Cell, Vol. 82, 287–296, July 28, 1995, Copyright © 1995 by Cell Press

Trypanosome Invasion of Mammalian Cells Requires Activation of the TGFβ Signaling Pathway

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Summary

Trypanosoma cruzi invades most nucleated mammalian cells by as yet unknown mechanisms. We report here that while T. cruzi attaches to epithelial cells lacking signaling transforming growth factor β (TGFβ) receptor I or II, the adherent parasites cannot penetrate and replicate inside the mutant cells, as they do in parental cells. Invasion of the mutants is restored by transfection with the TGFβ receptor genes, as are biological responses to TGFβ. Similar rescue of both TGFβ antiproliferative response and T. cruzi invasion was demonstrated in a hybrid of TGFβ-resistant bladder and colon carcinoma cells. In addition, T. cruzi did not efficiently invade epithelial cells with dysfunction of the intracellular signaling cascade caused by the constitutive expression of the cyclin-dependent kinase cdk4 or of the oncogene H-*ras*. Treatment with TGFβ, but not with other antiproliferative agents of nonphagocytic cells, greatly enhances T. cruzi invasion. Moreover, infective, but not noninfective, trypanosomes strongly induce a TGFβ-responsive reporter gene in TGFβ-sensitive, but not in TGFβ-insensitive, cell lines. Thus, T. cruzi itself may directly trigger activation of the TGFβ signaling pathway required for parasite entry into the mammalian cells.

Introduction

Trypanosoma cruzi is an obligate intracellular parasite, causing a chronic debilitating illness, Chagas' disease, in millions of people in Latin America. It infects most cell types, including fibroblasts, epithelial cells, endothelial cells, myocytes, and macrophages. Trypomastigote, the infective form of T. cruzi, adheres to specific receptors on the outer membrane of host cells as a prelude to intracellular invasion. Adhesion is mediated by at least two parasite ligands that react with carbohydrate receptor epitopes on the host cell surface (Pereira, 1994). One of the ligands is the trans-sialidase, which promotes attachment by reacting with α2,3-linked sialyl epitopes, and the other is penetrin, which reacts with host cell receptors containing heparan sulfate moieties.

Trypomastigote binding to the host cell surface does not, by itself, guarantee access of the parasite into the intracellular environment. In fact, it is possible to prevent adherent parasites from entering culture cells by blocking early metabolic responses that arise during host cell–parasite interactions (Tardieux et al., 1994; Moreno et al., 1994; Yakubu et al., 1994) and by altering lysosome traffic in the mammalian cells (Tardieux et al., 1992).

T. cruzi invasion may therefore require activation of specific signaling pathways critical for parasite entry into host cells. This hypothesis is consistent with current concepts of cellular invasion by virus and bacteria, which can activate surface membrane signaling molecules in their interplay with mammalian cell hosts (Gooding, 1992; Bliska et al., 1993). As an attempt to find the nature of these signaling pathways, we measured T. cruzi invasion of cell lines activated with various growth factors or genetically resistant to cytokine stimulation. We found that mink lung epithelial cells with a defect in the genes encoding signaling receptors I and II for transforming growth factor β (TGFβ) were resistant to TGFβ (Laiho et al., 1990; Wrana et al., 1992) as well as to T. cruzi invasion. Although T. cruzi could not invade the mutant cells, they attached to them at the same or at higher levels than to parental cells. Transfection of the mutants with plasmids carrying wild-type receptor genes restored response both to TGFβ action and T. cruzi invasion. T. cruzi invasion of other cell lines is also consistent with a requirement for TGFβ signaling. This signaling pathway may be activated by a factor secreted by infective trypomastigotes, as suggested by the results with a TGFβ-responsive reporter gene.

TGFβ induces signals in mammalian cells that lead to many types of biological activities, including regulation of cell proliferation and differentiation, extracellular matrix deposition, and modulation of many inflammatory and immune responses (Roberts and Sporn, 1990; Massagué, 1990; Miyazono et al., 1994). It is now apparent that TGFβ signaling in nonphagocytic cells is subverted by a microbial invader to gain access into a privileged niche, the host cell cytoplasm.

Results

Epithelial Cell Mutants Lacking TGFβ Receptor I or II Are Nonpermissive to T. cruzi Infection

Mink lung epithelial cells resistant to TGFβ action were originally isolated by chemical mutagenesis. One mutant phenotype, class R, expresses receptor II but not receptor I, while another phenotype, class DR, expresses receptor I but not receptor II (Laiho et al., 1990; Wrana et al., 1992).

To determine whether the R phenotype was permissive to T. cruzi infection, we incubated mutant and parental cells (Mv1Lu) with various concentrations of trypomastigotes at 37°C for 2 days to allow differentiation into, and replication of, intracellular amastigotes (Pereira, 1990). Remarkably, R-1B cells were extremely resistant to T. cruzi infection in a wide range of parasite concentrations

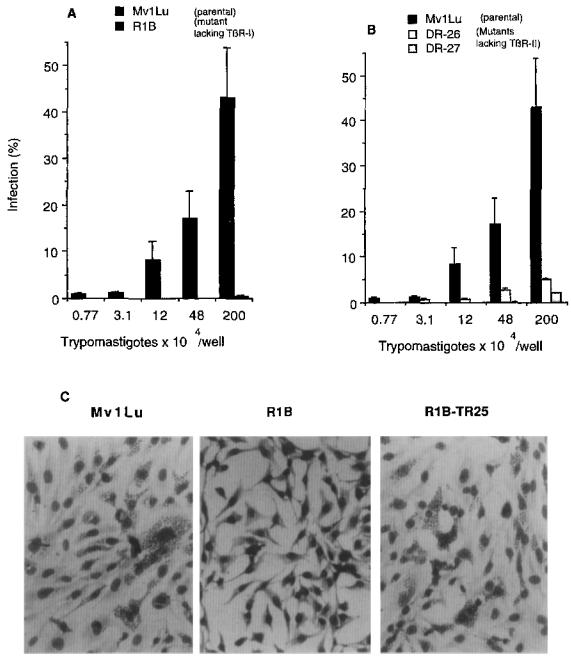

Figure 1. Invasion of Parental and TβR Mutant Cells by T. cruzi

Infection of parental and mutant cells R-1B (A) and parental and mutant cells DR-26 and DR-27 (B) with T. cruzi trypomastigotes. Cytology of the infection (40:1 ratio of T. cruzi to host cell) of parental Mv1Lu cells, R-1B cells, and R-1B cells transfected with *ALK-5* gene (clone R1B-TR25) 2 days after start of infection (C). Please note in (C) the abundance of intracellular amastigotes in most parental and transfected R-1B cells, in contrast with the rarity of parasites found in mutant R-1B cells. Magnification, 295×. Results similar to those above were reproducible in at least eight other experiments.

