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Hyperthermia Enhances CD95-Ligand Gene Expression in T Lymphocytes¹

Marco Cippitelli,^{2,3*†} Cinzia Fionda,^{3*†} Danilo Di Bona,[§] Mario Piccoli,^{*} Luigi Frati,^{*‡} and Angela Santoni^{*†}

Hyperthermia represents an interesting therapeutic strategy for the treatment of tumors. Moreover, it is able to regulate several aspects of the immune response. Fas (APO-1/CD95) and its ligand (FasL) are cell surface proteins whose interaction activates apoptosis of Fas-expressing targets. In T cells, the Fas-Fas-L system regulates activation-induced cell death, is implicated in diseases in which lymphocyte homeostasis is compromised, and plays an important role during cytotoxic and regulatory actions mediated by these cells. In this study we describe the effect of hyperthermia on activation of the *fas-L* gene in T lymphocytes. We show that hyperthermic treatment enhances Fas-L-mediated cytotoxicity, *fas-L* mRNA expression, and *fas-L* promoter activity in activated T cell lines. Our data indicate that hyperthermia enhances the transcriptional activity of AP-1 and NF- κ B in activated T cells, and this correlates with an increased expression/nuclear translocation of these transcription factors. Moreover, we found that heat shock factor-1 is a transactivator of *fas-L* promoter in activated T cells, and the overexpression of a dominant negative form of heat shock factor-1 may attenuate the effect of hyperthermia on *fas-L* promoter activity. Furthermore, overexpression of dominant negative mutants of protein kinase C ϵ (PKC ϵ) and PKC θ partially inhibited the promoter activation and, more importantly, could significantly reduce the enhancement mediated by hyperthermia, indicating that modulation of PKC activity may play an important role in this regulation. These results add novel information on the immunomodulatory action of heat, in particular in the context of its possible use as an adjuvant therapeutic strategy to consider for the treatment of cancer. *The Journal of Immunology*, 2005, 174: 223–232.

Increased body temperature associated with fever, which normally occurs during an infection, has been suggested to improve the immune response in a way that could be an advantage in clinical practice (1). The observation that hyperthermia, the therapeutic procedure used to raise the temperature of tissues or the whole body, seems to mimic fever, encouraged various in vitro and in vivo studies since the early 1980s of the effect of heat on lymphocytes, mostly focusing on the immunoregulatory functions of this treatment (2, 3).

Fever-range whole body hyperthermia (typically 39–40°C for 3–6 h) may induce changes in murine lymphocyte physiology indicative of an activation status, such as reorganization of the spectrin-based cytoskeleton and formation of uropodes, activation and relocation of different protein kinase C (PKC)⁴ isoforms, to-

gether with induction of heat shock proteins (HSPs) (4, 5). Moreover, hyperthermia enhances the adhesion of leukocytes to high endothelial venules by regulating L-selectin and $\alpha_4\beta_7$ integrin avidity in vitro and in vivo, and this effect correlates well with prominent lymphopenia together with increased leukocyte infiltration into secondary lymphoid tissues or tumor sites (6–10).

It has been hypothesized that the thermal microenvironment plays a critical role in regulating events in the immune response, and that an increase in the local temperature may act as a natural trigger or danger signal to the immune system. In this regard, the fever thermal component confers protection to the host in defense against microbial pathogens by enhancing the innate immune response and, in particular, activating neutrophils (11, 12). Moreover, hyperthermia may lead to direct maturation of dendritic cells in vitro and in vivo, and this effect correlates well with an improved immunostimulatory activity mediated by these cells (13–16). Despite these intriguing observations, temperature manipulation is rarely considered in the context of most experimental immunological investigations.

Fas ligand (Fas-L) is a type II transmembrane protein able to trigger apoptotic cell death by binding to its Fas receptor (CD95) (17, 18), a primary process for the maintenance of immune privilege and involved in the induction and regulation of certain organ-specific autoimmune diseases (19, 20) or in pathologies where normal peripheral lymphocyte homeostasis is compromised (21). Moreover, in the area of T lymphocyte-mediated cytotoxicity, the expression of Fas by tumor cells may enable their elimination by Ag-specific effector cells via Fas/Fas-L-dependent mechanisms (22, 23).

Additional regulatory functions for the Fas/Fas-L receptor-ligand pair have been described that expand the information

*Department of Experimental Medicine and Pathology, Istituto Pasteur-Fondazione Cenci Bolognetti, University La Sapienza, Rome, Italy; [†]Regina Elena Cancer Institute, Rome, Italy; [‡]Istituto Mediterraneo di Neuroscienze Neuromed, Pozzilli, Italy; and [§]Cattedra e Unità Operativa di Gastroenterologia, University of Palermo, Palermo, Italy

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² Address correspondence and reprint requests to Dr. Marco Cippitelli, Department of Experimental Medicine and Pathology, University La Sapienza, Viale Regina Elena 324, 00161 Rome, Italy. E-mail address: marco.cippitelli@uniroma1.it or cippitelli@ifo.it

³ M.C. and C.F. contributed equally to this paper.

⁴ Abbreviations used in this paper: PKC, protein kinase C; BCA, biconchonic acid; Fas-L, Fas ligand; Gal, galactosidase; HS, heat shock; HSF, heat shock factor; HSP, heat shock protein; Luc, luciferase; RSV, Rous sarcoma virus; WT, wild type; PP, protein phosphatase.

available on its complex regulatory activity. In fact, triggering of Fas-L is required for CTLs to achieve an optimal proliferation (24–26), and an activated Fas receptor may induce phenotypic and functional maturation of dendritic cells together with specific secretion of proinflammatory cytokines and a preferential local T cell polarization toward a Th1 phenotype (27). Furthermore, Fas ligation may contribute to T cell development and proliferation, suggesting important connections between cell proliferation and apoptosis (28, 29).

In this report we describe the effect of hyperthermia on activation of the *fas-L* gene in activated T lymphocytes and the regulatory actions of this treatment on the human *fas-L* promoter. We show that hyperthermia may enhance *fas-L* mRNA expression, promoter activity, and effector function in T cell lines such as the 2B4.11 T cell hybridoma and Jurkat cells. Progressive deletion analysis has identified a minimal promoter fragment spanning nucleotides from –195 bp immediately 5' of the translational start site, which is still sensitive to hyperthermia treatment. This correlates with an increased expression and activity of specific transcription factors, such as c-Jun, FosB, and NF- κ B, important regulators located in this position. Moreover, overexpression of a constitutive active form of heat shock factor-1 (HSF-1) significantly enhances *fas-L* promoter activity, and a dominant negative mutant of this transcription factor is able to decrease the effect of hyperthermia in activated T cells. Furthermore, cotransfection of dominant negative mutants of PKC ϵ and PKC θ may partially inhibit overall *fas-L* promoter activity and significantly decrease the specific enhancement mediated by hyperthermia.

The data described in this study provide novel findings on the complex effects of heat on the regulation of the immune response and indicate that the *fas-L* gene is a novel molecular target, adding to the variety of immunoregulatory activities mediated by hyperthermia. The physiological and therapeutical implications of these observations are discussed below.

