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Internalization of Factor J and Cellular Signalization after Factor J–Cell Interaction

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Factor J (FJ) is a cationic glycoprotein with inhibitory activity *in vitro* against both classical and alternative pathways of complement activation. Recently FJ has been implicated in adhesion to several cell lines, through a membrane receptor identified as nucleolin. In the present work we study the events that follow the binding of FJ to cells. After incubation of K562 with FJ, this protein was internalized actively and localized in the cytoplasm and nucleus. Adhesion to immobilized FJ induced tyrosine phosphorylation of several intracellular proteins in Jurkat cell line with a similar pattern to that induced by fibronectin (FN), an extracellular matrix protein. This effect was maximal at 5 min and decreased after 10 min, and inhibited by anti-FJ monoclonal antibody (mAb). These results suggest that the binding of FJ to cells may play an important role in transduction of biochemical signals across the plasma membrane to the cell interior.

We have recently described that FJ, a complement inhibitor of the classical and the alternative complement pathways, is able to adhere human cell lines *in vitro.* Nucleolin, a nuclear protein present at the cell membrane of several cell lines, was identified as the main receptor for FJ cellular adhesion by microsequencing analysis. The specificity of this interaction was established since antinucleolin mAb or purified nucleolin inhibited cellular adhesion (1).

Deng *et al.* (2) have described that an antinucleolin mAb was internalized by Hep2 cells after its binding to the membrane nucleolin. We have speculated whether FJ could be internalized by an active process. Previous studies have shown that nucleolin circulates between cytoplasm and nucleus suggesting that it could be involved in the transport of molecules along these cell compartments (3). Some authors have suggested that nucleolin could participate in nuclear translocation of the midkine (MK) and the heparin-binding growth associated molecule (HB-GAM) (4).

In the search for the mechanism of FJ-cell interaction we observed that the staining of FJ on the cell membrane disappeared after a three hour incubation, following this data, we considered the possibility of internalization. We analyzed this phenomena by flow cytometry and electron microscopy. We also examined if cellular adhesion induced by FJ increased tyrosine phosphorylation. For that, Western blot experiments were performed with cells adhered to FJ and the membrane was probed with an anti-phosphotyrosine mAb.

Abbreviations used: FJ, Factor J; FN, fibronectin; mAb, monoclonal antibody; RT, room temperature; MK, midkine; HB-GAM, heparin-binding growth associated molecule; FGF, fibroblast growth factor; NLS, nuclear localization signal.

MATERIALS AND METHODS

Reagents and antibodies. Factor J was purified from human urine following the original scheme with minor modifications as described before (5). Fibronectin and chloroquine were from Sigma (St. Louis, MO). MAJ2, an IgM mouse mAb anti-FJ, was previously described (6). Ascitic fluid containing monoclonal IgM of irrelevant specificity was a gift from Dr. M. Fresno (Centro de Biología Molecular, Madrid). Phycoerythrin (PE)-conjugated goat anti-mouse IgM $F(ab')_2$ (Immunotech, Marseille, France), gold-labeled anti-mouse IgG, IgM (15 nm, BioCell, Cardiff, UK), and anti-phosphotyrosine mAb (4G10) (Upstate Biotechnology, Inc., Lake Placid, NY) were purchased.

Culture of cell lines. The human cell lines Jurkat (T cell line) and K562 (chronic myelogenous leukemia line) (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 50 UI/ml penicillin and 50 μg/ml streptomycin (Flow Technologies, McLean, VA).

Internalization of FJ. To determine whether FJ was internalized after cell interaction by Jurkat and K562 cells flow cytometry and electron microscopy immunocytochemistry were used. The cells were obtained from cell cultures on exponential growth and resuspended at 2×10^6 cells/ml in RPMI. One hundred μl of these suspension was used for each experiment and incubated with 500 ng of FJ for 30 min on ice. Control experiments without FJ were carried out in the same way. For the analysis of the time course disappearance of FJ from the membrane, the cells were washed twice with ice-cold RPMI, treated

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with 50 μM chloroquine for 1 h at room temperature (RT), washed twice and maintained during 3 h at 37°C or no (control at 0 h).