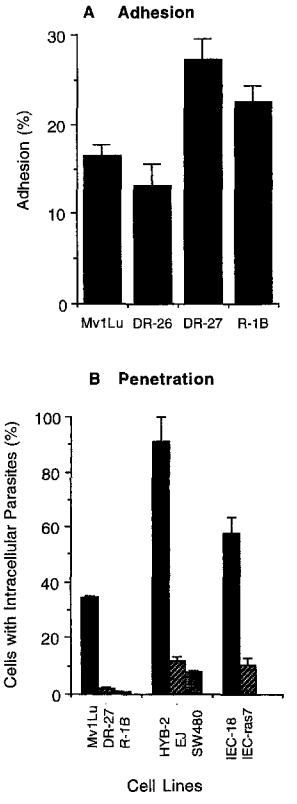

Figure 2. Adhesion and Penetration of T. cruzi into Epithelial Cell Lines

Adhesion (A) and penetration (B) of trypomastigotes to the indicated cell lines was determined as described in the Experimental Procedures.

(Figures 1A and 1C). At the highest trypomastigote inoculum (2×10^6 parasites/monolayer), 43.1% ± 10.7% of parental Mv1Lu cells were infected by T. cruzi compared with only 0.5% ± 0.1% for R-1B cells, representing >80-fold increase in resistance to infection in the R phenotype (Figures 1A and 1C).

Two types of DR mutant cells (DR-26 and DR-27) were tested for their potential to be infected by T. cruzi. The TGFβ receptor II (TβR-II) in DR-26 cells contains a single base mutation that transforms the seventh codon of the transmembrane region into a stop codon, and the TβR-II in DR-27 contains a single base mutation that converts the third cysteine of the extracellular domain to tyrosine (Wrana et al., 1992). The TβR-I in both DR-26 and DR-27 contains no mutation (Wrana et al., 1992). When these DR cell lines were tested for their potential to be infected by T. cruzi, they were found to be highly resistant to invasion (Figure 1B).

T. cruzi Attaches to, but Cannot Penetrate, Epithelial Cells Expressing Defective TβR-I or TβR-II

The resistance of R and DR mutants to being infected by T. cruzi could result from the inability of the parasites to attach to surface receptors, to enter the cells, or to differentiate and replicate intracellularly. To determine whether adherence was a mechanism of resistance, trypomastigotes were incubated with glutaraldehyde-fixed monolayers of parental and mutant cells. These cells should allow binding but not invasion of the parasites (Ortega-Barria and Pereira, 1991). We found that T. cruzi attached to the mutant cells at levels similar to or higher than those supported by parental cells (Figure 2A). Therefore, the inability of T. cruzi to infect the TβR mutant cells was not at the level of adhesion receptors.

To determine whether the adherent parasites were capable of penetrating the mutant cells, parasites were incubated with live monolayers of Mv1Lu, R, and DR cells for 1 hr to allow attachment and entry. The monolayers were washed to remove unbound trypanosomes and fixed with glutaraldehyde, making the outer membrane impermeable to macromolecules. In this way, residual extracellular parasites could be quantitated with polyclonal antibodies against T. cruzi, and intracellular and extracellular parasites with DNA stains such as DAPI (Schenkman et al., 1991). We found that trypomastigotes entered parental but not TβR mutant cells (Figure 2B). The inability of T. cruzi to grow in the R and DR mutants results, therefore, from a defect in penetration of the epithelial cells. Similar inability to penetrate was observed in the interaction of T. cruzi with TGFβ-resistant colon (SW480) and bladder (EJ)

carcinoma cell lines (Figure 2B; Geiser et al., 1992; see below). A TGFβ-susceptible hybrid of the two cell lines (HYB-2) was more permissive to T. cruzi penetration than parental cells (Figure 2B). Moreover, T. cruzi did not efficiently enter a TGFβ-resistant H-*ras*-transformed rat cell line (IEC–ras7) (Figure 2B), whereas it invaded the TGFβ-sensitive parental IEC-18 cells (Figure 2B; Filmus et al., 1992; see below).

Restoration of Permissiveness to T. cruzi Infection by Transformation with TβR Genes

To assess the ability of signaling TβRs to mediate T. cruzi invasion, we stably transfected DR-27 cells with a human TβR-II cDNA (Lin et al., 1992). Remarkably, transfected DR-27 cells became permissive hosts for T. cruzi invasion at levels comparable to those of wild-type Mv1Lu (Table 1). This confirmed that the nonpermissiveness of DR-27 cells was due to a defect in their signaling pathway at the level of the TβRs. The transfectant clones expressed human TβR-II on their surface because TGFβ1 inhibited [^{3}H]thymidine incorporation into DNA of the growing cells in response to TGFβ (Figure 3A). To prove this point further, the transfectants were treated with radioiodinated TGFβ1 and incubated with the chemical cross-linker disuccinimidyl suberate to label the TβRs (Massagué, 1987). The labeled receptors were then resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by autoradiography. While the DR-27 mutant showed a concomitant loss of receptors I and II, the transfectant clones showed a normal receptor labeling pattern (Figure 3B) as in reported results (Wrana et al., 1992).

Likewise, transfection of resistant R-1B cells with TβR-I gene (*ALK-5*) (Franzén et al., 1993) restored T. cruzi invasion to levels comparable to those of parental cells (Figure 1C). Transfected R-1B clones, such as R-1B–TR25 shown in Figure 1C, were growth arrested by TGFβ1 in a kinetics and dose response similar to that with parental Mv1Lu cells (M. M. and M. E. A. P., unpublished data).

Table 1. Transformation of DR-27 Mutant Cells with a Plasmid Encoding the Human TβR-II Gene Restored Permissiveness to T. cruzi Infection

Trypomastigote Inoculum[a] (Parasites/200 μl) × 10^4	Infection (%) ± SD[b]		
	Mv1Lu	DR-27	DR27-TR47[c]
0.8	0.3 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
3.1	1.6 ± 0.4	0.1 ± 0.0	0.9 ± 0.0
12.5	7.0 ± 1.0	0.6 ± 0.0	5.7 ± 0.7
50.0	24.5 ± 4.2	0.9 ± 0.0	15.8 ± 2.6
200.0	32.3 ± 2.3	4.9 ± 1.1	26.2 ± 1.1

[a] Cell lines were incubated with the indicated concentrations of T. cruzi for 1 hr and, after removing unbound parasites by washing, they were further incubated at 37° C for 3 days. Infection was quantitated by determining the percentage of cells containing more than two amastigotes. Results are representative of three others and represent an average of quadruplicate points.

