Human immunodeficiency virus type 1 interaction with the membrane of CD4+ cells induces the synthesis and nuclear translocation of 70K heat shock protein

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In the last few years a growing body of experimental evidence has indicated that the interaction of human immunodeficiency virus type 1 (HIV-1) surface glycoprotein (gp120) with the membrane of CD4+ cells may deliver negative signals, eventually leading to programmed cell death (apoptosis) of either mature CD4+ lymphocytes or CD34+ haematopoietic progenitor cells, in the absence of cell infection with HIV-1. However, information on the possible activation of the classical signal transduction pathway through gp120 engagement of cell surface CD4 is contradictory. Heat shock proteins (hsp) or 'stress' proteins' are involved in protecting cells from the deleterious effects of heat and other stresses and perform various cell roles. In mammalian cells there is evidence that hsp70 is involved in the transport of proteins to lysosomes, mitochondria and the nucleus. The results obtained in our study demonstrate that early (3 h) after the exposure of permissive CD4+ cells to HIV-1 (or to purified recombinant gp120) a peak of increased synthesis and nuclear translocation of a 70K hsp (and possibly other proteins) is observed. These data indicate that gp120 possesses the capacity to trigger a cascade of events through a transmembrane signalling activity.

The most detrimental consequences of infection with human immunodeficiency virus (HIV) type 1 are the functional defects and dramatic depletion of CD4+ T lymphocytes, which are the cause of the profound immunosuppression characteristic of AIDS (Fauci, 1988).

Despite some contradictions (Bagasra et al., 1992), the body of available experimental evidence indicates that the virus load in peripheral blood mononuclear cells is relatively low (Schnittman et al., 1989; Simmonds et al., 1990; Brinchmann et al., 1991). This suggests that the progressive decline in function and number of CD4+ T cells is unlikely to be a direct consequence of the presence of HIV-1 in infected cells and that an additional mechanism(s) may contribute to the depletion of CD4+ T lymphocytes characteristic of advanced HIV-1 infection. This depletion has been attributed to a wide range of immunological and autoimmune pathogenic mechanisms (for reviews, see Rosenberg & Fauci, 1989 and Pantaleo et al., 1993), but their role has yet to be established by more convincing experimental evidence.

HIV-1 envelope glycoproteins play a central role in HIV-1-induced cell killing, as demonstrated by the fact that a functional env gene, encoding the envelope glycoprotein precursor gp160, is a prerequisite for HIV-1-induced cytopathic effect (Stevenson et al., 1990; York-Higgins et al., 1990). Moreover, HIV-1 envelope glycoprotein interaction with CD4, in some circumstances at least, seems sufficient to induce irreversible cellular damage, since constructs expressing gp160 alone kill CD4+ but not CD4- T cells (Koga et al., 1990).

Besides the capacity to bind HIV-1 to CD44 receptor, gp120 has been shown to possess intrinsic biological activities of its own (Capron & Ward, 1991) as shown, for instance, by its ability to induce neuronal cell injury in the mammalian central nervous system (Brennemann et al., 1988; Dreyer et al., 1990) and by its capacity to induce interferon (Capobianchi et al., 1992).

In the last few years, a growing body of experimental data has focused on the central role of programmed cell death (apoptosis) as the cause of syncytium-independent, single-cell killing in HIV-1-infected subjects (Terai et al., 1991; Laurent-Crawford et al., 1991). This evidence supports the hypothesis that the simple interaction of HIV-1 envelope glycoprotein (gp120) with CD44 molecules present on the cell membranes may deliver negative signals, eventually leading to activation-induced apoptosis in the absence of HIV-1 cellular infection (Weinhold et al., 1989; Ameisen & Capron, 1991; Meyaard et al., 1992; Groux et al., 1992). It has...
been demonstrated that even picomolar concentrations of gp120, bound to human mature CD4+ T cells when cross-linked by anti-gp120 antibody, prime T cells for activation-induced cell death (Banda et al., 1992).

