, 1993).

CPE determinants have been mapped to the envelope (Env) glycoproteins of several retroviruses, including ALV-B (Dorner and Coffin, 1986), HIV-1 (reviewed by Siliciano, 1996), avian hemangioma virus (Resnick-Roguel et al., 1989), Cas-Br-E–murine leukemia virus (Paquette et al., 1989), feline leukemia virus C (Riedel et al., 1988), and the feline leukemia virus FeLV-FAIDS (Donahue et al., 1991). The CPE determinants of the ALV-B Env subunit surface (SU) also specify usage of the subgroup B viral receptor on chicken cells (Dorner and Coffin, 1986). This observation suggests that Env–receptor interactions might contribute directly to cell killing.

Cytopathic ALV-B and ALV-D and noncytopathic ALV-E are predicted to share a cellular receptor encoded by the chicken *tv-b* locus (reviewed by Weiss, 1993). A set of five different alleles of *tv-b* has been identified: *tv-b*^{s1}, *tv-b*^{s2a}, and *tv-b*^{s2b} permit infection by all three viral subgroups; *tv-b*^{s3} allows infection by ALV-B and ALV-D, but not ALV-E; and *tv-b*^{r} does not permit entry by any of these viruses (reviewed by Weiss, 1993).

To understand the mechanism of cell killing induced by cytopathic subgroups of ALV, we have cloned a chicken gene, presumably *tv-b*^{s3}, which encodes a cellular receptor for the subgroup B and D viruses. This receptor is a member of the tumor necrosis factor receptor (TNFR) family, and interaction of this protein with a subgroup B SU–Ig fusion protein promotes the death of avian cells. This result indicates that cytopathic ALV Env–receptor interactions might contribute directly to virus-induced cell death.

Results

Isolation of Genomic and cDNA Clones That Permit Infection by Cytopathic Subgroups of ALV

A gene transfer approach was used in an attempt to isolate the chicken *tv-b*^{s3} locus, predicted to encode cellular receptors for the cytopathic subgroups B and D ALV (Weiss, 1993). Mouse 3T3 cells were cotransfected with pMPHis plasmid DNA conferring histidinol resistance (Young et al., 1993) and with genomic DNA from chicken embryo fibroblasts (CEFs) homozygous for *tv-b*^{s3}. To identify which of the approximately 3,300 histidinol-resistant colonies were susceptible to ALV-B infection, we challenged them with the subgroup B–specific virus RCASH-B encoding hygromycin B phosphotransferase (Young et al., 1993). The resultant 19 hygromycin B–resistant colonies were challenged with another subgroup B–specific virus, RCASB-Neo, conferring resistance to G418. A single primary transfectant, designated 11B, was infected by both subgroup B viruses. The presence of approximately six copies of RCASH-B proviral DNA in the population of cells derived from transfectant

11B confirmed the occurrence of multiple infection events, consistent with receptor-mediated viral entry (data not shown). Southern blot analysis (Southern, 1975) demonstrated the presence of chicken genomic DNA in this transfectant (data not shown), as it contained multiple copies of B5/B6 avian repeat DNA (Stumph et al., 1981).

To segregate the putative ALV-B susceptibility gene from other transfected DNA sequences, we performed a second round of transfection and selection. Genomic DNA from primary transfectant 11B was cotransfected with pPur plasmid DNA encoding puromycin N-acetyltransferase into mouse 3T3 cells. The resulting 20,000 colonies were challenged with RCASB-Neo and RCASH-B, leading to 14 colonies resistant to G418 and hygromycin B.

The primary transfectant 11B contained multiple copies of pMPHIS plasmid DNA (data not shown), raising the possibility that plasmid sequences were linked to the ALV-B susceptibility locus. Indeed, Southern blot analysis demonstrated that 7 of 12 secondary transfectants contained a shared locus with a unique copy of pMPHIS plasmid DNA (Figure 1A). Thus, this plasmid DNA appeared to be linked to the susceptibility locus and was used as a molecular tag for cloning.

To isolate the chicken DNA sequences linked to this plasmid, a genomic DNA library prepared from secondary transfectant C12 was screened by hybridization with a probe specific for the histidinol dehydrogenase gene. The BK-1c clone was isolated (Figure 1A), and derivative restriction fragments were used as hybridization probes to isolate the overlapping genomic DNA clone BK-9 (Figure 1A). Southern blot analysis using probes derived from both of these clones revealed a 7.5 kb stretch of chicken genomic DNA shared between several independent secondary transfectants (Figure 1A). Human 293 cells transfected with the BK-9 genomic DNA clone could be infected by RCASH-B, indicating that the region of the shared chicken locus contained within this clone encodes the ALV-B susceptibility factor (data not shown).

To identify RNA transcripts encoding this factor, Northern blot analysis was performed using three probes derived from the shared region. A single 2.3 kb RNA transcript hybridized with all three probes (Figure 1B). Therefore, a cDNA library constructed from CEFs was screened with these probes. One clone, designated 7.6-2, which hybridized with all three probes, was isolated and conferred susceptibility to ALV-B infection upon COS-7 cells (Figure 1C). Furthermore, this clone increased by approximately 10-fold the susceptibility of these cells to infection by subgroup D viruses (Figure 1C), which are moderately mammal-tropic (Bova et al., 1988). These results indicate that the cloned gene is probably *tv-b*^{s3}, predicted to encode a factor allowing infection specifically by ALV-B and ALV-D (Weiss, 1993). As expected, the transfected COS-7 cells were not efficiently infected by subgroup A, C, or E viruses (Figure 1C), which do not use receptors encoded by this allele of *tv-b* (Weiss, 1993).

Preliminary studies have demonstrated that the candidate *tv-b* gene may be expressed ubiquitously in chicken tissues. Northern blot analysis using the 7.6-2 cDNA clone as a probe revealed an approximately 2.3 kb RNA species in several different chicken tissues including bursa, gizzard, liver, heart, and lung (data not shown). However, these transcripts appear to be much less abundant than those observed in CEFs.

The Cloned Factor Binds Specifically to the ALV-B SU Protein

The 7.6-2 cDNA clone was sequenced leading to the identification of a single long open reading frame that appears to encode a type I membrane protein with an estimated molecular mass of 39 kDa. This protein is predicted to contain a signal peptide, an extracellular domain with two putative N-linked glycosylation sites, a single transmembrane region, and a long cytoplasmic tail (Figure 2).

To test whether the cloned factor bound specifically to ALV-B Env, an immunoadhesin (SUB–rIgG) comprising a subgroup B SU protein fused to the Fc region of a rabbit immunoglobulin was constructed. The SUB–rIgG protein migrated at approximately 200 kDa under nonreducing conditions on an SDS–polyacrylamide gel (Figure 3A) but at approximately 120 kDa when reduced (data not shown). This is consistent with the formation of disulfide-linked homodimers similar to those of other immunoadhesins (Capon et al., 1989; Haak-Frendscho et al., 1993; Pitti et al., 1994). The SUB–rIgG protein precipitated two predominant proteins (approximately 42 kDa and 45 kDa in size) from lysates of human 293 cells transfected with the 7.6-2 cDNA clone (Figure 3B, right panel), but not from cells expressing Tva, the ALV-A receptor (Figure 3B, left panel). These two proteins were not precipitated by a control immunoadhesin (SUA-rIgG) containing the SU portion of a subgroup A Env protein, which instead precipitated the heterogeneously modified Tva proteins (Bates et al., 1993; Figure 3B). Endoglycosidase H digestion of the 42 kDa and 45 kDa proteins generated a single 40 kDa protein species (M. M. R., H. Adkins, and J. A. T. Y.; unpublished data), demonstrating that the major proteins precipitated by SUB–rIgG were glycosylated forms of the cloned factor. Flow cytometry demonstrated that human 293 cells transfected with the 7.6-2 cDNA clone specifically bound SUB–rIgG (data not shown). The ability of the cloned factor to bind directly to ALV-B SU is an activity expected of a specific viral receptor; we therefore designated this protein CAR1 (cytopathic ALSV receptor).