[b] Mv1Lu is the parental cell line of mutant DR-27, which lacks TβR-II; DR27-47 is the DR-27 mutant transfected with a plasmid encoding TβR-II (see Figure 3).

[c] Similar results were obtained with the transfectant DR27-TR50 (see text).

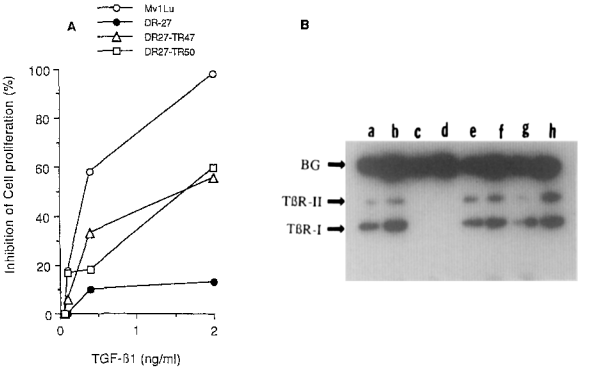

Figure 3. Characteristics of the Parental Mv1Lu, Mutant DR-27, and DR-27 Clones (DR27-TR47 and DR27-TR50) Transfected with Human TβR-II

(A) Inhibition of DNA synthesis by TGFβ1. Subconfluent monolayers of the various cell lines were incubated for 48 hr with the indicated concentrations of TGFβ1 and then for 18 hr with [^{3}H]thymidine. Results are plotted as percent inhibition of the [^{3}H]thymidine incorporation of cells grown in the absence of TGFβ1. Each point is the average of quadruplicate samples, and standard deviation was less than 5%.

(B) Cross-linking of [^{125}I]TGFβ1 to Mv1Lu, DR-27, and DR-27 cells transfected with human TβR-II. Cells were incubated with 50 pM [^{125}I]TGFβ1 and cross-linked with disuccinimidyl suberate before analysis by SDS–PAGE as described in the Experimental Procedures. Lanes a and b, Mv1Lu cells; lanes c and d, DR-27 cells; lanes e and f, transfectant DR27-TR47; lanes g and h, transfectant DR27-TR50. Lanes a, c, e, and g represent one experiment, and lanes b, d, f, and h represent another experiment. The three types of TβRs are indicated by arrows on the left (BG, β-glycan or receptor III).

Activation of Mammalian Cells with TGFβ1 Enhances Infection in a Specific Manner

The above results suggest that the TβRs are required for T. cruzi invasion. This opens the possibility that signaling through the receptors directs parasite invasion. As a first approach to investigate this possibility, we asked whether activation of the receptors facilitated T. cruzi invasion. For this, Mv1Lu cells were treated with TGFβ1 (1.0 ng/ml) for 2, 8, and 24 hr, washed to remove residual cytokine, and assayed for their ability to be infected by T. cruzi. The results show that TGFβ1-primed cells supported T. cruzi infection much more efficiently than nonsensitized cells (Figure 4A). Similar increase in infection was obtained by priming Mv1Lu cells with another isoform of TGFβ, TGFβ2 (data not shown). The rise in infection resulting from the sensitization with TGFβ required signal transduction through the TβRs because DR-27 cells did not become a better host after priming with TGFβ (Figure 4A). Furthermore, clones of DR-27 transfected with TβR-II behave like parental cells upon sensitization with TGFβ1 (Figure 4A). Sensitization with epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) did not affect T. cruzi invasion (data not shown).

TGFβ-activated cells did not become more permissive to another intracellular protozoan parasite, Toxoplasma gondii (Figure 4B), which resides inside cytoplasmic vesicles of a variety of mammalian cells (Schwab et al., 1994). Furthermore, T. gondii invaded DR-27 cells to the same extent as parental mink lung cells (Figure 4B). Thus, the

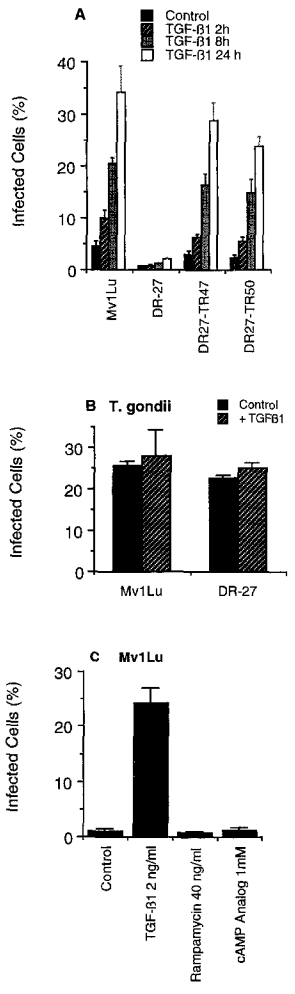

Figure 4. Specific Enhancement of T. cruzi Infection by TGFβ Sensitization of Epithelial Cells

(A) Parental Mv1Lu, mutant DR-27, and stable transfectants DR27-TR47 and DR27-TR50 were grown in the absence (control) or in the presence of TGFβ1 (2 ng/ml) for the indicated times and infected with T. cruzi (1×10^5 trypomastigotes/well for 2 hr, washed, and then further incubated for 2 days at 37°C).

(B) Parental and DR-27 cells without priming (control) or primed with TGFβ1 (1 ng/ml, 24 hr) were infected with T. gondii for 2 days.

(C) Mv1Lu cells were grown for 24 hr in the absence (control) or in the presence of the indicated concentrations of TGFβ1, rapamycin, and a cAMP analog (Bt2cAMP), and then infected with T. cruzi for 2 days as in (A).

Cell cycle distribution of subconfluent Mv1Lu cells treated with rapamycin (40 ng/ml for 21 hr) was as follows: G1 = 93.0%, S = 6.3%, and G2/M = 0.7%, compared with G1 = 48.8%, S = 16.9%, and G2/M = 34.3% for the control cells not treated with rapamycin.

increase in invasion by activation with TGFβ may be specific for T. cruzi.

Mv1Lu cells treated with TGFβ1 under the conditions described in Figure 4 are arrested at mid- to late-G1 phase of the cell cycle (Massagué, 1990). To determine whether the increased susceptibility to T. cruzi infection was a direct consequence of the G1 arrest, regardless of the cellular stimulus, we treated Mv1Lu cells with cyclic AMP (cAMP) or rapamycin for 24 hr prior to using them as host for T. cruzi. Under these conditions, cAMP arrests cell growth at G1 and G2 (Boynton and Whitfield, 1983; M. E. E., unpublished data) and rapamycin only at G1 (see legend to Figure 4C). Treatment of Mv1Lu cells with a cAMP analog or with rapamycin did not significantly increase T. cruzi invasion, compared with the sharp rise induced by TGFβ1 sensitization (Figure 4C). Because priming with rapamycin, which arrests the cell cycle at G1, and with the growth factors EGF and PDGF, which stimulate cell multiplication, did not enhance invasion of T. cruzi, the enhancement in TGFβ-activated Mv1Lu cells appeared to be specific for the antiproliferative cytokine.

