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Interferon increases the abundance of submembranous microfilaments in HeLa-S_3 cells in suspension culture

(immunofluorescence microscopy/electron microscopy/heavy meromyosin/plasma membrane/actin filament)

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ABSTRACT **Human β (fibroblast) interferon inhibits the proliferation of human HeLa-S_3 carcinoma cells in suspension culture. Accompanying this effect, the lateral mobility of cell surface receptors for concanavalin A is decreased and the rigidity of the plasma membrane lipid bilayer is increased. The present findings show a marked increase in the number of polymerized actin-containing microfilaments 3 days after treatment of HeLa-S_3 cells with β-interferon (640 units/ml). The cortical region of the treated enlarged cells contains a thick and dense meshwork of 40–70 Å microfilaments. The actin nature of the filaments was verified by their ability to bind heavy meromyosin. These results support the concept that β-interferon induces a coordinated response in the plasma membrane and the underlying microfilaments in both tumor and normal cells.**

Interferon-treated cells support viral replication poorly (1–3). Moreover, the proliferative capacity of both normal and tumor cells decreases after interferon treatment (1–3). The mechanism of action of interferon has been likened to that of various polypeptide hormones (4) and viewed as antagonistic to that of a number of cell growth factors (5–7). Accompanying the decrease in the proliferative activity of interferon-treated human fibroblasts, there is a parallel decrease in cell locomotion (8, 9). The treated cells become enlarged (3), the plasma membrane lipid bilayer shows increased rigidity (5), the microfilament organization is abnormally increased (9), and fibronection becomes distributed over much of the cell surface (9). The enlarged well-spread fibroblasts display striking changes in actin polymerization and assembly that result in the formation of numerous large actin fibers. We have suggested that interferon triggers a response pathway in fibroblasts and that this pathway involves coordinated changes in the plasma membrane and the underlying actin-containing microfilaments (8–10).

We have demonstrated that β-interferon treatment results in the slowing of the proliferation of human carcinoma cells (HeLa-S_3 line) in suspension culture (1). The rigidity of the plasma membrane lipid bilayer of treated cells shows a transient early increase (unpublished results), followed by a persistent increase that develops within 24 hr from the beginning of treatment (11). Interferon treatment also impairs the ability of HeLa-S_3 cells to redistribute cell surface receptors for concanavalin A to one pole of the cell. Although interferon markedly inhibits "capping" of the receptors, it has no detectable effect on "patching" (10). As the movements of receptors within the plane of the lipid bilayer are thought to be mediated by the submembranous microfilaments (12), it appeared important to determine whether interferon treatment alters the microfilament organization in HeLa-S_3 cells.

The present study shows that interferon treatment causes the actin-containing filaments to become more organized in HeLa-S_3 cells, as it does in human fibroblasts. The outstanding feature of the organization of microfilaments in interferon-treated HeLa-S_3 cells is the formation of a dense submembranous meshwork of filaments. Correlating this evidence with other aspects of interferon action in HeLa-S_3 cells, we postulate that, in tumor cells growing in suspension, interferon likewise elicits a coordinated response involving the cell membrane with its associated cytoskeletal proteins.

MATERIALS AND METHODS

Cells. Human HeLa cell subclone S_3 (epithelial carcinoma) was grown as described (10). The initial cell concentration was $2–4 \times 10^4$/ml. At 1 day after subculturing, human β (fibroblast) interferon was added 640 units/ml; control cultures received no interferon.

Interferon. Two interferon preparations produced in human fibroblasts superinduced with poly(I-C) were used. A partially purified preparation of interferon from Rentschler, Fed. Rep. of Germany, was provided by J. K. Dunnick of the National Institute for Allergy and Infectious Diseases. Preparations of β_1 interferon, which had been purified to homogeneity and had a specific activity of $>2 \times 10^8$ units/mg of protein, were provided by E. Knight, Jr., of Du Pont. Interferon activity was assayed by a microtitration procedure using vesicular stomatitis virus as described (15) with a human fibroblast reference standard (National Institutes of Health catalog no. G-023-902-527) for comparison. Activity is expressed in terms of international reference units/ml.

