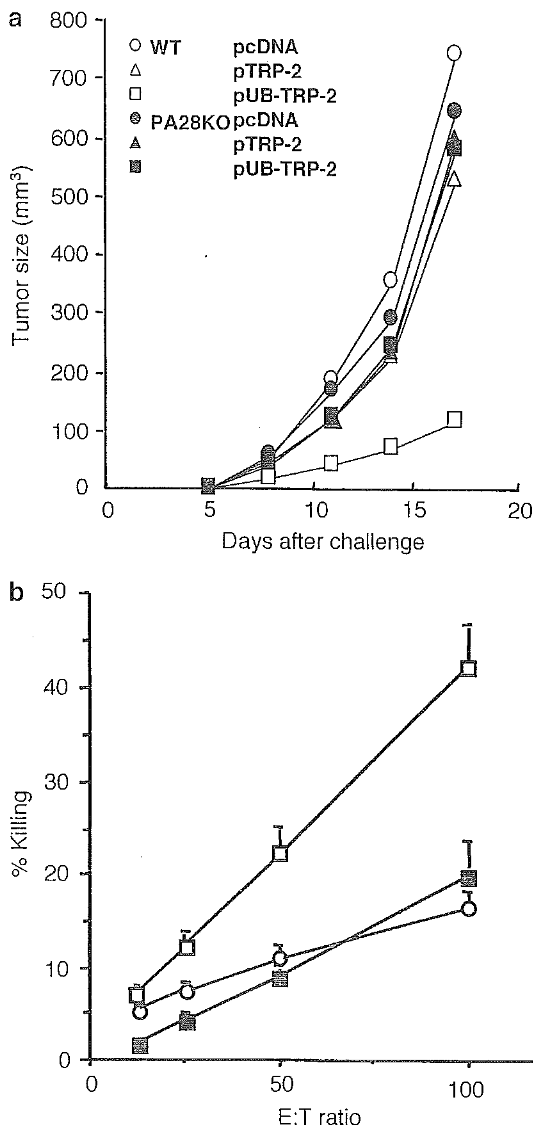

Figure 5 Impaired antitumor immunity by pUB-TRP-2 vaccination in PA28αβ−/− mice. C57BL/6 mice and PA28αβ−/− mice vaccinated with pTRP-2 or pUB-TRP-2 were challenged with B16F10 cells. Course of tumor growth was monitored (a) and CTL activity was analyzed (b). Data are mean of six mice in each group and s.d. are less than 15% of the mean. This experiment was repeated three times.

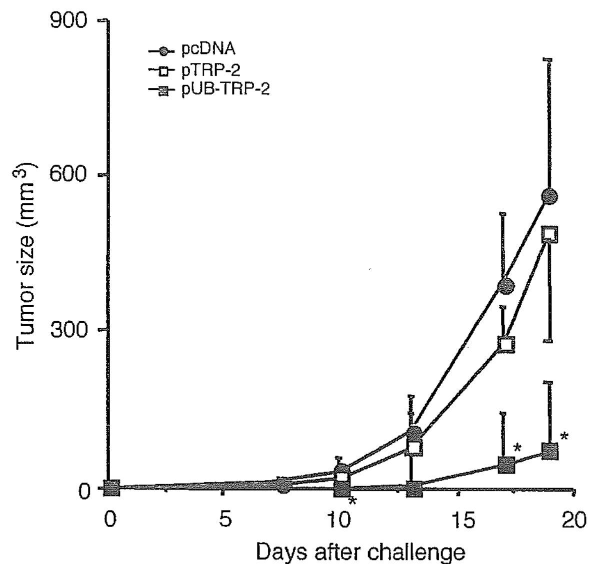

*Figure 6 Therapeutic effect of vaccination of pUB-TRP-2. C57BL/6 mice implanted with B16F1 melanoma cells were treated with pcDNA (closed circles), pTRP-2 (open squares) or pUb-TRP-2 (closed squares). Tumor growth was monitored as in Figure 2. Data are mean ± s.d. of eight mice in each group. *P<0.05 compared with the other two by unpaired Student's t-test. This experiment was repeated three times.*

following increased processing of epitopes by the proteasome.

Several investigators have approached to break peripheral T-cell tolerance using genes encoding heterologous melanocytic antigens such as human TRP-2 or chimeric genes encoding fused proteins between murine TRP-2 and foreign proteins like GFP. In this type of genetic immunization, $CD4^+$ T cells recognizing those kinds of heterologous/foreign antigens are activated and then break peripheral tolerance of $CD8^+$ T cells. In the present study, however, $CD4^+$ T cells appeared not to be required. It is important to elucidate how $CD8^+$ T cells are activated without support from $CD4^+$ T cells in our system, although there are some reports that some professional DC directly activate $CD8^+$ T cells without the support of $CD4^+$ T cells.[7,9] Further, Leitner *et al* recently reported that naked DNA vaccine encoding alphavirus replicon and mTRP-1 provides immunity to B16 melanoma. In this system, $CD8^+$ T cells are also final effector cells and $CD4^+$ T cells are not required, although they do not focus on 'ubiquitin–proteasome pathway', and speculate that such $CD8^+$ T cells are induced through activating innate antiviral pathways such as TLR3.[29]

There exist many reports that C57BL/6 mice show prominent vitiligo when mice have acquired antimelanoma immunity according to immunization with heterologous melanoma antigens/DNAs such as human TRP-2 and gp100.[30,31] Strangely, however, vitiligo was scarcely seen in the present study, in which mice were immunized with DNA encoding a fusion protein between murine ubiquitin and murine TRP-2. The difference between our observation and theirs may be the difference in contribution of $CD4^+$ T cells. In their system, $CD4^+$ T cells as well as $CD8^+$ T cells are required, while $CD4^+$ T cells were not required in the present system. One possibility is that vitiligo is caused by nonspecific inflammation induced by those activated $CD4^+$ T cells. It is worth noting that our previous finding should be documented. That is, we observed that C57BL/6 mice immunized with DNA encoding a fusion protein between GFP and murine TRP-2 also acquired a potent antimelanoma immunity mediated by $CD4^+$ T cells as well as $CD8^+$ T cells. These mice showed remarkable vitiligo (manuscript submitted). Antimelanoma immunity may not always correlate with vitiligo as reported by Bronte *et al*.[32]

Melanoma is one of the most common cancers and is the most serious type of skin cancer. In this study, we explored the potential of this DNA vaccine for melanoma treatment and demonstrated that this strategy was useful even after implantation of the melanoma cells in mice (Figure 6). We propose here that genetic immunization with naked chimeric DNA encoding a fusion protein between full-length human TRP-2 and human ubiquitin is one of the possible strategies for clinical trial aimed at inducing potent protective immunity in melanoma

patients. Immunization with full-length autologous TRP-2 could offer many advantages over ordinary vaccine strategies using 8- to 9-mer peptides such as human TRP-$2_{181-188}$. First, the ubiquitin-fused self-antigen(s) are rapidly degraded by the ubiquitin–proteasome pathway, resulting in efficient production of a variety of peptides including many CTL epitopes that might be presented by many types of MHC class I molecules. In other words, this type of vaccination would be available for patients with various types of MHC class I molecules. Second, the incidence of side effects should be low compared with vaccines that use vector viruses or heterologous peptides/genes. The latter types of vaccines could trigger certain side effects such as neutralizing antibodies, anaphylaxis mediated by homocytotropic antibodies and systemic inflammatory diseases mediated by $CD4^+$ T cells that recognize foreign/heterologous antigens or vector virus antigens included in vaccines. Third, immunoactivators such as complete Freund adjuvant or cytokines like IL-12 are not always required. Fourth, construction of naked DNA is easy and inexpensive. Finally, one can design the DNA vaccine depending on the type of cancer, implicating applicability to most types of cancers.

Materials and methods

Animals and tumors

We performed mouse studies in accordance with the institutional guidelines of Kyushu University. Female C57BL/6 mice (8 weeks of age) were purchased from SLC (Hamamatsu, Japan). Proteasome activator, PA28, knockout ($PA28\alpha\beta^{-/-}$) mice of C57BL/6 background were established by our group.[18] B16F1 and B16F10, murine melanoma cell lines, were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/streptomycin, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 50 mM $NaHCO_3$ and 2 mM L-glutamine. Lung carcinoma 3LL cell line derived from B6 mice was used as the control tumor.

Plasmid construction

Plasmid encoding mTRP-2 was constructed as follows: total RNA was separated from B16F1 cells and reverse-transcribed to cDNA. TRP-2 cDNA was then amplified by PCR using sense 5′-GAATCCAAGCGGCCGCCAATT AAGAAGGCATGGGCCTTG-3′ and antisense 5′-CCAT GGATCCTATTAGAGGCTAGCGTAATCCGGAACATCG TATGGGTAGGCTTCCTCCGTGTATCTCTTGCT-3′ primers. PCR product of TRP-2 cDNA was inserted into *Not*I and *Bam*HI site of pcDNA 3.1 (−) vector (Invitrogen, San Diego, CA, USA). Gene encoding mutant ubiquitin, whose C-terminal Gly residue was replaced by Ala (G76A), was amplified by PCR from genomic DNA obtained from the liver of BALB/c mice and was inserted into *Eco*RI and *Bam*HI site of pcDNA 3.1 (−). Gene encoding TRP-2 was amplified again by PCR using sense 5′-TAGGATCCGGCCTTGTGGGATGGGGGCTT CT-3′ and the same antisense primers. The mutant ubiquitin cDNA was then ligated to the 5′ of the gene encoding TRP-2-in-frame and inserted into *Bam*HI site of pcDNA 3.1 (−).

In vivo gene transfer and implantation of melanoma cells

We used a Helios Gene Gun (BioRad, NY, USA), as described previously.[11,33,34] Briefly, plasmid DNA was precipitated onto 1.6 μm gold particles and coated onto the inner surface of the tubing by a tube loader. The final tubing segment resulted in delivery of 0.125 mg gold particles and 2 μg plasmid DNA per transfection. pTRP-2, pUB-TRP-2 or pcDNA was transferred to three different portions of shaved abdominal skin three times at 1-week intervals. A total of 18 μg plasmid was administered into each mouse. At 1 week after the last vaccination, 2×10^5 melanoma cells in 0.1 ml of PBS were implanted subcutaneously into the center of the abdomen of B6 mice. Tumor size was measured twice a week using a caliper and was calculated as $\pi/6\times\{(a\times b)^{1/2}\}^3$, where a and b are two perpendicular major diameters of the tumor.[35] For the experiment of lung metastasis, B6 mice were intravenously challenged with 2×10^5 B16F10 cells in 0.2 ml of PBS. At 2 weeks after the challenge, mice were killed, all lobes of both lungs were dissected out and metastatic tumors were counted. For the above experiments, each group consisted of six mice. For therapeutic trials, 5×10^4 B16F1 cells in 0.1 ml of PBS were implanted subcutaneously in the abdominal area of B6 mice on day 0. pTRP-2, pUb-TRP-2 or pcDNA was transfected into three different portions of shaved abdominal skin three times, one of which was the tumor-implanted site. Treatment commenced on day 1 and was applied twice a week for 2 weeks (total five times). Each group consisted of eight mice. A total of 18 μg plasmid was administered into each mouse for one treatment.