Materials and Methods

Cell lines, reagents, and treatments

Jurkat cells and 2B4.11 murine T hybridoma cells were maintained as described previously (30). PMA and ionomycin were purchased from Sigma-Aldrich. Anti-CD3 ϵ -chain mAb (clone 145-2C11) was purchased from BD Pharmingen. CAY10410 (9,10-dihydro-15-deoxy- $\Delta^{12,14}$ -PGJ₂) was purchased from Cayman Chemical. For hyperthermia, culture flasks were sealed with Parafilm (American National Can) and immersed in a water bath at the indicated temperature and time. Control cells were left in the incubator (sealed with Parafilm) at 37°C. After hyperthermia treatment, cells were returned to the incubator (37°C) for recovery and appropriate stimulation.

Cytotoxicity assay

Wild-type and Fas-transfected L1210 cells (10⁶ cells; provided by Dr. Hueber, Institute of Signaling, Developmental Biology, and Cancer Research, Nice, France) were labeled with 0.2 mCi of ⁵¹Cr (Amersham Biosciences) at 37°C. Target cells (2 × 10³) were cocultured with 2B4.11 effector cells in 200 μ l of complete medium in U-bottom, 96-well plates for 4 h. 2B4.11 effector cells were previously activated with 20 ng/ml PMA and 0.5 μ g/ml ionomycin in the absence of pretreatment or after pretreatment at 42°C for 1 h or 39.5°C for 4 h, followed by a 0.5-h recovery period at 37°C in complete medium. Cells were then harvested, washed twice in complete medium, and cocultured with ⁵¹Cr-labeled target cells. After 4 h, 100 μ l of supernatant was removed from each well and counted in a gamma counter for determination of ⁵¹Cr release. Maximal and spontaneous release were determined by incubating ⁵¹Cr-labeled target cells with 1% Nonidet P-40 or medium alone, respectively. The percent specific killing was calculated as 100% (experimental ⁵¹Cr release – spontaneous ⁵¹Cr release)/(maximal ⁵¹Cr release – spontaneous ⁵¹Cr release). All determinations were made in triplicate, and E:T cell ratios ranged from 80:1 to 20:1, as indicated.

Northern blot analysis

Total RNA was extracted from 2B4.11 hybridoma T cells by TRIzol (Invitrogen Life Technologies). Equal amounts of RNA (15 μ g/lane) were fractionated on a 1.5% agarose-formaldehyde gel. The specific mRNA was detected by hybridization of Nytran membranes (Schleicher & Schuell) with a ³²P-labeled cDNA probe specific for the murine *fas-L* gene. The RNA-containing membranes were prehybridized for 20 min and hybridized for 2 h at 65°C with QuikHyb hybridization solution (Stratagene). The membranes were then washed twice in 2× SSC containing 0.1% SDS and twice in 0.1× SSC containing 0.1% SDS at 60°C (20 min each time) and exposed to X-OMAT AR films (Eastman Kodak) at –70°C with intensifying screens.

Plasmid constructions

The human Fas-L promoter luciferase (Luc) reporter pFasL-486, the distal NFAT binding mutant (Δ NFAT-Dist), and the response element 3/Fas-L regulatory element (RE3/FLRE) binding mutant were provided by Dr. G. A. Koretzky (University of Pennsylvania, Philadelphia, PA) (31, 32). pGL3-FasL-luc has been already described (33). The different deletions of the human Fas-L promoter –453 Fas-L(pGL2), –237 Fas-L(pGL2), and –195 Fas-L(pGL2) were provided by Dr. C. V. Paya (Mayo Clinic, Rochester, MN) (34). The CMV- β -galactosidase (CMV- β -Gal) expression vector pEQ176 and the Rous sarcoma virus (RSV)-Gal expression vector have been previously described (30). The expression vector for human constitutively activated HSF1 S303A/S307A double mutant (pcDNA3-HSF1-S303A/S307A) was provided by Dr. R. I. Morimoto (Northwestern University, Evanston, IL).

To prepare the expression vector for HSF-1 dominant negative (deletion of aa 453–529, the C-terminal sequences including the C-terminal region element, which is part of the transcription activation domain) (35), the appropriate deletion fragment was generated by RT-PCR according to standard methods from an HSF-1 expression vector containing the human HSF-1 gene and cloned in the pReRSV expression vector (Invitrogen Life Technologies, Carlsbad, CA). The following primers were used for amplification: HSF-1 forward, 5'-ATCGGAATTCCTCGAGAATTCGAGATG GATCTGCCCCGTGG-3'; and HSF-1 reverse, 5'-ACTATCTAGACTCCT GGGGAGACAGGAGCTC-3'.

Luc reporter vectors for the transcription factors AP-1 (pAPI-TA-Luc), c-Myc (pMyc-TA-Luc), NF- κ B (pNF- κ B-TA-Luc), and the control vector containing the minimal TATA box from the herpes simplex virus thymidine kinase promoter pTA-Luc, were purchased by BD Clontech. The dominant negative (K/R) expression vectors for human PKC θ (K409R), rat PKC ϵ (K436R), and the control vector (pEFneo) were provided by Drs. G. Baier and F. Uberall (University of Innsbruck, Innsbruck, Austria). The cDNA for murine Fas-L was provided by Dr. R. De Maria (Istituto Superiore di Santà, Rome, Italy).

DNA transfections

Transfections of Jurkat cells were conducted using the DEAE-dextran method as described previously (30). To decrease variations in the experiments due to different transfection efficiencies, cells were transfected in single batches, which were then separated into different drug treatment groups. A CMV- β -Gal expression vector was cotransfected to normalize DNA uptake. After 24 h, cells were treated with different combinations of stimuli, and after an additional 24 h, cells were harvested, and protein extracts were prepared for the Luc and β -Gal assays as described previously (30). Protein concentration was quantified by the bicinchoninic acid (BCA) method (Pierce). Luc activity was read using the Luc assay system (Promega) following the manufacturer's instructions. β -Gal activity was determined as described previously (30).

EMSA

Nuclear proteins were prepared as described previously (30). The protein concentration of extracts was determined using the BCA method (Pierce). Nuclear proteins (10 μ g) were incubated with radiolabeled DNA probes in a 20- μ l reaction mixture containing 20 mM Tris (pH 7.5), 60 mM KCl, 2 mM EDTA, 0.5 mM DTT, 1–2 μ g of poly(dI-dC), and 4% Ficoll. Nucleo-protein complexes were resolved as described previously (30). Oligonucleotides were purchased from Invitrogen Life Technologies. Complementary strands were annealed and end-labeled as described previously (30). Approximately 3 × 10⁴ cpm of labeled DNA was used in a standard EMSA reaction. The following double-strand oligomers were used as specific labeled probes: human Fas-L-NF- κ B proximal, 5'-AGAGAAAGACAG AGGTGTTTCCCTT-3'; human Fas-L-AP-1 proximal, 5'-GGGCTGGC CTGACTCACCAGCTGC-3'; and octamer (human histone H2b), 5'-agCTCTTCACCTTATTTGCATAAGCGAT-3'.

Western blot analysis

For Western blot analysis, nuclear proteins were prepared as described previously (33). Protein concentration of nuclear extracts was determined by the BCA method (Pierce). Forty micrograms of nuclear extract was run on 12% denaturing SDS-polyacrylamide gels. Proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell) and blocked in 3% milk in TBST. Immunoreactive bands were visualized on the nitrocellulose membranes, using HRP-coupled goat anti-rabbit or goat anti-mouse Igs and the ECL detection system (Amersham Biosciences) following the manufacturer's instructions. Abs against RelA, Oct-1, c-Jun, and FosB were purchased from Santa Cruz Biotechnology.