CAR1 Is a Member of the TNFR Family

Several features identified CAR1 as a member of the TNFR family that includes TNFR1, TNFR2, Fas, p75^{NTR}, OX-40, CD40, CD27, and CD30 (reviewed by Beutler et al., 1994). The predicted extracellular region of CAR1 contains two TNFR-like cysteine-rich domains (CRDs) (Figure 4A). Both domains contain six highly conserved cysteines, which in TNFR1 are organized into three intradomain disulfide bonds (Banner et al., 1993). The second CRD of CAR1 contains two additional cysteines (residues 109 and 115; Figure 4A), as do the third CRDs of Fas (Itoh et al., 1991), TNFR2 (Smith et al., 1990), and CD40 (Stamenkovic et al., 1989). Additional conserved amino acid residues were identified in both CRDs of

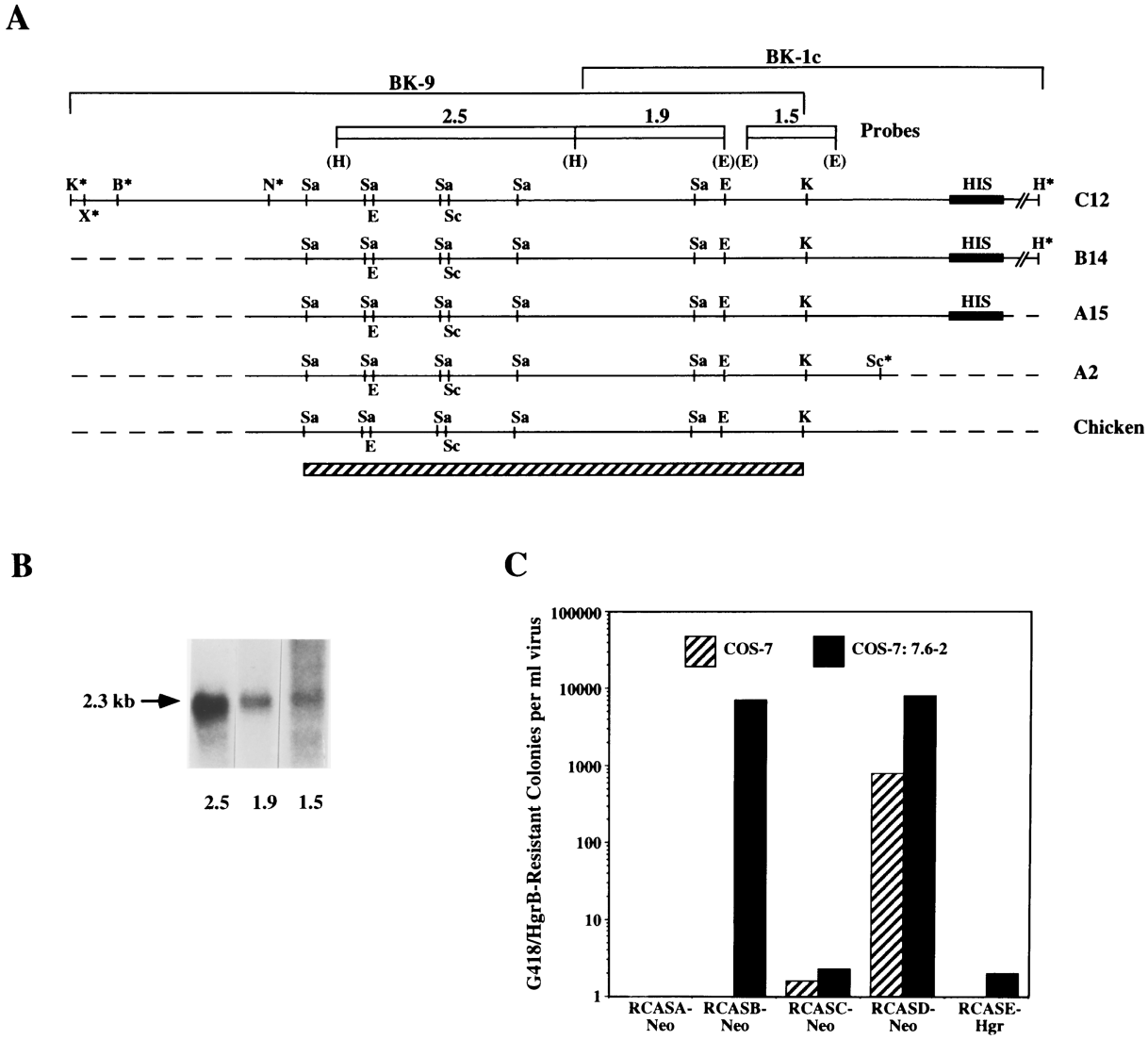

Figure 1. Isolation of a cDNA Clone Encoding an ALV-B Susceptibility Factor

(A) Restriction enzyme site map of a 7.5 kb chicken DNA locus shared between independent secondary transfectants (A2, A15, B14, and C12) that were susceptible to ALV-B. The 2.5 kb and 1.5 kb fragments (open boxes) were used as hybridization probes. The pMPHIS plasmid is indicated by a black box. The location of BK-9 and BK-1c clones is indicated. The shared 7.5 kb region of chicken genomic DNA is indicated by a striped box. Enzyme abbreviations: B, BglII; E, EcoRI; H, HindIII; N, NdeII; K, KpnI; Sa, SacI; Sc, ScaI; X, XbaI. Unique sites are indicated by asterisks. Sites in parentheses were used to generate probes but were not tested in every transfectant.

(B) Northern blot analysis of total RNA from chicken cells that were homozygous for *tv-b*^{s3} was performed using the 2.5 kb, 1.9 kb, and 1.5 kb fragments as hybridization probes.

(C) COS-7 cells transfected with the 7.6-2 cDNA clone were susceptible to ALV-B infection and showed an increased susceptibility to infection by mammal-tropic subgroup D viruses (Bova et al., 1988). The transfected cells were challenged with subgroup A, B, C, and D viruses conferring resistance to G418 and with a subgroup E virus conferring resistance to hygromycin B. The resultant numbers of G418 or hygromycin B-resistant colonies are indicated per ml virus.

CAR1, some of which in TNFR1 are required for proper folding of the structural core of these motifs (Banner et al., 1993; Figure 4A). Compared with the extracellular domains of other TNFR–like proteins, the regions of CAR1 with the most amino acid spacing differences (residues Thr-68 to Lys-76; Arg-81 to Gln-87; Cys-109 to Met-117; Pro-126 to Gln-129) correspond to two variable loop regions in the CRDs of TNFR1 (Banner et al., 1993).

The predicted cytoplasmic tail of CAR1 contained a region strikingly similar to the defined death domains of TNFR1 and Fas (Figure 4B), which are important for the ability of these receptors to induce apoptosis (Itoh and Nagata, 1993; Tartaglia et al., 1993). Similar death domains have been described in other apoptosis-inducing proteins including p75^{NTR} (Chapman, 1995), TRADD, FADD/MORT-1, and RIP (Figure 4B; reviewed by Cleveland and Ihle, 1995). The putative death domain of CAR1 was most highly related to the death domain of human TNFR1 (31% identity). Significantly, six residues in the death domain of TNFR1 shown to be critical for cell