(a) For flow cytometry, the cells were then pelleted by centrifugation, washed twice in cold RPMI and incubated with MAJ2 (20 μg) followed by the addition of phycoerythrin labeled secondary Ab (diluted 1:1000); both incubations were performed for 30 min at 4°C. The cells were washed and analyzed by flow cytometry on a FACSsort Cytometer using Cell Quest software (Becton–Dickinson). Three thousand cells per sample were acquired.

(b) For electron microscopy analysis, cells were fixed for 10 min with 4% paraformaldehyde in PBS, washed in PBS and blocked with 5% nonfat dry milk in PBS. Afterwards, the material was washed, dehydrated with methanol by progressively lowering the temperature from 4°C to −30°C. The samples were embedded in Lowicryl K4M and polymerized with UV light for 24 h at −30°C and 48 h at RT. The dehydration, inclusion and polymerization were performed with a Leyca EM AFS processor (Austria). Ultrathin sections were placed on drops of 0.2 M Tris-buffer (pH 7.8) containing 0.1% glycine and 1% BSA. Then they were incubated for 2 h at RT with MAJ2 (800 ng). After washing with Tris-buffer, sections were incubated with 15 nm gold-labeled goat anti-mouse IgG, IgM diluted 1:100 in 20% goat serum in PBS pH 7.6 for 2 h a RT. Finally, sections were washed with Tris-buffer and counterstained with uranyl acetate for 20 min at RT. The specificity was tested with an irrelevant IgM ascitic fluid. For the quantitative study, 20 electron microscopic fields (144 μm^2) from each group were selected at random. In each of these areas the number of immunogold particles in the nucleus, cytoplasm and in extracellular locations was counted. The results were expressed as the number of immunogold particles per 10 μm^2. From the average values obtained, the mean and SD for each location were calculated. The significance of differences between the obtained values was evaluated by using the Fischer and Behrens' test.

Cell adhesion and preparation of cell lysates. Cell adhesion assays were performed as previously described (1). Briefly, flat-bottom 96-well microtiter plates were incubated with 10 μg/ml of FJ (100 μl) for 2 h at 37°C, then incubated with 1% BSA in PBS for 1 h at 37°C and washed twice with PBS. In most of the experiments, FN was used as a positive control. Jurkat cells resuspended in RPMI at 2×10^6 (100 μl) were plated on FJ coated plates and incubated for 30 min at 37°C. Unbound cells were removed by two washings with RPMI solution. Bound cells were removed with a buffer containing 20 mM Tris, 140 mM NaCl, 1mM sodium orthovanadate, 1 mM sodium molibdate, 1 mM leupeptin, 1 mM benzamidine, 1 mM iodoacetamide, 1 mM PMSF, pH 7.4. Cells were pelleted by centrifugation at 4°C and then solubilized with lysis buffer containing 20 mM PO_4Na_3, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% deoxicolate, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM sodium molibdate, 30 mM pyrophosphate, 50 mM NaF, 1 mM leupeptin, 1 mM benzamidine, 1 mM iodoacetamide, pH 7.2, for 15 min on ice. After removing insoluble materials by centrifugation at 12,000g for 15 min, protein concentrations in the supernatant were determined using a micro BCA protein assay kit (Pierce, Rockford, IL). Cell lysates were stored at −70°C until use.

Immunoblotting. Cell lysates (10 μg/well) were loaded into 10% SDS–polyacrylamide gels under reducing conditions. Proteins on the gel were electrotransferred to a nitrocellulose membrane in Tris–glycine–methanol containing buffer. After blocking the membrane with 3% BSA in PBS, protein bands were visualized by incubation with anti-phosphotyrosine 4G10 mAb (1:2000) overnight at 4°C. Ab was detected with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:3000) followed by the use of enhanced chemiluminescence technique ECL according to the manufacturer's directions (Amersham, UK). To evaluate the kinetics of the reaction, the cell incubation on FJ coated plates was performed at different times (2, 5 and 10 min).

