Immunelectron Microscopic Localization of the pX Gene Products in Human T-Cell Leukemia Virus Type 1-producing Cells

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ABSTRACT

The location of the pX gene products in human T-cell leukemia virus type 1-producing cells, MT-2 and HUT 102, was studied by immunelectron microscopy using the direct and indirect peroxidase-labeled antibody methods. Fab'-peroxidase conjugates were prepared for the direct method with a maleimide compound from antisera to the carboxy-terminal region of the pX gene products. Positive immunostaining in MT-2 cells was detected in the endoplasmic reticulum, the outer and inner leaflets of the nuclear membrane, and inside their cisternae, but not in the plasma membrane and viral particles. Staining in the nucleus was faint. On the other hand, positive immunostaining in HUT 102 cells was detected diffusely in the euchromatin regions of the nucleus but not in the nucleoli, nuclear envelope, and cellular membrane systems. The location of the positive immunostaining in the HUT 102 nuclei was reconfirmed by the reaction in isolated nuclei. On the basis of both the immunelectron microscopic and immunoblotting analyses of the pX gene products, it is suggested that the M, 40,000 to 42,000 protein (p40) is localized mainly in the euchromatin regions of the nucleus of human T-cell leukemia virus type 1-producing cells, and the M, 68,000 protein (p68) is localized mainly in the nuclear envelope and the endoplasmic reticulum of MT-2 cells. p68 detected in MT-2 cells with the anti-p40 serum was deduced to be a protein consisting of p40 and a part of env gene products and to share epitopes in common with p40.

INTRODUCTION

HTLV-1 is a retrovirus etiologically associated with ATL, which is endemic in southwest Japan and some other countries (1-7). HTLV-1 is capable of transforming normal human T-lymphocytes in vitro by cocultivation with virus-producing cells (8), but it does not contain a cell-derived oncogene. However, the putative transforming gene of this virus, termed pX, is located between the env gene and the 3' LTR (7). The proteins encoded by this gene have been identified as p40 and p68 (9-13). On the basis of both subcellular fractionation techniques and immunocytochemical analysis at the light microscopic level, p40 has been shown to be located mainly in the nucleus and partly in the cytoplasm of HTLV-1-infected cells (14-18). It has been suggested that the pX gene product (p40) participates in trans-acting transcriptional activation of the LTR of HTLV-1 and is associated with virus replication as well as with the initiation and maintenance of cell transformation (16, 19-21). It is important to know the detailed intracellular distribution of these proteins to clarify the molecular mechanism of cell transformation by HTLV-1.

To locate the pX gene products (p40 and p68) in the ultrastructure of HTLV-1-producing human lymphocytes, we have carried out immunelectron microscopic observations of the pX proteins in MT-2 cells and HUT 102 cells by the direct and indirect peroxidase-labeled antibody methods.

MATERIALS AND METHODS

Cells. The HTLV-1-producing lymphocyte cell lines used for this work were MT-2, established by cocultivating normal human cord leukocytes and leukemic T-cells of an ATL patient (8), HUT 102, established from a cutaneous T-cell lymphoma (1), and ATL-S, established from leukemic T-cells of an ATL patient. Uninfected control T-cell lines Molt-4 and Jurkat, both derived from acute lymphoblastic leukemia, were used as control cell lines (22). Cells of these cell lines were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum.

Isolation of Nuclei. Cells in suspension culture were pelleted by centrifugation for 10 min at 200 x g. The pellet was washed with Hank's solution twice, suspended in Buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 1 mM CaCl2, and 2 mM MgCl2) containing 0.5% Nonidet P-40. The suspension was layered on 2.2 M sucrose in Buffer A and centrifuged for 90 min at 130,000 x g at 4°C. The pelleted nuclei were washed once with 0.25 M sucrose in Buffer A and used as isolated nuclei.

Preparation of Fab'-Peroxidase Conjugates Using a Maleimide Compound. IgG was obtained from the antisera and preimmune sera by adsorbing on a Protein A column, eluting with 0.1 M citrate buffer, pH 3.0, and concentrating with Amicon Centriflo ultrafiltration membrane cones. The pH was adjusted to 5.0 with 0.1 M acetate buffer, pH 5.0, and 0.15 M sucrose containing 10% Nonidet P-40. The conjugate was layered on a 2.2 M sucrose in Buffer A and incubated for 90 min at 4°C. The pelleted nuclei were washed once with 0.25 M sucrose in Buffer A and used as isolated nuclei.