Moreover, TGFβ1 priming of monkey fibroblasts (Vero cells) and bovine endothelial cells enhanced T. cruzi infection significantly, and the enhancement was blocked by TGFβ-neutralizing antibodies (M. M. and M. E. A. P., unpublished data), suggesting that T. cruzi may use the TGFβ signaling pathway to invade a variety of cell types. However, TGFβ-activated human or mouse macrophages did not become better hosts for T. cruzi (M. M. and M. E. A. P., unpublished data), suggesting that the TGFβ signaling pathway may not be critical for T. cruzi invasion of phagocytic cells.

Restoration of T. cruzi Invasion in a Hybrid of Two Nonresponsive Human Carcinoma Cell Lines

Further evidence that efficient T. cruzi invasion of host cells requires TGFβ signaling was provided by the response of a hybrid of two TGFβ-resistant human carcinoma cell lines. Previous studies showed that this hybrid cell line expresses much higher levels of surface TβR-II than parental EJ bladder carcinoma and the SW480 colon adenocarcinoma cells (Geiser et al., 1992). Consequently, the cell hybrid, but not the parental cell lines, is growth arrested in response to TGFβ (Figure 5A) (Geiser et al., 1992). When these cell lines were tested for permissiveness to T. cruzi invasion, the difference in host potential between the TGFβ-resistant carcinoma cells and a TGFβ-sensitive hybrid (HYB-2) cell line was very dramatic indeed (Figures 2B, 5B, and 5C). As already discussed above, trypomastigotes penetrated TGFβ-resistant parental carcinoma cells inefficiently compared with their invasion of the TGFβ-sensitive hybrid (Figure 2B). Moreover, T. cruzi infected poorly (less than 10%) parental carcinoma cells and robustly (nearly 80%) HYB-2 cells (Figures 5B and 5C). Furthermore, T. cruzi infected nearly all TGFβ-activated HYB-2 cells and only about 10% of TGFβ-activated parental carcinoma cells (Figure 5B). These findings further underscore the critical role of TGFβ signaling in promoting T. cruzi invasion of mammalian cells.

Constitutive Expression of the Cyclin-Dependent Kinase Cdk4 or Transformation with the Oncogene H-*ras* Renders Epithelial Cells Resistant to Both TGFβ Activation and T. cruzi Invasion

Normal progression through the G1 phase of the cell cycle is promoted by the activity of the cyclin-dependent protein kinases cdk2 and cdk4 (Sherr, 1993). In mink lung cells, TGFβ inhibits cellular proliferation largely by down-regu-

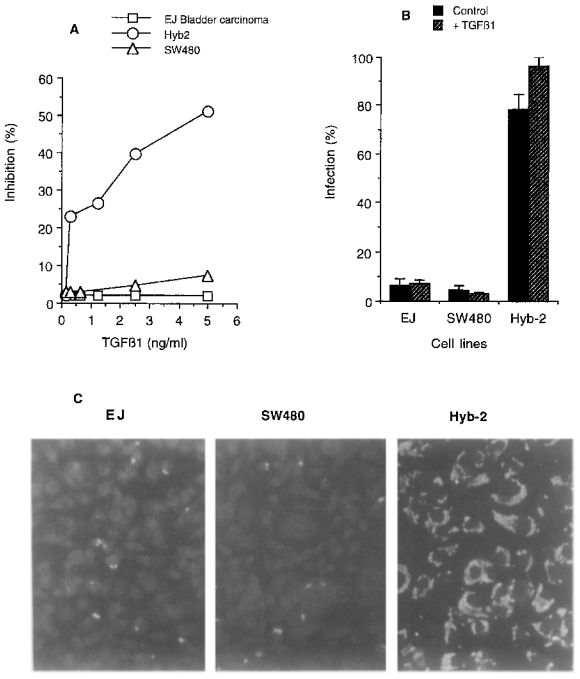

Figure 5. Response of EJ Bladder Carcinoma Cells, of SW480 Colon Carcinoma Cells, and of a Hybrid of the Two Carcinoma Cells to TGFβ1 and T. cruzi

(A) Inhibition of DNA synthesis in the three types of cell lines grown in the presence of the indicated concentrations of TGFβ1.

(B) T. cruzi infection of the three cell lines without (control) and with TGFβ1 priming (2 ng/ml, 24 hr). After priming or mock priming (absence of TGFβ1), cells were washed and infected with T. cruzi for 2 days.

(C) Fluorescence microscopy of EJ, SW480, and hybrid cell line HYB-2 infected with T. cruzi. Two days after infection, monolayers were fixed with formaldehyde and permeabilized with 0.1% Triton X-100, and the parasites (amastigotes) were visualized by indirect immunofluorescence (first antibody, IgG from chronic Chagasic patients; second antibody, an FITC-labeled rabbit anti-human IgG). Please note abundant intracellular fluorescent amastigotes in most of the HYB-2 cells, in contrast with the paucity of parasites in the parental carcinoma cells. Magnification, 295×.

These experiments were repeated more than four times with similar results.

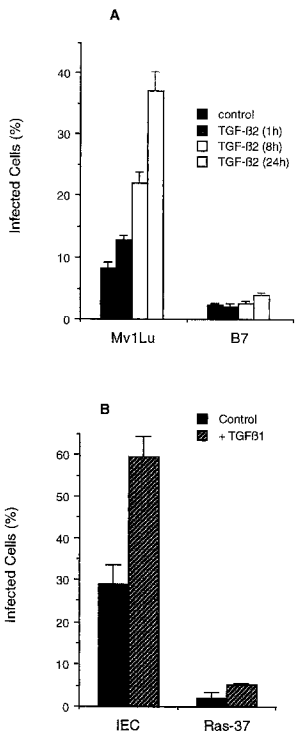

Figure 6. Cell Lines Overexpressing Cdk4 kinase or H-*ras* Oncogene Are Nonpermissive to T. cruzi Invasion

(A) Parental Mv1Lu cells and cdk4-B7 clone (overexpressing cdk4 kinase) were grown with TGFβ2 (2 ng/ml) for the indicated times, and then infected with T. cruzi for 2 days.