Immunofluorescence Microscopy. Actin filaments were visualized by indirect immunofluorescence microscopy. At 72 hr after the beginning of treatment, $2–5 \times 10^5$ cells were removed from control and interferon-treated cultures, pelleted at 800 $\times g$, and fixed at room temperature for 10–20 min in 3% paraformaldehyde in phosphate-buffered saline (P_i/NaCl)/1 mM $MgCl_2$. After pelleting at 800 $\times g$ and washing with P_i/NaCl, the cells were rendered permeable by treatment with 0.1% Triton X-100 in P_i/NaCl for 10 min. The cells were then incubated with antiserum specific for actin and prepared for microscopic examination as described (8, 10, 14).

Electron Microscopy. Cultured HeLa-S_3 cells were prepared for electron microscopy in the suspended state. Aliquots containing $2–5 \times 10^6$ cells were washed in P_i/NaCl and fixed in 1% glutaraldehyde in P_i/NaCl for 30 min at room temperature. The cells were then processed for electron microscopic studies as described (9, 15, 16).

Glycerination and Heavy Meromyosin Staining. The actin nature of submembranous microfilaments was confirmed by *in situ* localization of heavy meromyosin (HMM)-decorated filaments, which was performed according to the procedure of Szent-Gyorgi (17, 18) and Pollard and Weihing (19). At 72 hr after the beginning of treatment, $2–5 \times 10^6$ cells were removed from control and interferon-treated HeLa-S_3 cultures, washed

Abbreviations: P_i/NaCl, phosphate-buffered saline; HMM, heavy meromyosin.

three times with P_i/NaCl, and prepared for HMM decoration and electron microscopy.

RESULTS

Human carcinoma (HeLa-S_3) cells in suspension are spherical (Fig. 1 *a* and *c*). Unlike fibroblasts in monolayer culture, these cells do not contain large phase-dense actin fibers. Actin-containing microfilaments in HeLa-S_3 cells form a sparse network of filaments located adjacent to the plasma membrane. Such a network, observed by indirect immunofluorescence microscopy, is usually seen as a faint band of fluorescence in the cortical region of cells (Fig. 1 *b* and *d*). In addition, there is also diffuse staining of actin throughout the cytoplasm, which represents in part a pool of unpolymerized actin molecules and in part sparse microfilaments oriented in a crisscross network.

Treatment of HeLa-S_3 cells with human β-interferon (640 units/ml) for 3 days causes a 48% increase in the modal cell volume (unpublished results). The increased size of interferon-treated cells is evident in Fig. 1 *e* and *g*, which also illustrates the fact that the number of binucleated cells is increased after interferon treatment. In a sample of 525 control cells, 1% were binucleated whereas, among 519 treated cells, 4% were binucleated. The nuclei in interferon-treated cells, unlike those in control cells, are frequently lobed. Submembranous actin in the treated cells is visualized as a distinct band of fluorescence with projections that indicate the locations of microvilli and microspikes.

The results of immunofluorescence microscopy show an increased intensity of membrane-associated fluorescence, possibly due to the increase in the amount of actin filament in the cortical region. Nevertheless it is hard to distinguish between fluorescence due to actin staining and autofluorescence of the membrane itself. We therefore have analyzed the distribution and organization of actin-containing microfilaments by electron microscopy.

Fig. 2 illustrates the organization of actin-containing microfilaments in control and interferon-treated cells as shown by thin-section electron microscopy. In the membrane region of control cells, few organized cytoskeletal components can be seen (Fig. 2*A*). Electron-dense polyribosomes are found throughout the cytoplasm, extending to the proximity of the plasma membrane. Some microfilaments can be seen in this region. In contrast, the interferon-treated HeLa cells contain a prominent dense submembranous network of 40–70 Å microfilaments. Unlike the control cells, most of the polyribosomes are excluded from this fibrous region. Interestingly, numerous microtubules are found in the area adjacent to the microfilament network in the treated cells. The presence of a thick layer of microfilaments has been observed in ≈80% of the cells in the interferon-treated population. Representative fields from ultrastructural examination of 10 cells each from the control and interferon-treated cultures are shown.