Results

Hyperthermia enhances Fas-L-dependent target lysis mediated by activated 2B4.11 T cells

To investigate on the functional effect of hyperthermia on Fas-L expression/function in activated T cells, cytotoxicity assays were performed using the 2B4.11 T cell hybridoma in the presence of ^{51}Cr -labeled, Fas-transfected, L1210-Fas⁺ target cells. As shown in Fig. 1A, 2B4.11 T cells activated by a combination of PMA and ionomycin after pretreatment at 42°C for 1 h, followed by a recovery period at 37°C for 0.5 h (indicated as heat shock (HS) treatment), significantly increased specific killing compared with the cytotoxicity elicited by 2B4.11 cells treated at normal temperature (37°C). This result was also confirmed in a different setting of mild hyperthermia (pretreatment of cells at 39.5°C for 4 h), as shown in Fig. 1C.

The effects of HS and mild hyperthermia on L1210-wild-type (L1210-WT) target cells (used in these experiments as a negative control for Fas-L-mediated killing) are shown in Fig. 1, B and D. Treatment of 2B4.11 cells with HS or mild hyperthermia in the absence of PMA and ionomycin stimulation was unable to induce any specific killing, at least in this experimental system. Thus, hyperthermia enhances Fas-L-mediated cytotoxicity in activated 2B4.11 T cells.

Hyperthermia increases fas-L gene expression and promoter activation in 2B4.11 T cells

We investigated whether hyperthermia could affect induction of the *fas-L* gene in activated T cells. Total RNA was isolated from 2B4.11 T cells 5 h after activation and analyzed for *fas-L* mRNA expression by Northern blot assay. As shown in Fig. 2A, *fas-L* mRNA was induced by PMA plus ionomycin stimulation and was

significantly increased by HS pretreatment, indicating that *fas-L* is a molecular target of hyperthermia in T cells. Hyperthermia without PMA and ionomycin was unable to induce *fas-L* mRNA in this experimental system. The effect of hyperthermia was also confirmed by stimulating 2B4.11 T cells with plate-bound anti-CD3 and was verified in mild hyperthermia, as shown in Fig. 2, B and C, respectively.

To determine whether one of the mechanisms involved in the hyperthermia-mediated increase in *fas-L* gene activation could be a direct effect on the transcriptional activity of its promoter, transient transfection assays were performed in Jurkat cells. As shown in Fig. 2D, the activity of a human *fas-L* promoter fragment, consisting of 486 bp immediately 5' of the translational start site, was induced by PMA plus ionomycin treatment and was significantly enhanced by HS or mild hyperthermia in Jurkat cells. HS or mild hyperthermia in the absence of PMA and ionomycin stimulation was unable to activate this promoter, at least in this experimental system. Thus, *fas-L* mRNA expression and promoter activation are enhanced by hyperthermia in activated T cells.

Hyperthermia enhances fas-L gene expression in activated T cells: promoter analysis

To gain further insight into the mechanism(s) involved in *fas-L* promoter enhancement, we investigated the possible presence of a *fas-L* promoter region(s) involved in the hyperthermia-mediated action on this gene. We analyzed the activity of specific internal mutations and progressive deletions of the *fas-L* promoter by transient transfection assays in Jurkat cells. Transfection of *fas-L* promoter constructs bearing internal mutations that abrogate the binding of important transactivators, such as NF-AT ($\Delta\text{NFAT-Dist.}$) or early growth response factors ($\Delta\text{RE3/FLRE}$), considerably decreased activation after stimulation with PMA and ionomycin as previously reported (31, 32, 36). However, in this context hyperthermia was still able to significantly increase residual promoter activation compared with the wild-type *fas-L* promoter reporter (Fig. 3, A–C). Progressive deletion of these enhancer elements could delineate a minimal promoter fragment spanning nucleotides from –195 bp immediately 5' of the translational start site, which was still sensitive to the hyperthermia treatment (Fig. 3, D–F). These data suggest that signaling events triggered by hyperthermia can augment *fas-L* promoter activation by mechanisms that cooperate at the level of the enhancer activity of its first –195 bp immediately 5' of the translational start site.

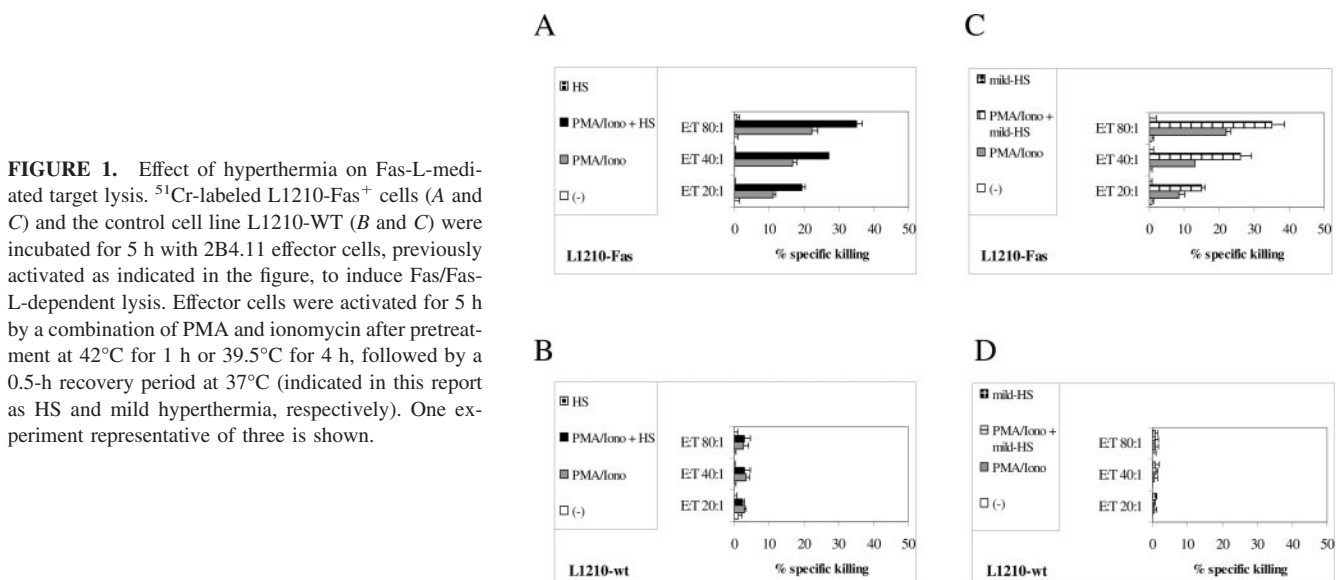
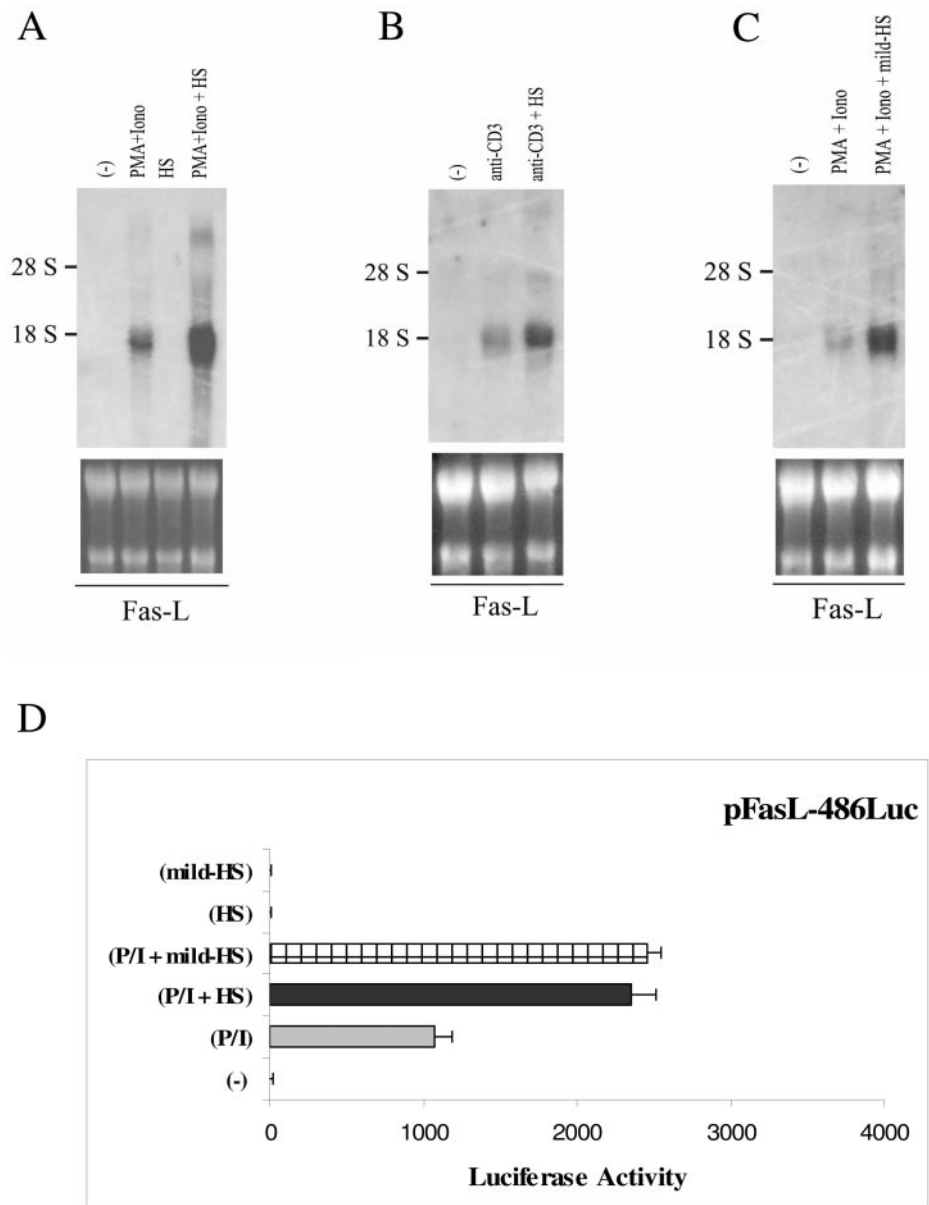