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1 To whom requests for reprints should be addressed.

The abbreviations used are: HTLV-1, human T-cell leukemia virus type 1; ATL, adult T-cell leukemia; LTR, long terminal repeat; p40, M, 40,000 to 42,000 protein; p68, M, 68,000 protein (24, p19, and p28 defined similarly); gp46, M, 46,000 polypeptide (gp21, gp62, and gp68 defined similarly); HRP, horseradish peroxidase; PFA, paraformaldehyde; PBS, 0.1 M phosphate buffer solution; DAB, 0.03% 3',3'-diaminobenzidine tetrahydrochloride.

4 Miyamoto et al., unpublished observation.
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Fig. 1. A and B. immunoelectron micrographs of MT-2 cells, fixed with PFA, frozen sectioned, and incubated directly with Fab'-peroxidase conjugates prepared from antiserum to bGH-p40*, showing positive immunostaining in the outer and inner leaflets of nuclear membranes, endoplasmic reticulum, and inside their cisternae. Plasma membrane and virus particles (B) are not stained. N, nucleus; V, virus particles. A, X 15,600; B X 19,900. C. MT-2 cells, fixed with glutaraldehyde and osmium tetroxide, sectioned, and stained with uranyl acetate and lead citrate, showing a large number of virus particles in the extracellular space. X 46,200. Scale line, 0.5 µm.

Table 1 Results of immunoelectron microscopic observations and Western blot analysis of the pX gene products with the anti-p40* serum

<table>
<thead>
<tr>
<th>Location of immunoelectron microscopic staining</th>
<th>MT-2</th>
<th>HUT 102</th>
<th>Molt-4</th>
</tr>
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<tbody>
<tr>
<td>Endoplasmic reticulum</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nuclear envelope</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Euchromatin regions</td>
<td>+</td>
<td>++</td>
<td>-</td>
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<thead>
<tr>
<th>Western blot analysis of the pX gene products</th>
<th>p68</th>
<th>p40</th>
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<tbody>
<tr>
<td>MT-2</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>HUT 102</td>
<td>+</td>
<td>++</td>
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<tr>
<td>Molt-4</td>
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PBS and incubated with Fab'-HRP conjugates (Fab', 160 µg; HRP, 140 µg/ml) at 4°C overnight. After the immunological reaction, the specimens were washed with PBS, fixed in 1% glutaraldehyde, washed with PBS, and preincubated in Graham-Karnowsky's substrate solution containing DAB in 0.05 M Tris-HCl buffer, pH 7.6, without H2O2 for 30 min and then in Graham-Karnowsky's substrate solution containing 0.03% DAB and 0.005% H2O2 in 0.05 M Tris-HCl buffer, pH 7.6, for 2–5 min at room temperature (25). After the reaction, the specimens were fixed with 2% OsO4, dehydrated in an ethanol series and propylene oxide, and embedded in epoxy resin on the slides. The embedded specimens were removed from the slides by brief heating, ultrathin sectioned, and examined with a Hitachi HU-11C electron microscope.

RESULTS

Electron Microscopy of Immunoperoxidase Staining. The results of immunoelectron microscopic observations are summarized in Table 1. Positive immunostaining in MT-2 cells with antiserum to bGH-p40* was detected in the endoplasmic reticulum, the outer and inner leaflets of the nuclear membranes, and inside their cisternae, but not in the plasma membrane and viral particles by both the direct (Fig. 1, A and B) and indirect method (Fig. 2). Staining in the nucleus was extremely faint and ambiguous. A conventional electron micrograph of MT-2...
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Fig. 2. MT-2 cells, fixed with PFA, frozen sectioned, incubated with antiserum to bGH-p40*, and peroxidase stained by the indirect method, showing similar immunostaining as in Fig. 1. × 6,300.