To obtain additional evidence concerning the distribution of 40–70 Å microfilaments in the submembranous region and to verify that these filaments contain actin, we have investigated the interaction of these filaments with purified HMM (19). The characteristic morphology of HMM–actin complexes is represented by "arrowhead" structures along the length of the individual 40–70 Å microfilaments. A representative submembranous area of a control cell that has been glycerinated and incubated with HMM contains a few microfilaments (Fig. 3*a*). At higher magnification, some HMM-decorated microfilaments with readily recognizable sidearm structures are seen in the vicinity of the plasma membrane. In interferon-treated cells, a prominent area occupied by HMM-decorated filaments is observed in the proximity of the plasma membrane (Fig. 3*c*).

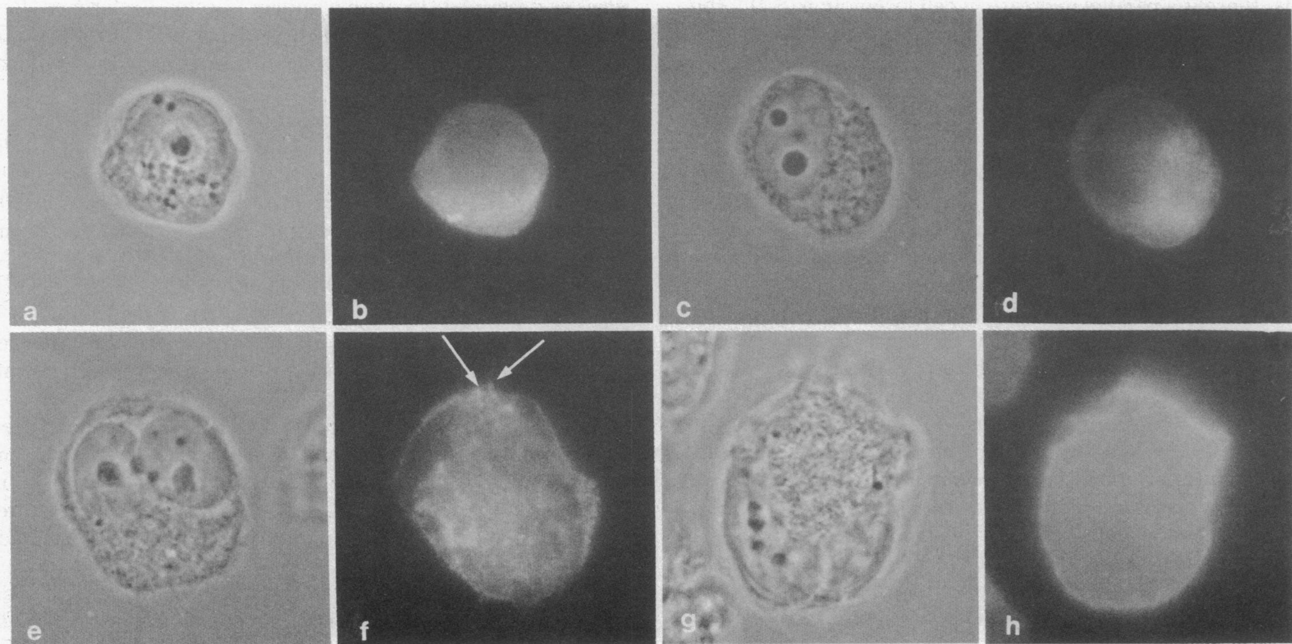

FIG. 1. Effect of interferon treatment on the abundance of actin in the region of the plasma membrane of HeLa-S_3 cells as determined by immunofluorescence staining. Phase-contrast (*a*) and fluorescence (*b*) micrographs of the same cell in suspension culture of control human carcinoma (HeLa-S_3) cells. Note the diffuse fluorescence pattern showing the generalized distribution of actin in the cytoplasm. There is a very faint band of fluorescence in the plasma membrane region. (*c* and *d*) Another example of control cells. Phase-contrast (*e*) and fluorescence (*f*) micrographs of a cell in the HeLa-S_3 suspension culture treated with interferon (640 units/ml) for 3 days. In addition to the diffuse distribution of actin in the cytoplasm, note the distinct band of fluorescence in the cell cortex and small surface projections (arrows) that have stained for actin. (*g* and *h*) Another example of interferon-treated cells. (×900.)