FIGURE 2. Hyperthermia enhances *fas-L* mRNA expression and promoter activation in T cells. **A**, Northern blot analysis of total mRNA obtained from 2B4.11 hybridoma cells untreated (–) or stimulated with 20 ng/ml PMA and 0.5 μ g/ml ionomycin for 5 h in the absence or the presence of HS treatment. **B**, Northern blot analysis of total mRNA obtained from 2B4.11 hybridoma cells untreated (–) or stimulated with plate-bound anti-CD3 ϵ -chain mAb for 5 h in the absence or the presence of HS treatment. **C**, Northern blot analysis of total mRNA obtained from 2B4.11 hybridoma cells untreated (–) or stimulated with 20 ng/ml PMA and 0.5 μ g/ml ionomycin for 5 h in the absence or the presence of mild HS treatment (pretreatment at 39.5°C for 4 h, followed by a 0.5-h recovery period at 37°C). The migration of the ribosomal RNA (18S and 28S) is indicated. The experiments shown in A–C are representative of different independent experiments with similar results. **D**, Jurkat cells were transfected with 15 μ g of the indicated Luc reporter vector as described in *Materials and Methods*. Twenty-four hours after transfection, cells were left untreated (–) or were stimulated with 20 ng/ml PMA and 0.5 μ g/ml ionomycin (P/I) after or without HS or mild HS treatment for 24 h. Cells were then harvested, and protein extracts were prepared for the Luc assay. Results are expressed as relative Luc activity normalized to protein concentration and represent the mean \pm SE from at least three experiments.



A number of studies have shown that the fragment from –195 to +1 bp of the human *fas-L* promoter contains several important enhancer elements that cooperate for the transcription of this gene. Among these, a major transcription initiation site at –181 bp from the first ATG (34), a NF- κ B/SP-1 binding site located –150 bp from the first ATG (37), and a noncanonical c-Myc binding element localized in a position originally described as a putative TATA box sequence (38) have been described. Moreover, an AP-1 binding site in the proximity of the noncanonical c-Myc binding element at position –16 bp from the first ATG (37, 39) has been recently identified (Fig. 4A).

To determine whether the effect of hyperthermia on the *fas-L* promoter activation could involve an altered activity of these transactivators, we transfected Luc multicopy reporter vectors driven by NF- κ B, c-Myc, and AP-1 transcription factors in Jurkat cells. As shown in Fig. 4, B–D, the PMA- plus ionomycin-induced activity of these reporter vectors was differently modulated by hyperthermia. The transcriptional activity of AP-1 and, to a lesser extent, of NF- κ B was significantly increased by hyperthermia (Fig. 4, B and D). On the contrary, the multicopy reporter vector driven by c-Myc

was unaffected, similar to the basal activity of the control vector containing only the minimal TATA box from the herpes simplex virus thymidine kinase promoter (Fig. 4, C and E). Thus, hyperthermia differently affects the enhancer activity of the *fas-L* promoter transactivators AP-1 and NF- κ B, but not c-Myc, in activated T cells.

Hyperthermia enhances AP-1 and NF- κ B transcription factor expression/nuclear translocation in activated T cells

To investigate the mechanisms involved in hyperthermia-mediated increase in AP-1 and NF- κ B transcriptional activity, mobility shift and Western blot assays were performed on nuclear extracts prepared from activated Jurkat cells treated with hyperthermia. As shown in Fig. 5, A and B, activation of Jurkat cells induced specific FasL-NF- κ B and AP-1 binding complexes that were significantly enhanced by hyperthermia. As a control for equal protein loading, the same amount of nuclear extract was tested in the presence of an octamer factor(s)-specific probe. The DNA binding of octamer-1 was unaltered in hyperthermia-treated cells (Fig. 5C).