In vitro transfection, Western blotting and pulse–chase analyses

A total of 500 000 of COS-7 cells in a 2.5 cm dish (Nunc, Roskilde, Denmark) were transfected with 2 μg pTRP-2 by using Lipofectamine (Invitrogen). At 24 h after transfection, cell lysates were prepared by adding 200 μl of lysis buffer (50 mM Tris-HCl, 1% nonidet P-40/1% sodium dodecyl sulfate (SDS), 1 μM leupeptin/100 μM phenylmethylsulfonylfluoride, 1 μM pepstatin A and 100 μM ethylenediamine tetraacetic acid). In some experiments, a specific inhibitor of proteasomes, MG132, was used at a concentration of 10 μM. A 10 μg portion of protein was used for Western blot with anti-HA antibody (mouse monoclonal antibody clone 12CA5, Roche, Mannheim, Germany) as the first antibody. Peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (H+L) (Zymed Laboratories, San Francisco, CA, USA) was used as the second antibody. Binding antibody was detected by using enhanced chemiluminescence (ECL) reagents (Amersham Life Science, Buckinghamshire, UK). Some transfectants were labeled with ^{35}S-labeled-amino acids (0.12 mCi/ml) for 60 min 18 h after transfection (pulse). Cells were washed and harvested after incubation for up to 40 min (chase). Then, cells were lysed, and the protein extracts were adsorbed with protein G-agarose beads for 2 h followed by immunoprecipitation with 1 μl of anti-HA and 20 μl of the beads overnight. Beads were washed four times and suspended with SDS-PAGE sample buffer. A 15 μl portion of the supernatant was separated on SDS-PAGE under nonreducing conditions. Gels were

soaked in Amplify Fluorographic Reagent (Amersham) for 30 min, dried and exposed to photographic film.

Cytotoxicity assay

Mice were killed at the time of tumor challenge and their spleen cells (4×10^7) were cocultured with mTRP-$2_{181-188}$ peptide (4 μg/ml) in six-well culture plates in complete RPMI 1640 medium. After 5-day culture, graded numbers of viable effector cells and [^{3}H]thymidine-labeled EL-4 cells (10^4) pulsed with mTRP-$2_{181-188}$ peptide (4 μg/ml) for 2 h were placed into round-bottomed 96-well plates. After 4.5 h incubation, the media were harvested onto glass-fiber filters and radioactivity was counted using a β scintillation counter, and specific killing was calculated as described previously.[36]

In vivo depletion of T-cell subsets

Anti-CD4 mAb (clone GK1.5) or anti-CD8 mAb (clone 53-6.72) was injected intraperitoneally at 0.5 mg/mouse on days −3, −1, 1 and 3 of tumor challenge. Tumor cells were inoculated on day 0. Depletion of each T-cell subset was confirmed by flow cytometry; over 98% of the appropriate cell subset was depleted.

Detection of intracelluar IFN-γ or antigen-specific $CD8^+$ T cells by flow cytometry

One million splenocytes were cultured in RPMI 1640 complete medium with phorbol 12-myristate 13-acetate (50 ng/ml), calcium ionophore (1 μg/ml) and brefeldin A (1 μg/ml) for 4 h in a 12-well plate, then harvested and washed once with staining buffer. After centrifugation, cells were stained with allophycocyanin-labeled anti-CD4 antibody (clone GK1.5, BD Pharmingen) and PE-labeled anti-CD8 antibody (clone 53-6.72, BD Pharmingen) at 4°C for 30 min and then washed with staining buffer. Cell pellets were fixed with 100 μl of 4% paraformaldehyde at 4°C for 20 min. After washing, cells were stained again with FITC-labeled anti-IFN-γ antibody (clone XMG1.2, BD Pharmingen), which was diluted in permeabilization buffer (1% heat-inactivated FCS, 0.1% sodium azide, 0.1% saponin in PBS) at 4°C. To detect antigen-specific $CD8^+$ T cells, lymph node cells were stained with FITC-CD8 antibody and PE-conjugated H-$2K^b$-TRP-$2_{181-188}$ tetramers (Proimmune, Springfield, VA, USA). After washing twice, cells were collected on a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA), and data were analyzed using CellQuest version 3.1 software (BD Biosciences).

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Trypanosoma cruzi Posttranscriptionally Up-Regulates and Exploits Cellular FLIP for Inhibition of Death-inducing Signal

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Intracellular persistence of the protozoan parasite, *Trypanosoma cruzi*, is an aggravating cause of Chagas' disease, involving that the protozoan infection specifically inhibits death receptor-mediated apoptosis of host cells. Here we demonstrate that the parasite dramatically up-regulates cellular FLICE inhibitory protein (c-FLIP), the only known mammalian inhibitor specific for death receptor signaling, in infected cells by an unusual, posttranscriptional stabilization of the short-lived protein. We also show that c-FLIP is accumulated in *T. cruzi*–infected mouse heart muscle cells in vivo. Stimulation of death receptor Fas in infected cells induces recruitment of c-FLIP to block the procaspase-8 activation at the most upstream caspase cascade. c-FLIP knock-down with a small interfering RNA significantly restores Fas-mediated apoptosis in infected cells. Taken together, our findings indicate that *T. cruzi* posttranscriptionally up-regulates and exploits host c-FLIP for the inhibition of death-inducing signal, a mechanism that may allow parasites to persist in host cells.

INTRODUCTION

Apoptosis is used as a defense strategy for the elimination of virus-, bacteria-, and parasite-infected cells by the immune system, as well as in cell selection during the development and maintenance of tissue homeostasis (Vaux *et al.*, 1994). Pathogen-infected apoptotic cells are recognized and phagocytosed by macrophages, and the pathogen is eliminated, together with host cells, in an immunologically silent manner (Ren and Savill, 1998). In contrast, pathogens have been found to antagonize apoptotic death of invaded host cells, prolonging their survival and allowing them more time to replicate (Moss *et al.*, 1999). Typically, viruses inhibit host cell apoptosis through the expression of antiapoptotic genes, such as viral FLIP (v-FLIP) in herpesvirus, p35 in baculovirus, E1B 19K in adenovirus, and CrmA in cow pox virus (Benedict *et al.*, 2002). Little is known, however, regarding the molecule(s) through which intracellular parasites interfere with host cell apoptosis (Heussler *et al.*, 2001; James and Green, 2004).

Trypanosoma cruzi, the protozoan parasite that causes Chagas' disease in Latin America (Morel and Lazdins, 2003), occurs as two forms in mammalian hosts. The nondividing trypomastigote form circulates in the bloodstream and invades a wide variety of nucleated cells, preferably heart muscle cells. Once in the host cell cytoplasm, the parasite transforms into the amastigote form. The intracellular parasite multiplies by binary fission, kills the host cell, and returns to the circulation as trypomastigotes that propagate the infection (Brener, 1973). $CD8^+$ T lymphocytes are involved in *T. cruzi* infection, killing infected cells by triggering their death through the interaction of Fas ligand with its receptor and of tumor necrosis factor (TNF)-α with its receptor, TNFR (Tarleton *et al.*, 1992; Rottenberg *et al.*, 1993; Locksley *et al.*, 2001). *T. cruzi*, however, can persist for many years in the mammalian host as intracellular amastigotes, suggesting that the parasite antagonizes apoptotic death of the invaded host cells. Chagas' disease is characterized by two distinct phases (Brener, 1973). The acute phase, which lasts 2–4 mo, involves a number of parasites detected in the blood stream as well as in host tissues, followed by a lifelong chronic phase in up to 30% of the patients. In chronic phase of Chagas' disease, *T. cruzi* persists in human with a nearly undetectable parasite load, and then the ultimate cause of the disease has been still hotly debated. However, currently accumulating evidence indicates that inefficient immune response to the parasites results in increased parasite load and increased incidence of chronic phase of Chagas' disease and that intracellular persistence of the parasites is an aggravating cause of even chronic phase of Chagas' disease (Tarleton and Zhang, 1999; Higuchi Mde *et al.*, 2003). Therefore, how *T. cruzi* persists in host cell is one of the most important studies to understand the pathogenicity.

On binding of the trimeric Fas ligands or agonistic antibodies, Fas receptors recruit adaptor molecules (FADD) and procaspase-8 to form the death-inducing signaling complex (DISC). In this complex, procaspase-8 is activated to caspase-8, triggering the proteolytic cascade of effecter caspases leading to cell death (Medema *et al.*, 1997). We previously demonstrated that induction of Fas- and TNFR-mediated apoptosis was more strongly inhibited in *T. cruzi*–infected cells than in uninfected cells and that caspase-8

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Abbreviations used: c-FLIP, cellular FLICE inhibitory protein; c-$FLIP_L$, c-FLIP long; DISC, death-inducing signaling complex; FADD, Fas-associated death domain-containing protein; siRNA, small interfering RNA oligoribonucleotide.

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activity could not be measured upon Fas stimulation of *T. cruzi*–infected cells (Nakajima-Shimada *et al.*, 2000). We also showed that x-ray, H_2O_2, cholchicine, and etoposide, respectively, induced an essentially same degree of apoptosis between *T. cruzi*–infected and uninfected cells. These findings indicate that parasite infection inhibits one of the earliest steps of death receptor-mediated apoptosis.

Here we report that *T. cruzi* uses the host's cellular FLICE inhibitory protein (c-FLIP), the only known inhibitor specific for death receptor–mediated apoptosis in mammals (Thome and Tschopp, 2001), for the inhibition of Fas-mediated apoptosis by posttranscriptional up-regulation. This finding indicates that *T. cruzi* modulates and exploits a host molecule to counteract death receptor signaling, a finding consistent with the view that parasites hijack the host cell, placing themselves in the driver's seat (Beverley, 1996).

MATERIALS AND METHODS

Cells and Parasites

HT1080 cells, a human fibrosarcoma cell line obtained from Japan Health Sciences Foundation (Tokyo, Japan), were cultured in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum. HT1080 cells (2×10^5) were infected with 4×10^5 *T. cruzi* trypomastigotes (Tulahuen strain) and cultured for 4 d. A cell population with an infection rate greater than 80% was considered as infected cells. The infection complex of HT1080 cells and *T. cruzi* was maintained as described (Nakajima-Shimada *et al.*, 2000).

Antibodies

Rabbit anti-c-FLIP polyclonal antibodies specific for amino acid residues 2–17 was obtained from Upstate Biotechnology (Lake Placid, NY). Mouse anti-c-FLIP monoclonal antibody (mAb; Dave-2) was from Alexis Biochemicals (San Diego, CA). Mouse anti-FADD mAb (clone 1) was purchased from BD Biosciences Clontech (Palo Alto, CA). Rabbit anti-caspase-8 polyclonal antibody (GD-13) was from Sigma-Aldrich (St. Louis, MO) and mouse anti-caspase-8 mAb (12F5) from Alexis Biochemicals. Mouse anti-p53 mAb (antibody-6) was from Oncogene (San Diego, CA). Mouse anti-Fas mAb (APO1–3) was purchased from Wako (Osaka, Japan). Rabbit anti-actin polyclonal antibody was from Sigma-Aldrich.

Western Blotting

Cell lysates were prepared in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride [PMSF]), and the protein concentration was determined by the Bradford assay. Proteins were resolved by SDS-PAGE and transferred to Immobilon Transfer Membranes (Millipore, Bedford, MA) by electroblotting. Immunoblot analyses were performed with the indicated antibodies. Bound primary antibodies were visualized with alkaline phosphatase–conjugated specific antibodies and with CSPD (Roche, Mannheim, Germany).

Northern Blotting

Total RNA was isolated using TRIzol (Life Technologies, Tokyo, Japan) according to the manufacturer's instruction. Total RNA was size-fractionated, and blotting was performed using the VacuGene XL protocol (Amersham Biosciences, Piscataway, NJ). The c-FLIP probe was DIG-labeled using PCR Probe Synthesis Kit (Roche) in the presence of 5′-GAGTTGGAGAAACTA-AAT-3′ and 5′-ACACTCTGGGAGCCTCCT-3′, the forward and reverse primers, respectively.