Fig. 3. MT-2 cells, fixed with PFA, frozen sectioned, and incubated directly with Fab'-peroxidase conjugates prepared from sera of patients with ATL, presented as a positive control. Positive staining is observed on the viral particles, plasma membranes, endoplasmic reticulum, the outer and inner leaflets of nuclear membranes, and inside their cisternae. × 9,600.

cells, stained with uranyl acetate and lead citrate, showed a large number of virus particles in the extracellular space (Fig. 1C). Immunoperoxidase reaction in cell suspension confirmed that the plasma membrane and viral particles were not stained with antiserum to bGH-p40*. Immunoperoxidase staining in MT-2 cells with sera from patients with ATL as a positive control was detected on the viral particles, plasma membranes, endoplasmic reticulum, the outer and inner leaflets of nuclear membranes, and inside their cisternae (Fig. 3). Staining in the nucleus was also extremely faint and ambiguous.

On the other hand, positive immunostaining in HUT 102 cells with the same antiserum to bGH-p40* was detected diffusely in the euchromatin regions of the nucleus but not in the nucleoli, nuclear envelope, or cellular membrane systems (Fig. 4). The location of the positive immunostaining in the HUT 102 cell nuclei was also confirmed by the reaction in isolated nuclei (Fig. 5). Positive immunostaining in isolated nuclei appeared diffusely in euchromatin regions. Nucleoli were not stained. Immunoperoxidase staining in HUT 102 cells by the indirect method with sera from patients with ATL was detected on the viral particles, plasma membranes, endoplasmic reticulum, the outer and inner leaflets of nuclear membranes, and inside their cisternae (Fig. 6). The nucleus was weakly stained.

Neither MT-2 cells nor HUT 102 cells were stained with sera from normal guinea pigs and normal persons. Control cells, Molt-4 (Fig. 7) and Jurkat, were not stained with antiserum to bGH-p40*. Endogenous peroxidase staining was not observed in either MT-2 or HUT 102 cells under the present reaction conditions. Although similar positive staining was obtained by both the direct and indirect immunoperoxidase methods, staining by the direct method appeared more clearly than that by the indirect method. The direct immunoperoxidase staining with Fab'-peroxidase conjugates prepared using a maleimide compound seems to be an excellent method for performing highly sensitive immunostaining with a low background and specific binding.

Western Blot Analysis of the pX Gene Products. Western blot analysis of the pX gene products with antiserum against bGH-p40* revealed that p40 and p68 were present in the extracts of MT-2 cells and their isolated nuclei, but only p40 was present in the extracts of HUT 102 cells and their isolated nuclei (Fig. 8; Table 1). The p40 band was faint compared to the p68 band in MT-2 cells. These protein bands were not detected with preimmune guinea pig serum in these cell extracts nor with the same antiserum in the control cell extracts. Immunoreactivity of the protein bands of p40 and p68 did not change after absorption of antiserum with bovine growth hormone, but was completely blocked after preabsorption of the antiserum with bGH-p40* (data not shown). These results indicate the specific detection of p40 and p68 as the pX gene products.

DISCUSSION

By immunoelectron microscopy using the direct and indirect peroxidase-labeled antibody methods, we demonstrated for the first time the pX gene products in the euchromatin regions of HUT 102 cell nuclei and in the nuclear envelope and endoplasmic reticulum of MT-2 cells. The results of immunoelectron microscopic observations correlated well with the results of Western blot analysis with the anti-p40* serum, as shown in Table 1. On the basis of both the immunoelectron microscopic
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Fig. 4. HUT 102 cells, fixed with PFA, frozen sectioned, and incubated directly with Fab'-peroxidase conjugates prepared from antiserum to bGH-p40*, showing positive immunostaining in euchromatin regions of the nucleus. The nuclear envelope and cellular membrane systems are not stained. × 7,800.