We also confirmed these results by Western blot assay using Abs specific for NF- κ B/RelA, c-Jun, and FosB, on these nuclear

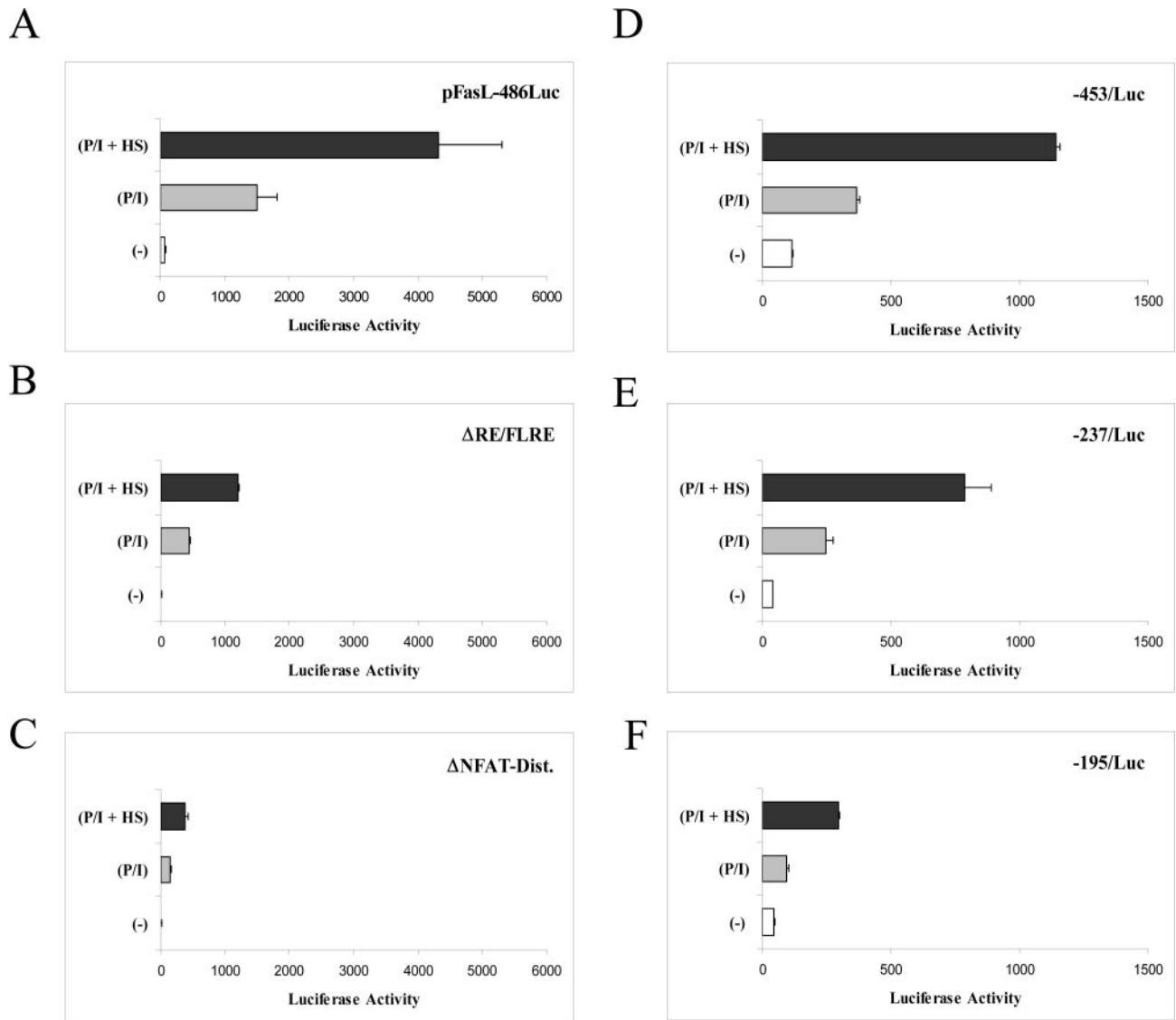


FIGURE 3. The *fas-L* promoter activation is enhanced by hyperthermia. Promoter analysis. *A–F*, Jurkat cells were cotransfected with 15 μg of the indicated Luc reporter vector plus 4 μg of pEQ176 CMV- β -Gal expression vector as described in *Materials and Methods*. Twenty-four hours after transfection, cells were left untreated (–) or were stimulated with 20 ng/ml PMA and 0.5 $\mu\text{g}/\text{ml}$ ionomycin (P/I) in the absence or the presence of HS treatment for 24 h. Cells were then harvested, and protein extracts were prepared for Luc and β -Gal assays. Results are expressed as relative Luc activity normalized to protein concentration as well as to β -Gal activity produced by the internal control plasmid and represent the mean \pm SE from at least four experiments.

extracts. As shown in Fig. 5, *D–F*, activation of Jurkat cells induced RelA nuclear translocation together with c-Jun and FosB expression. Hyperthermic treatment significantly increased c-Jun and FosB expression and, to a lesser extent, RelA nuclear translocation, in agreement with the data relating to the transcriptional activity and DNA-binding activity of these transactivators. As shown in these figures, the same filters were stained with an Ab specific for the octamer-1 transcription factor as a control for equal protein loading. Thus, hyperthermia increases the expression and nuclear translocation of AP-1 and NF- κ B in activated T cells.

Hyperthermia enhances fas-L gene expression in activated T cells: role of HSF-1

Hyperthermic stress has been shown to activate protective HS response genes in many different experimental models, including T cells (40–44). This evolutionarily conserved phenomenon, called cellular stress response, is mediated by a family of transactivators, the HSFs. They are endowed with heat-inducible DNA-binding

activity and transactivation (40, 41) and regulate the induction of a set of proteins, the HSPs, that is able to increase stress tolerance (we also confirmed these pathways in our cell lines; data not shown). Although four closely related *hsf* genes have been described (*hsf-1* to *hsf-4*), HSF-1 is the primary transactivator responsible for the HS response (40, 41).

To investigate whether HSF-1 could directly modulate the activity of the *fas-L* promoter, we cotransfected an expression vector encoding a constitutively active mutant of human HSF-1 in Jurkat cells. As shown in Fig. 6*A*, the expression vector encoding the constitutively active mutant of the human HSF-1 significantly increased *fas-L* promoter activity at normal temperature, suggesting that HSF-1 is able to cooperate for transactivation in this context. Interestingly, the internal promoter mutation that abrogates binding of early growth response factors (Δ RE3/FLRE) was more responsive to HSF-1 overexpression, whereas the mutant that abrogates NF-AT binding at the distal site (Δ NFAT-Dist.) lost responsiveness to HSF-1 (Fig. 6, *B* and *C*). Progressive deletion of these enhancer elements could confirm that the