(B) Rat intestinal IEC cells and a IEC-ras7 clone (overexpressing H-*ras* oncogene) were grown in the absence (control) and presence of TGFβ1 (2 ng/ml) for 24 hr, and then infected with T. cruzi for 2 days.

lating the activity of cdk2 and cdk4 kinases (Ewen et al., 1993; Koff et al., 1993). TGFβ inactivates cdk4 directly by decreasing the level of cdk4 expression, and constitutive expression of cdk4 turns Mv1Lu cells resistant to the effects of TGFβ1 (Ewen et al., 1993). We tested the permissiveness to T. cruzi invasion of the Mv1Lu-derived cdk4 clone (B7), which expresses cdk4 constitutively at relatively high levels (Ewen et al., 1993). Remarkably, this Mv1Lu–cdk4 clone was not only resistant to T. cruzi invasion, but their low permissiveness was not significantly enhanced by TGFβ2 priming, contrary to the steep rise in T. cruzi invasion of TGFβ-sensitized parental cells (Figure 6A).

Another type of intracellular dysfunction that confers resistance to TGFβ results from overexpression of the activated human oncogene H-*ras* (Filmus et al., 1992). As already discussed, the rat intestinal cell IEC-18 is growth arrested by TGFβ, but after transformation with H-*ras*, it may become insensitive to the growth factor (Filmus et al., 1992). We found that parental, TGFβ-sensitive IEC-18 cells were invaded by T. cruzi in a TGFβ-enhanceable manner, whereas an H-*ras*-transformed, TGFβ-resistant derivative of IEC-18 cells (IEC–ras7 clone) was not (Figure 6B). Resistance to infection was due to a defect in T. cruzi penetration of TGFβ-resistant IEC–ras7 (Figure 2B). Thus, because cdk4 clone and IEC–ras7 cells express normal TGFβ surface receptors (Ewen et al., 1993; Filmus et al., 1992), the results with the cdk4 and H-*ras* clones indicate that T. cruzi invasion of epithelial cells requires intact TGFβ signaling pathway and not just the availability of functional TβRs.

Invasive, but Not Noninvasive, T. cruzi and Parasite Conditioned Medium Activate a TGFβ-Responsive Reporter Gene

The above results suggest that downstream signaling proteins are important for T. cruzi invasion. To test the possibility that T. cruzi directly activates signal transduction through the TβRs, we incubated live parasites with monolayers of Mv1Lu cells stably transfected with a plasminogen activator inhibitor-1 (*PAI-1*) construct (Abe et al., 1994). The construct is a plasmid containing a truncated *PAI-1* promoter fused to the firefly luciferase reporter gene.

Transfected cells increase luciferase activity to a great extent after stimulation with TGFβ, but minimally with other inducers of *PAI-1* expression and growth factors (Abe et al., 1994). We found that invasive trypomastigotes induced luciferase activity in transfected Mv1Lu cells in a dose-dependent manner, whereas noninvasive epimastigotes had no effect (Figure 7A). *PAI-1* promoter activation by live trypomastigotes was also demonstrated in cells transiently transfected with another *PAI-1* promoter–luciferase construct, p3TP–Lux (Wrana et al., 1992; data not shown).

We then determined whether proteins shed by T. cruzi would contain the *PAI-1* promoter activator. For this, we prepared an extract of live parasites by incubating them

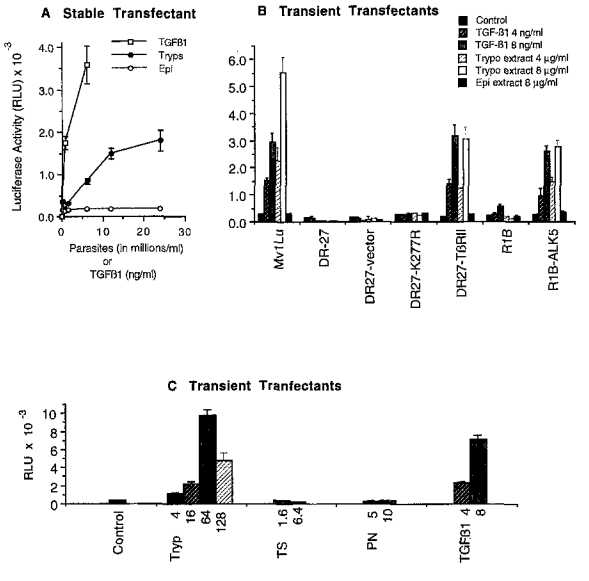

Figure 7. Transcriptional Activation of *PAI-1* Promoter Constructs by T. cruzi

(A) Induction of luciferase in Mv1Lu cells stably transfected with a *PAI-1*–luciferase construct. Monolayers were treated with the indicated concentrations of TGFβ1 or live trypomastigotes (Tryps) and epimastigotes (Epi). Luciferase activity (relative light units or RLU) in the cell lysates was determined in a luminometer 20 hr after addition of TGFβ1 or parasites.

(B) Restoration of responsiveness to TGFβ and T. cruzi in two classes of TGFβ-resistant cells by transient transfection with human TβR-II and TβR-I. Transfectants consisted of the *PAI-1* construct, p3TP–Lux in the cell lines Mv1Lu, DR-27, and R-1B, as indicated in the abscissa. Cotransfectants consisted of p3TP–Lux plus empty pCDNA I in DR-27 (DR27-vector), p3TP–Lux plus kinase-deficient TβR-II in DR-27 cells (DR27-K277R), p3TP–Lux plus human TβR-II in DR-27 cells (DR-27-TβRII), and p3TP–Lux plus human TβR-I in R-1B cells (R1B-ALK5). Luciferase activity was determined in cell monolayers that had been incubated for 20 hr in the absence (control) and in the presence of the indicated concentrations of TGFβ1 and trypomastigote extract (Trypo extract) and epimastigote extract (Epi extract).

(C) Lack of *PAI-1* promoter activation by trans-sialidase and penetrin. Mv1Lu cells transiently transfected with p3TP–Lux were grown in the absence (control) and in the presence of the indicated concentrations of trypomastigote extract (Tryp, in µg/ml), purified trans-sialidase (TS, in ng/ml), penetrin (PN, in µg/ml), and TGFβ1 (in ng/ml). Note that TS and PN did not induce *PAI-1* response while the induction of the *PAI-1* promoter by the trypomastigote extract in this experiment was even greater than that in the experiment shown in (B).

at 4°C for 2 days in RPMI medium containing a cocktail of protease inhibitors followed by centrifugation and filtration through 0.22 µm pores. This cell-free RPMI extract contains mostly surface and secreted proteins (Cavallesco and Pereira, 1988). Remarkably, extract from invasive trypomastigotes, but not from noninvasive epimastigotes (4 and 8 µg/ml), induced luciferase activity to a great extent (Figure 7B).