Immunohistochemistry

Five-week-old female BALB/c mice (Japan SLC, Hamamatsu, Japan) were intraperitoneally infected with *T. cruzi* trypomastigotes (5000 parasites/mouse) recovered from the preceding infected mice. Fourteen days later, heart samples were collected and frozen in blocks of optimal cutting temperature compound (OTC, Sakura, Tokyo, Japan) in liquid nitrogen according to standard procedure. The OTC block was cut in 4-μm sections onto silane-coated slide glasses. The sections were incubated with 10% normal goat blocking serum and then with anti-c-FLIP mAb G-11 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted to 1:100. After washing with phosphate-buffered saline (PBS), the sections were incubated with secondary antibody (horse anti-mouse-FITC, Vector Laboratories, Burlingame, CA). After washing with PBS, the specimens were counterstained with 10 μM Hoechst 33342 (Calbiochem, La Jolla, CA) to visualize DNA. The quantitation of the fluorescence intensity of *T. cruzi*–infected and uninfected cells, the latter locating near the infected cells, were manually assessed by Image-Proplus ver. 4.0 software (Media Cybernetics, Silver Spring, MD). Background fluorescence intensity of the thin section was subtracted from the sample cell fluorescence intensity. Statistical evaluation was performed by SigmaPlot software (Systat Software, Point Richmond, CA).

Apoptosis Induction and Cell Death Assay

Cells were incubated with anti-Fas CH11 mAb (0.5 μg/ml; MBL, Nagoya, Japan) to induce apoptosis through Fas. The cells were washed in PBS, fixed with methanol, stained with Hoechst 33342, and photographed under a fluorescence microscope. Condensed nuclei were scored as apoptotic.

Immunoprecipitation

Immunoprecipitation was performed using magnetic beads as described by the manufacturer (New England Biolabs, Beverly, MA). Cells were lysed with IP buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 1% Triton X-100, and 0.5% Nonidet P-40). The crude cell extract was preincubated with Protein G Magnetic Beads (New England Biolabs), and the resulting supernatant was incubated with the indicated antibody and the beads. Magnetic beads were washed with IP buffer and suspended in SDS sample loading buffer. After incubation at 70°C for 5 min, each sample was subjected to SDS-PAGE and Western blotting as described above.

DISC analysis was performed using a standard method (Krueger *et al.*, 2001). Briefly, cells (1×10^7) were trypsinized, collected by centrifugation, and then resuspended in 5 ml of DMEM. Fas receptor was stimulated with 2 μg/ml anti-Fas mAb (APO1–3) for 20 min. Cells were washed with ice-cold PBS and lysed in 1 ml of lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1% Triton X-100, and 10% glycerol) for 30 min at 4°C. Protein A-Sepharose beads (Amersham Bioscience) was added to the lysate. As a negative control, APO1–3 and protein A-Sepharose beads were added to the lysate of unstimulated cells. Sample and negative control lysates were incubated at 4°C on a rotator for 3 h. The beads were washed four times in lysis buffer and suspended and boiled in SDS sample loading buffer, and then each sample was subjected to SDS-PAGE and Western blotting as described above.

siRNA Experiment

For depletion of c-FLIP, we used siRNA generated in vitro by RNase III from *Escherichia coli* (Yang *et al.*, 2002). To produce the long double-stranded RNA (dsRNA), c-FLIP gene (nucleotides 89–698 downstream from the start codon) were amplified by PCR using the specific primers 5′-gcgtaatacgactcactatagg-gagaagatgtggttccacctaatg-3′ (forward) and 5′-gcgtaatacgactcactatagg-gagagcttctgattcctgaatgga-3′ (reverse), which also contain a T7 promoter and a leader sequence. Double-stranded (ds) RNA was generated using TurboScript T7 Transcription Kit (Gene Therapy Systems, San Diego, CA). The dsRNA was converted to siRNA using ShortCut RNase III (New England Biolabs). The siRNAs were precipitated in ethanol and then dissolved in nuclease-free water. The concentration of the siRNAs was determined spectrophotometrically and by ethidium bromide staining in 3% agarose gels. As a negative control, we used luciferase siRNA (Luciferase GL2, Fasmac, Kanagawa, Japan).

Transfection of siRNAs (200 ng/ml) into 40–50% confluent HT1080 cells cultured in wells of a 12-well plate was performed using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instruction. After cultivation for 24–30 h, the cells were infected with 2×10^6 trypomastigotes as previously described (Nakajima-Shimada *et al.*, 2000), incubated for 24 h further, and harvested for Western blotting or apoptosis induction followed by cell death assay.

RESULTS

T. cruzi *Infection Up-regulates c-FLIP$_L$ Protein Expression*

Because our previous study has demonstrated that caspase-8 activity could not be measured upon Fas stimulation of *T. cruzi*–infected cells (Nakajima-Shimada *et al.*, 2000), expression of c-FLIP protein in *T. cruzi*–infected and uninfected cells was examined by Western blotting using an anti-c-FLIP antibody that recognizes both c-FLIP long (c-FLIP$_L$) and c-FLIP short (c-FLIP$_S$; Figure 1A). Both c-FLIP$_L$ and c-FLIP$_S$, which are generated by alternative splicing, block procaspase-8 activation at the DISC and consequently inhibit death receptor–mediated apoptosis (Thome and Tschopp, 2001). Although we did not detect any 26-kDa c-FLIP$_S$ in either infected or control cells, the level of c-FLIP$_L$ (55 kDa) was dramatically up-regulated in infected cells when compared with uninfected cells. These two cell populations showed an essentially same level of FADD. We confirmed that, after immunoprecipitation by a c-FLIP-specific mAb

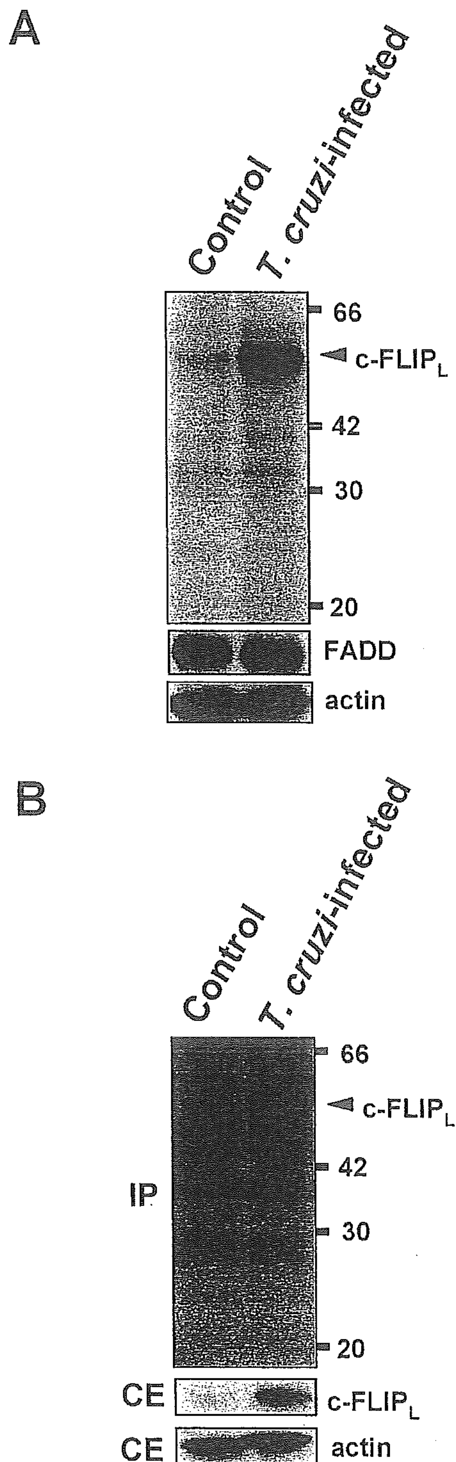

Figure 1. Expression of c-FLIP protein in *T. cruzi*–infected cells. (A) The cells (2×10^5) were infected with *T. cruzi* trypomastigotes as described in *Materials and Methods*. Cell extracts (100 μg protein/lane) were resolved on 12.5% SDS-PAGE, and Western blots were probed with anti-c-FLIP antibody specific for amino acid residues 2–17 and with anti-FADD antibody. Actin was used for loading control. (B) Cell extracts were immunoprecipitated (IP) using anti-c-FLIP mAb (Dave-2). Precipitated samples were analyzed by Western blotting with anti-c-FLIP antibody described in A. Cellular extracts (CE) were also analyzed by Western blotting (10 μg protein/lane). Numbers on the right indicate kilodaltons.

(Dave-2, Alexis Biochemicals), Western blotting of the precipitated proteins using the above anti-c-FLIP antibody (see Figure 1A) also showed markedly up-regulated c-FLIP$_L$ protein level in *T. cruzi*–infected cells (Figure 1B).

Posttranscriptional Up-regulation of c-FLIP Protein in T. cruzi–infected Cells

We examined whether the increase in c-FLIP$_L$ protein was due to the elevation of the corresponding mRNA in *T. cruzi*–infected cells. Northern blots showed that *T. cruzi*–infected cells had a level of c-FLIP mRNA (2.1 kb) nearly equivalent to that in uninfected cells (Figure 2A). In infected cells, mRNA of ~1 kb was slightly up-regulated, but we did not elucidate whether this mRNA was translated. Importantly, c-FLIP$_L$ mRNA level does not parallel the increased protein level (see Figure 1).

Because c-FLIP is a short-lived protein, inhibitors of protein synthesis lower its expression level in a variety of cell lines (Imanishi *et al.*, 2000a). In contrast to uninfected cells, *T. cruzi*–infected cells treated with cycloheximide did not show a rapid decrease in c-FLIP$_L$ protein (Figure 2B). The level of expression of p53, another short-lived protein regulated by the ubiquitin-proteasome pathway (Zhang *et al.*, 2004), however, was rapidly decreased by cycloheximide treatment in both *T. cruzi*–infected and uninfected cells, indicating that *T. cruzi* infection selectively inhibits the degradation of c-FLIP$_L$ protein. Taken together, these results indicate that c-FLIP$_L$ is posttranscriptionally up-regulated by *T. cruzi* infection.

c-FLIP Is Also Up-regulated in T. cruzi–infected Cardiomyocytes In Vivo

We also examined whether c-FLIP protein is increased in *T. cruzi*–infected mouse heart muscle cells, in which Chagasic myocarditis and amastigotes are often observed (Figure 3A). The monoclonal anti-c-FLIP antibody (G-11), with specificity for c-FLIP in immunohistochemical analysis (Kim and Seong, 2003; Mathas *et al.*, 2004), revealed strong staining throughout the cytoplasm of an amastigote-dwelling cardiomyocyte, with obscure staining in uninfected cells. The fluorescence intensity of 58 each of *T. cruzi*–infected and uninfected cells was measured for statistical evaluation (Figure 3B). Up-regulation of c-FLIP was observed in all *T. cruzi*–infected cells, showing the fluorescence intensity significantly ($p < 0.01$) higher in the infected than in the uninfected cells. These results indicate that c-FLIP is also up-regulated in *T. cruzi*–infected cells in vivo when compared with uninfected cells.

The fluorescence intensity of the infected cardiomyocytes in vivo did not clearly parallel in vitro studies (see Figure 1A), probably because of the lightly and heavily infected host cells in vivo and in vitro, respectively, with average parasite numbers of 5.5 and 15–20 per infected cell. Additionally, the *T. cruzi*–infected heart muscle cell, cut into a thin section, may exhibit a partial fluorescence staining of the whole cell. These results are consistent with a suggestion that the degree of the fluorescence intensity in host cells in vivo is lower than that of Western blot in vitro.