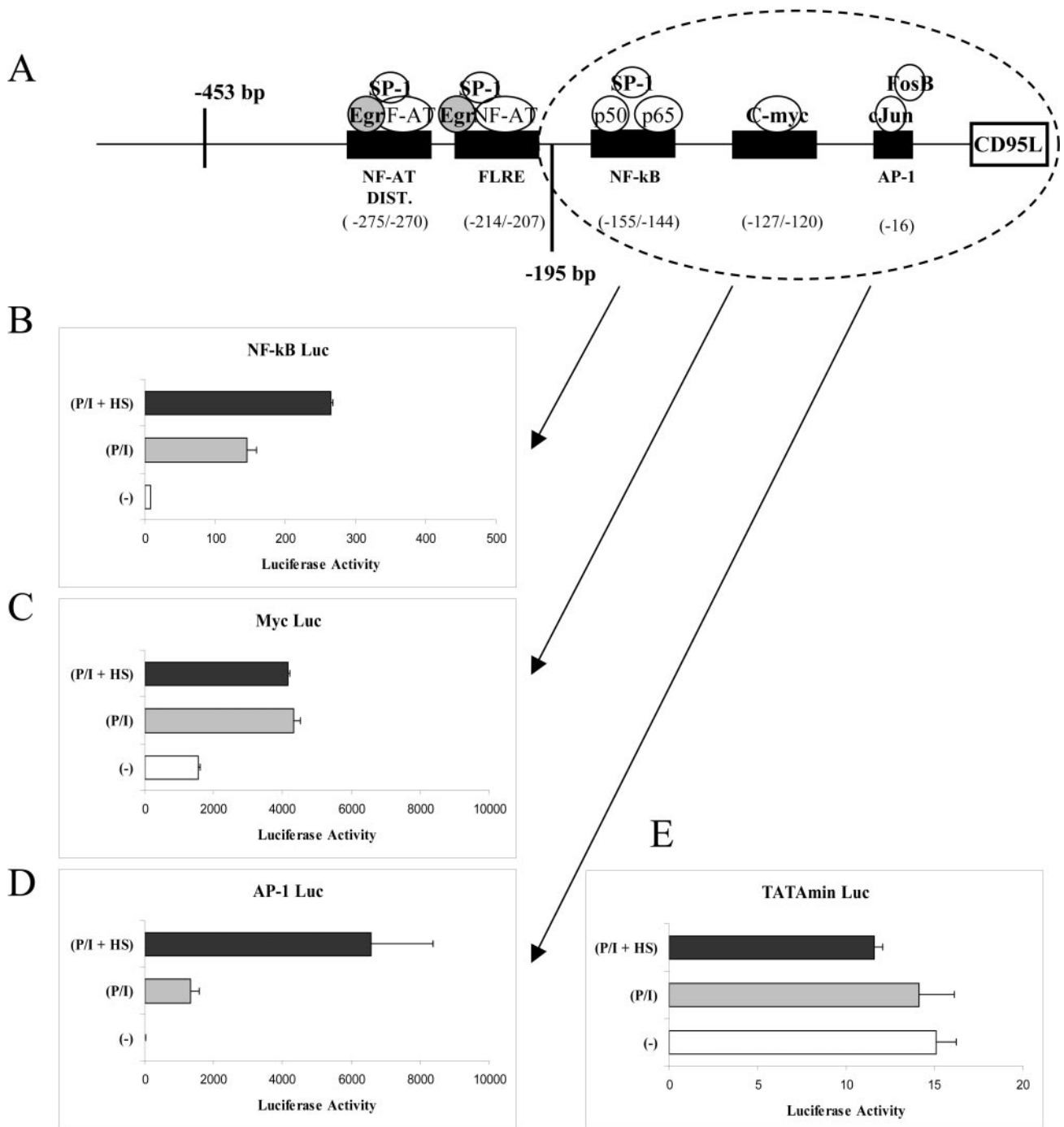


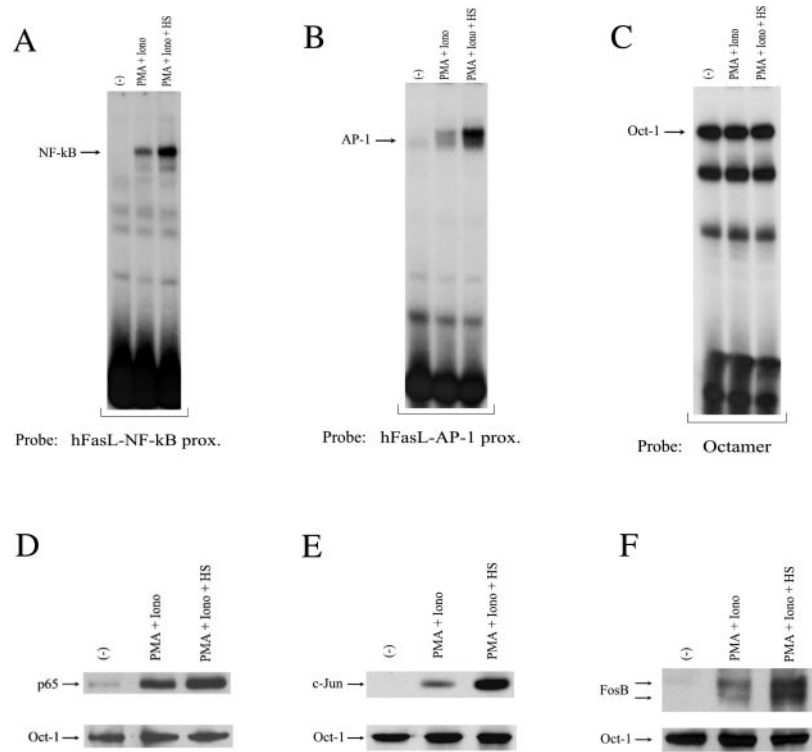
FIGURE 4. Hyperthermia enhances the activity of specific *fas-L* promoter transactivators. *A*, Schematic representation of the *fas-L* promoter. *B–E*, Jurkat cells were cotransfected with 10 μg of the indicated reporter vector plus 4 μg of pEQ176 CMV- β -Gal expression vector as described in *Materials and Methods*. Activation and sample harvesting were conducted as described in Fig. 3. Results are expressed as relative Luc activity normalized to protein concentration as well as to β -Gal activity produced by the internal control plasmid and represent the mean \pm SE from at least four experiments.

distal NF-AT binding site in this promoter represents a critical enhancer element for HSF-1-mediated transactivation in activated T cells (Fig. 6, *D–F*). Moreover, the overexpression of a deleted form of HSF-1 that constitutively binds HSE enhancers, but is unable to transactivate even under stress conditions (35, 45), could partially abrogate the effect of hyperthermia on *fas-L* promoter activation (Fig. 7). These data indicate that activation of HSF-1 plays a role in *fas-L* promoter regulation mediated by hyperthermia in activated T cells and identify the distal NF-AT binding site as an important element for this mechanism.

Hyperthermia enhances fas-L gene expression in activated T cells: role of PKC

Hyperthermia has been shown to affect a number of immune functions, including lymphocyte homing and activation (3–5, 7, 8). In particular, hyperthermia treatment results in a notable reorganization of the cytoskeleton in T lymphocytes, with HSP70, the cytoskeletal protein spectrin, the adaptor protein receptor for activated C kinase 1, and different PKC isoform colocalization in cytoplasmic aggregates and at the uropod (4, 5). Moreover,

FIGURE 5. Hyperthermia enhances AP-1 and NF- κ B transcription factor expression/nuclear translocation in activated T cells. *A–C*, EMSAs were performed using the indicated 32 P-labeled oligonucleotide as a probe in the presence of nuclear extracts (10 μ g) from unstimulated (–) or PMA/ionomycin-treated Jurkat cells (3 h) in the absence or the presence of HS. The arrow represents the DNA binding of specific complexes. *D–F*, Western blot assay of nuclear extracts (30 μ g) from unstimulated (–) or PMA/ionomycin-treated Jurkat cells (3 h) in the absence or the presence of HS. Specific bands corresponding to p65/RelA, c-Jun, FosB, and octamer-1 (used in this study as a control for equal protein amount loading) are indicated by arrows. The EMSAs and Western blots shown are representative of at least three independent experiments with similar results.



heat exposure can increase PKC activity in T lymphocytes, indicating that PKCs may be involved in hyperthermia-induced signal transduction events, leading to lymphocyte activation and function (3–5). Among the PKC isoforms involved in this context, PKC θ and PKC ϵ are particularly interesting because they are important regulators of the *fas-L* gene and promoter in activated T lymphocytes (46–48). To investigate whether the hyperthermia-mediated increase in *fas-L* promoter activation could also involve PKC activity, transient transfection

assays were performed in Jurkat T cells using expression vectors encoding dominant negative mutants of PKC θ and PKC ϵ . As shown in Fig. 8A, hyperthermia significantly enhanced activation of the human *fas-L* promoter in Jurkat cells. Overexpression of a dominant negative mutant of PKC ϵ and PKC θ partially inhibited promoter activation as previously described (46–48) and, more importantly, could significantly reduce the enhancement mediated by hyperthermia. To verify that the action of these dominant negative vectors was