In addition, the survival/proliferation capacity of human haematopoietic progenitor (CD34+) cells, stimulated by interleukin-3 (IL-3) in liquid culture, and the number of committed progenitors are severely impaired by exposure to HIV-1, or to purified recombinant gp120, in the absence of productive and/or latent HIV-1 infection (Zauli et al., 1992a, b). These events are very likely to be the consequence of gp120 interaction with small amounts of CD4 molecules expressed at the surface of CD34+ cells (Zauli et al., 1992c). This mechanism may also operate in vivo; CD34+ cells from HIV-1-seropositive patients, although apparently free from productive and/or latent HIV-1 infection, show a significantly reduced colony formation involving all committed progenitor lineages and, when placed in liquid cultures containing IL-3, appear committed to growth suppression (Zauli et al., 1992c, d). These findings together with the increased production of negative regulators of haematopoiesis, induced by HIV-1 tat gene product (Zauli et al., 1992e), may help to elucidate the pathogenesis of impaired haematopoiesis, resulting in various types of peripheral blood cytopenias, frequently observed during the course of HIV-1 infection.

However, the mechanisms involved in priming apoptotic cell death are not known, and information on the possible activation of the classic signal transduction pathway through gp120 engagement of cell surface CD4 is contradictory (Linette et al., 1988; Kornfeld et al., 1988; Dreyer et al., 1990; Horak et al., 1990; Cohen et al., 1992; Kaufmann et al., 1992).

Heat-shock and other types of cell stress, as agents triggering or facilitating HIV-1 replication and diffusion, have been investigated (Geelen et al., 1988; Re et al., 1989; Furlini et al., 1990; Stanley et al., 1990; Legrand-Poels et al., 1990). Heat-shock proteins (hsp) present at the surface of HIV-1-infected cells have also been implicated in AIDS pathogenesis (Di Cesare et al., 1992).

Hsp belonging to the 70K family have been shown recently to be involved in the active transport of proteins from the cytoplasm to the nucleus (Shi & Thomas, 1992). Therefore we investigated the behaviour of hsp70 in CD4+ lymphoblastoid T cell cultures after exposure to infectious HIV-1, heat-inactivated HIV-1 or purified recombinant gp120.

The 'A3.01' clone of a human lymphoblastoid T cell line (Folks et al., 1985) was grown in RPMI 1640 (Gibco BRL) supplemented with 10% fetal calf serum (FCS) and passaged twice a week. The virus was obtained from the supernatant of H9/HTLVIIIIB cell cultures (Popovic et al., 1984). The supernatant, preclarified by low speed centrifugation, was ultracentrifuged at 100000 g for 1 h at 4 °C and the pellet resuspended in one-tenth of the original volume. Under our experimental conditions, the virus preparation incorporated 4 x 106 c.p.m./ml, measured in a reverse transcriptase assay (Lee et al., 1988), and contained 400 ng/ml of p24 HIV-1 core antigen as measured by a commercial immunoassay (DuPont).

Different aliquots of cell cultures (8 ml of a cell suspension seeded at a density of 2.5 x 106 cells/ml in complete growth medium) were centrifuged and the cell pellets separately treated as follows: (i) resuspended in 1 ml of the infectious virus stock; (ii) resuspended in the same amount of virus stock pre-inactivated by heating for 1 h at 56 °C; (iii) resuspended in the same amount of complete RPMI medium containing 10 μg/ml of purified recombinant gp120 produced in insect cells using a baculovirus expression vector (MycoGenesys); (iv) resuspended in the same amount of supernatant of uninfected H9 cells (mock-treated controls). All the preparations were incubated for 2 h at 37 °C and then washed by low speed centrifugation, resuspended in 10 ml of fresh growth medium and incubated at 37 °C.

Two ml aliquots of the various preparations were collected at different intervals (after 1, 3, 6, 12 and 24 h of incubation), counted to evaluate the total number of cells, pelleted, and stored frozen at -80 °C until examination.

For immunoblot analysis, each cell pellet was resuspended in 100 μl of electrophoresis sample buffer (0.05 M-Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.0025% bromophenol blue) and heated at 100 °C for 2 min. This resulted in complete cell lysis and solubilization of proteins. Alternatively, cell pellets were resuspended in lysing solution no. 1 (20 mM-Tris–HCl pH 7.5, 50 mM-NaCl, 0.5% NP40, 0.1 M-PMSF) and incubated for 5 to 10 min on ice, in order to lyse and solubilize the cytoplasmic fraction. Cell extracts were then centrifuged at 10000 g for 10 min to pellet the nuclei which were separately lysed and solubilized by resuspension in 100 μl of electrophoresis sample buffer and heating at 100 °C for 2 min.