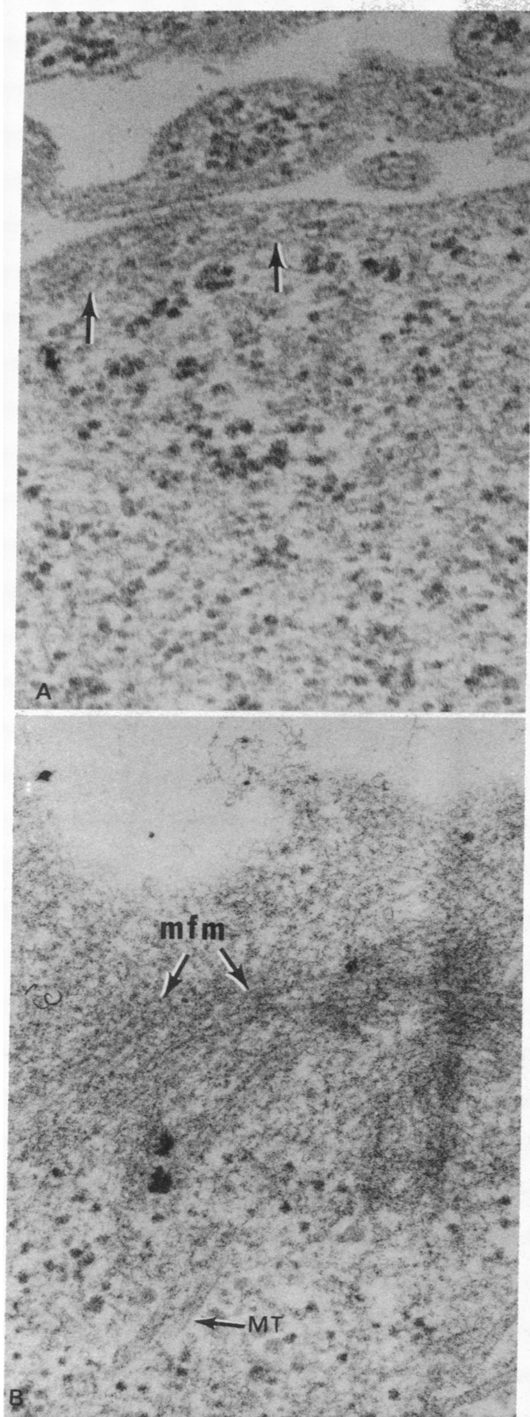

FIG. 2. Effect of interferon treatment on the distribution of microfilaments in the cortical region of HeLa-S_3 cells as visualized by electron microscopy. (*A*) Control HeLa-S_3 cell. Note the absence of a thick and dense microfilament meshwork in the submembranous region (arrows). (*B*) Interferon-treated HeLa-S_3 cell. Note the thick and dense submembranous mesh of microfilaments (mfm; arrows). In addition, numerous microtubules (mt) are seen in the area adjacent to the microfilament mesh. (×79,900.)

The arrowheads representing the HMM bound to the filaments are clearly distinguishable at higher magnification (Fig. 3*d*). Fig. 3 *f* and *h* show that the HMM-arrowhead decoration can be removed by the addition of sodium pyrophosphate, which dissociates the complex between HMM and actin (20). These results establish that the microfilaments found in the submembranous network in interferon-treated cells are composed of polymerized actin. The contrasting distribution of such filaments in Fig. 3 *a* and *c* and, similarly, in Fig. 3 *e* and *g* indicates that interferon treatment increases the abundance of microfilaments in the submembranous region and causes the formation of a prominent network of such filaments.