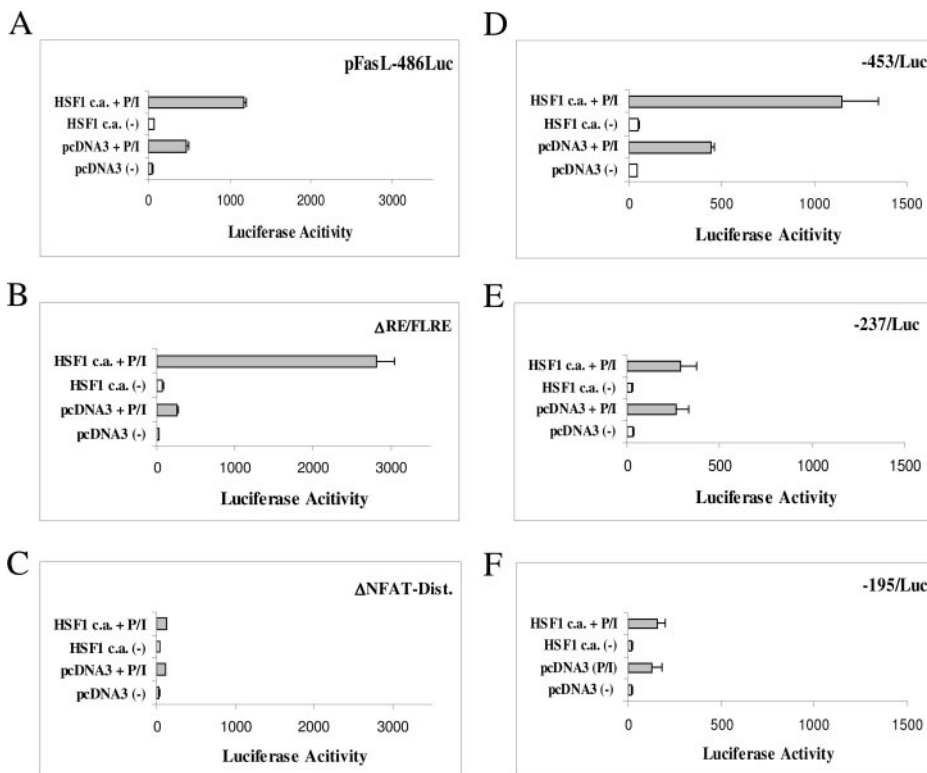


FIGURE 6. HSF-1 transactivates *fas-L* promoter in activated T cells. *A–F*, Jurkat cells were cotransfected with 15 μ g of the indicated reporter vector plus 5 μ g of RSV-Gal expression vector as described in *Materials and Methods*. Where indicated, 3 μ g of an expression vector encoding a constitutive active form of HSF-1 (pcDNA-HSF1 c.a.) or the pcDNA3 empty control vector was added to the cotransfection setting. Activation and sample harvesting were conducted as described in Fig. 3. Results are expressed as relative Luc activity normalized to protein concentration as well as to the β -Gal activity produced by the internal control plasmid and represent the mean \pm SE from at least three experiments.

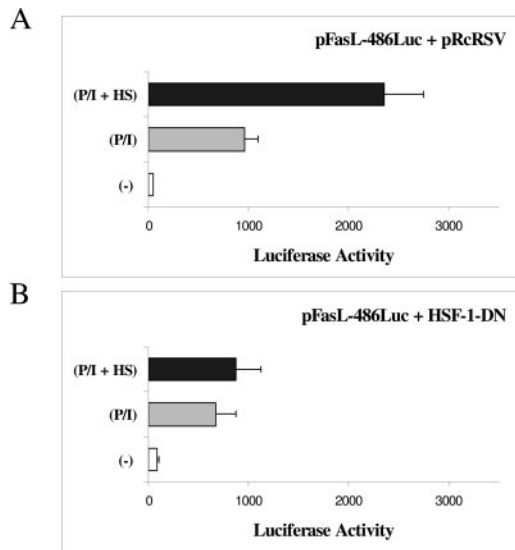


FIGURE 7. Effect of dominant negative HSF-1 on *fas-L* promoter activation after hyperthermia. *A* and *B*, Jurkat cells were cotransfected with 15 μ g of the indicated reporter vector plus 5 μ g of RSV-HSF1 dominant negative (DN) or pRcRSV empty control vector, as described in *Materials and Methods*. Activation and sample harvesting were conducted as described in Fig. 3. Results are expressed as the relative Luc activity normalized to protein concentration as well as to β -Gal activity produced by the internal control plasmid and represent the mean \pm SE from at least three experiments.

not simply a generalized reduced responsiveness of the cotransfected cells, we performed activation in the presence of the prostanoid CAY10410 (a 15-deoxy- $\Delta^{12,14}$ -PGJ₂ molecular analog). As shown in a previous study (33), this molecule significantly increased *fas-L* promoter activity, but cotransfection of the two PKC dominant negative isoforms could not reduce this effect (Fig. 8*B*). Thus, the activity of PKC may play an important role in the enhancement of *fas-L* promoter activation by hyperthermia.

Discussion

It has long been known that a significant number of cases of spontaneous remission in cancer patients often coincide with feverish infection. Since the late 19th century, Coley et al. (49, 50) made a number of clinical observations indicating that high fevers induced by intentional bacterial infections significantly correlated with improved cancer disease course. After those clinical studies until modern times, there has been little clinical interest in using the febrile response to bacterial infections as a therapeutic option for neoplastic diseases. However, since the 1970s, treatments of systemic whole body and regional hyperthermia have been reconsidered as a therapeutic tool for patients with malignant disease and have been used as a direct treatment or as an adjunct to classical radio-/chemotherapy protocols (2, 51, 52).

Besides the well-studied effects of hyperthermia to enhance the actions of several antineoplastic drugs (2), a great deal of attention has focused on the ability of heat to regulate the immune system and to contribute to its therapeutic actions (2, 3, 53–55).

Over the last few years, our laboratory has investigated the molecular mechanisms involved in normal physiological regulation and pharmacological intervention applied to the modulation of cytokine expression in activated T lymphocytes. In particular, regulation of the *fas-L* gene has been studied in these cells (30, 33), given its relevance for the maintenance of immune privilege, in the induction and regulation of organ-specific autoimmune diseases, and for T lymphocyte-