Signals were also detected near the cells lacking the parasite infection in the section shown in Figure 3A. Importantly, because these cells are not stained throughout the cytoplasm, the fluorescence would not be c-FLIP signal. We experienced that the mouse blood vessel was nonspecifically stained with anti-c-FLIP antibody (G-11). Therefore, it may be possible that blood vessels near uninfected cells were nonspecifically detected with this antibody. Alternatively, it may be possible that, because heart muscle cells are very

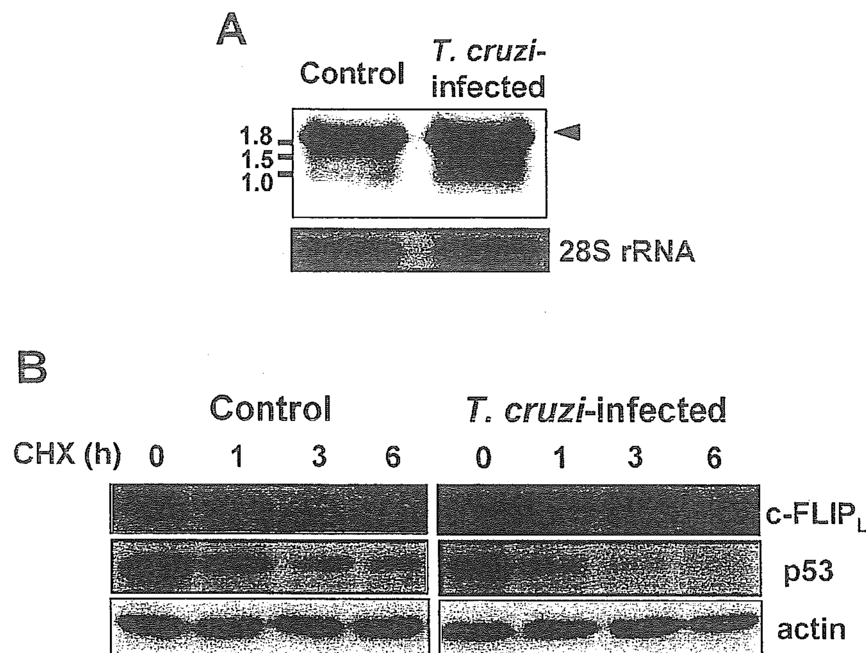

Figure 2. Expression of c-FLIP mRNA and effect of cycloheximide treatment. (A) Total RNA (2.5 μg/lane) was resolved on 1% agarose gel, Northern-blotted, and probed for c-FLIP. The band corresponding to c-$FLIP_L$ mRNA is indicated by arrowhead. Numbers on the left indicate kilobases. (B) Cells (70–80% confluent) were incubated with 100 μM cycloheximide for the indicated time, and the expression of c-$FLIP_L$ and p53 was analyzed by Western blotting. Actin was used for loading control.

long, a part of infected cells, whose nuclei and infected parasites were not seen, was stained with the antibody.

T. cruzi Infection Inhibits Fas-stimulated Procaspase-8 Activation by Recruitment of c-$FLIP_L$ into the DISC

At the DISC, procaspase-8 is cleaved and converted to p43/41 fragments, and these are further processed to active caspase-8, a heterotetramer composed of two p18 and two p10 polypeptides (Medema *et al.*, 1997). In the presence of a large amount of c-$FLIP_L$, procaspase-8 and c-$FLIP_L$ are recruited into the DISC, the p43/41 cleavage products of caspase-8 and p43 cleavage product of c-$FLIP_L$ are generated, and then the cleavage intermediates remain bound to the DISC and can no longer be replaced by procaspase-8 (Scaffidi *et al.*, 1999; Krueger *et al.*, 2001). Because c-$FLIP_L$ protein is highly expressed in *T. cruzi*–infected cells, we examined procaspase-8 processing in infected and control cells following Fas stimulation (Figures 4, A and B).

Five hours after Fas stimulation of control and infected cells, followed by immunoprecipitation with a caspase-8-specific antibody (12F5), Western blot visualized with an anti-caspase-8 antibody (GD-13) showed that p43/41 could be detected in both control cells and *T. cruzi*–infected cells (Figure 4A, top panel). However, in control cells, but not in infected cells, p18 was produced (Figure 4A, bottom panel). Then, we monitored processing of procaspase-8 in control and *T. cruzi*–infected cells after Fas stimulation (Figure 4B). The anti-caspase-8 antibody we used (GD-13) does not differentiate between procaspase-8a (55 kDa) and -8b (53 kDa; Scaffidi *et al.*, 1997; indicated by arrowhead). We found that, 3 h after Fas stimulation, only 14% of procaspase-8 was processed in *T. cruzi*–infected cells, whereas 82% was processed in control cells. These results indicate that procaspase-8 is recruited into the DISC, but that activation of the cytoplasmic procaspase-8 pool is strongly prevented in infected cells, upon Fas stimulation.

Next, we analyzed the DISC composition in *T. cruzi*–infected cells to know whether c-$FLIP_L$ is recruited into the DISC (Figure 4C). Both full-length c-$FLIP_L$ and p43 cleavage fragment of c-$FLIP_L$ were detected in Fas-stimulated *T. cruzi*–infected cells. In control cells, we could not detect both c-$FLIP_L$ and p43 c-$FLIP_L$ after Fas stimulation, which may be due to the low expression of c-$FLIP_L$. Procaspase-8 was hardly detectable both in infected and control cells, but p43/p41 cleavage fragments of caspase-8 were detected in both infected and control cells after Fas stimulation. It has been shown that recruitment of FADD into the DISC is reduced in cells overexpressing c-$FLIP_L$ (Krueger *et al.*, 2001). Consistent with this, FADD detected in our experiment was reduced in infected cells when compared with control cells. Taken together, our results indicate that *T. cruzi* infection inhibits the activation of procaspase-8 into caspase-8, due to the recruitment of c-$FLIP_L$ to the DISC, eventually leading to the inhibition of Fas-mediated apoptosis.

c-FLIP Knock-down with siRNA Restores Fas-mediated Apoptosis in T. cruzi–infected Cells

A selective c-FLIP knock-down with siRNA was carried out to clarify the crucial role of this protein in the inhibition of Fas-mediated apoptosis in *T. cruzi*–infected cells. Transfection of c-FLIP-specific siRNA (siFLIP) reduced the levels of c-$FLIP_L$ protein in infected cells when compared with a negative control siRNA specific for luciferase (siLuc; Figure 5, inset).

When we tested the effects of RNA interference against c-FLIP on the rate of apoptosis induced by Fas stimulation, we found that the control siRNA (siLuc) induced the apoptosis of 20.0 and 5.4% of the uninfected and *T. cruzi*–infected cell samples, respectively (Figure 5), indicating that *T. cruzi* infection strongly inhibited Fas-mediated apoptosis (Nakajima-Shimada *et al.*, 2000); the difference is statistically significant ($p < 0.01$). In contrast, siFLIP resulted in a markedly and significantly increased apoptosis (from 5.4 to 17.8%) in *T. cruzi*–infected cells ($p < 0.05$), with a slight increase in apoptosis (from 20.0 to 24.6%) in uninfected cells ($p = 0.188$). Importantly, the rate of apoptosis was not significantly different between the infected cells transfected

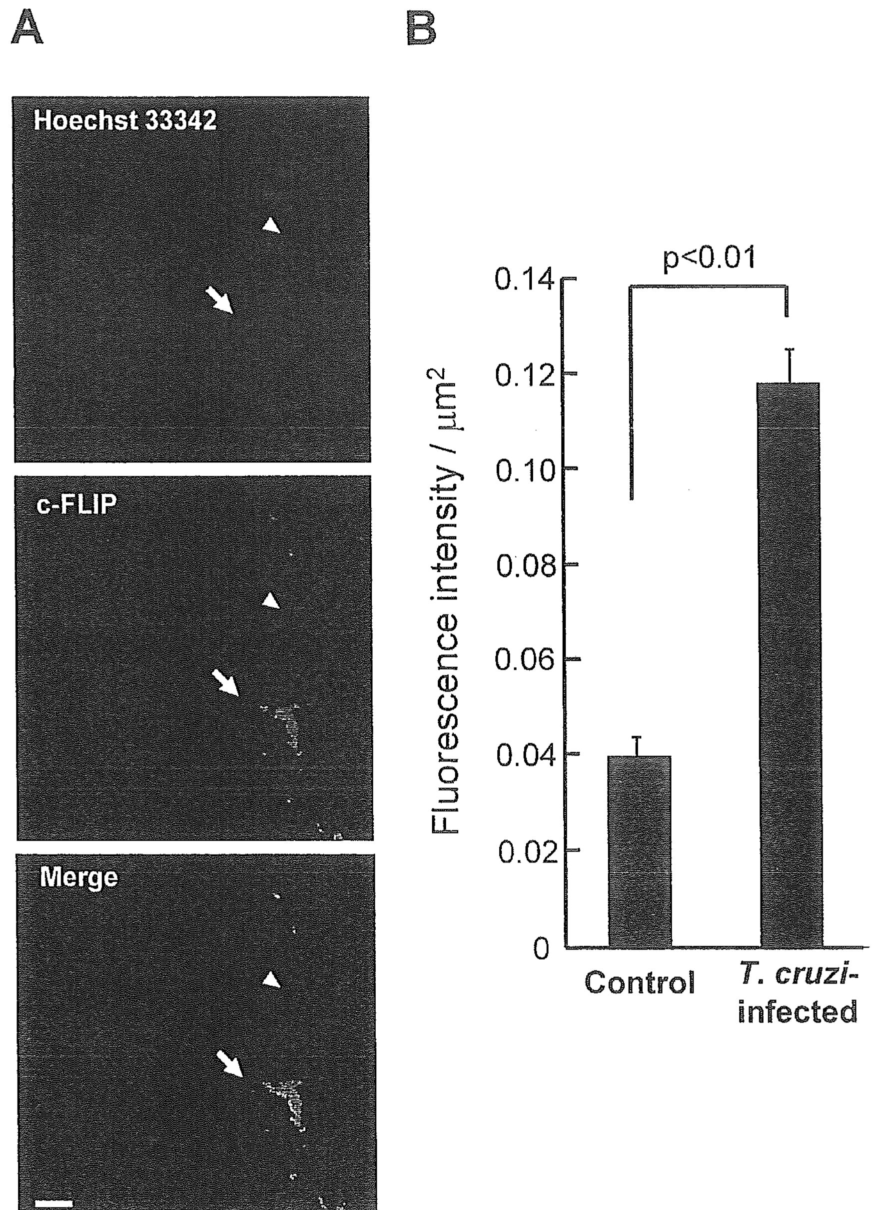

Figure 3. Immunohistochemical detection of c-FLIP in the cardiomyocytes of mice infected with *T. cruzi*. (A) Thin sections prepared from *T. cruzi*–infected mouse hearts were stained with Hoechst 33342 and with anti-c-FLIP mAb (G-11). Details are described in *Materials and Methods*. Fluorescence images and their merger in the same field in a typical section are shown. The arrow points toward a *T. cruzi*-dwelling cardiomyocyte. The arrowhead points at a typical uninfected cell. Bar in Merge, 10 μm. (B) The fluorescence intensity of 58 each of *T. cruzi*–infected and uninfected heart muscle cells was measured for a quantitation of expression level of c-FLIP. Statistical significance was assessed by Student's *t* test.

with siFLIP and the uninfected cells transfected with siLuc or siFLIP. The result implies that c-FLIP knock-down in *T. cruzi*–infected cells yielded the recovery of Fas-mediated apoptosis to a level equivalent to that in uninfected cells. From these findings, we conclude that c-$FLIP_L$ protein plays a key role in the inhibition of Fas-mediated apoptosis in *T. cruzi*–infected cells.

DISCUSSION

Inhibition of death receptor–mediated apoptosis is highly likely to play an important role for the survival of the intracellular protozoan parasite, *T. cruzi*, in infected cells. We have shown here that *T. cruzi* posttranscriptionally up-regulates and exploits the host protein, c-FLIP, to interfere with Fas-mediated host cell apoptosis. This is the first report showing a crucial molecule for a eukaryotic intracellular pathogen, which is much more complex an organism than virus or bacteria, to inhibit death-inducing signal. Concerning the death receptors and their signals examined to date, c-FLIP displays a protective role against apoptosis mediated by all these receptors (Thome and Tschopp, 2001). Therefore, the host c-FLIP would be a good target to be "manipulated" by intracellular protozoan parasites.