A

```
1      ATG CGC TCA GCT GCG CTC CGG TTG TGC CCC GTT CTA CTG CTG CTC TTC GCG GAG GTT CAG
1       M   R   S   A   A   L   R   L   C   P   V   L   L   L   L   F   A   E   V   Q
61     TTG GGA TCT GCT GCA GCA GTG AAG AAG AGG GCA GAC AGG TCA GAC CTC CAG AAG CCA GAC
21      L   G   S   A   A   A   V   K   K   R   A   D   R   S   D   L   Q   K   P   D
121    CTC TAC AGA AGG AAG TGT CCT ATG GGC ACC TAT GAG GCA AAT GAC TCC ATC CAG TGC CTC
41      L   Y   R   R   K   C   P   M   G   T   Y   E   A   N   D   S   I   Q   C   L
181    CCA AGT AAG AAA GAC GAG TAC ACC GAG TAT CCA AAT GAC TTT CCC AAG TGC CTG GGC TGC
61      P   S   K   K   D   E   Y   T   E   Y   P   N   D   F   P   K   C   L   G   C
241    CGG ACG TGT AGG GAA GAC CAG GTG GAG GTG AGT CCC TGC ATC CCC ACC AGG AAC ACG CAG
81      R   T   C   R   E   D   Q   V   E   V   S   P   C   I   P   T   R   N   T   Q
301    TGC GCT TGC AAG AAC GGC ACC TTC TGC TTA CCT GAC CAC CCC TGT GAG ATG TGC CAA AAG
101     C   A   C   K   N   G   T   F   C   L   P   D   H   P   C   E   M   C   Q   K
361    TGC CAG ACC GAG TGC CCC AAA GGA CAA GTG AGG TTA GCT CCG TGC ACG CAA CAC AGC GAC
121     C   Q   T   E   C   P   K   G   Q   V   R   L   A   P   C   T   Q   H   S   D
421    CTG CTG TGC GGT CCA CCC TTG GAA ATC TCC TCC AGC TCC TCC ACT TTA TGG ATC ATC ATC
141     L   L   C   G   P   P   L   E   I   S   S   S   S   S   T   L   W   I   I   I
481    ACC TTC ACC GTG CTG CTG GCT GTG ATC CTG GGG CTC GTG CTG GTG TTC TGG AAG AGG TGC
161     T   F   T   V   L   L   A   V   I   L   G   L   V   L   V   F   W   K   R   C
541    TCC TCC AGA CAC CAC GGT GCA GGG GAT GAT GGA GAG CTG AGC TGG AAG CCC AGC GCC GTG
181     S   S   R   H   H   G   A   G   D   D   G   E   L   S   W   K   P   S   A   V
601    GTG AAC AGA CTG TTG CAG CGG CTG GGG ATT CAG GAC AAC AGA TGC AAT GAG CAG ATC TAC
201     V   N   R   L   L   Q   R   L   G   I   Q   D   N   R   C   N   E   Q   I   Y
661    CAG AAC CAG CAG CAG CAG GAG CTG CTT TTC ACA GCG CAG GGC TCA GAG GTT CCC CAT GGT
221     Q   N   Q   Q   Q   Q   E   L   L   F   T   A   Q   G   S   E   V   P   H   G
721    GTG GAG ATG GAG GGG ACG GAA CGA AGA ACC CCA GAT CCC AAA GTG GAA ACC CAG AGG AAG
241     V   E   M   E   G   T   E   R   R   T   P   D   P   K   V   E   T   Q   R   K
781    CTG GTT CCA GTG CTA GGA GAG AAC CCC ATA GCC CTT TTG CAT CGC TCT TTC AAC ACC TTT
261     L   V   P   V   L   G   E   N   P   I   A   L   L   H   R   S   F   N   T   F
841    GTC GAC TAT GTG CCC TTC CCG GAA TGG AAG AGA TTT GGC CGA GCC CTC GAC CTG CAG GAA
281     V   D   Y   V   P   F   P   E   W   K   R   F   G   R   A   L   D   L   Q   E
901    AAC GAC CTT TAT CTG GCA GAG CAG CAC GAC AGG GTC TCA TGT GAG CCG TTC TAT CAG ATG
301     N   D   L   Y   L   A   E   Q   H   D   R   V   S   C   E   P   F   Y   Q   M
961    CTC AAC ACG TGG CTC AAC CAA CAG GGC AGC AAA GCC TCT GTG AAT ACG CTG CTG GAG ACC
321     L   N   T   W   L   N   Q   Q   G   S   K   A   S   V   N   T   L   L   E   T
1021   CTG CCC CGC ATC GGC CTC AGC GGC GTG GCA GAC ATA ATT GCA TCC GAA CTC ATT AGC AAG
341     L   P   R   I   G   L   S   G   V   A   D   I   I   A   S   E   L   I   S   K
1081   GGC TAT TTC CAG TAC GAG GTG AGC TGA
361     G   Y   F   Q   Y   E   V   S   *
```

B

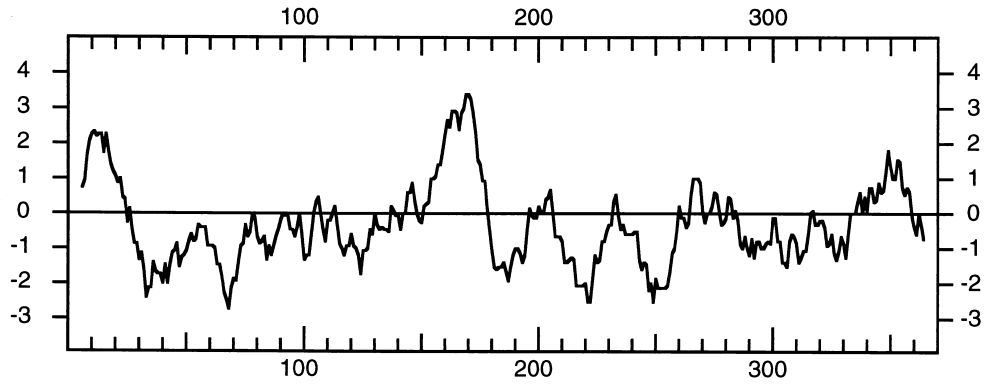

Figure 2. The 7.6-2 cDNA Clone Encodes a Type I Membrane Protein

(A) The long open reading frame contained within the 7.6-2 cDNA clone. The putative transmembrane domain is underlined, and two potential N-linked glycosylation sites (N-X-S/T) are boxed. The predicted leader peptidase cleavage site (von Heijne, 1986) is marked by an arrow.
(B) A hydropathy plot of the protein created using the algorithm of Kyte and Doolittle (1982).

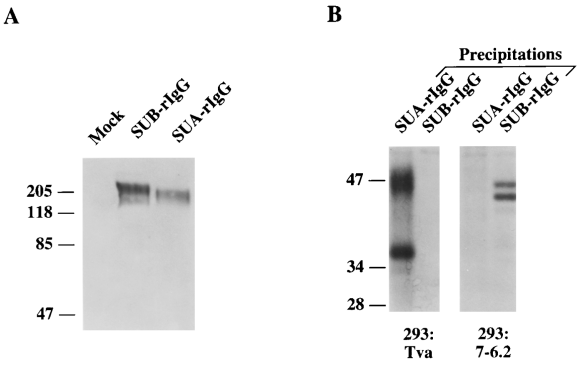

Figure 3. The Cloned Factor Bound Specifically to an ALV-B SU–Ig Fusion Protein

(A) Extracellular supernatants containing no immunoadhesin (mock), SUA–rIgG, or SUB–rIgG were subjected to electrophoresis under nonreducing conditions on a 7.5% polyacrylamide gel containing SDS and were immunoblotted using a horseradish peroxidase–coupled antibody specific for rabbit immunoglobulins.

(B) ^{35}S-labeled proteins from lysates of human 293 cells that had been transfected either with an expression vector encoding Tva (Zingler et al., 1995) or with the 7.6-2 cDNA clone were precipitated by the SU-immunoadhesins and protein A–Sepharose and analyzed by SDS–polyacrylamide gel electrophoresis. The sizes of the molecular mass markers are given in kDa.

killing (Tartaglia et al., 1993) appear to be absolutely conserved in this region of CAR1 (Figure 4B). Therefore, the cytoplasmic domain of CAR1 contains a region with many of the hallmarks of a functional death domain.

CAR1 Induces Apoptosis in Avian Cells

TNFR1 and Fas are known to signal apoptosis following receptor cross-linking upon binding to their cognate trimeric ligands or to receptor-specific antibodies (reviewed by Nagata and Golstein, 1995). Therefore, we decided to test whether the dimeric SUB–rIgG fusion protein that is able to bind CAR1 could elicit apoptosis. First, we determined if incubation with SUB–rIgG affected the viability of avian cells that expressed subgroup B viral receptors. CEFs (homozygous for the *tv-b*^{s2a} allele) that express subgroup B viral receptors and chicken cells (homozygous for the *tv-b*^{r} allele) that presumably do not express these receptors were used for these studies. These cells were incubated with increasing amounts of SUB–rIgG in the presence of a protein biosynthesis inhibitor, cycloheximide, which is routinely included in apoptosis assays (e.g., Laster et al., 1988). Following a 6 day incubation period with SUB–rIgG, the cell population that expressed subgroup B viral receptors showed a marked reduction in the numbers of adherent cells (Figure 5A). The magnitude of this effect was dependent upon the concentration of SUB–rIgG added (Figure 5A) and required cycloheximide. In contrast, the SUB–rIgG protein did not affect the numbers of adherent cells observed with the cell population not expressing functional subgroup B viral receptors (Figure 5A).