Furthermore, with the aim to demonstrate that phosphorylation was induced by cellular adhesion, experiments were performed with adhered and non adhered cells. For that, non adhered and FJ adhered cells were lysed, submitted to Western blot and probed with 4G10 mAb.

Moreover, we performed experiments blocking the plates with MAJ2 in order to test the specificity of tyrosine phosphorylation. For that, immobilized FJ or FN were incubated with an excess of MAJ2. After washing the plates, cells were added and incubated for 30 min at 37°C. After that, cells were lysed and analyzed for tyrosine phosphorylation as described above.

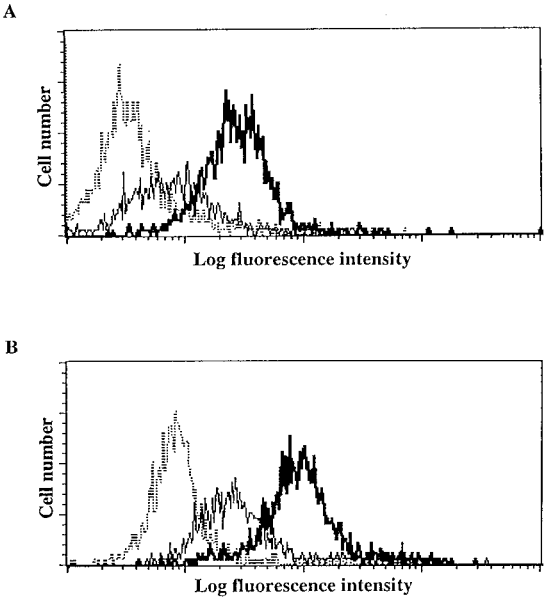

FIG. 1. Disappearance of FJ from cell membrane after three hours incubation. Jurkat (A) or K562 (B) cells (200,000 cells/tube) were incubated at 4°C for 30 min with 500 ng of FJ, washed twice and kept at 37°C for 3 h. The presence of FJ on the membrane was tested with MAJ2 followed by the addition of a secondary PE-labeled Ab and analyzed by flow cytometry. Solid wide line is the profile at 0 h incubation. Solid thin line is the profile after a 3 h incubation. Broken line represents the profile of the cells without FJ after 3 h incubation (negative control).

RESULTS

FJ Disappears from Plasma Membrane

Previous studies using flow cytometry have shown that FJ binds to Jurkat and K562 cell lines in a dose-dependent and specific manner (1). Here, we analyzed the presence of FJ on the membrane after several incubation times by flow cytometry experiments. We observed that after one hour incubation, fluorescence decreased on the membrane until 50% (data not shown), and if we further continued these experiments, we observed that most of the membrane staining on both Jurkat and K562 cell surfaces diminished after 3 h incubation at 37°C (Fig. 1). This result led us to investigate whether FJ was shed from the membrane or internalized into the cell after cellular interaction.

To determine whether FJ disappearance from plasma membrane was an active process, we examined

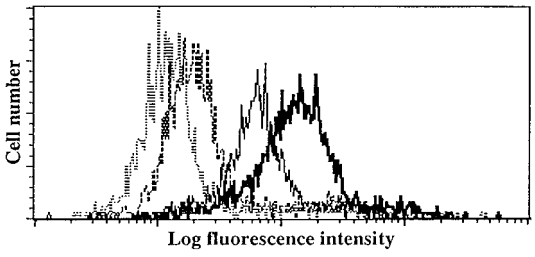

FIG. 2. Comparison of FJ disappearance from cellular membrane at 4°C and 37°C incubation. Experiments were performed with K562 cells as indicated in Fig. 1. Solid wide line is the profile at 0 h. Solid thin line is the profile at 4°C after 3 h incubation. Broken line is the profile at 37°C after 3 h incubation. Dotted line is the profile of the control cells processed at 37°C for 3 h in the absence of FJ.

the presence of FJ on cell surface at different temperatures. As shown in Fig. 2, this process is temperature-dependent because it was significantly reduced when the incubation was carried out at 4°C instead of at 37°C. At 37°C, cell surface staining was very similar to that observed on cells incubated without FJ, demonstrating the notable disappearance of FJ bound to plasma membrane. These results suggested that FJ could be internalized after binding to FJ-receptor on plasma membrane.