Several viruses encode v-FLIP (Thome and Tschopp, 2001), ensuring their survival and propagation. However, a BLAST search of TcruziDB, an integrated genome database for *T. cruzi*, shows that there is no FLIP homologue in the *T. cruzi* genome (Luchtan *et al.*, 2004). The method by which the parasite benefits from exploitation of host c-FLIP, but not from exploitation of own FLIP homologue, is not yet known. Perhaps c-FLIP in infected cells is unable to become a target of $CD8^+$ cytotoxic T lymphocytes, or posttranscriptional up-regulation of c-FLIP is more efficient and effective than expression of its own FLIP homologue in the inhibition of death-inducing signals.

Some tumor cells highly express c-FLIP and its level has correlated with cellular resistance to death receptor–medi-

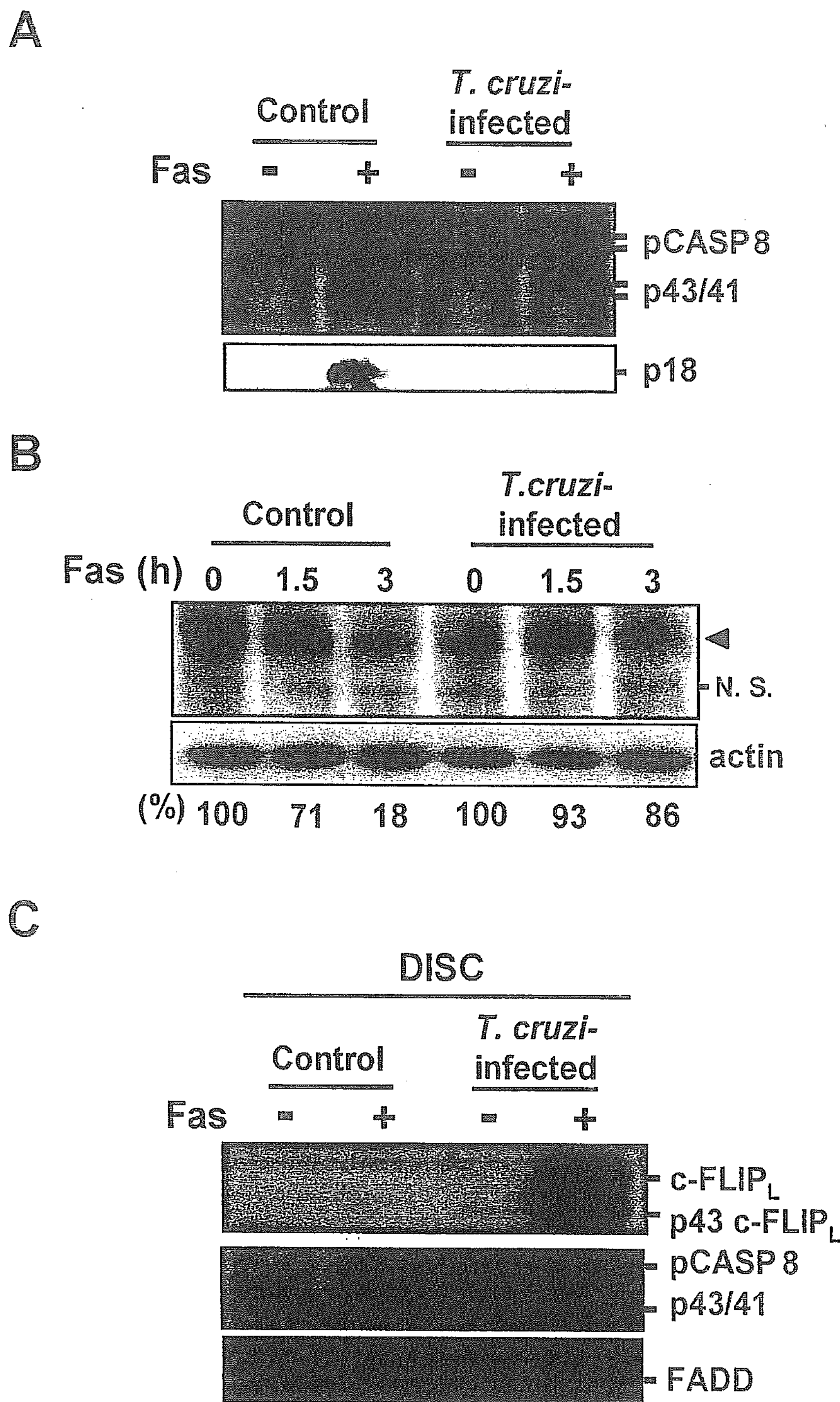

Figure 4. Processing of procaspase-8 and c-FLIP$_L$ in *T. cruzi*–infected cells. (A) Top: cells were incubated in the presence or absence of anti-Fas antibody for 5 h, and the cell lysates were immunoprecipitated with anti-caspase-8 mAb (12F5). The precipitated protein was analyzed by Western blotting with rabbit anti-caspase-8 polyclonal antibody (GD-13). Bottom: cell extracts (200 μg protein/lane) were fractionated on 12.5% SDS-PAGE and Western-blotted with anti-caspase-8 antibody (GD-13). p18 form processed from p43/41 form of caspase-8 is shown. (B) Cells were stimulated with anti-Fas antibody for 0, 1.5, and 3 h. Each cell extract (55 μg protein/lane) was fractionated on 15% SDS-PAGE and Western-blotted with anti-caspase-8 antibody. Arrowhead indicates the position of pro-caspase-8. Western blots of the panel were scanned using RFLP-scan (Scanalytics, Billerica, MA) and quantified for procaspase-8. The levels of procaspase-8 are shown at the bottom. N.S., nonspecific cross-reactive band. (C) *T. cruzi*–infected and uninfected cells were stimulated with anti-Fas antibody or were left untreated. After lysis of these cells, DISC or unstimulated Fas was immunoprecipitated by anti-Fas antibody (APO1–3) and analyzed by Western blotting using anti-caspase-8 antibody (GD-13), anti-c-FLIP antibody described in Figure 1A, and anti-FADD antibody. The positions of the proteins and the respective cleavage fragments are indicated.

ated apoptosis (Igney and Krammer, 2002). Using siRNA specific for c-FLIP, it was recently shown that this protein is a key molecule in death receptor resistance in Hodgkin/Reed-Sternberg cells (Mathas *et al.*, 2004). However, c-FLIP expression is reportedly regulated by transcriptional mechanism for most of these cells. By contrast, *T. cruzi* posttranscriptionally stabilizes the host c-FLIP protein (see Figure 2B), a unique strategy that the parasite takes advantage of the short-lived nature of the target protein.

T. cruzi infection has been shown to protect mammalian cells from apoptotic death caused by growth factor deprivation (Clark and Kuhn, 1999; Chuenkova and Pereira, 2000; Chuenkova *et al.*, 2001). This phenomenon, however, has not been investigated from the viewpoint of inhibition of death receptor–mediated apoptosis. Interestingly, *T. cruzi* trans-sialidase activates phosphatidylinositol 3-kinase (PI3K)/Akt protein kinase signaling, which is utilized as a survival pathway in a variety of cell types (Chuenkova *et al.*, 2001). Additionally, c-FLIP expression depends on the PI3K/Akt protein kinase activity in many cell lines, where its up-regulation may take place at a transcriptional level (Panka *et al.*, 2001). By contrast, our data clearly show that c-FLIP, at

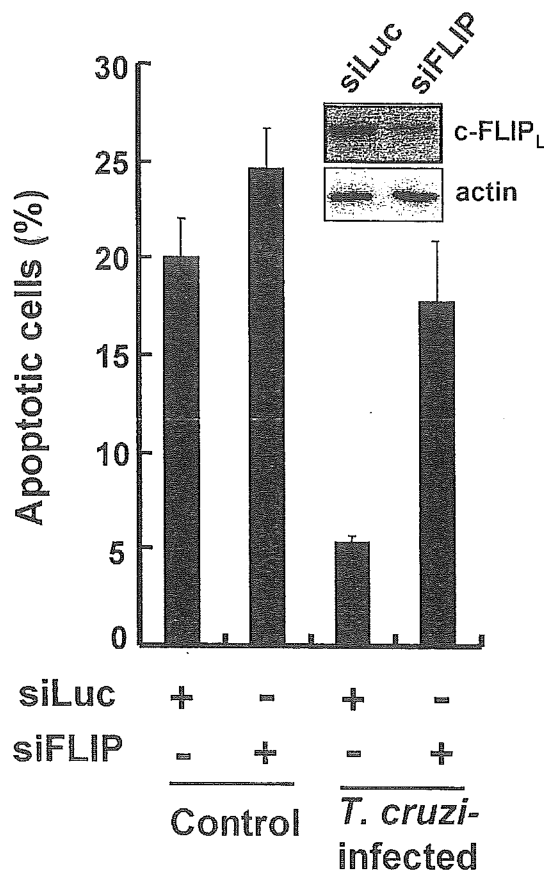

Figure 5. c-FLIP knock-down and recovery of apoptosis in *T. cruzi*–infected cells. Preparation of siLuc (negative control) and si-FLIP, transfection of these siRNAs into cells, *T. cruzi* infection, and Fas stimulation were carried out as described in *Materials and Methods*. siRNA-transfected cells (70–80% confluent), which were subsequently infected with *T. cruzi* and incubated for 24 h, were stimulated for 5 h with anti-Fas antibody. The percentage of apoptotic cells was determined. In each experiment, more than 350 uninfected or 250 *T. cruzi*–infected cells were examined. The values are means of three separate experiments and the bars represent standard deviations. The inset shows the expression level of c-FLIP$_L$ and actin in *T. cruzi*–infected cells (15 μg protein/lane). Because of the low expression level of c-FLIP$_L$ in control cells, the protein was not detected under the conditions examined.

least c-FLIP$_L$, is up-regulated posttranscriptionally in *T. cruzi*–infected cells, implying that its up-regulation is independent on PI3K/Akt protein kinase signaling. Indeed, treatment of *T. cruzi*–infected cells with LY294002, a specific and permeable PI3K inhibitor, at 50 μM for 24 h did not affect c-FLIP expression or sensitivity to Fas stimulation (our unpublished data).

How *T. cruzi* selectively inhibits the degradation of c-FLIP protein is an important issue. As a short-lived protein, c-FLIP level would be regulated by the ubiquitin-proteasome pathway, in which the specific ubiquitin ligase (E3) is involved. In general, however, identification of physiologically functional E3 is important, but difficult, in that the endogenous expression level of c-FLIP is very low (Scaffidi *et al.*, 1999), and in that c-FLIP interact with various key proteins, including procaspase-8, FADD, TNFR-associated factors 1 and 2, and Rip and Raf kinases (Kataoka *et al.*, 2000). Because of these factors, it would therefore be difficult to pinpoint the role of the E3 associated with the posttranscriptional up-regulation of c-FLIP. Nevertheless, we believe that further molecular and cellular pursuit of insight into how *T. cruzi* stabilizes c-FLIP contributes to identify the specific E3.