Unequivocal demonstration that T. cruzi activates the TβR signaling pathway was provided with DR and R mutant cells transiently transfected with p3TP–Lux. Neither TGFβ1 nor the trypomastigote extract activated the *PAI-1* promoter in DR-27 and R-1B cells (Figure 7B). However, when DR-27 cells and R-1B cells were transiently cotransfected with p3TP–Lux and with plasmids containing TβR-II or TβR-I genes, respectively, they produced high luciferase activity upon stimulation with either TGFβ1 or trypomastigote extract, but not with epimastigote extract (Figure 7B). Cotransfection of DR-27 mutants with p3TP–Lux plus empty pcDNA I (Figure 7B, DR27-vector) or with a plasmid containing a kinase-deficient receptor II construct (Chen and Weinberg, 1995; Figure 7B, DR27-K277R) did not produce promoter response to TGFβ1 nor to T. cruzi. Like TGFβ, which inhibits DNA synthesis and cell proliferation in many cell types (Massagué, 1990), trypomastigote extract inhibited DNA synthesis ([^{3}H]thymidine incorporation) in TGFβ-responsive Mv1Lu cells but not in TGFβ-resistant DR-27 and R-1B cells (data not shown). Noninvasive epimastigotes did not inhibit DNA synthesis in Mv1Lu cells. These results further confirm the stimulation of the TGFβ signaling pathway by T. cruzi. This stimulation was selective for T. cruzi because a similar extract from two other protozoan parasites, Plasmodium falciparum ($\sim 10^8$ trophozoites plus schizont stages/ml) and T. gondii ($\sim 10^8$ tissue culture forms/ml), did not activate the *PAI-1* promoter (M. M. and M. E. A. P., unpublished data).

Trans-sialidase and penetrin are surface proteins implicated in parasite adhesion to mammalian cells (Pereira, 1994). Because these proteins are present in the trypomastigote extract, it is possible that they will bind to cell surface sialyl and heparan sulfate epitopes and activate the TGFβ signaling pathway. However, purified trans-sialidase, at concentrations similar to those in the trypomastigote extract, did not trigger *PAI-1* promoter response in transiently transfected Mv1Lu cells (Figure 7C), nor did purified penetrin at relatively high concentrations (Figure 7C).

We have partially purified the TGFβ-like factor from trypomastigote extracts through several steps of conventional protein fractionation procedures. The factor did not react with a variety of commercial TGFβ monoclonal and polyclonal antibodies, as determined by neutralization of biological activity, immunoblot, and ELISA (M. M. and M. E. A. P., unpublished data).

Discussion

TGFβ is a cytokine that alters many functions in nearly all higher eukaryotic cells (Roberts and Sporn, 1990). The

nature of the TGFβ action depends on many parameters, including type and state of differentiation of the cell targets, growth conditions, and presence of other growth factors. TGFβ controls extracellular matrix production, regulation of myogenesis, immune response, angiogenesis, and embryogenesis. Here, we report that utilization of the TGFβ signaling pathway by a pathogenic microbe is essential for the invader to gain access and thrive in the cytosol of nonphagocytic host cells.

Many types of evidence support the hypothesis that T. cruzi invasion requires activation of the TGFβ signaling pathway. T. cruzi could not infect, or infected extremely inefficiently, mink lung epithelial cells with a genetic defect in TβR-I or TβR-II (Figures 1A and 1B). Reconstitution of TβR function by transfection with human receptor I or II restored sensitivity of the mutants to TGFβ and permissiveness to T. cruzi invasion (Figure 1C; Table 1). Similarly, T. cruzi did not efficiently invade the human EJ bladder carcinoma cell line nor the human SW480 colon adenocarcinoma cells (Figure 5B). These cells express relatively low levels of TβR-II and thus are resistant to TGFβ-induced growth arrest (Figure 5A; Geiser et al., 1992). However, fusion of EJ cells with SW480 cells produced a hybrid cell sensitive to TGFβ due to high level expression of receptor II (Figure 5A; Geiser et al., 1992). Rescue of TGFβ response in the hybrid cell line correlated with a remarkable permissiveness to T. cruzi invasion (Figure 5B). Another approach to demonstrate the importance of the TGFβ signaling for T. cruzi invasion was to infect cells genetically engineered to constitutively overexpress cdk4 kinase and H-*ras* oncogene, which make them growth resistant to TGFβ (Ewen et al., 1993; Filmus et al., 1992). Consistent with the other results summarized above, mink and rat epithelial cells with deregulated cdk4 and H-*ras* expression were very poor hosts for T. cruzi (Figures 6A and 6B).

The conclusion from these genetic studies was consistent with the enhanced T. cruzi invasion of TGFβ-sensitized wild-type cells from different animal species and of various lineages (Figure 4A; unpublished data). Enhancement of invasion was specific for TGFβ, since priming of the cells with other growth factors (EGF and PDGF) and with molecules capable of eliciting a G1 growth arrest (rapamycin and cAMP) did not increase invasion.

Our findings are consistent with early studies demonstrating a dependency of T. cruzi invasion on the phase of the host cell cycle. Specifically, T. cruzi invasion of synchronized cultured cells increased as the cells progressed from G1 to the S phase of the cell cycle and dropped sharply as the cells entered G2/M (Dvorak and Crane, 1981). Accordingly, Mv1Lu and other cells arrested at mid- to late-G1 by TGFβ (Massagué, 1990) were much better hosts for T. cruzi than asynchronous culture cells (Figure 4). However, cell arrest at G1 per se did not necessarily lead to enhanced trypanosome invasion, as suggested by the results with rapamycin, which blocks cells such as Mv1Lu at G1 (see legend to Figure 4C), through a mechanism distinct from the TGFβ arrest (Kato et al., 1994). In addition, cells growth arrested at G0/G1 by contact inhibition are much poorer hosts for T. cruzi than subconfluent cells progressing through the cell cycle (Henriquez et al., 1981; Dvorak and Crane, 1981). Thus, contrary to the TGFβ arrest, contact inhibition and rapamycin-induced G1 block did not turn the cells into better hosts for T. cruzi, underscoring the specificity of the TGFβ signaling pathway in directing trypanosome invasion.

Our results also provide a logical explanation for early observations about the effect of T. cruzi and TGFβ on skeletal muscle differentiation. Thus, T. cruzi infection of L6E9 rat myoblasts prevented the myoblasts from fusing into multinucleated myotubes (Rowin et al., 1983), an effect similar to that of purified TGFβ (Massagué et al., 1986). Furthermore, T. cruzi reduced the expression of muscle-specific mRNA (Rowin et al., 1983), as did TGFβ (Massagué et al, 1986). Moreover, the L6E9 cells, once committed to myogenic differentiation, became resistant to the inhibitory effect of both T. cruzi and TGFβ, even though the differentiated cells were still able to be infected by T. cruzi and to be bound by TGFβ (Rowin et al., 1983; Massagué et al., 1986). Thus, in view of the parallel actions of T. cruzi and TGFβ on skeletal muscle differentiation in vitro, we hypothesize that the parasite-induced inhibition of myogenic differentiation results from trypomastigote activation of TβRs.