The polypeptides present in normalized (on the basis of the protein content/ml) samples of various whole cell lysates or of different cytoplasmic and nuclear fraction lysates were separated by SDS–PAGE using 0.75 mm thick slab cells (10% acrylamide and 2.6% bisacrylamide) containing 0.025 M-Tris, 0.186 M-glycine (pH 8.3) and 0.1% SDS. The polypeptides were then electrotransferred to nitrocellulose membranes, in 0.025 M-Tris–HCl, 0.192 M-glycine (pH 8.2) containing 20% (v/v) methanol, using a Bio-Rad Trans-Blot electrophoretic cell at a constant current of 220 mA (40 to 50 V) for 4 h at 4 °C.

The blots, quenched for 1 h at room temperature in a
buffer containing 10 mM-Tris-HCl pH 7.4, 50 mM-KCl, 5 mM-MgCl₂, and 3% gelatin, were then analysed for the presence of hsp70 by overnight incubation at room temperature in mouse anti-hsp70 monoclonal antibody (MAb) (Boehringer Mannheim) diluted 1:100 in blotting buffer (0.02 M-Tris-HCl pH 7.4, 0.1 M-NaCl, heat-inactivated normal goat serum and 5% non-fat dry milk). The blots were then washed three times in 0.02 M-Tris-HCl pH 7.4, containing 0.1 M-NaCl and 0.3% Tween 20, and the specifically bound antibody was visualized in situ using goat anti-mouse IgG conjugated with biotin, avidin conjugated with horseradish peroxidase and the enzyme substrate 4-chloro-1-naphthol. The reaction was terminated after 20 to 30 min by extensive washing with water and the blots were immediately recorded photographically.

For pulse-chase experiments, the cells were washed three times with PBS and resuspended at a density of 5 x 10⁶ cells/ml in RPMI lacking methionine, cysteine and FCS, and containing 100 μCi/ml of [35S]methionine–cysteine (Amersham). At the end of the incubation period the cells were washed by low-speed centrifugation and resuspended in prewarmed complete growth medium. Two types of experiments were performed. In the first, the cells were incubated for 2 h in the presence of radioactive amino acids and then washed and incubated for 2 h in complete growth medium before mock treatment or treatment with infectious virus, heat-inactivated virus, or recombinant gp120, as previously described. In the second type of experiments the cells were radioactively pulsed for 2 h starting at the beginning of the various treatments.

At different times, 2 ml aliquots were collected centrifuged, and resuspended in cold lysing solution no. 1 for 10 min on ice to lyse and solubilize the cytoplasmic fraction. The nuclei, collected by centrifugation, were resuspended in 200 μl of lysing solution no. 2 (50 mM-Tris–HCl pH 8.0, 150 mM-NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% NP40, 0.5% sodium deoxycholate) and sonicated twice for 30 s with a sonifier (Branson).

Nuclear fractions were precleared by adding 60 μl of 1:10 (w/v) of killed, fixed *Staphylococcus aureus* (Cowan strain; SAC) and 15 μl of normal mouse serum, incubated for 1 h at 0 °C and centrifuged at 12000 g for 5 min in a microfuge.

 Supernatants were harvested and mixed with 5 μl of mouse anti-hsp70 MAb and incubated overnight at 4 °C in the presence of 60 μl of SAC. The immunoprecipitates were collected by centrifugation at 13000 g for 15 min, washed three times by the same procedure, electrophoresed in polyacrylamide gels and transferred to a nitrocellulose sheet, as previously described. The blots were then exposed to Kodak X-Omat films for 48 to 72 h at −80 °C.