FJ Is Internalized by K562 Cells

To further explore the possibility that FJ was internalized and to determine its intracellular localization, experiments were performed in which the cells were incubated for 3 h at 37°C, probed with MAJ2, and then stained with gold labeled secondary Ab. Samples were examined by electron microscopy. We showed that following incubation at 37°C for 3 h there was an increase in the amount of gold staining localized intracellularly (Fig. 3A) when compared with cells at 0 h (Fig. 3B). The distribution of gold staining indicated that FJ was localized both in the cytoplasm and nucleus. However, we could not relate this localization to any subcellular region or compartment. The quantitative study (Table I) revealed that the number of gold particles significantly increased on cells incubated with FJ for 3 h in both nucleus and cytoplasm, compared with cells at 0 h. But there was not significant difference between nucleus and cytoplasm. The values obtained from cells incubated without FJ for 0 h and 3 h were similar to background and control.

Jurkat Cell Adhesion to FJ Induces Tyrosine Phosphorylation of Multiple Proteins

Recent studies have documented that in cellular adhesion mediated by integrins, tyrosine phosphorylation occurs. Therefore, we studied the potential tyrosine phosphorylation induced by cell adhesion to FJ by anti-phosphotyrosine immunoblot analysis. We observed an increase in tyrosine phosphorylation of cellular proteins with an apparent molecular weight of 150, 125, 105, 70, 60, 55 and other around of 45 kDa in cells adhered to FJ-coated plates (Fig. 4A, lane 1) when compared with control (BSA-coated plates) (Fig. 4A, lane 3). As positive control, we examined the tyrosine phosphorylation of proteins in cells adhered to FN under identical conditions (Fig. 4A, lane 2), showing the same pattern observed with FJ. Phosphorylation was a rapid process, was evident at 2 min, reached a maximum levels at 5 min, and persisted until 10 min (Fig. 4B).

Tyrosine Phosphorylation Induced by Cell Adhesion to FJ Is Specific

To study whether tyrosine phosphorylation was specifically increased by adhesion phenomena, we analyzed the induced phosphorylation in Jurkat adhered cells to FJ-coated plates and compared this with that observed in cells that remained non-adhered. Figure 5 shows the specificity of tyrosine phosphorylation, since the pattern observed in the non adhered cells (lane 1) was very similar to the negative control (lane B, BSA-coated plates) (Fig. 5), by opposite adhered cells to FJ showed enhanced tyrosine phosphorylation (lane 2).

To further investigate the involvement of FJ in the increase of tyrosine phosphorylation after cellular adhesion, we performed inhibitory experiments with anti-FJ mAb using the same procedure described above. This phosphorylation was specifically inhibited when FJ-coated plates were incubated with MAJ2 before adding cells (Fig. 6, FJ), but not on FN-coated plates (Fig. 6, FN). These results clearly demonstrated the specificity of tyrosine phosphorylation induced by FJ.

DISCUSSION

In this paper we performed experiments with the aim to further investigate the events which happen after FJ-cell interaction (1). First, we showed that FJ disappeared on the cell membrane after 3 h incubation (Fig. 1). This disappearance suggested the presence of FJ at the interior of the cell as evidenced by electron microscopy (Fig. 3).