Although we found that c-FLIP was highly expressed in infected cardiomyocytes in vivo, it is not clear that the protein is the only molecule that inhibits death receptor–mediated apoptosis in vivo. Because c-FLIP knock-out mice do not survive past day 10.5 of embryogenesis (Yeh *et al.*, 2000), the role of c-FLIP in adult mammals is not well understood. However, it has been shown that c-FLIP is highly expressed in the adult human and murine heart when compared with other organs and suggested that down-regulation of c-FLIP sensitized cardiac myocytes to apoptotic death (Rasper *et al.*, 1998). Furthermore, c-FLIP is abundant in normal cardiomyocytes from failing human hearts, but the protein is absent from apoptotic cardiac myocytes (Imanishi *et al.*, 2000b). It is, therefore, strongly suggested that c-FLIP functions as a strong antiapoptotic factor in *T. cruzi*–dwelling cardiomyocytes in hearts from infected animals. Because the differences in biological features of *T. cruzi* amastigotes in acute and chronic phase of infection are poorly understood, whether the chronic stage amastigotes also up-regulate c-FLIP is interesting. However, these amastigotes could not be observed by standard histochemical techniques because of the very low parasite density. To address this question, we need experimental techniques that allow more exhaustive analysis of a whole mouse heart and we need more detailed information about the cell biological features of the chronic stage amastigotes (e.g., their method of replication and the spot(s) they preferably persist). On the other hand, it has been proposed that inadequate clearance of the acute phase parasites due to a substandard immune response leads to chronic phase of Chagas' disease. Therefore, c-FLIP may be a clue to understanding how the intracellular parasite persists in mammalian cells, eventually giving rise to pathogenicity at the molecular level.

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Gene expression profiles in response to Fas stimulation in *Trypanosoma cruzi*-infected host cells☆

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Abstract

To determine the molecular mechanism by which apoptosis is inhibited in *Trypanosoma cruzi*-infected host cells, we used human cDNA apoptosis chips to compare the gene expression profiles in response with 'death ligands target' (Fas) stimulation in infected and uninfected cells. Of the 164 apoptosis-related genes examined, 20, including those encoding both pro- and anti-apoptotic proteins, were highly up-regulated in the infected group. Genes encoding caspases and apoptosis inhibitors were optimally expressed 10–30 min after induction of apoptosis, whereas genes involved in transcriptional regulation and cell proliferation were up-regulated after 2–24 h. These results suggest that host anti-apoptotic gene(s) may play a crucial role in the inhibition of Fas-mediated apoptosis in *T. cruzi*-infected cells.
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Keywords: Apoptosis; DNA chip; Fas; Gene expression; *Trypanosoma cruzi*

1. Introduction

Trypanosoma cruzi, an intracellular protozoan parasite, is responsible for Chagas' disease, which affects 16–18 million people in South America (Urbina and Docampo, 2003). The parasite has a complex life cycle involving multiple hosts. In mammals, the infective trypomastigote form invades a wide variety of host cells, including heart muscle and nerve cells, and transforms into the amastigote form in the host cell cytoplasm. The amastigotes subsequently divide several times by binary fission and transform again into trypomastigotes which are released into the blood circulation, reinitiating the cycle.

Apoptosis plays a major role in the removal of damaged or harmful cells during development and tissue homeostasis, as well as in the regulation of the host immune response. Apoptosis is also an important host defense mechanism against viral, bacterial, and parasitic pathogens in innate and adaptive immunity (Williams, 1994). Several pathogens, however, have evolved strategies to modulate host cell apoptosis (Moss et al., 1999; Vaux et al., 1994). Thus, an understanding of the mechanism of apoptosis inhibition in pathogen-infected cells contributes to an understanding of pathogenicity.

Death ligands, including Fas ligand (FasL) and tumor necrosis factor α (TNF-α), are potent regulators of apoptosis, which act at various points in the immune response, from inflammation to cell death (Nagata and Golstein, 1995). The FasL/Fas system constitutes an important cellular pathway that mediates apoptosis during the course of infection. When FasL binds to Fas on a target cell, procaspase-8 is recruited via an adaptor protein, Fas-associated death domain (FADD), forming a death-inducing signaling complex (DISC) (Nagata, 1997). In this DISC, procaspase-8 is autocatalytically processed into caspase-8, which initiates a caspase cascade, including the activation of the effector caspases-3, -6, and -7, as well as mitochondrial release of cytochrome *c* (Kuwana et al.,

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1998). Cytochrome *c* binds and activates the adaptor protein, apoptotic protease activating factor 1 (Apaf-1), which recruits and activates caspase-9 (Kroemer and Reed, 2000; Li et al., 1997). In addition, this pathway may be indirectly activated by caspase-8 through the proteolysis of Bid, a pro-apoptotic member of the Bcl-2 family protein (Li et al., 1998).

Apoptotic lymphocytes have been observed during the acute phase of infection with *T. cruzi* in animal models (Leguizamon et al., 1999; Zhang et al., 1999). The FasL/Fas system has been found to be functional in activated T cells from infected mice (Lopes et al., 1999), and interferon (IFN)-γ is thought to be involved in the up-regulation of FasL (Martins et al., 1998). In contrast, Acosta-Rodriguez et al. (2003) reported that IL-4 mediates down-regulation of FasL in *T. cruzi*-infected mice. *Trypanosoma cruzi* infection, however, has been found to protect host cells, including murine fibroblasts, from apoptosis in vitro, although the mechanism of this inhibition is not yet known (Clark and Kuhn, 1999). A *T. cruzi* trans-sialidase has been shown to induce apoptosis in the spleen, thymus, and ganglia of mice (Leguizamon et al., 1999), but to prevent apoptosis in neuronal cell lines (Chuenkova and Pereira, 2000). In Schwann cells, the protozoan trans-sialidase acts as a survival factor via the phosphatidylinositol 3-kinase/Akt signaling pathway (Chuenkova et al., 2001). Previously, we reported that *T. cruzi* infection inhibits one of the earliest steps of Fas-mediated apoptosis in host cells (Nakajima-Shimada et al., 1998), and we found that host cell factor(s) were involved in this inhibition (Nakajima-Shimada et al., 2000). We proposed that this inhibition of apoptosis is a parasite defense strategy, which it uses to escape from the host immune response, but little is known about Fas-mediated death signaling in *T. cruzi*-infected cells. Using cDNA apoptosis chips, we report here the time-course of transcriptional changes in apoptosis-related genes responsive to Fas stimulation in *T. cruzi*-infected cells.

2. Materials and methods

2.1. Cell culture and infection

The Tulahuen strain of *T. cruzi* and the human HT1080 fibrosarcoma cell line were maintained as described previously (Nakajima-Shimada et al., 1994, 2000). Briefly, exponentially growing HT1080 cells ($1–3\times10^4$ cells) were incubated for 2 days, infected with *T. cruzi* trypomastigotes ($3–5\times10^6$ parasites), and further incubated for 3 days. We used a cell preparation as the infected group in which each infected cell contained more than 10 amastigotes on average, with a rate of infection greater than 86%. Under these conditions, amastigotes were retransformed to trypomastigotes, and the latter were released from the host cells on average 6 days p.i.

2.2. Induction of apoptosis

Apoptosis was induced in *T. cruzi*-infected and uninfected HT1080 cells by the addition of 0.5 μg/ml anti-human Fas monoclonal antibody (anti-Fas mAb, CH-11, MBL, Nagoya, Japan). At various times (0, 10, 30, 60, 120, 180, 360, 600 and 1440 min) after induction, the cells were washed once with PBS, trypsinised and collected by centrifugation. Apoptotic uninfected cells that became rounded at 360 min and had started to detach from the flask at 600 min were also collected.

2.3. RNA extraction

For each collection point, total RNA was isolated from *T. cruzi*-infected and uninfected cells using TRIzol (Gibco, BRL, Tokyo, Japan); the RNA concentration was determined spectrophotometerically (BioSpec-1600, Shimadzu, Kyoto, Japan) and the purity of each preparation was confirmed by gel electrophoresis.

2.4. Probe labeling and microarray hybridization

Fluorescence-labeled cDNAs were prepared from total RNA using an RNA Fluorescence Labeling Core Kit (Takara, Kyoto, Japan) according to the manufacturer's instruction. Briefly, each 100 μg aliquot of total RNA from infected cells was labeled with Cy3, and each aliquot from uninfected cells was labeled with Cy5. The two labeled probes were mixed and hybridised to a human apoptosis chip slide (IntelliGene human Apoptosis chip Ver. 1.1, http://www.takara.co.jp/bio/goods.html, Takara, Kyoto, Japan), which contained 164 apoptosis-related genes spotted in pairs at two different positions on the slide. Since the results of individual sets of data were comparable, a representative data set for each gene expression profile was shown. The DNA chip was specific for human genes, and cross hybridization with parasite cDNA was negligible (see Table 2), since human and *T. cruzi* orthologs had less than 70% identity, except for ubiquitin, which was 78.1% identical (http://tcruzidb.org/). After washing, each glass slide was scanned in a GMS418 scanner (Takara, Kyoto, Japan) and the intensity of each spot was calculated and exported to Excel files using a computer package, ImaGene Ver. 3.0 (BioDiscovery Inc., LA, USA).

2.5. Data analysis

Data were further analysed using a computer package, GeneSpring™ Ver. 6.0 (Silicon Genetics, Redwood City, CA, USA). After subtracting the background intensity, the sum of Cy3 intensities of all spots on each slide was adjusted to that of Cy5 intensities of all spots on the same slide for normalization. To support the validity of this normalization, it was confirmed that the control spot for tubulin β2 gene on each DNA chip showed essentially

almost the same intensities between Cy3 and Cy5 signals. For the comparisons between Fas treatment groups, scaling factors were applied to normalise the sum of intensities of all spots on each DNA chip slide. For each time point after Fas stimulation, the ratio of expression of each gene in the infected group relative to that in the uninfected group was calculated and subjected to K-mean cluster analysis. Genes having a fluorescence intensity ratio appearing at a minimum of four of the nine time points and filtered by the 'Expression level (0.01–94.3) program' ($n=134$) were subjected to 'One-way t-test' algorithm in GeneSpring™ for the calculation of P-values.

2.6. Probes for Northern blot analysis

Probes for human apoptosis inhibitor 1 (c-IAP1), cellular FLICE inhibitory protein (c-FLIP), xeroderma pigmentosum, complementation group G (XPG), caspase-4 and GAPDH were generated by PCR using the primer sets listed in Table 1. Total RNA (15 μg) from HT1080 cells was converted to cDNA by reverse transcription using random hexamer primers. The probes were amplified by PCR from the cDNA fragment, DIG-labeled by random priming using Probe Synthesis Kit (Roche, Mannheim, Germany), and heated to 95 °C for use for Northern blots.

2.7. Northern blot analysis

Total RNA (10 μg) aliquots were electrophoresed on formaldehyde-containing 1% agarose gels in MOPS buffer. The gels were stained with ethidium bromide to confirm RNA integrity and normalization, and the RNA was transferred to nylon membranes (Roche, Mannheim, Germany). Each membrane was prehybridised for 1 h, and DIG-labeled probe was added at a final concentration of 10 ng/ml. The prehybridization and hybridization temperatures were 42 °C. After hybridization for 16 h, the membrane was washed three times for 5 min each at 42 °C with 2×SSC containing 0.1% SDS, once with 1× SSC containing 0.1% SDS for 5 min at 42 °C, and three times for 5 min each with 2×SSC at room temperature.

Table 1
Primers for probes of Northern blot analysis

Target gene	Sense (S) and antisense (AS) primers	Sequence
c-IAP1	S	5′-ccaggtccctcgtatcaaaa-3′
	AS	5′-aaaccagcacgagcaagact-3′
c-FLIP	S	5′-gagttggagaaactaaat-3′
	AS	5′-acactctgggagcctcct-3′
XPG	S	5′-cagacacagctccgaattga-3′
	AS	5′-ttctgggtttttcgttttgc-3′
caspase-4	S	5′-gaaggacaaacccaaggtca-3′
	AS	5′-acttcctctaggtggcagca-3′
GAPDH	S	5′-gtcagtggtggacctgacct-3′
	AS	5′-tgagcttgacaaagtggtcg-3′

Substrate was developed using the CSPD chemiluminescence detection system (Roche, Mannheim, Germany) according to the manufacturer's instructions.