If T. cruzi invasion requires activation of the TGFβ signaling pathway, then what turns the pathway on, the host TGFβ, the parasite-specified TGFβ-like factor, or both? Previous studies suggest that spleen cells from mice with acute T. cruzi infections produced elevated levels of TGFβ in vitro (Silva et al., 1991). This elevation could result from T. cruzi activating host-derived TGFβ precursor into the active 25 kDa peptide. Other studies showed that proteases and sialidases can process the TGFβ precursor into the active peptide (Miyazono and Heldin, 1989). Interestingly, T. cruzi secretes both protease (Cazzulo et al., 1989) and sialidase (Pereira, 1983) proteins. Therefore, the intriguing possibility that T. cruzi can correctly process host TGFβ precursor molecules should be further investigated.

On the other hand, we found that T. cruzi itself, specifically its invasive trypomastigote form, activates a TGFβ-responsive extracellular matrix protein (*PAI-1*) promoter of transfected Mv1Lu epithelial cells (Figure 7; Abe et al., 1994). Trypomastigotes release a factor that engages the TGFβ signaling receptors, as judged by the activation of another *PAI-1* promoter construct (p3TP–Lux) in Mv1Lu cells (Wrana et al., 1992) by soluble proteins shed by invasive trypomastigotes. This T. cruzi–derived TGFβ-like factor is distinct from host TGFβ by many criteria, including physical properties like resistance to boiling (30 min at 100°C; M. M. and M. E. A. P., unpublished data). Thus, it may also be that T. cruzi invasion is directed by a parasite-specified product that activates the TGFβ signaling pathway.

It is becoming evident that intracellular microbes can bind to cell surface receptor signaling proteins during their interaction with host cells. The bovine papilloma virus protein E5 dimerizes in the plasma membrane, activates receptors for several growth factors (Petti and DiMaio, 1992),

and binds to a vacuolar H^+-ATPase to affect receptor recycling (Goldstein et al., 1991). The Epstein–Barr virus transforming protein LMP1 engages signaling proteins coupled to the tumor necrosis factor receptor family (Mosialos et al., 1995), and the HIV virus binds to CD4 receptors on lymphocytes as a requirement to invasion (Olshevsky et al., 1990). These and other interactions (Gooding, 1992) may be important not only in facilitating parasite growth but also in allowing the virally infected cells to evade cellular host defense mechanisms. Identification of relevant parasite ligands and characterization of the cellular changes induced by ligand–host cell interactions is an exciting and flourishing area of current microbiological research (Bliska et al., 1993).

On the basis of the results presented here and elsewhere (Pereira, 1994), we propose that T. cruzi invasion starts with trypomastigotes attaching to host cells through at least two pathways involving trans-sialidase/sialic acid and penetrin/heparan sulfate recognition. These protein–carbohydrate interactions do not induce cellular invasion, which, at least in cells growth arrested by TGFβ, is driven by the activation of the TGFβ signaling pathway through ligands distinct from trans-sialidase and penetrin. TGFβ signaling may be turned on either by host TGFβ, by a TGFβ-like peptide released into the environment by the invading trypomastigotes, or by both. It will be of great interest to determine the mechanism used by T. cruzi to trigger TGFβ signaling, whether that is the pathway that leads to G1 arrest, the pathway that induces extracellular matrix deposition, or both. It is likely that activation of the antiproliferative pathway is a requirement for invasion, as judged by the correlation between TGFβ-induced inhibition of DNA synthesis in various mammalian cell types and T. cruzi invasion. On the other hand, T. cruzi and T. cruzi extracts strongly activate a promoter for an extracellular matrix protein (Figure 7), suggesting that this pathway may be activated as well. Ascertaining these problems are not just of academic interest, for a T. cruzi–specified activation of the TGFβ pathway might underlie responses such as immunosuppression (Wahl et al., 1988) and abnormal extracellular matrix deposition (Border and Ruoslahti, 1992), each a dominant feature in humans and experimental animals with acute (Brener and Krettli, 1990) and chronic Chagas' disease (Koberle, 1968; Andrade, 1983), respectively.

Experimental Procedures

Parasites

The Silvio X-10/4 and Tulahuen clones of T. cruzi were obtained by infection of cultured Vero cells as previously described (Cavallesco and Pereira, 1988). The T. cruzi cell-free RPMI extract was obtained by incubating trypomastigotes and epimastigotes (from LIT medium) (2×10^8/ml) for 2 days at 4°C in RPMI containing a cocktail of protease inhibitors (10 μM pepstatin A, 10 μM leupeptin, 10 mU/ml aprotinin, 5 mM EDTA, and 10 mM iodoacetamide), centrifuging the suspension ($1000 \times g$, 10 min), and filtering the supernatant through 0.22 μm pores. A RPMI extract of human red blood cells infected with P. falciparum (containing a mixture of schizonts and trophozoites at ~10^8/ml) was obtained in a manner similar to that of T. cruzi (gift from Drs. G. Pasvol and B. Clough).

Cell Lines and Cell Culture

The Mv1Lu mink lung cell line was from the ATCC (CCL-64), and the Mv1Lu-derived TβR mutants DR-26, DR-27, and R-1B were provided by Dr. J. Massagué (Laiho et al., 1990; Wrana et al., 1992). The SW480 colon adenocarcinoma cell line was from ATCC (through Dr. J. Sharon), and the EJ bladder carcinoma cell line and the hybrid (HYB-2) of SW480 with EJ cells were provided by Dr. A. Geiser (Geiser et al., 1992). The rat intestinal cell line IEC-18 and an IEC-18 clone transformed with human H-*ras* oncogene (IEC–ras7) were provided by Dr. J. Filmus (Filmus et al., 1992). The cdk4 B7 clone was the cell line used in previous studies (Ewen et al., 1993). Cell lines were maintained as described in the references cited above.

Infection Assay

Infection of culture cells was carried out as described previously (Cavallesco and Pereira, 1988). Subconfluent monolayers were incubated for 1–2 hr at 37°C with various concentrations of trypomastigotes in 0.1 ml of RPMI-BSA, washed with RPMI to remove swimming parasites, and further incubated for 2–3 days at 37°C in RPMI-1640 containing 5% FCS. Diff-Quik-stained preparations were examined under a microscope to estimate the ratio of infected (i.e., cells containing more than one intracellular amastigote) to noninfected cells.

A similar protocol was used to infect monolayers with T. gondii, RH strain, provided by Dr. K. Joiner (Schwab et al., 1994).