Some proteins involved in cellular adhesion, as CAP-37 and VCAM-1, are internalized. CAP-37 is a neutrophil-derived heparin-binding protein, that increases the LPS-induced release of proinflammatory cytokines (TNF-α, IL-1, and IL-6). Moreover, it has a high affinity to monocytes and is internalized within 30 min (7). VCAM-1 is involved in receptor/counter-receptor interactions with $\alpha_4\beta_1$ (CD49d/CD29) integrin, and this interaction is an essential step in the sequence of events leading to the extravascular migration of T lymphocytes (8). VCAM-1 is internalized by a

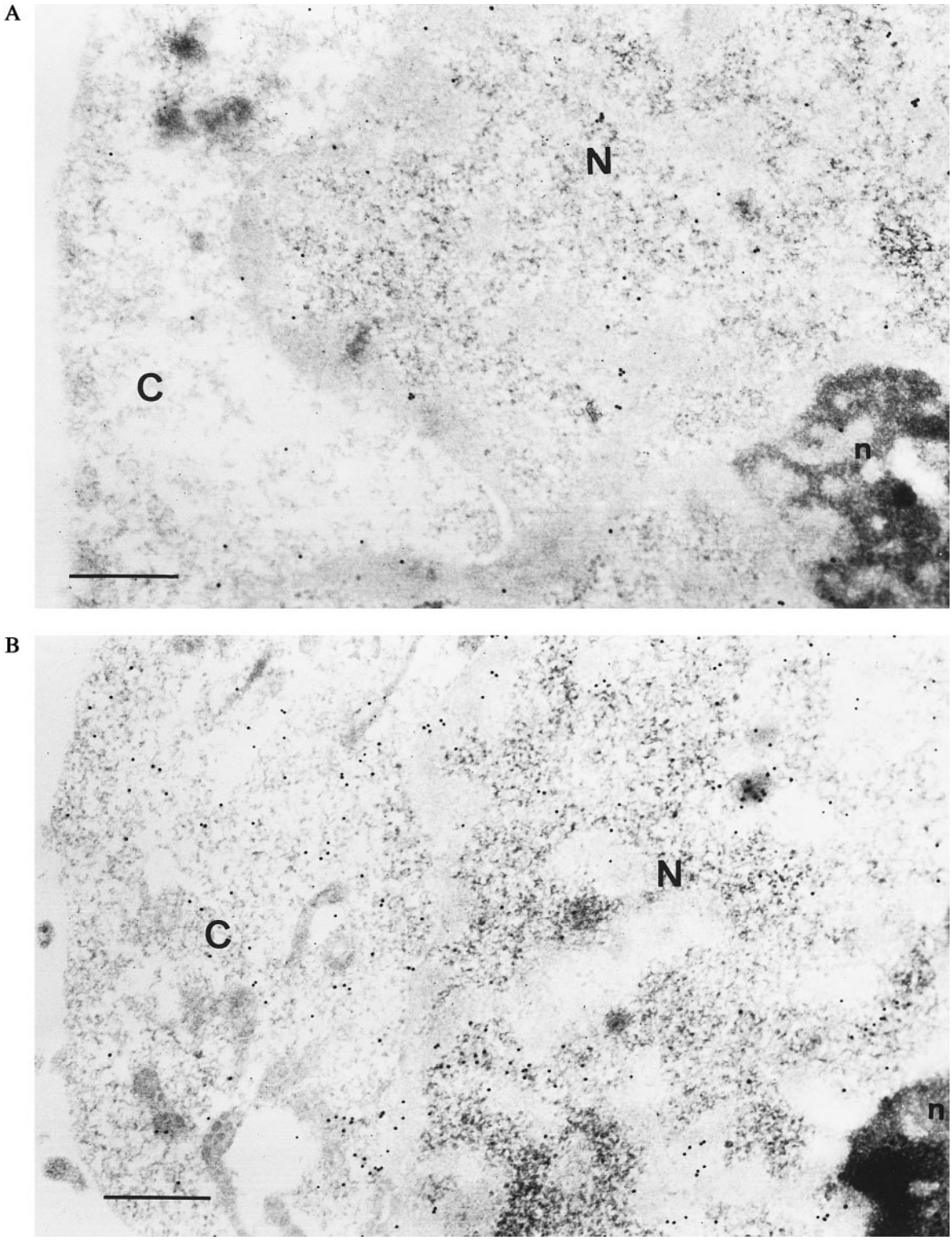

FIG. 3. Electron micrograph of FJ internalization to the cytoplasm and nucleus using MAJ2 and 15 nm immunogold particles. K562 cells were incubated for 30 min at 4°C with 500 ng of FJ and washed to remove free FJ. After two washings with RPMI, cells were incubated with 50 μM chloroquine for 60 min. Then cells were washed, resuspended in 100 μl of RPMI and incubated for 3 h at 37°C. Cells were fixed for electron microscopy as described under Materials and Methods. Control without FJ was included. After blocking with nonfat dry milk, cells were dehydrated, included and polymerized. Then, sections were incubated with MAJ2 followed by gold-labeled goat anti-mouse IgG and IgM. Nanogold particles are indicated by arrows. (A) At 0 h of incubation. (B) After 3 h of incubation at 37°C. C: cytoplasm, N: nucleus, n: nucleolus. Scale bar: 0.5 μm.

TABLE I

Quantitative Analysis of FJ Cellular Internalization

Group	Nucleus	Cytoplasm	Background
−FJ (0 h)	7.21 ± 6.03[a,b]	8.16 ± 5.43[a,b]	5.05 ± 2.02[a,b]
−FJ (3 h)	13.84 ± 9.31[b]	10.69 ± 8.08[b]	6.16 ± 2.01[a,b]
+FJ (0 h)	80.39 ± 35.60[c]	53.71 ± 30.26[c]	10.03 ± 3.92[b]
+FJ (3 h)	228.47 ± 32.07[d]	202.98 ± 40.23[d]	9.12 ± 3.25[b]
Control	3.92 ± 1.44[a]	4.08 ± 2.04[a,b]	3.18 ± 1.21[a]

Note. Number of immunogold particles per 10 μm^2 in nucleus, cytoplasm and extracellular locations (background) in each group. As control, irrelevant mAb was used. Values are expressed as mean ± SD. Values with different superscript letters differ significantly ($p \leq 0.05$). Values with the same superscript letters do not differ significantly.

clathrin-related pathway in human endothelial cells (ECV 304 and HUVEC).