The nonadherent cells that resulted from incubations with SUB–rIgG and cycloheximide contained nucleosomal genomic DNA ladders, indicating that they had undergone apoptosis (data not shown). To obtain further evidence that SUB–rIgG induced apoptosis in a manner that was dependent on expression of the subgroup B viral receptor, this immunoadhesin was incubated with CEFs (homozygous for the *tv-b*^{s3} allele) that were either uninfected or chronically infected by the subgroup B virus RCASH-B. Cells chronically infected by this virus have presumably survived any virus-induced cell death (Weller et al., 1980; Weller and Temin, 1981) and are resistant to viral superinfection owing to functional down-regulation of the subgroup B viral receptor (Weiss, 1993). For control purposes, these experiments were also performed with CEFs chronically infected by the subgroup A virus RCASH-A and with the SUA–rIgG protein, which was not expected to induce apoptosis. To quantitate the degree of apoptosis induced in each cell population, we used an ELISA–based assay to measure the level of cytoplasmic nucleosomal DNA fragments. The uninfected cells and the subgroup A virus–infected cells showed significantly increased apoptosis in the presence of SUB–rIgG and cycloheximide, compared with incubation with SUA–rIgG and cycloheximide (Figure 5B). However, cells chronically infected with the subgroup B virus did not die preferentially when incubated with SUB–rIgG, presumably because subgroup B viral receptors had been functionally down-regulated (Weiss, 1993). These data provided further evidence that the subgroup B viral receptor was important for inducing cell death.

To obtain direct evidence that the subgroup B viral receptor CAR1 can induce apoptosis, a cloned line of transfected quail QT6 cells stably expressing this factor was incubated with SUB–rIgG protein that had been purified to 80%–90% homogeneity using a protein A column (Zingler and Young, 1996). CAR1 expression was confirmed in these cells by immunoblot analysis (data not shown). As expected, these cells were infected by RCASH-B in contrast to wild-type QT6 cells (Figure 5C), which are normally resistant to subgroup B viral infection (Weiss, 1993). Only the QT6 cells expressing CAR1 were induced to undergo apoptosis in response to SUB–rIgG (Figure 5D). Similar results were obtained with a second independent clone of QT6 cells that expressed CAR1 (data not shown). These results demonstrate that SUB–rIgG/CAR1 interactions can lead to the death of avian cells.

Discussion

A gene was cloned encoding a TNFR–related protein, CAR1, which permitted specific infection of mammalian cells by ALV-B and ALV-D. This protein bound selectively to a subgroup B ALV SU–Ig fusion protein (SUB–rIgG). Taken together, these observations indicate that CAR1 most likely mediates viral entry by serving as a specific receptor for viral subgroups B and D. CAR1 is apparently not related to any other known retroviral receptor; these include Tva, a receptor for another avian viral subgroup (ALV-A), which is a member of the low density lipoprotein receptor family; CD4, a member of the immunoglobulin protein superfamily; and transporter proteins that contain multiple membrane-spanning regions (Weiss and Tailor, 1995).

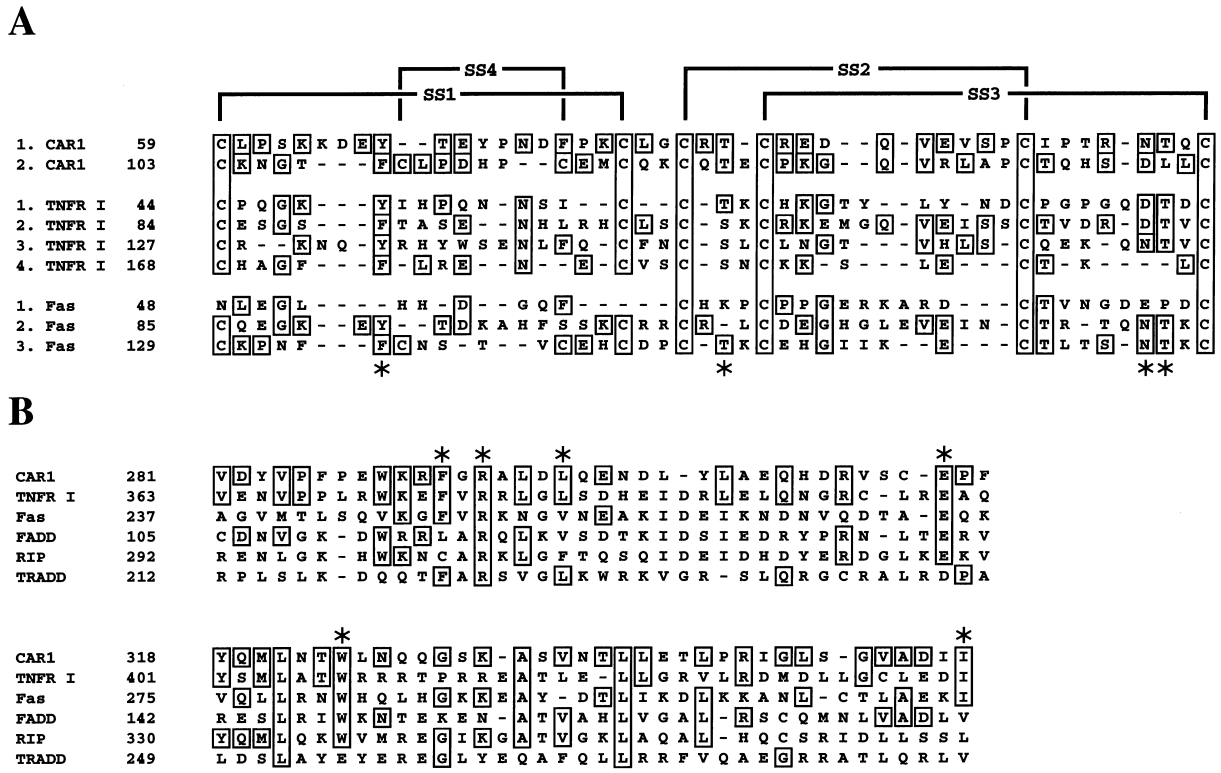

Figure 4. CAR1 Is a Member of the TNFR Superfamily

(A) Alignment of the extracellular CRDs of CAR1 with the corresponding regions of the human TNFR1 (Loetscher et al., 1990; Schall et al., 1990) and human Fas (Itoh et al., 1991). Boxed amino acids indicate amino acids identical to CAR1. A set of three disulfide bonds in TNFR1 (Banner et al., 1993) is indicated as SS1–SS3. SS4 represents a putative disulfide bond found in CRDs of some TNFR–related proteins including the second CRD of CAR1. Asterisks indicate amino acid residues which, in addition to the cysteines, stabilize the conformation of TNFR1 CRDs.

(B) CAR1 contains a putative death domain. Alignment of a cytoplasmic region of CAR1 with the death domains of other proteins. Boxed amino acids indicate amino acids identical to CAR1, and asterisks indicate residues of TNFR1 that are essential for cell killing (Tartaglia et al., 1993).

CAR1 contains two extracellular CRDs similar to those found in the TNFR family. Based on the known structure of the four domains of TNFR1 (Banner et al., 1993), the six cysteines in the first CRD of CAR1 are predicted to form three intradomain disulfide bonds (Figure 4A). The eight cysteines in the second CRD of CAR1 are predicted to have the same pattern of disulfide bonds with an additional putative bond formed between Cys-109 and Cys-115 (Figure 4A). It is intriguing that the ALV-A interaction site of Tva is contained within a low density lipoprotein receptor–related motif that is similar in size to TNFR–like CRDs and is predicted to contain three intradomain disulfide bonds (Bélanger et al., 1995; Daly et al., 1995; Zingler et al., 1995). This similarity suggests that viral interaction sites of Tva and CAR1 might have common features.

The CRDs of TNFR1 are known to adopt similar core structures (Banner et al., 1993), and residues important for the stability of this structure have been identified. These include highly conserved Asp/Asn–Thr residues (located before the last cysteine residue), which are hydrogen-bonded to a conserved serine or threonine residue located after the third cysteine (Figure 4A). Also, a conserved aromatic residue (Tyr or Phe) located approximately five residues after the first cysteine provides further stability by interacting both with the second disulfide bridge and the Asp/Asn:Ser hydrogen bond bridge (Banner et al., 1993). A number of these important amino acid residues are also found in CAR1 (Tyr-67, Thr-82, Asn-98, Thr-99, Phe-108, Thr-123, and Asp-140), suggesting that both CRDs of the ALV receptor are structurally related to those of TNFR1 (Figure 4A).