DISCUSSION

Knowledge of interferon-induced alterations in the plasma membrane and the submembranous cytoskeletal components such as the microfilaments appears to be of fundamental importance for understanding the macromolecular mechanisms involved in interferon action. The changes in the plasma membrane–cytoskeletal complex may be brought about directly by interferon action or may be secondary consequences of the slowing of cell cycle traverse caused by some action of interferon that is not yet understood. In either case, such changes in cell structure may, in and of themselves, impede cell cycling.

The development of numerous thick long bundles of microfilaments (actin fibers) in β-interferon-treated fibroblasts that have become enlarged and well spread on the substrate (3) can be explained by postulating that there is a cell–substrate contact-mediated signal that initiates the formation of microfilament bundles. Such an explanation would be compatible with results of previous studies with hamster and mouse fibroblasts (21, 22). However, such an explanation would address only one aspect of interferon action on cells.

In general, animal cells grown in suspension culture do not contain large actin fibers. Instead, polymerized F-actin in these cells forms a sparse meshwork consisting of a crisscross arrangement of 40–70 Å filaments (23). These microfilaments have often been noted in the immediate vicinity (a few thousand angstroms) of the plasma membrane and have been identified as being membrane associated (24). In agreement with these findings, we have observed that control HeLa-S_3 cells in suspension culture display a thin layer of microfilaments adjacent to the plasma membrane. As a result of interferon treatment, however, there develops a thick mesh of microfilaments in the cortical region. Thus, interferon treatment abnormally increases the organization of microfilaments in cells that either are attached to a substrate (in monolayer) or not so attached (in suspension). These findings exclude any explanation of the action of interferon on microfilament organization linked solely to the state of spreading or shape of the treated cells. However, they still leave open the question of whether interferon effect is direct or indirect. It is possible that the development either of microfilament bundles in flat substrate-attached cells or of a cortical microfilament mesh in round cells in suspension is at least in part an epiphenomenon associated with the interferon-induced inhibition of cell proliferation. However, it is clear that the precise expression of the interferon-induced increase in the organization of microfilaments is conditioned by the cell system on which interferon is acting. Furthermore, it appears likely that the altered organization of microfilaments in interferon-treated cells interferes with cytokinesis. The fraction of bi- or multinucleated HeLa-S_3 cells as well as fibroblasts (3) is increased after interferon treatment. Time-lapse observation of fibroblasts has shown that most of these cells arise through abortive mitosis (3). The finding of nuclear lobation also suggests that interferon treatment may disturb nuclear processes during mitosis.

Membrane-associated microfilaments are thought to be involved in the lateral mobility of surface receptors for multivalent ligands such as concanavalin A (10, 12, 25–30). We have demonstrated previously that interferon treatment inhibits the movement of concanavalin A receptors to one pole on the HeLa cell surface (10). After 3 days of treatment with β interferon at