3. Results

3.1. Transcriptional responses to T. cruzi infection

Gene expression profiles of *T. cruzi*-infected group were compared with those of uninfected group; a list of raw data is available on the web site http://www.med.juntendo.ac.jp/kenkyu/16-kiseichu/top.htm. The 164 human apoptosis-related genes analysed by DNA chips had a signal intensity spanning $10–10^5$ fluorescent units. Ten of the genes (13 spots) were outside the filtering criteria, indicating more than three-fold higher (putatively up-regulated) or lower (putatively down-regulated) fluorescence intensity (Fig. 1, red spots). In the absence of Fas stimulation, *T. cruzi*-infected group had 3.6- to 3.9-fold higher fluorescence intensities of genes encoding TNF-α (TNF superfamily member 2, NCBI UniGene ID: Hs. 241570), insulin-like growth factor binding proteins 5 (Hs. 380833) and 6 (Hs. 274313), caspase-4 (Hs. 74122), mitogen-activated protein

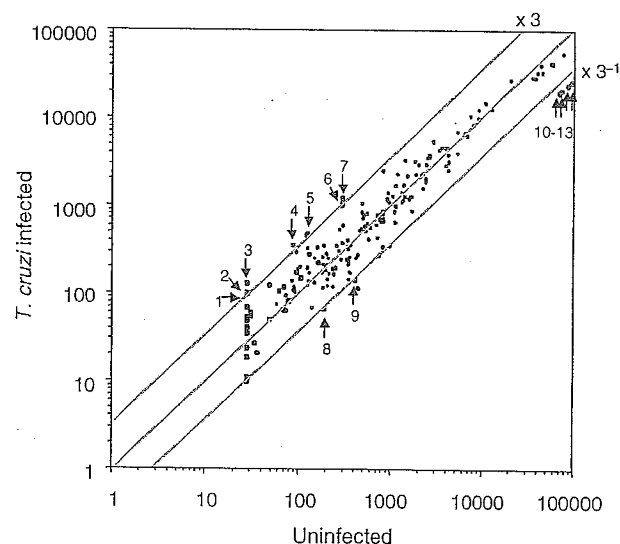

Fig. 1. Scatter plot analysis of a set of human apoptosis-related genes in *Trypanosoma cruzi*-infected and uninfected groups. The hybridization signal for each gene was plotted as a single point for probes generated from *T. cruzi*-infected and uninfected cell samples. Fas-stimulated apoptosis was not induced. Each point represents the ratio of Cy5 (horizontal axis) to Cy3 (vertical axis) fluorescence signals (log scale). Ten genes (13 spots) with greater than three-fold differences in expression levels induced by *T. cruzi* are represented by individual red spots: 1, insulin-like growth factor binding protein 5 (Hs. 380833); 2, mitogen-activated protein kinase 10 (Hs. 151051); 3, TNF-α (Hs. 241570); 4, insulin-like growth factor binding protein 6 (Hs. 274313); 5, c-FLIP (Hs. 195175); 6, cyclin A2 (Hs. 85137); 7, caspase-4 (Hs. 74122); 8, cyclin D2 (Hs. 75586); 9, ectodermal-neural cortex (Hs. 104925); 10, 11, 12, and 13, GAPDH (Hs. 169476). The reproducibility of the hybridization data was confirmed by performing a second identical experiment.

kinase 10 (MAPK10, Hs. 151051), c-FLIP (Hs. 195175), and cyclin A2 (Hs. 85137) relative to expression in uninfected group (Fig. 1). In contrast, the expression of GAPDH (Hs. 169476) gene was 3.5 times lower in infected group compared with uninfected group in the absence of Fas stimulation. In addition, the expression of genes encoding cyclin D2 (Hs. 75586), ectodermal-neural cortex (Hs. 104925) and β-actin (Hs. 520640) were down-regulated by *T. cruzi* infection. Transcriptional levels of most other genes were not affected significantly by *T. cruzi* infection (Fig. 1).

3.2. Gene expression in T. cruzi-infected cells after Fas stimulation

When we assayed gene expression in uninfected HT1080 group in response to an apoptosis signal, we found that the level of expression of most of genes was not changed after Fas stimulation in the uninfected group. In contrast, the expression of genes encoding ubiquitin (Uba 80, NCBI UniGene ID: Hs. 311640, 6.0-fold higher at 10 min), superoxide dismutase 1 (Hs: 443914, 4.7-fold higher at 120 min), GAPDH (6.7-fold lower at 60 min), and β-actin (3.1-fold lower at 120 min) (http://www.med.juntendo.ac.jp/kenkyu/16-kiseichu/top.htm) were significantly altered by the induction of apoptosis. In *T. cruzi*-infected group, Fas stimulation resulted in four distinct gene expression profiles (Fig. 2). Curiously, both pro- and anti-apoptotic genes were associated with clusters 1–4. Of the genes having more than 20-fold higher fluorescence intensities in *T. cruzi*-infected compared with the uninfected group at any point, eight were pro-apoptotic, nine were anti-apoptotic, and three fell into other functional categories (Table 2).

In the apoptosis signaling pathway, Fas activates the caspase cascade, resulting in cell death. We found that, in *T. cruzi*-infected group, the level of expression of caspases-1, -4, and -7, were up-regulated 43.5-, 43.3-, and 31.8-fold, respectively, 10 min after Fas stimulation (Fig. 2). Caspase-3, an effector caspase, was classified into cluster 1 and found to be up-regulated 3.44-fold 180 min after apoptosis induction in *T. cruzi*-infected

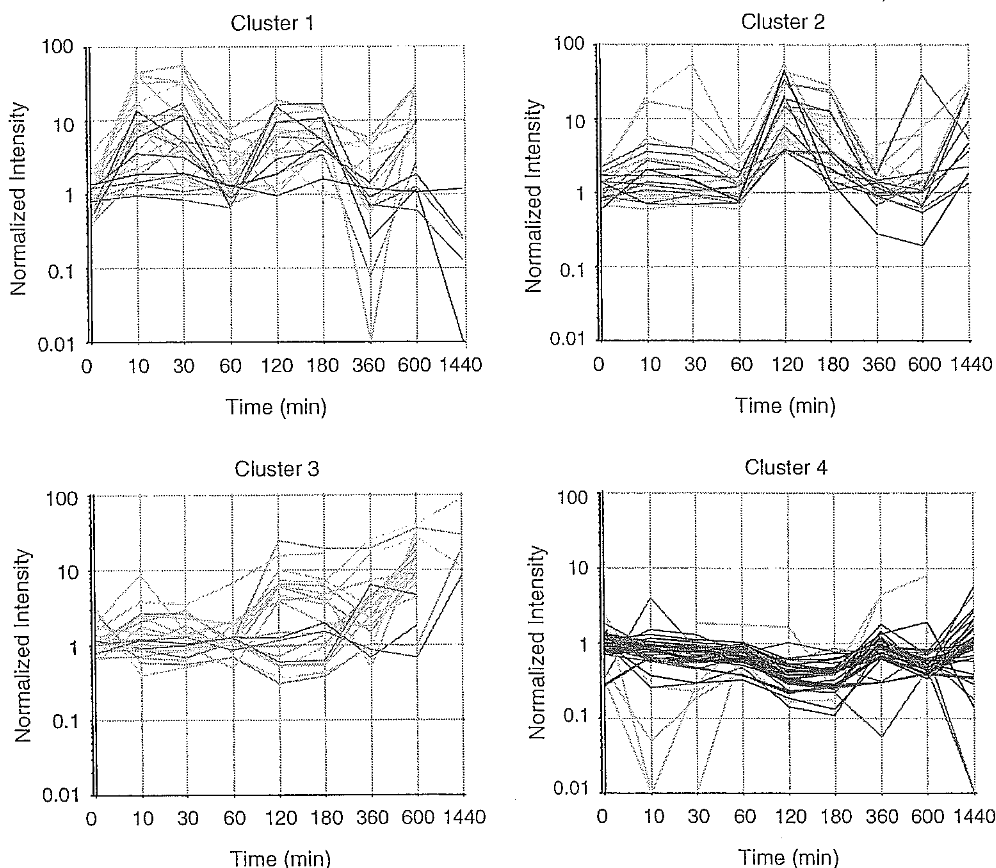

Fig. 2. Gene expression profiles clustered according to similarities of time-course expression patterns using K-mean cluster analysis. In response to Fas stimulation, the 134 genes were classified into five clusters. Cluster 1 included 26 genes whose level of expression increased rapidly (10 and 30 min) after the induction of apoptosis; cluster 2 included 25 genes showing maximum expression at 120 and 180 min; cluster 3 included 21 genes up-regulated at >240 min after Fas stimulation; and cluster 4 included 55 genes that showed no alteration in expression level in response to Fas stimulation. A fifth, unclassified cluster included seven genes that had poor quality on individual DNA chip slides. Note that genes for β-actin, tubulin, ubiquitin, and GAPDH were classified into cluster 4 and that the level of expression of GAPDH mRNA was lower in *Trypanosoma cruzi*-infected than in uninfected group at all time points.

Table 2
Up-regulated or down-regulated genes in response to Fas stimulation in *Trypanosoma cruzi*-infected cells

Gene name	UniGene ID	Maximal expression		Cluster	*P*-value[a]	% Homology with *T. cruzi*[b] (length in a.a.)	*T. cruzi* TSKTSCC ID
		Fold of change	Peak in the time-course (min)				
Up-regulated genes							
Pro-apoptotic genes							
Promotion of Fas mediate apoptosis cascade							
caspase-1	Hs. 2490	56.5	30	1	0.061	none	
caspase-4	Hs. 74122	59.0	1440	3	0.059	none	
caspase-7	Hs. 9216	31.8	10	1	0.015	none	
Fas (TNF receptor superfamily, member 6)	Hs. 82359	36.2	600	3	0.066	25% (143)	7038
Inhibition of NF-κB activation							
NF-κB inhibitor, ε	Hs. 458276	34.1	120	2	0.028	33% (106)	4791
v-rel avian reticuloendotheliosis viral oncogene homolog B (I-REL)	Hs. 307905	53.8	60	2	0.065	31% (125)	5891
TRAF family member-associated NF-κB activator (I-TRAF)	Hs. 146847	56.4	30	1	0.053	35% (53)	4808
Inhibition of cell proliferation							
menage a trios 1 (CAK assembly factor)	Hs. 72870	29.6	120	2	0.048	30% (158)	8797
Anti-apoptotic genes							
Apoptosis inhibition							
apoptosis inhibitor 1 (c-IAP1)	Hs. 289107	40.8	10	1	0.065	29% (116)	4687
c-FLIP (caspase-8 and FADD-like apoptosis regulator)	Hs. 355724	33.2	30	1	0.078	None	
bcl-w (bcl2-like2)	Hs. 410026	28.9	30	1	0.090	None	
DNA repair							
xeroderma pigmentosum, complementation group G (XPG)	Hs. 258429	43.8	120	2	0.037	29% (274)	7172
Activation of NF-κB							
NF-κB 1 enhancer, p105 (KBF1)	Hs. 160557	55.3	120	2	0.052	31% (232)	7635
Promotion of cell proliferation							
cell division cycle 2 (CDC2)	Hs. 334562	20.7	1440	3	0.087	54% (291)	7051
CDC-like kinase 3 (Clk3)	Hs. 511790	22.5	1440	2	0.073	35% (353)	8603
cyclin A2	Hs. 85137	33.3	1440	3	0.064	34% (269)	7062
junB proto-oncogene	Hs. 25292	26.7	60	2	0.051	34% (103)	8447
Others							
prostaglandin E synthase	Hs. 146688	41.5	10	1	0.063	53% (28)	6711
DNA-damage-inducible transcript 3	Hs. 392171	44.6	120	2	0.037	45% (131)	4660
growth factor receptor-bound protein 2	Hs. 411366	31.1	120	2	0.055	40% (52)	6866
Down-regulated genes							
β-actin	Hs. 520640	−3.8	180	4	0.557	70% (375)	8434
GAPDH	Hs. 169476	−4.6	120	4	0.534	64% (331)	8328
Ubiquitin	Hs. 311640	−4.3	180	4	0.544	96% (76)	6526

[a] *P*-value of One-way *t*-test in up-regulated genes <0.09.
[b] Blast search of each ortholog was performed in TcruziDB (http://tcruzidb.org/).

group. Up-regulation of the gene encoding Fas was detected 120–600 min after Fas stimulation of infected group. In addition, genes inhibiting activation of a transcriptional factor, NF-κB, were up-regulated 10–120 min after exposure to Fas.