The presence of a cytoplasmic region in CAR1 similar to the death domain of Fas and TNFR1 (Figure 4B) led to experiments demonstrating that this ALV receptor could induce apoptosis in avian cells. CEFs that expressed subgroup B viral receptors underwent apoptosis in response to the ALV-B SU immunoadhesin, whereas cells that lacked these receptors did not. In addition, preinfection of CEFs by ALV-B led to protection against apoptosis induced by this immunoadhesin. Presumably, this protection is due to subgroup B viral receptor down-regulation in these cells (Weiss, 1993), although subgroup B virus infection might have selected for a cell population resistant to SUB–rIg-induced apoptosis. Furthermore, the subgroup B SU immunoadhesin caused apoptosis in quail QT6 cells that stably expressed CAR1, but not in wild-type QT6 cells. These data demonstrate that cytopathic ALV Env–receptor interactions can lead to cell death and might

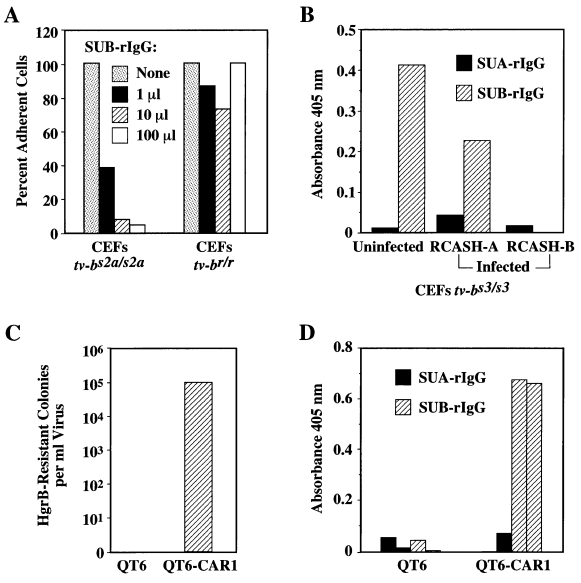

Figure 5. CAR1-Mediated Apoptosis

(A) CEFs susceptible to ALV-B infection are preferentially killed in response to SUB–rIgG. A representative experiment in which approximately 2×10^5 CEFs that either expressed (*tv-b*$^{s2a/s2a}$) or lacked (*tv-b*$^{r/r}$) functional subgroup B viral receptors were incubated with increasing amounts (1, 10, and 100 μl) of extracellular supernatants containing SUB–rIgG (Figure 3A) in the presence of 7.5 μg/ml of cycloheximide. The numbers of adherent cells were counted after 6 days and corrected relative to the numbers of cells remaining after incubation with cycloheximide alone.

(B) CEFs chronically infected by ALV-B are protected from SUB–rIgG-induced apoptosis. A representative experiment in which extracellular supernatants containing SUA–rIgG or SUB–rIgG (Figure 3A) were incubated with 2×10^4 CEFs that express subgroup B viral receptors (*tv-b*$^{s3/s3}$) and that were either uninfected or chronically infected with the subgroup A virus RCASH-A or with the subgroup B virus RCASH-B. These experiments were performed for 2 days in the presence of 10 μg/ml of cycloheximide. Cells were assayed for evidence of apoptosis using an ELISA–based assay that measures photometrically the amount of cytoplasmic histone-associated DNA fragments. The apparent reduction in the level of SUB–rIgG-induced apoptosis obtained with RCASH-A–infected cells probably reflects their observed reduced plating efficiency in these experiments.

(C) A cloned line of QT6 cells stably expressing CAR1 was susceptible to infection by the RCASH-B virus. The number of hygromycin B-resistant colonies that resulted from viral infection is shown.

(D) Quail QT6 cells that expressed CAR1 underwent apoptosis in response to SUB–rIgG. Approximately 2×10^4 QT6 cells or QT6 cells expressing CAR1 were incubated with 16 ng of either purified SUB–rIgG or purified SUA–rIgG in the presence of 2.5 μg/ml of cycloheximide. The apoptosis assays were performed as described in (B) above, and the results were from two independent experiments.

explain why cells killed by these viruses contain "ladders" of degraded genomic DNA fragments (Weller and Temin, 1981) that presumably result from apoptosis (e.g., Itoh et al., 1991).

It seems likely that CAR1 triggers cell death in a manner similar to Fas and TNFR1; cross-linking of these receptors by their trimeric ligands leads to aggregation of their death domains, resulting in apoptosis (Itoh and Nagata, 1993; Nagata and Golstein, 1995; Smith et al., 1994; Tartaglia et al., 1993; Watanabe-Fukunaga et al., 1992). Cytoplasmic proteins have been identified that bind directly to the wild-type death domain of either Fas or TNFR1 and induce or potentiate apoptosis. These include TRADD (Hsu et al., 1995), FADD/MORT1 (Chinnaiyan et al., 1995; Boldin et al., 1995), and RIP (Stanger et al., 1995). It seems reasonable to expect that similar types of proteins interact with the death domain of CAR1 and may be involved in signaling apoptosis by this cellular receptor.

The fact that CAR1 permits specific infection of mammalian cells by ALV subgroups B and D but not E indicates that it is most likely the product of *tv-b*^{s3}, the chicken locus predicted to encode cellular receptors for these two ALV subgroups (Weiss, 1993). If future gene-mapping studies confirm this locus designation, it will be important to isolate and characterize any products of tv-b^r (which do not allow infection by cytopathic subgroup B and D viruses; Weiss, 1993). Any differences between these proteins and CAR1 might help identify the viral interaction determinants of the receptor. Comparison of *tv-b* alleles that encode receptors for noncytopathic subgroup E viruses (Weiss, 1993) with CAR1 might provide crucial insights into the mechanisms of cell killing associated with ALV-B and ALV-D.

Viruses have evolved various strategies to evade host-cell killing by encoding inhibitors of apoptosis (reviewed by Thompson, 1995). These include the Bcl-2 homologs BHRF1 and LMW5-HL of Epstein-Barr virus and African swine fever virus, respectively (Neilan et al., 1993; Henderson et al., 1993); the cowpox virus crmA protein, which is a specific inhibitor of IL-1β–converting enzyme (Ray et al., 1992); and the myxoma virus T2 protein (a soluble TNFR–like protein), which can protect cells from lysis by TNF (Schreiber and McFadden, 1994). Given that other viruses have devised strategies to evade apoptosis, it seems counterintuitive that cytopathic subgroups of ALV would utilize a cellular receptor that could induce apoptosis in the host cell. A hypothesis that might explain this apparent paradox is that functional down-regulation of the subgroup B viral receptor that follows ALV-B infection (Weiss, 1993) might provide a selective advantage for the virus. For example, given that Fas is important for the elimination of virus-infected cells by cytotoxic T lymphocytes (Lowin et al., 1994; reviewed by Nagata and Golstein, 1995) and that TNFR1 has been shown to be necessary for clearing Listeria monocytogenes infection (Pfeffer et al., 1993), CAR1 might also be involved in immune clearance of virus-infected cells. If so, functional down-regulation of CAR1 following infection by subgroup B or D ALV might protect the host cell from apoptosis by this pathway. Consistent with this hypothesis, cells infected by ALV-B were protected from apoptosis induced by SUB–rIgG (Figure 5B). This would be a novel mechanism for evading apoptotic elimination of infected cells.