Nucleolin, the receptor involved in FJ-cellular adhesion, has been found to shuttle between cytoplasm and nucleus, and it is considered to be involved in the transport of molecules between these cell compartments. Furthermore, some authors identified nucleolin as a binding protein for midkine (MK) and heparin-binding growth associated molecule (HB-GAM) which is structurally unrelated to fibroblast growth factors (FGF) (4) and suggested that nucleolin may be involved in nuclear translocation of these molecules. By analogy, nucleolin could participate in FJ translocation from the plasma membrane to the nucleus. Furthermore, Deng *et al.* 1996 (2) demonstrated that an anti-nucleolin antibody bound to its target on the plasma membrane was internalized into the nucleolus. The mechanism that accounts for FJ internalization remains to be elucidated. The entry of molecules larger than M_r 40,000 into the nucleus is a highly selective process (9) depending on a short stretch of basic amino acids called nuclear localization signal (NLS). Beside the nuclear import of many nuclear proteins based on the presence of a peptidic NLS, other non-peptidic nuclear localization signals could exist. Sugars could be one of the non-peptidic NLS. Indeed, numerous cytosolic and nuclear proteins are glycosylated. On the other hand, several sugar-binding proteins (lectins) have been characterized inside the nucleus and in the cytosol (10). In accordance with this, FJ is a protein having a high sugar content and a p*I* ≥9.6 (11).

Factor J induced a phosphorylation pattern very similar to that observed after FN interaction. FN is an extracellular matrix component that is a ligand for integrins (12, 13). The binding of integrins to their ligands induces the phosphorylation of different proteins in tyrosine, and this is one of the ways of intracellular signalization of these membrane receptors (14). When we compared phosphorylation pattern after cellular adhesion to FJ, it was found a very similar to that observed after FN interaction (Fig. 4A). Western-blot analysis showed an increase in tyrosine phosphorylation on several proteins with apparent molecular weights of 150, 125, 105, 70-72, 60, 55 and lower than 45 kDa.