The expression of anti-apoptotic genes was also higher 10 and 30 min after Fas stimulation of infected group (Fig. 2). For example, the fluorescence intensity of cellular inhibitor of apoptosis protein (c-IAP1) cDNA was 40.8- and 33.9-fold higher after 10 and 30 min, respectively, and the level of expression of c-FLIP, an inhibitor of procaspase-8 activation, was 33.2-fold higher at 30 min. When caspase-8 is activated, the apoptosis signal is transmitted to mitochondria, which release cytochrome *c*, followed by activation of procaspase-9. Bcl-w (Bcl2-like 2), a member of the Bcl-2 family, is an anti-apoptotic regulator involved in the release of cytochrome *c* from mitochondria. Following induction of apoptosis of *T. cruzi*-infected cells, the expression of bcl-w gene was up-regulated after 30 and 600 min, and the expression of the bcl-2 gene was 3.8-fold up-regulated after 60 min. In addition, genes related to cell cycle promotion, including CDC2, CDC-like kinase 3, and cyclin A2, were up-regulated, whereas genes encoding GAPDH and β-actin were down-regulated, in infected cells. In all time points after Fas stimulation, the expression of tubulin β2 gene was stable in both the *T. cruzi*-infected and uninfected groups.

3.3. Validation of microarray data by Northern blot analysis

To confirm the results of microarray hybridization, we performed Northern blot analysis, an independent method of measuring levels of gene expression. We assayed the expression of four genes found to be up-regulated and one found to be down-regulated in *T. cruzi*-infected cells by microarray analysis. The expression of c-IAP1 and c-FLIP, which had been classified into cluster 1, appeared to be higher at 0, 60, and 180 min after Fas stimulation of infected cells (Fig. 3). In addition, we detected alternative spliced variants of c-FLIP mRNA, which ranged in size from 1.0 to 2.2 kb, all of which were up-regulated in infected cells. At 180 min after Fas stimulation, expression of the XPG gene, which had been classified into cluster 2, was markedly higher in infected cells compared with uninfected cells. At most time points, the expression of mRNA encoding caspase-4, a cluster 3 gene, was at higher levels in infected cells than in uninfected cells. In contrast, the level of mRNA encoding GAPDH, which was classified into cluster 4, was lower in infected than in uninfected cells. These results were therefore in substantial agreement with the microarray data.

4. Discussion

Expression profiles of apoptosis-related genes in cells infected with *T. cruzi* may provide important clues as to how

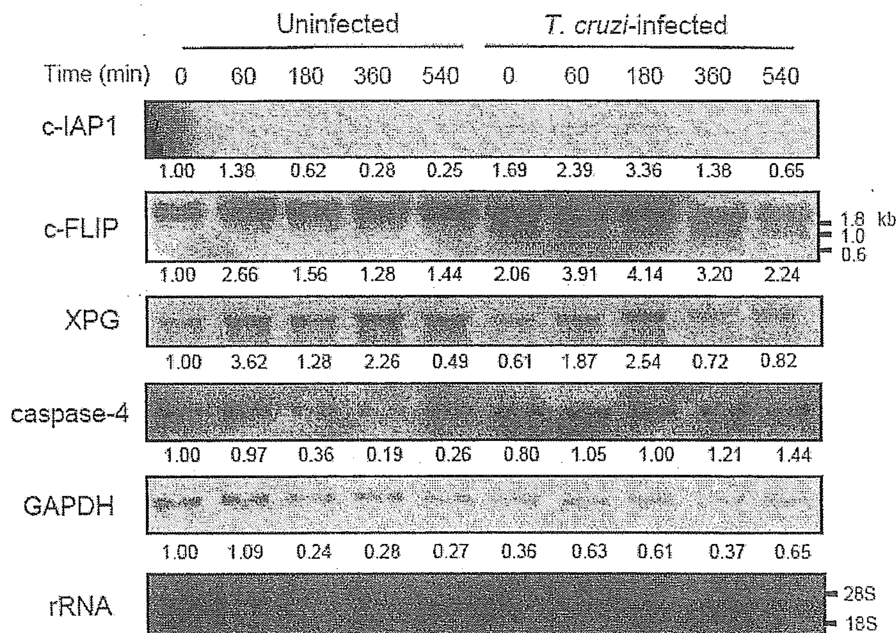

Fig. 3. Validation of transcription patterns of c-IAP1, c-FLIP, XPG, caspase-4 and GAPDH genes in uninfected and *Trypanosoma cruzi*-infected HT1080 cells after Fas stimulation. Cells were incubated with anti-Fas Ab (0.5 μg/ml) for the indicated times; 10 μg aliquots of total RNA isolated from the cells were fractionated on 1% agarose gels, and the blots were hybridised with DIG-labeled probes for each gene. Messages encoded by the c-IAP1 (cluster 1), c-FLIP (cluster 1), XPG (cluster 2), and caspase-4 (cluster 3) genes showed higher levels of expression at most time points in *T. cruzi*-infected than in uninfected cells, where the level of GAPDH mRNA (cluster 4) was lower in infected cells. Blots were densitometrically analysed by MasterScan (Scanalytics, Billerica, MA), and the intensity of each band was normalised relative to that of 28S rRNA in each sample and further normalised relative to band intensity in uninfected cells at time 0. For c-FLIP, the values are the sum of the intensities of both isoforms at each time point.

host cells defend against protozoan infections and respond to apoptosis-triggering signals. Although the expression profile of *T. cruzi*-infected cells has been reported (Moore-Lai and Rowland, 2004; Vaena de Avalos et al., 2002), this study is the first, to our knowledge, to use microarray analysis to compare the time-course of apoptosis-related gene expression in *T. cruzi*-infected and uninfected groups after Fas stimulation. Responses after apoptosis induction may assess secondary gene activation, the consequences of which can offer insights into control mechanisms and their downstream effects. In addition, assays of time-course profiles rather than one time point may help determine the dynamics of gene expression in *T. cruzi*-infected cells after the induction of apoptosis.

Prior to Fas stimulation (time 0), the levels of expression of GAPDH and β-actin were lower in the *T. cruzi*-infected than in the uninfected group. Cell growth was shown to be delayed in heavily infected cells, which contain more than 10 parasites per host cell (Nakajima-Shimada et al., 1994). Although very little change in transcription has been reported in cells infected with at least one parasite per cell (Vaena de Avalos et al., 2002), we detected 10 genes with three-fold higher or lower fluorescence intensity in the infected group. These differences may be due to the degree of infection, in that transcriptional responses to *T. cruzi* infection may depend on the number of parasites per host cell. Our finding, that TNF-α mRNA was highly expressed in the *T. cruzi*-infected group prior to Fas stimulation (time 0), was similar to results obtained in the experimental Chagasic model mice (Chandrasekar et al., 1996). That is, heart cells of infected mice express high levels of mRNA encoding the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 (Chandrasekar et al., 1996). *Trypanosoma cruzi* infection down-regulates TNFR1 (Moore-Lai and Rowland, 2004) in the mouse fibroblasts.

Unexpectedly, we found that both pro- and anti-apoptotic genes were up-regulated in the *T. cruzi*-infected group after Fas stimulation. Among the genes up-regulated in response to Fas stimulation were those encoding caspases. The caspase-4 gene is located within the caspase-1 (IL-1β converting enzyme) gene cluster on chromosome 11q22-23, and both caspases are activated during inflammatory responses (Martinon et al., 2002). Thus, these genes may be involved in proinflammatory responses rather than apoptosis. Caspase-3 gene expression was not up-regulated significantly after Fas stimulation, and the caspase-8 gene was not assayed in these experiments. Although all of these caspase genes were found to be up-regulated in rat brains in response to ischemia (Harrison et al., 2001), their responses to Fas signals were not assayed. In human intestinal epithelial cells, Fas stimulation did not induce the expression of caspase-3 protein (Martin and Panja, 2002).

We previously showed that Fas-mediated apoptosis was inhibited by *T. cruzi* infection, suggesting that anti-apoptotic gene(s) may play a crucial role in infected cells. We found that expression of three apoptosis inhibitory genes, c-IAP1, c-FLIP and bcl-w, were up-regulated soon after Fas stimulation. The c-IAP1 gene product directly inhibits caspases-3 and -7 (Roy et al., 1997) and can prevent TNF-α-induced apoptosis by degradation of TNF receptor-associated factor 2 (TRAF2) (Li et al., 2002).

One proximal regulator of the Fas-induced death program is c-FLIP. FLIP was originally identified as a virus-encoded apoptosis-inhibitory protein, and its cellular homolog (c-FLIP) also has the capacity to interfere with DISC function (Tschopp et al., 1998) and has a key role in the regulation of apoptosis. The c-FLIP gene has been reported to have 11 spliced variants (Djerbi et al., 2001) and alternative splicing generates two isoforms of c-FLIP mRNA: a long form (c-$FLIP_L$), which contains a caspase-like domain but is devoid of caspase catalytic activity; and a short form (c-$FLIP_S$), which lacks the caspase-like domain. Since the alternatively spliced variants could not be detected by microarray technique analysis or reverse transcription-PCR, we performed Northern blot experiments. We observed that *T. cruzi* infection up-regulated both c-$FLIP_L$ (2.2 kb) and c-$FLIP_S$ (1.0 kb) mRNA. Our previous finding, that caspase-8 activity was inhibited in *T. cruzi*-infected cells after Fas stimulation (Nakajima-Shimada et al., 2000), suggests that c-FLIP may be a candidate molecule in the inhibition of apoptosis.

Bcl-w, a member of bcl-2 gene family, is an anti-apoptotic regulator that prevents the release of cytochrome *c* from mitochondria (Djerbi et al., 2001). Various apoptotic stimuli trigger the integration of Bcl-w into the mitochondrial membrane (Wilson-Annan et al., 2003; Borner, 2003), and Bcl-w has been shown to mediate the survival of prostate sertoli cells in mice (Kaufmann et al., 2004; Print et al., 1998). However, the involvement of Bcl-w in the inhibition of Fas-mediated apoptosis is not known.

We also found that the expression of bcl-2 mRNA was up-regulated slightly after the induction of apoptosis. A *T. cruzi* trans-sialidase has been shown to trigger bcl-2 gene expression in growth factor-deprived neuronal cells (Chuenkova and Pereira, 2000). However, the level of expression of Bcl-2 protein did not increase in *T. cruzi*-infected murine fibroblasts (Clark and Kuhn, 1999). We found that the level of expression of other anti-apoptotic genes, such as c-IAP2 or apoptosis inhibitor 4 (survivin), were not significantly altered. Although it is difficult to determine which anti-apoptotic genes act in *T. cruzi*-infected cells, any of these four genes, c-IAP1, c-FLIP, bcl-w or bcl-2, would be a promising candidate for involvement in the inhibition of host cell apoptosis. There is also the possibility that the *T. cruzi* genome possesses a gene that inhibits host cell apoptosis. Indeed, if the parasite modulates the regulatory mechanism of apoptosis and utilises the apoptosis inhibitory machinery of host cells, it would be an ingenious strategy to escape from host defense mechanisms.

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