Fig. 5. Nuclei isolated from HUT 102 cells, fixed with PFA, frozen sectioned, incubated with antiserum to bGH-p40*, and peroxidase stained by the indirect method, showing positive immunostaining in euchromatin regions of the nuclei in the same manner as in HUT 102 cells. × 11,500.

observations and immunoblotting analysis, it is strongly suggested that p40* is localized mainly in the euchromatin regions of the nuclei of HTLV-1-producing cells, and p68* is localized mainly in the nuclear envelope and the endoplasmic reticulum of MT-2 cells. Although the p40* band was detected in all HTLV-1-producing cells examined, the p68* band was detected only in MT-2 cells and T-cells infected with HTLV-1 by cocultivation with MT-2. Miwa et al. (10) reported on the basis of protein labeling in the presence of tunicamycin that p68 is a glycoprotein with a protein moiety having a molecular weight of 60,000, and that p68 might be the translation product from uniquely spliced mRNA of the complete provirus or from the rearranged defective provirus present in MT-2 cells (4, 7). Thus, p68* detected in MT-2 cells with the anti-p40* serum was deduced to be a protein consisting of p40* and a part of env gene products and to share epitopes in common with p40*. Clarification of the precise molecular structure of the p68*-encoding gene has to await further study.

Immunoelectron microscopic detection of p40* in the nucleus is consistent with previous light microscopic observations of immunofluorescence and immunoperoxidase staining and subcellular fractionation data (14–18). However, detection of the pX gene product, probably p68*, in the nuclear membrane, endoplasmic reticulum, and inside their cisternae is a unique finding in the present study. We have observed a similar distribution of the pX gene products in other HTLV-1-infected T-cell lines, in which p68* was detected by Western blot analysis. Although the plasma membranes and viral particles were not stained with anti-bGH p40* in either frozen-sectioned specimens or cell suspension, they were strongly stained with sera from patients with ATL. This staining seems to be mainly due to antibodies to viral envelope proteins present in sera of patients with ATL. However, it is difficult to deduce from the present study what viral antigens were involved in the immunostaining of the endoplasmic reticulum and nuclear envelope in MT-2 cells and HUT 102 cells with sera from ATL patients, because the patient’s serum contained various kinds of polyclonal antibodies in different amounts in each patient. The immunostaining of these organellae was usually stronger in MT-2 cells than in HUT 102 cells. p68* will be one of the factors of the staining in MT-2 cells. env proteins or their precursors might also be involved in the staining of these organellae in HTLV-1-infected cells, but it has to be proven with monospecific or monoclonal antibodies against these proteins. The molecular weights of polypeptides detectable in most of the HTLV-1-producing cell lines are 62,000, 46,000, 40,000, 24,000, 21,000, and 19,000 (30). In addition to these, M, 68,000 and 28,000 polypeptides were frequently detected in MT-2 and some cell lines transformed by cocultivation with MT-2 but not in any other cell line unrelated to MT-2 cells (30). The gp46 has been thought to be a viral envelope protein (31, 32), gp62 to be a precursor of gp46 (32), and gp21 to be a minor envelope component (33). gp68 in MT-2 cells has also been thought to be a precursor of envelope protein gp46 (30, 32). However, the relationship between this gp68 and p68* detected by anti-p40* serum has to be clarified in the future. We previously showed by immunoelectron microscopy with monoclonal and polyclonal antibodies that HTLV-1 gag gene-encoded protein, p24, is localized in the viral core and that p19 and p28, detectable in MT-2 cells by a monoclonal antibody to p19 (34), are localized in the viral core and envelope and some parts of the plasma membrane adjacent to virus clusters (26). These gag
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Fig. 6. HUT 102 cells, fixed with PFA, frozen sectioned, incubated with sera from patients with ATL, and peroxidase stained by the indirect method, showing positive immunostaining on the viral particles, plasma membranes, endoplasmic reticulum, the outer and inner leaflets of nuclear membranes, and inside their cisternae. The nucleus is weakly stained. × 7,900.

gene-encoded proteins have not been detected in the endoplasmic reticulum nor in the nuclear envelope.

The presence of the pX gene products inside the cisternae of the nuclear envelope and endoplasmic reticulum might suggest the possibility of excretion of the pX gene products. A significant amount of the pX gene products (p68*) was actually detected in the supernatant fraction of serum-free culture medium of MT-2 cells. However, the possibility that this is due to cell degradation and lysis cannot be ruled out. The functional relationships of the pX gene products in the nucleus, nuclear envelope and endoplasmic reticulum, and extracellular fluid need to be clarified before the molecular mechanism of cell transformation by HTLV-1 can be understood.

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