In conclusion, our results indicate that subgroups B and D ALV Env–CAR1 interactions may contribute to the virus-associated CPE by inducing apoptosis. This model would explain why the determinants of subgroup B viral receptor usage on ALV-B SU Env proteins appear to be the same as those responsible for the cytopathic effect (Dorner and Coffin, 1986). Presumably, cell-surface expression of functional subgroup B viral receptors is important for the viral CPE as well as for the associated

viral superinfection. This would explain why chronically infected cells, which have functionally down-regulated subgroup B viral receptors, are resistant both to viral superinfection and to cell killing (Weller et al., 1980; Weller and Temin, 1981). It is possible that viral superinfection contributes to ALV–induced cell killing (Weller et al., 1980; Weller and Temin, 1981), although multiple rounds of viral infection may simply be a consequence of cell death. Env–receptor interactions have also been implicated in HIV-1–induced CPE (Lu et al., 1994; Cao et al., 1996; Banda et al., 1992), although the exact details of the mechanisms of cell killing are probably different because of the absence of a death domain in the viral binding receptor, CD4. It remains to be seen whether other proteins containing death domains are used as cellular receptors by different cytopathic retroviruses, including ALV-F (Weller et al., 1980; Weller and Temin, 1981), spleen necrosis virus (Keshet and Temin, 1979), and subgroup C feline leukemia viruses (Riedel, 1988).

Experimental Procedures

Cells and Viruses

Mouse NIH 3T3 cells, Quail QT6 cells, Monkey COS-7 cells, Human 293 cells, and primary CEFs were grown and selected as previously described (Young et al., 1993; Bates et al., 1993; Connolly et al., 1994; Zingler and Young, 1996). Chicken fibroblasts were obtained from the Avian Disease and Oncology Laboratory, USDA Poultry Laboratories (East Lansing, Michigan). Cells homozygous for *tv-bs*^{3} were derived from line 0 chickens, those homozygous for *tv-b*^{r} were derived from a cross between line 100B and line 7 chickens, and those homozygous for *tv-b*^{s2a} were derived from line 15B1 chickens. The subgroup B–specific RCASH-B virus containing the hygromycin B phosphotransferase gene driven by the HSV thymidine kinase (tk) promoter was described previously (Young et al., 1993). The subgroup E–specific RCASH-E virus was derived from RCASH-B by replacing a 1.1 kb KpnI–SalI *env* fragment with that from the RAV-0 viral strain. The subgroup A–specific RCASA-neo, subgroup B–specific RCASB-Neo, and subgroup D–specific RCASD-neo viruses were generated from previously described proviral DNAs (Connolly et al., 1994) by digestion with ClaI followed by end-filling with Klenow polymerase to generate blunt-ended cloning sites. An end-filled 1.2 kb XhoI HincII fragment containing the HSV-tk promoter and the neomycin phosphotransferase gene from plasmid pMCI.neo PA− (gift from M. Feinberg) was introduced into the blunt-ended cloning site of each viral vector. The subgroup C–specific RCASC-neo virus was derived by first subcloning a similar XhoI–HindIII fragment from plasmid pMCI.neo PA− into plasmid SACla12Nco (Hughes et al., 1987). A ClaI–ClaI fragment containing these DNA sequences was then used to replace the hygromycin B phosphotransferase gene of the RCASH-C virus (Connolly et al., 1994).

DNA Transfections, Radioactively Labeled Probes, and Nucleic Acid Hybridizations

All DNA transfections were performed by the calcium phosphate precipitation method (Wigler et al., 1977). The radioactively labeled probes were prepared by the random priming method (Feinberg and Vogelstein, 1984). DNA and RNA samples that were transferred to a Genescreen plus (DuPont) membrane were hybridized with probes at 65°C using standard conditions (Church and Gilbert, 1984) unless otherwise indicated. The membranes were exposed for autoradiography to Kodak XAR-5 film with intensifying screens at −80°C.

Genomic DNA Transfections and Infection by Subgroup B ALV Vectors

A total of 6×10^6 mouse BALB/3T3 cells were cotransfected with 120 μg of genomic DNA (sheared to an average size of approximately 20 kb) prepared from CEFs homozygous for *tv-b*^{s3} and with 12 μg of pMPHis plasmid DNA encoding histidinol dehydrogenase (Young et al., 1993). After 48 hr, the cells were selected in histidine-free medium containing 1 mM histidinol. Cells derived from these colonies were challenged over a 3 day period with a total of 6×10^6 infectious units of RCASH-B virus and 1 day later were selected in medium containing 300 μg/ml of hygromycin B. Cells derived from the resultant hygromycin B–resistant colonies were plated out at 20% confluency in 6-well plates, challenged with 10^4 infectious units of RCASB-neo virus, and 1 day later were selected in medium containing 300 μg/ml of G418.

A total of 1.4×10^7 mouse BALB/3T3 cells were transfected with 200 μg of genomic DNA (from an aliquot of primary transfectant 11B cells that were infected only by RCASH-B) and with 20 μg of plasmid pPur encoding puromycin-N-acetyl-transferase (Clontech). After 2 days, secondary transfectants were selected in media containing 2 μg/ml of puromycin. Cells derived from the puromycin-resistant colonies were challenged with a total of 2×10^7 infectious units of the RCASB-neo virus and 1 day later were selected in medium containing 300 μg/ml of G418. Cells derived from the resultant G418-resistant colonies were plated at 20% confluency in 6-well plates, challenged with 5×10^5 infectious units of subgroup B–specific RCASH-B virus, and 1 day later were selected in medium containing 300 μg/ml of hygromycin B.

Genomic DNA Clones

HindIII–HindIII restriction fragments approximately 6.5–8 kb in size and KpnI–KpnI restriction fragments approximately 8–10 kb in size (derived from C12 secondary transfectant genomic DNA) were size-selected by agarose gel electrophoresis and introduced into the λZAP vector (Stratagene), generating libraries of approximately 300,000 and 150,000 recombinant clones, respectively. The library of HindIII fragments was subjected to three rounds of screening using a 2 kb PvuII–PvuII fragment from the pMPHIS plasmid as a hybridization probe. Following a standard phagemid excision protocol (Stratagene), the BK-1c clone was isolated. The library of KpnI fragments was subjected to three rounds of screening by hybridization with the 1.9 kb EcoRI–HindIII and 1.5 kb EcoRI–EcoRI fragments from clone BK-1c. This led to the isolation of the genomic DNA clone BK-9.

Northern Blot Analysis and cDNA Cloning

Samples of approximately 10 μg of total RNA prepared from CEFs homozygous for *tv-b*^{s3} were subjected to 1% agarose gel electrophoresis in the presence of formaldehyde (Ausubel et al., 1992), transferred to a nylon membrane, and subjected to hybridization with radioactively labeled probes.

Approximately 5 μg of polyadenylated mRNA from these CEFs was reverse-transcribed to generate cDNA using a commercially available kit (ZAP Express cDNA synthesis kit; Stratagene) and introduced into the λZAP Express vector (Stratagene). Approximately 225,000 recombinant clones were transferred to nylon membranes and subjected to three rounds of screening by hybridization with the radioactively labeled 2.5 kb, 1.9 kb, and 1.5 kb probes derived from the BK-9 and BK-1c genomic clones. Following hybridization, the membranes were washed at 56°C with 2 × SSC prior to autoradiography. Using a standard phagemid excision protocol (Stratagene), plasmid pBK7.6-2 was isolated, and the cDNA clone was subsequently sequenced by the chain termination method (Sanger et al., 1977).

Construction, Purification, and Analysis of SU-Immunoadhesins

The SUB–rIgG protein comprised amino acids 1–344 of the subgroup B SU protein derived from the RCASH-B virus (Connolly et al., 1994) fused in-frame to the Fc region (amino acids 96–323) of a rabbit IgG heavy chain (SwissProt accession number P01870). The SUA–rIgG protein comprised amino acids 1–338 of the subgroup A ALV SU protein derived from the RCASH-A virus (Young et al., 1993) fused to the same immunoglobulin sequences. The corresponding DNA sequences were fused together following the introduction of in-frame BamHI sites in each construct by standard polymerase chain reaction–based mutagenesis protocols (Ausubel et al., 1992). The resultant *SUA–rIgG* and *SUB–rIgG* genes were introduced into the

pCB6 mammalian expression vector (Bates et al., 1993). These proteins were released in the extracellular supernatants of transiently transfected human 293 cells and purified as described elsewhere (Zingler and Young, 1996).

Samples of 25 μl transfected cell supernatants were subjected to 7.5% SDS–polyacrylamide gel electrophoresis under nonreducing conditions, and the SU-immunoadhesins were detected by immunoblotting with a horseradish peroxidase–coupled donkey antibody specific for rabbit immunoglobulins and by enhanced chemiluminescence (Amersham) as described elsewhere (Zingler and Young, 1996). The SU-immunoadhesins were purified using a protein A column as described elsewhere (Zingler and Young, 1996).