Total RNA was isolated by the RNezol B method (Cinna/Biotec) performed according to the manufacturer’s instructions and the amount of hsp70-specific mRNA was examined by Northern blotting using a probe consisting of an hsp70 mRNA-specific 2.3 kb cDNA [excised from the pH2.3 plasmid (Hunt & Komiyama, 1985) obtained from Dr E. Lalli, Immunology and Genetics Department, Cevilla-Putti Research Institute, Bologna, Italy] labeled by random priming (Feinberg & Vogelstein, 1983) with digoxigenin–dUTP (Boehringer Mannheim).

For Northern blot analysis, samples of 10 μg of total RNA were electrophoresed in 1% agarose–formaldehyde gels and then transferred onto nylon membranes (Sambrook et al., 1989). RNA blots were prehybridized in 50% formamide, 5 x SSC, 5 x Denhardt’s solution, 0.2% SDS and 200 μg/ml of calf thymus DNA, at 45 °C for 3 h. The hybridization was performed in the same solution containing 100 ng/ml of digoxigenin–labeled, hsp70-specific, denatured probe and the reaction was carried out at 45 °C overnight. At the end of the hybridization periods the blots were washed twice in 0.2 x SSC, 0.1% SDS at 55 °C for 30 min. Hybrids were detected with a probe consisting of a 780 bp glyceraldehyde-3-phosphate dehydrogenase mRNA–specific cDNA from the PGl1 plasmid (Lalli et al., 1992), labelled and used as described above.

As shown in Fig. 1, the results of immunoblot analysis of whole cell lysates showed no significant differences in
Fig. 2. Separate immunoblot analysis with anti-hsp70 mouse MAb of cytosolic (C) and nuclear (N) fractions of cells harvested at different times after exposure to (a) mock treatment, heat-inactivated HIV-1, infectious HIV-1, 10 μg/ml recombinant gp120 or heat shock; (b) mock treatment, infectious HIV-1, infectious HIV-1 pre-incubated with neutralizing anti-gp120 antibody or soluble (s) CD4.

the various samples of cells treated with infectious virus, heat-inactivated virus or recombinant gp120, with respect to mock-treated controls. Only mock-treated heat-shocked (1 h at 41.5 °C) cells showed a clear-cut increase in the amount of hsp70 present in whole cell lysate in the samples harvested after 12 and 24 h of incubation.

When cytoplasmic and nuclear extracts were analysed separately (Fig. 2a), however, an interesting phenomenon was consistently observed in the samples harvested after 3 h of incubation from heat-shocked cells or cells treated with infectious virus, heat-inactivated virus or recombinant gp120. Almost all the immunologically detectable hsp70 in these samples was present in the nuclear fractions, and only in cells treated with purified gp120 alone was hsp70 still detectable at the cytoplasmic level.

To study the specificity of the observed phenomenon, the experiments with infectious HIV-1, heat-inactivated HIV-1 and recombinant gp120 were repeated pre-incubating the inocula with 20 μg/ml of soluble CD4 (American Biotechnology) for 30 min at 4 °C or with NEA-9305 neutralizing anti-gp120 mouse MAb (DuPont-NEN) diluted 1:100, for 1 h at 4 °C. In both instances the appearance of hsp70 in the nucleus was inhibited in samples harvested 3 h after the beginning of the experiments. The data obtained with infectious HIV-1 are shown in Fig. 2 (b).

In pulse-chase experiments, we tried to verify whether the nuclear influx of hsp70 was due to a protein synthesized de novo or to the translocation of a protein already present in the cytoplasm. The results (Fig. 3) demonstrate that when the first type of labelling was used (2 h of radioactive pulse, followed by a chase of 2 h, before starting the various treatments) the same amount of radioactive hsp70 was present in all the nuclear samples, indicating a constant nuclear influx of hsp70 synthesized before the analyses. On the contrary, when the radioactive pulse (2 h) was performed at the beginning of the experimental treatments, there was a clear-cut increase in radioactively labelled hsp70 in the nuclear fractions collected 3 h after exposure to heat-inactivated HIV-1, infectious HIV-1 or recombinant gp120, at variance with mock-treated cells. These results were confirmed after autoradiography when the same
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Fig. 4. (a) Northern blot analysis of hsp70-specific mRNA in total RNA preparations from: lanes 1, 4, 6, mock-treated cells; lanes 2, 5, 7, cells exposed to infectious HIV-1; lanes 3, 6, 9, cells exposed to heat-inactivated HIV-1, harvested at various times (1, 3 and 6 h) after the different treatments. (b) The same preparations as in (a), hybridized with the GAPDH mRNA-specific probe.