Furthermore, crosslinking T cells by VLA-4 antibody or the CS-1 region from FN stimulate phosphorylation on tyrosines of phospholipase Cγ (PLCγ) (pp140), local adhesion kinase (FAK) (pp125), paxillin (pp70-50), fyn

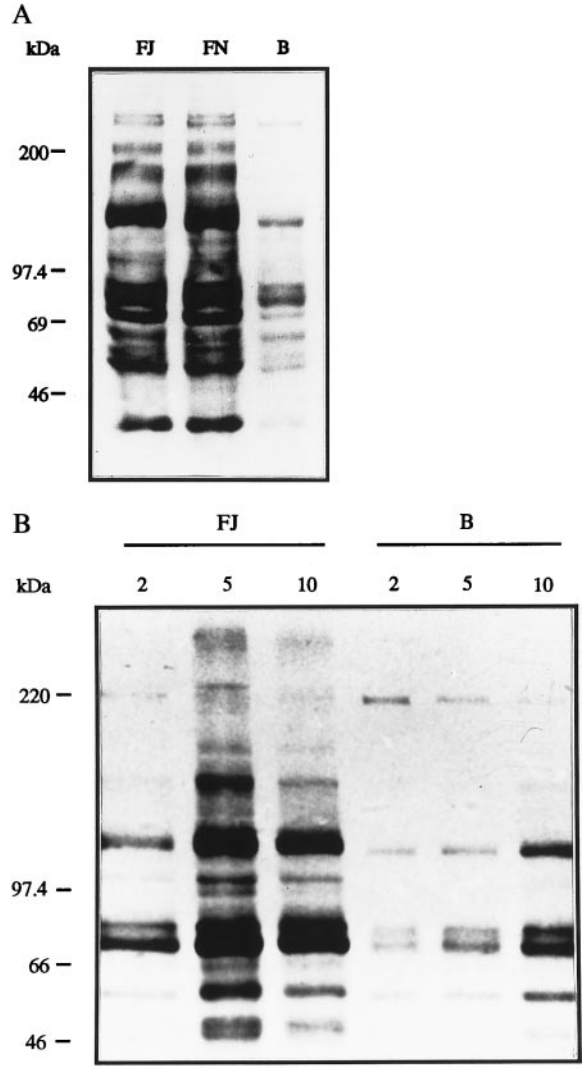

FIG. 4. Tyrosine phosphorylation induced after cellular adhesion to FJ in Jurkat cell line. (A) Comparison of the induced phosphorylation after cellular adhesion to FJ and FN. Cells were incubated for 30 min at 37°C with plates coated with 1 μg of FJ, FN or BSA (B). After that, adhered cells were recovered with a rubber policeman and lysed with an appropriate buffer. Lysates were quantified in protein and a sample of each (10 μg) was loaded on 10% SDS–PAGE. After that, proteins were electrotransferred and probed with anti-phosphotyrosine mAb. A secondary Ab labeled with peroxidase was added and the membrane was developed by chemiluminescence using ECL. (B) Kinetics of the phosphorylation. Cells were incubated at different times (2, 5 or 10 min) with coated FJ. After washing the plates, adhered cells were lysed and analyzed for tyrosine phosphorylation. A control experiment (B) was performed in the same way with BSA-coated plates.

and lck (pp60-55), mitogen-activated protein (MAP kinase or ERK1/2) (pp45), and additional non identified protein of 105 kDa (15).

The protein of 150 kDa, on which phosphorylation of tyrosine is induced after FJ cell adhesion on Jurkat cells, could be PLCγ induced by several growth factors, which stimulates enzymatic activity that cleaves membrane phosphoinositides to yield dyacilglycerol as well as 1,4,5-triphosphate (16). The 125 kDa protein could be FAK, a tyrosine kinase which is present at the focal contacts (17). FAK is one of the main substrata of the tyrosine phosphorylation induced by integrins and whose principal function is the regulation of signal transduction (18). This molecule is expressed in a number of established cell lines, in most tissues examined, in erythroid cells and platelets, and in some populations of B and T cells, and there are several growth factors and hormones that induce phosphorylation on tyrosine of FAK (19, 20). Moreover, in 1997 Gismondi *et al.* (21) showed that crosslinking of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ FN receptors on human NK cells stimulates tyrosine phosphorylation of a protein of about 105–115 kDa identified as proline-rich tyrosine kinase-2. The possibility remained that the protein of 105 kDa which was phosphorylated after cellular adhesion on Jurkat cells by FJ and FN could be PYK-2.