Adhesion Assay

Adhesion of trypomastigotes to monolayers of glutaraldehyde-fixed cells was performed as previously described (Ortega-Barria and Pereira, 1991; Ming et al., 1993). In brief, trypomastigotes (10^5/well/0.1 ml of RPMI-BSA) were incubated at 37°C with glutaraldehyde-fixed monolayers in 16-well Lab-Tek chamber slides. After 1 hr, swimming and loosely bound parasites were removed by dipping the slides 10 times in PBS. Adherent parasites were fixed for 10 min with 4% paraformaldehyde in PBS containing 7% sucrose. After rinsing with PBS, the slides were stained with Diff-Quik and examined under a light microscope to assess T. cruzi adherence. A minimum of 300 host cells were counted for each point.

Penetration Assay

Monolayers of the various cell types, equilibrated in RPMI-BSA medium, were incubated with trypomastigotes (6×10^6/ml) for 1 hr at 37°C, washed in cold HEPES-buffered (pH 7.2) RPMI, fixed in 2% paraformaldehyde in 100 mM lysine for 20 min, and blocked with 50 mM ethanolamine (Schenkman et al., 1991). The monolayers were fixed one more time in 1% glutaraldehyde for 1 hr at room temperature and blocked with 1% BSA overnight at 4°C. The monolayers were then reacted for 30 min with IgG (20 μg/ml) purified from human Chagasic sera (Cavallesco and Pereira, 1988) and washed several times with PBS, and the bound IgG were detected with an FITC-labeled second antibody (Sigma). After washing with PBS, the DNA of the host cells and the parasites was stained with DAPI (Sigma) at 5 μg/ml and observed under a Nikon epifluorescence microscope.

Receptor Affinity-Labeling Assay

These assays were performed precisely as described by Massagué (1987), except that [^{125}I]TGFβ1 (specific activity 137 μCi/μg) was from a commercial supplier (Dupont–New England Nuclear).

Growth Inhibition Assay

Cells were seeded in 96-well flat-bottomed plates, grown overnight at 37°C in 10% FCS in RPMI, and incubated with various concentrations of recombinant human TGFβ1 (R & D Systems) or TGFβ2 (Genzyme), EGF or PDGF (R & D Systems), BtcAMP (Sigma), rapamycin, or T. cruzi RPMI extract. After 2 days, cells were labeled with 1 μCi/ml [^{3}H]thymidine (specific activity 16.8 Ci/mmol; Dupont–New England Nuclear) for 18 hr, washed three times with cold PBS, extracted with 0.2 N NaOH for 30 min, and counted in a scintillation counter. Each assay was carried out in triplicate or quadruplicate.

Transfection Assays

DR-27 and R-1B cells stably expressing human TβR-II tagged with FLAG epitope in pRK5 (Chen et al., 1993) (gift of Dr. R. Derynck) or

TβR-I (*ALK-5*) in pSV7d (Franzén et al., 1993) (gift from Drs. C.-H. Heldin and K. Miyazono), respectively, were generated by transfection with lipofectin as recommended by the manufacturer (GIBCO-BRL). In brief, cells growing in 60 mm dishes were incubated overnight in serum-free medium containing 2 μg of DNA and 10 μg of lipofectin and then washed into media containing 10% FCS and antibiotics. After 2 days, cells were subcultured into several 75 cm^2 flasks and grown in the presence of 25 μg/ml mycophenolic acid (GIBCO-BRL) for 2–3 weeks with a change in media every 4–5 days. Mycophenolic acid–resistant cells were cloned in microtiter wells by limited dilution in the presence of 10% FCS. After 2–3 weeks, each colony was tested for sensitivity to the antiproliferative action of TGFβ1, and those growth arrested by the cytokine were incubated with [^{125}I]TGFβ1 and ascertained for the presence of TβRs with the chemical cross-linker disuccinimidyl suberate. Transfectants sensitive to TGFβ were continuously maintained in hygromycin. In this way, we generated two clones of DR-27 cells expressing human TβR-II (DR27-47 and DR27-50) and six clones of R-1B expressing *ALK-5* (R1B-TR9, RIB-TR11, RIB-TR16, RIB-TR20, RIB-TR22, and RIB-TR25).

Transient Transfections and Luciferase Assays

Parental cells were plated to semiconfluency in 6-well tissue culture plates, and 24 hr later transfected with lipofectin in serum-free medium using 2.0 μg of reporter plasmid (p3TP–Lux) (gift of Dr. J. Massagué, see Wrana et al., 1992). For DR and R cells, they were transfected with 2.0 μg of p3TP–Lux without and with 0.5 μg of TβR-II in pcDNA-1 vector (Lin et al., 1992) or kinase-deficient TβR-II (Chen and Weinberg, 1995) (gifts from Dr. R. Weinberg) or *ALK-5* in pSV7d vector (Franzén et al., 1993), respectively. After overnight incubation at 37°C in a 5% CO_2 atmosphere, the medium was replaced with 10% FCS in RPMI and incubated for a further 48 hr at 37°C, followed by TGFβ, T. cruzi, or RPMI extract of T. cruzi and P. falciparum. After 20 hr, cells were harvested and assayed for luciferase activity using the luciferase assay system described by the manufacturer (Promega). Light emission was measured during the initial 30 s of the reaction using a luminometer (MONOLIGHT 2010, Analytical Luminescence Laboratory) (courtesy of Drs. A. Leiter and A. Kopin).

For introduction of reporter construct in transfected DR-27 and R-1B clones (see above), a procedure similar to the one described above was followed, except that the TβR constructs were ommitted.

Determination of reporter activity in Mv1Lu cells stably transfected with *PAI-1* promoter construct (gift from Dr. D. Rifkin) was as described by Abe et al. (1994).

Other Procedures

Penetrin and trans-sialidase were isolated from T. cruzi by heparin affinity chromatography (Herrera et al., 1994) and a combination of immunoaffinity and sieving chromatography (Scudder et al., 1993), respectively. Cell cycle determination was determined as previously described (Ewen et al., 1993).

Acknowledgments

Correspondence should be addressed to M. E. A. P. We thank Drs. J. Massagué, F. Filmus, A. Geiser, C.-H. Heldin, K. Miyazono, D. Rifkin, J. Sharon, R. Derynk, and R. Weinberg for various reagents acknowledged in the Experimental Procedures. We also thank J. Sharon for useful comments, K. Zhang for technical assistance, and S. Wong for typing the manuscript. This work was supported by grants from National Institutes of Health (to M. E. A. P.) and by a grant from Sandoz/Dana–Farber Drug Discovery Programs (to M. E. E.).

Received March 28, 1995; revised June 1, 1995.

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