Tva and CAR1 Binding to Immunoadhesins

Human 293 cells plated at approximately 20% confluence on 100 mm tissue culture plates were transfected with 15 μg of a plasmid encoding Tva (Zingler et al., 1995), 15 μg of plasmid pBK7.6-2 encoding CAR1, or no DNA. The transfected cells were metabolically labeled for 2 hr with Dulbecco's modified Eagle's medium without cysteine and methionine containing 1% dialyzed fetal calf serum and 100 μCi/ml ^{35}S-cysteine (ICN Chemicals). Cell lysates were prepared in a lysis buffer containing NP-40 as described previously (Bélanger et al., 1995). Aliquots (one-tenth) of each sample were then incubated at 4°C for 45 min with 10 μl of protein A–Sepharose beads (Sigma) and 30 μl of Sepharose CL-4B (prebound to 1 ml of extracellular supernatants containing SUA–rIgG or SUB–rIgG for 45 min). The bound proteins were collected by centrifugation, and aliquots of these samples were subjected to electrophoresis on a 10% SDS–polyacrylamide gel under reducing conditions. The gel was dried and exposed to Kodak XAR-5 film at room temperature.

Infection of Transfected COS-7 Cells with Different Subgroups of ALV

COS-7 cells were transfected with 20 μg of 7.6-2 plasmid pBK7.6-2 Amp (derived from pBK7.6-2 by replacement of the neomycin phosphotransferase gene with the ampicillin resistance gene of pBluescript KS− [Stratagene]). Transfected cells were challenged with serial 10-fold dilutions of the virus stocks. The titers of the undiluted stocks of RCASA-neo, RCASB-neo, RCASC-neo, and RCASD-neo viruses were approximately 10^5 infectious units/ml as determined by limiting dilution infection of CEFs. The titer of the undiluted stock of RCASH-E virus was approximately 10^3 infectious units/ml as determined by limiting dilution infection of turkey embryo fibroblasts. Between 24–36 hr after infection, the cells were selected in media containing 300 μg/ml of either hygromycin B or G418. Drug-resistant colonies were counted 11–14 days after infection and the numbers corrected per ml of undiluted virus.

Cell-Killing Assays

CEFs chronically infected with either RCASH-A or RCASH-B were generated by infecting 5×10^5 cells with approximately 10^5 infectious units of virus. Following two cell passages to allow viral spread, infected cells were selected in medium containing 80 μg/ml of hygromycin B. Cell-killing experiments were performed with uninfected and infected CEFs derived from the same embryo that had been passaged in culture for the same period of time (13 days). QT6 cells expressing CAR1 were generated by transfection with 15 μg of plasmid pBK7.6-2 followed by selection in medium containing 300 μg/ml of G418. Individual clones were tested for CAR1 expression by immunoblot analysis that employed SUB–rIgG and a horseradish peroxidase–coupled antibody specific for rabbit immunoglobulins. The cell-killing experiments were performed using the Cell Death Detection Plus™ ELISA kit (Boehringer Mannheim), in which cytoplasmic nucleosomes are purified by a histone-specific antibody and detected photometrically.

Acknowledgments

Correspondence should be addressed to J. B. We would like to thank Lyman Crittenden and colleagues at the USDA Poultry Laboratories for providing chicken cells, and we also thank our colleagues Heather Adkins, Vincent Solomon, Michael Starnbach, Cindy Murphy, and Louise Bergeron for stimulating discussions and critical reading of the manuscript. This work was supported by National Institutes of Health grants CA62000 and CA70810, by a grant from the Milton Fund at Harvard Medical School, and by funds provided by the Gladstone Institute of Virology and Immunology. J. B. is an Ernst Fellow at Harvard Medical School. M. M. R. is a predoctoral fellow of the Howard Hughes Medical Institute.

Received August 8, 1996; revised October 7, 1996.

References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds. (1992). Short Protocols in Molecular Biology (New York: Greene Publishing Associates; John Wiley and Sons).

Banda, N.K., Bernier, J., Kurahara, D.K., Kurrie, R., Haigwood, N., Sekaly, R.-P., and Finkel, T.H. (1992). Cross-linking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J. Exp. Med. *176*, 1099–1106.

Banner, D.W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H.J., Broger, C., Loetscher, H., and Lesslauer, W. (1993). Crystal structure of the soluble human 55 kd TNF receptor–human TNF β complex: implications for TNF receptor activation. Cell *73*, 431–445.

Bates, P., Young, J.A.T., and Varmus, H.E. (1993). A receptor for subgroup A Rous sarcoma virus is related to the LDL receptor. Cell *74*, 1043–1051.

Bélanger, C., Zingler, K., and Young, J.A.T. (1995). Importance of cysteines in the LDLR–related domain of the subgroup A avian leukosis and sarcoma virus receptor for viral entry. J. Virol. *69*, 1019–1024.

Bergeron, L., and Sodroski, J. (1992). Dissociation of unintegrated viral DNA accumulation from single-cell lysis induced by human immunodeficiency virus type 1. J. Virol. *66*, 5777–5787.

Beutler, B., and Van Huffel, C. (1994). An evolutionary and functional approach to the TNF receptor/ligand family. Ann. NY Acad. Sci. *730*, 118–133.

Boldin, M.P., Varfolomeev, E.E., Pancer, Z., Mett, I.L., Camonis, J.H., and Wallach, D. (1995). A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. J. Biol. Chem. *270*, 7795–7798.

Bova, C.A., Olsen, J.C., and Swanstrom, R. (1988). The avian retrovirus Env gene family: molecular analysis of host range and antigenic variants. J. Virol. *62*, 75–83.

Cao, J., Park, I.W., Cooper, A., and Sodroski, J. (1996). Molecular determinants of acute single-cell lysis by human immunodeficiency virus type 1. J. Virol. *70*, 1340–1354.

Capon, D.J., Chamow, S.M., Mordenti, J., Marsters, S.A., Gregory, T., Mitsuya, H., Byrn, R.A., Lucas, C., Wurm, F.M., Groopman, J.E., and others. (1989). Designing CD4 immunoadhesins for AIDS therapy. Nature *337*, 525–531.

Chapman, B.S. (1995). A region of the 75 kDa neurotrophin receptor homologous to the death domains of TNFR-I and Fas. FEBS Lett. *374*, 216–220.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M. (1995). FADD, a novel death domain–containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505–512.

Connolly, L., Zingler, Z., and Young, J.A.T. (1994). A soluble form of a receptor for subgroup A avian leukosis and sarcoma viruses (ALV-A) blocks infection and binds directly to ALV-A. J. Virol. *68*, 2760–2764.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA *81*, 1991–1995.

Cleveland, J.L., and Ihle, J.N. (1995). Contenders in FasL/TNF death signaling. Cell *81*, 479–482.

Daly, N.L., Scanlon, M.J., Djordjevic, J.T., Kroon, P.A., and Smith, R. (1995). Three-dimensional structure of a cysteine-rich repeat from the low-density lipoprotein receptor. Proc. Natl. Acad. Sci. USA *92*, 6334–6338.

Donahue, P.R., Quackenbush, S.L., Gallo, M.V., deNoronha, C.M., Overbaugh, J., Hoover, E.A., and Mullins, J.I. (1991). Viral genetic

determinants of T-cell killing and immunodeficiency disease induction by the feline leukemia virus FeLV-FAIDS. J. Virol. *65*, 4461–4469.

Dorner, A.J., and Coffin, J.M. (1986). Determinants for receptor interaction and cell killing on the avian retrovirus glycoprotein gp85. Cell *45*, 365–374.

Feinberg, A.P., and Vogelstein, B. (1984). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *137*, 266–267.

Haak-Frendscho, M., Marsters, S.A., Chamow, S.M., Peers, D.H., Simpson, N.J., and Ashkenazi, A. (1993). Inhibition of interferon-γ by an interferon-γ receptor immunoadhesin. Immunology *79*, 594–599.

Henderson, S., Huen, D., Rowe, M., Dawson, C., Johnson, G., and Rickinson, A. (1993). Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. Proc. Natl. Acad. Sci. USA *90*, 8479–8483.