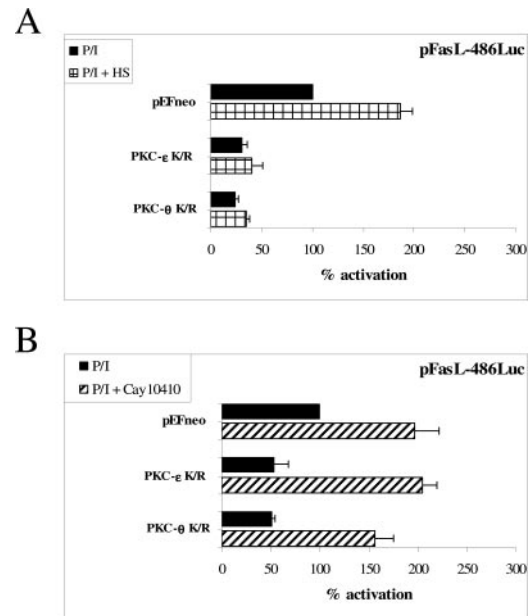


FIGURE 8. Effect of dominant negative PKC ϵ and PKC θ on *fas-L* promoter activation after hyperthermia. *A*, Jurkat cells were cotransfected with 15 μ g of the indicated reporter vector plus 10 μ g of PKC ϵ or PKC θ dominant negative K/R or pEF-Neo empty control vector, as described in *Materials and Methods*. *B*, Jurkat cells were cotransfected as described above. Twenty-four hours after transfection, cells were left untreated (–) or were stimulated with 20 ng/ml PMA and 0.5 μ g/ml ionomycin (P/I) in the absence or the presence of 10 μ M Cay10410 for 24 h. Activation and sample harvesting was conducted as described in Fig. 3. Results are expressed as the percent activation relative to the PMA- plus ionomycin-activatable Luc activity of the pEF-Neo empty control vector cotransfection (considered in this study as 100%) and represent the mean \pm SE from at least three experiments.

mediated cytotoxicity, a mechanism by which Ag-specific effector cells may eliminate different targets, such as tumor cells, via Fas-dependent mechanisms (19, 20, 22, 23).

In this study, the effect of hyperthermia on *fas-L* gene activation has been investigated in human and murine T cell lines. We have shown that hyperthermia may enhance Fas-L-mediated cytotoxicity, *fas-L* mRNA expression, and promoter activity in 2B4.11 T cell hybridoma and Jurkat cells. These effects have been observed using different modalities of T cell activation (PMA plus ionomycin, plate-bound anti-CD3) and with two experimental setting of hyperthermia (HS, 42°C for 1 h; mild hyperthermia, 39.5°C for 4 h). Our data also indicate that a minimal promoter fragment spanning nucleotides from –195 bp immediately 5' of the translational start site is still responsive to hyperthermia; this correlates with increased expression and transcriptional activity of specific transcription factors such as c-Jun, FosB, and NF- κ B, important regulators of the *fas-L* gene, located in this position.

Many cellular effects of hyperthermia have been described. High temperature may induce multiple actions on cell structure and physiology, the most remarkable of which are the pathways involved in activation of the stress response, resulting in the induction of a particular set of proteins, the HSPs (2). HSPs may cooperate together with accessory co-chaperones to protect the cell from dangerous stress, regulating different aspects of cell homeostasis, such as altered protein folding, or interacting with various signaling molecules to form active complexes (40, 41). This evolutionarily conserved phenomenon is mediated by the activation of a pivotal transcriptional factor called HSF-1, which is endowed with heat-inducible, DNA-binding activity and transactivation, in many different experimental

models, including T lymphocytes (42) (we have confirmed this pathway in our cell lines; data not shown). In this regard, the overexpression of a constitutively active mutant of human HSF-1 could significantly increase *fas-L* promoter activity at normal temperature in our cotransfection assays, suggesting that HSF-1 is able to cooperate for transactivation in this context. Moreover, the overexpression of a dominant negative HSF-1, unable to transactivate even under stress conditions, could partially abrogate the effect of hyperthermia on *fas-L* promoter activation. These results indicate that activation of HSF-1 plays a role in *fas-L* promoter regulation mediated by hyperthermia in activated T cells.

Hyperthermia may also affect the stability and fluidity of cellular membranes and may induce various changes in cytoskeletal organization in different cell types that may affect important functions, such as signaling events, cell shape, and migration (2, 56). In this regard, fever-range whole body hyperthermia may induce different changes in lymphocyte physiology indicative of an activation condition, such as reorganization of the cytoskeleton and cellular redistribution of several PKC isoforms with increased enzymatic activity (3–5).

Among the PKC isoforms involved in this effect, PKC θ and PKC ϵ are particularly interesting because they are important regulators of the *fas-L* gene and promoter in activated T lymphocytes (46–48). In addition, in T cells these two PKC isoforms are differently redistributed to the membrane or associated with the cytoskeleton after hyperthermia, and this correlates with increased PKC activity associated with these cellular fractions, suggesting a possible role in heat-induced signal transduction events leading to lymphocyte activation and function (4, 5). Our cotransfection assays have shown that overexpression of dominant negative mutants of the PKC θ and PKC ϵ isoforms partially inhibited promoter activation as previously described (46–48) and, more importantly, could significantly reduce the specific enhancement mediated by hyperthermia. These data indicate that PKC may play an important role in this regulation. Interestingly, increased *fas-L* promoter activity correlates with increased expression and activity of transcription factors, such as AP-1 and NF- κ B, which are critical transactivators regulated by PKC (57–60) and are located in the minimal promoter fragment (from –195 bp), still sensitive to hyperthermia.

The thermal component of fever and the HS response may act in the context of different pathologies in several ways. Besides its direct action on the viability of the pathogens, hyperthermia may increase their immunogenicity via HSP production or differently regulate important pathways of the immune response (61). The experiments described in this report, showing that hyperthermia increases *fas-L* gene expression in activated T cells, add further evidence of the multiple regulatory effects of heat on the immune system; they may help in our understanding of the complex pathways that operate during adjuvant hyperthermia treatments of malignant disease (2, 51, 52). Various laboratories have already shown that triggering of death receptors, such as Fas/CD95 or TNF receptor type 1, may inhibit activation of the HSF-1/hsp70-mediated stress response; they activate protein phosphatase (PP) 1/PP2a- and PP2b-specific phosphatases that inhibit HSF-1 hyperphosphorylation and DNA-binding activity, thus increasing the sensitivity of the cell to heat-induced apoptosis (62, 63). In contrast, hyperthermia may activate caspase-8 and down-regulate FLIP levels, which sensitizes different cell lines to Fas/CD95-mediated apoptosis (64). Our observation, that hyperthermia increases *fas-L* gene expression in activated T cells could well fit with these regulatory pathways that involve death receptor signaling, stress response to heat, and modulation of apoptosis. Appropriate

schedules of hyperthermia treatment (or hyperthermia together radio-/chemotherapy) might render specific target cells more susceptible to Fas-mediated apoptosis and, in parallel, increase *fas-L* gene expression and action in activated CTL. This might, in turn, further sensitize targets to heat by inhibiting the HSF-1/hsp70 stress response via Fas receptor signaling. Associated with these effects is the ability of elevated temperatures to induce immature dendritic cells to mature phenotypically and functionally, both in vitro and in vivo (also in the absence of any exogenous inflammatory stimuli), and this correlates with the improved immunostimulatory ability of these cells (13–15). These observations indicate that enhanced dendritic cell activation and function might represent an additional strategic use of heat to enhance the potency of immunotherapy schedules or to improve the activity of vaccines consisting of relatively weak Ags, such as cancer vaccines (65). In this context, the ability of Fas-L to elicit an optimal proliferation of CTLs (24–26) and the effect of an appropriate Fas receptor triggering on the phenotypic and functional maturation of dendritic cells should also be considered (27).

In summary, this study has identified *fas-L* as a novel molecular target of hyperthermia that extends knowledge about the immunoregulatory actions of heat. Additional work will be necessary to further characterize the effects of hyperthermia on the molecular mechanisms of T cell activation and to support these data using nontransfected systems and/or in vivo models to improve the potential for thermal therapy in the clinic, particularly in the context of cancer immunotherapy.

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