Fig. 5. Comparison of the relative amounts of hsp70-specific mRNA in (a) cells exposed to heat-inactivated HIV-1, harvested 6 h after the beginning of treatment, (b) cells exposed to infectious HIV-1 and (c) mock-treated cells. Each row represents 6 μg of total RNA serially diluted from 1:2 to 1:64 (columns 1 to 6 respectively), spotted onto nylon membranes and hybridized with the hsp70 mRNA-specific probe as described in the text.

blots were treated with anti-hsp mouse MAb and stained as previously described (not shown).

These findings demonstrated that the hsp70 detected in the nuclear fractions 3 h after the various treatments was mainly represented by a protein synthesized de novo.

In another set of experiments we then analysed the rate of accumulation of hsp70-specific mRNA. These experiments were limited to samples of mock-treated cells and cells exposed to infectious or heat-inactivated HIV-1. As shown in Fig. 4, only 6 h after the start of experiments a consistent increase (about four times the baseline value; see Fig. 5) in hsp70-specific mRNA content was observed in the cells treated with infectious or heat-inactivated HIV-1, whereas the amount of GAPDH-specific mRNA remained constant in all samples examined. These data indicate that the bulk of hsp70 translocated in the nuclei 3 h after treatment with infectious HIV-1, heat-inactivated HIV-1 or recombinant gp120, although apparently synthesized de novo, is derived from the translation of mRNAs already present in the cell, rather than from an increased specific transcription.

In conclusion, our data support the idea that the simple interaction of HIV-1 with the membrane of CD4+ cells is able to trigger a signal detectable at nuclear level. This signal is revealed by an enhanced transient nuclear translocation of hsp70 3 h after virus/cell contact.

The phenomenon is particularly evident in cells exposed to infectious HIV-1, but occurs also in cells exposed to heat-inactivated whole HIV-1 and is detectable in cells exposed to purified recombinant gp120 alone, and therefore seems independent of the presence of a viral infectious process. Some of the increased nuclear hsp70 probably derives from the nuclear influx of hsp70 already present in the cytosolic fraction, as demonstrated by its disappearance from the cytoplasm of cells treated with infectious or heat-inactivated HIV-1 and by the decreased amount present in the cytoplasmic fraction of gp120-treated cells. However, much of the increased nuclear content of hsp70 seems to be the consequence of the nuclear translocation of the product of a de novo rapid synthesis of inducible members of the hsp70 family, as shown by the results of radioimmunoprecipitation assays performed with radioactive labelling at the beginning of cell treatments.

Moreover, the absence of an increase in specific mRNA accumulation during the first 3 h after the various treatments indicates that the newly synthesized hsp70 in this situation derives mainly from the translation of constitutively expressed mRNAs which are stored in the cytoplasm ready to be used in an emergency. The subsequent increase in specific mRNA accumulation represents the cellular answer to this enhanced turnover and may constitute a prelude to the elevation of intracellular contents of hsp observed late after infection by other authors (Poccia et al., 1992).

The question of whether gp120 binding to CD4 triggers a transmembrane signalling process is an important issue in understanding the mechanism underlying the ‘toxicity’ of HIV-1 envelope glycoprotein (gp120) demonstrated by a substantial body of experimental evidence.

Analysis of the classical pathway of transmembrane signal transduction in CD4+ T cells exposed to HIV-1 or to purified gp120 has led only to inconclusive and contradictory results. The present findings clearly con-
firm the definite biological role of gp120 and uncover its capacity to trigger the nuclear translocation of hsp70 in a transmembrane signalling activity in the absence of cellular infection. As mentioned before, hsp70 is involved in the active transport of proteins from the cytoplasm to the nucleus.

Further experiments are needed in order to clarify the molecular events associated with the nuclear influx of hsp70 (and possibly other proteins) and the possible links of this phenomenon to the priming of apoptotic cell death, in the absence of a productive and/or latent HIV-1 infection.

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References


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