Hughes, S.H., Greenhouse, J.J., Petropoulos, C.J., and Sultrave, P. (1987). Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. J. Virol. *61*, 3004–3012.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995). The TNF receptor 1–associated protein TRADD signals cell death and NF-κ B activation. Cell *81*, 495–504.

Itoh, N., and Nagata, S. (1993). A novel protein domain required for apoptosis: mutational analysis of human Fas antigen. J. Biol. Chem. *268*, 10932–10937.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S.-I., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell *66*, 233–243.

Keshet, E., and Temin, H.M. (1979). Cell killing by spleen necrosis virus is correlated with a transient accumulation of spleen necrosis virus DNA. J. Virol. *31*, 376–388.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. *157*, 105–132.

Laster, S.M., Wood, J.G., and Gooding, L.R. (1988). Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. J. Immunol. *141*, 2629–2634.

Laurent-Crawford, A.G., and Hovanessian, A.G. (1993). The cytopathic effect of human immunodeficiency virus is independent of high levels of unintegrated viral DNA accumulated in response to superinfection of cells. J. Gen. Virol. *74*, 2619–2628.

Loetscher, H., Pan, Y.-C.E., Lahm, H.-W., Gentz, R., Brockhaus, M., Tabuchi, H., and Lesslauer, W. (1990). Molecular cloning and expression of the human 55 kDa tumor necrosis factor receptor. Cell *61*, 351–359.

Lowin, B., Hahne, M., Mattmann, C., and Tschopp, J. (1994). Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature *370*, 650–652.

Lu, Y.Y., Koga, Y., Tanaka, K., Sasaki, M., Kimura, G., and Nomoto, K. (1994). Apoptosis induced in CD4+ cells expressing gp160 of human immunodeficiency virus type 1. J. Virol. *68*, 390–399.

Nagata, S., and Golstein, P. (1995). The Fas death factor. Science *267*, 1449–1456.

Neilan, J.G., Lu, Z., Afonso, C.L., Kutish, G.F., Sussman, M.D., and Rock, D.L. (1993). An African swine fever virus gene with similarity to the proto-oncogene bcl-2 and the Epstein-Barr virus gene BHRF 1. J. Virol *67*, 4391–4394.

Paquette, Y., Hanna, Z., Savard, P., Brousseau, R., Robitaille, Y., and Jolicoeur, P. (1989). Retrovirus-induced murine motor neuron disease: mapping the determinant of spongiform degeneration within the Envelope gene. Proc. Natl. Acad. Sci. USA *86*, 3896–3900.

Pauza, C.D., Galindo, J.E., and Richman, D.D. (1990). Reinfection results in accumulation of unintegrated viral DNA in cytopathic and persistent human immunodeficiency virus type 1 infection of CEM cells. J. Exp. Med. *172*, 1035–1042.

Pfeffer, K., Matsuyama, T., Kundig, T.M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P.S., Kronke, M., and Mak, T.W. (1993). Mice deficient for the 55 kDa tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell *73*, 457–467.

Pitti, R.M., Marsters, S.A., Haak-Frendscho, M., Osaka, G.C., Mordenti, J., Chamow, S.M., and Ashkenazi, A. (1994). Molecular and biological properties of an interleukin-1 receptor immunoadhesin. Mol. Immunol. *31*, 1345–1351.

Ray, C.A., Black, R.A., Kronheim, S.R., Greenstreet, T.A., Sleath, P.R., Salveson, G.S., and Pickup, D.J. (1992). Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1β–converting enzyme. Cell *69*, 597–604.

Resnick-Roguel, N., Burstein, H., Hamburger, J., Panet, A., Eldor, A., Vlodavsky, I., and Kotler, M. (1989). Cytocidal effect caused by the Envelope glycoprotein of a newly isolated avian hemangioma-inducing retrovirus. J. Virol. *63*, 4325–4330.

Riedel, N., Hoover, E.A., Dornsife, R.E., and Mullins, J.I. (1988). Pathogenic and host range determinants of the feline aplastic anemia retrovirus. Proc. Natl. Acad. Sci. USA *85*, 2758–2762.

Robinson, H.L., and Zinkus, D.M. (1990). Accumulation of human immunodeficiency virus type 1 DNA in T cells: results of multiple infection events. J. Virol. *64*, 4836–4841.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467.

Schall, T.J., Lewis, M., Koller, K.J., Lee, A., Rice, G.C., Wong, G.H.W., Gatanaga, T., Granger, G.A., Lentz, R., Raab, H., Kohr, W.J., and Goeddel, D.V. (1990). Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell *61*, 361–370.

Schreiber, M., and McFadden, G. (1994). The myxoma virus TNF–receptor homologue (T2) inhibits tumor necrosis factor-α in a species specific fashion. Virology *204*, 692–705.

Siliciano, R.F. (1996). The role of CD4 in HIV Envelope-mediated pathogenesis. Curr. Topics Microbiol. Immunol. *205*, 159–179.

Smith, C.A., Davis, T., Anderson, D., Solam, L., Beckman M.P., Jerzy, R., Dower, S.K., Cosman, D., and Goodwin, R.G. (1990). A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science *248*, 1019–1023.

Smith, C.A., Farrah, T., and Goodwin, R.G. (1994). The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell *76*, 959–962.

Somasundaran, M., and Robinson, H.L. (1987). A major mechanism of human immunodeficiency induced cell killing does not involve cell fusion. J. Virol. *61*, 3114–3119.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. *98*, 503–517.

Stamenkovic, I., Clark, E.A., and Seed, B. (1989). B-lymphocyte activation molecule related to the nerve growth factor and induced by cytokines in carcinomas. EMBO J. *8*, 1403–1410.

Stanger, B.Z., Leder, P., Lee, T.H., Kim, E., and Seed, B. (1995). RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell *81*, 513–523.

Stevenson, M., Meier, C., Mann, A.M., Chapman, N., and Wasiak, A. (1988). Envelope glycoprotein of HIV induces interference and cytolysis resistance in CD4+ cells: mechanism for persistence in AIDS. Cell *53*, 483–496.

Stumph, W.E., Kristo, P., Tsai, M.J., and O'Malley, B.W. (1981). A chicken middle-repetitive DNA sequence which shares homology with mammalian ubiquitous repeats. Nucl. Acids Res. *9*, 5383–5397.

Tartaglia, L.A., Ayres, T.M., Wong, G.H., and Goeddel, D.V. (1993). A novel domain within the 55 kDa TNF receptor signals cell death. Cell *74*, 845–853.

Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. Science *267*, 1456–1462.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. Nucl. Acids Res. *14*, 4683–4690.

Watanabe-Fukanaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A., and Nagata, S. (1992). Lymphoproliferation disorder in mice

explained by defects in Fas antigen that mediates apoptosis. Nature *356*, 314–317.

Weiss, R.A. (1993). Cellular receptors and viral glycoproteins involved in retrovirus entry. In The Retroviridae, Volume 2, J.A. Levy, ed. (New York: Plenum Press), pp. 1–107.

Weiss, R.A., and Tailor, C.S. (1995). Retrovirus receptors. Cell *82*, 531–533.

Weller, S.K., and Temin, H.M. (1981). Cell killing by avian leukosis viruses. J. Virol. *39*, 713–721.

Weller, S.K., Joy, A.E., and Temin, H.M. (1980). Correlation between cell killing and massive second-round superinfection by members of some subgroups of avian leukosis virus. J. Virol. *33*, 494–506.

Wigler, M., Silverstein, S., Lee, L.S., Pellicer, A., Cheng, Y.C., and Axel, R. (1977). Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell *11*, 223–232.

Young, J.A.T., Bates, P., and Varmus, H.E. (1993). Isolation of a chicken gene that confers susceptibility to infection by subgroup A avian leukosis and sarcoma viruses. J. Virol. *67*, 1811–1816.

Zingler, K., Belanger, C., Peters, R., Agard, D., and Young, J.A.T. (1995). Identification and characterization of the viral interaction determinants of the subgroup A avian leukosis virus receptor. J. Virol. *69*, 4261–4266.

Zingler, K., and Young, J.A.T. (1996). Residue Trp-48 of Tva is critical for viral entry but not for high affinity binding to the SU glycoprotein of subgroup A avian leukosis and sarcoma viruses. J. Virol. *70*, 7510–7516.