The 70 kDa polypeptide could be paxillin, a cytoskeleton component involved in focal adhesion and that is susceptible to being phosphorylated on tyrosine after cellular adhesion to FN (22, 23). Many stimuli induce tyrosine phosphorylation of the FAK and paxillin, including adhesion to extracellular matrix proteins and stimulation with a platelet growth factor, bombesin and lipophosphatidic acid (18). Adhesion of NK cells to

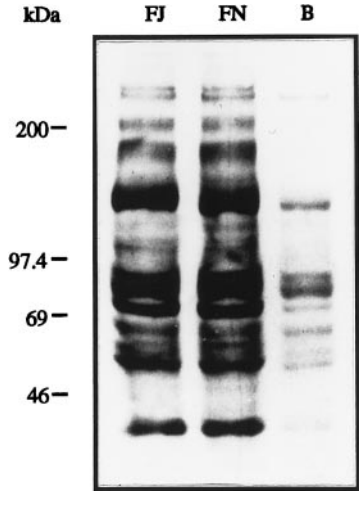

FIG. 5. Comparison between adhered and nonadhered Jurkat cells to FJ. Cells were incubated with coated FJ plates and after 30 min incubation, nonadhered cells (lane 1) were recovered, and adhered cells (lane 2) were separated with a rubber policeman. In both cases cells were lysed and processed as described. B represents the lysed cells in contact with BSA alone.

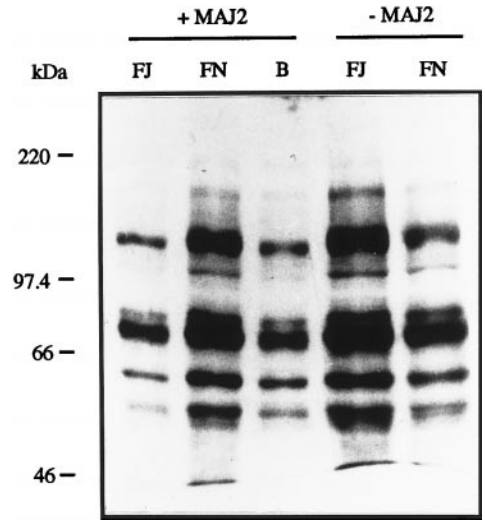

FIG. 6. Effect of MAJ2 on tyrosine phosphorylation. Plates coated with FJ, FN, or BSA were incubated with MAJ2. After that, 200,000 cells were added to each well, incubated for 30 min at 37°C and removed with a rubber policeman. All the cells were lysed and tested for tyrosine phosphorylation by Western blot with 4G10 Ab as described.

FN induces phosphorylation on tyrosine of PTK FAK, Zap-70, fyn and lyn by cross-linking of β_1 integrins (24). In this context it is probable that the 70 kDa protein could be Zap-70.

The proteins of 60 and 55 kDa could be fyn and lck, respectively, and the protein of 45 kDa, could be a member of MAP kinases (ERK 1/2; p44/p42), which are activated in the response to integrin mediated adhesion (25, 26). MAP kinases phosphorylate and regulate the activity of enzymes and transcription factors. The activation of MAP kinases is regulated by tyrosine phosphorylation, but it works as a serine-threonin-kinase.

In conclusion, the internalization of FJ following cellular interaction is demonstrated here; this process is temperature dependent. Furthermore, cellular adhesion induced tyrosine phosphorylation as showed by the existence of different phosphorylated substrate after protein interactions. Further studies are necessary to identify the proteins that are phosphorylated after FJ-cell interaction. These studies are going to elucidate the signals involved after adhesion induced by FJ.

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