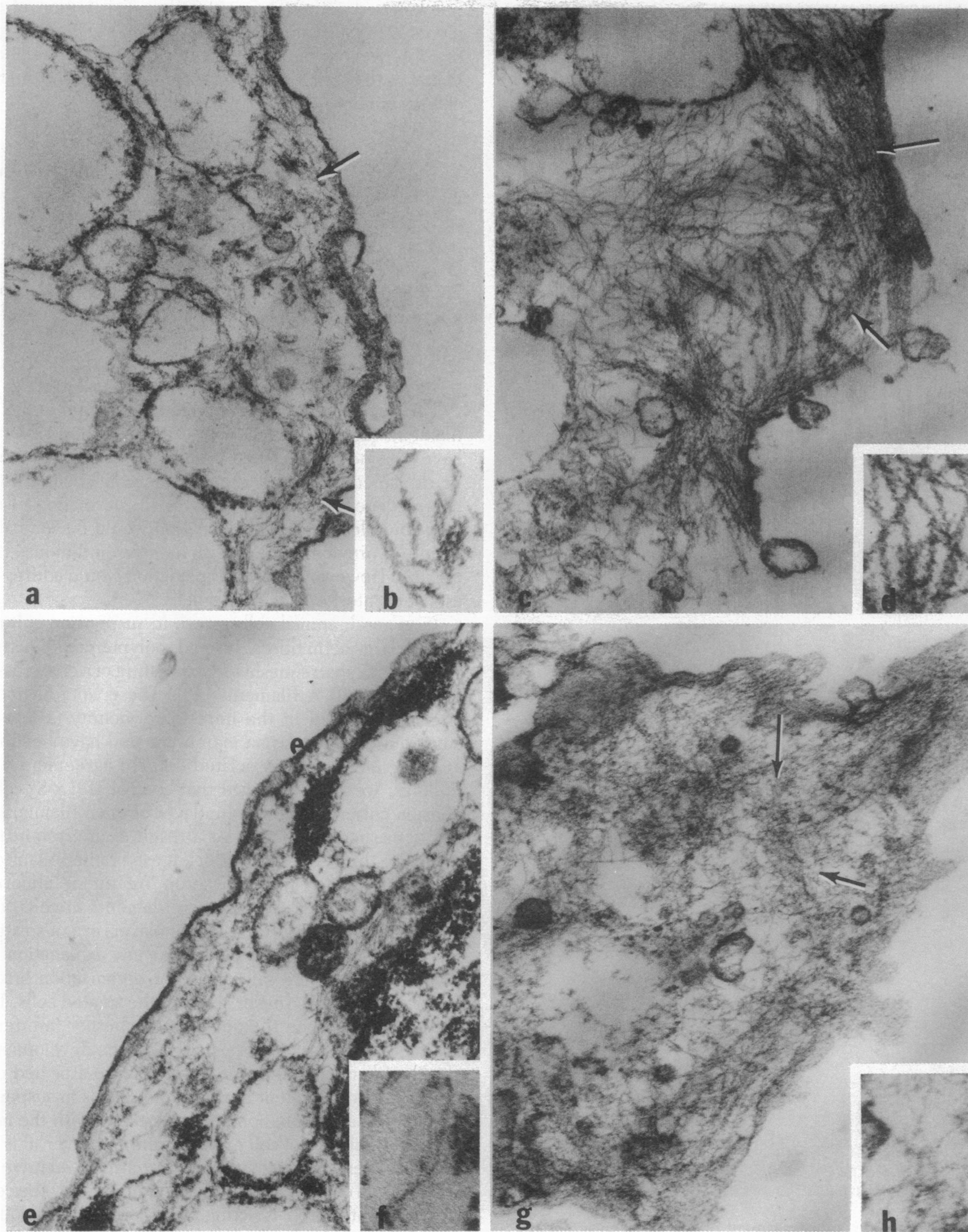

FIG. 3. Effect of interferon treatment on the distribution of actin-containing microfilaments in the cortical region of HeLa-S_3 cells as determined by electron microscopy of glycerinated HMM-treated HeLa-S_3 cells. (*a* and *b*) Control cells show few HMM-decorated microfilaments (arrows) in the cortical region; the "arrowhead" structure of HMM–microfilament complexes is clearly distinguishable at high magnification; the large vacuoles represent rough endoplasmic reticulum structures altered by glycerination. (×32,300 and 89,300, respectively.) (*c* and *d*) Interferon-treated cells show a prominent submembranous layer composed of a network of HMM-decorated filaments (arrows); the actin nature of the 40–70 Å filaments is evidenced by the arrowhead structures of bound HMM (arrow) along the individual filaments; a group of 10-nm filaments that are larger and unable to interact with HMM can also be seen. (×30,600 and 89,300, respectively.) Control (*e* and *f*) and interferon-treated (*g* and *h*) cells illustrating that incubation with sodium pyrophosphate removes HMM from actin filaments; there is a prominent microfilament meshwork in interferon-treated cells (*g*) but not in control cells (*e*). (×36,600, 95,200, 36,600, and 74,000, respectively.)

640 units/ml, ≈80% of the cells fail to exhibit capping of these receptors (10). The fact that we now find an increase in submembranous filaments in ≈80% of interferon-treated cells suggests that the abnormal abundance of submembranous microfilaments and the failure of capping of cell surface receptors may be related phenomena. In our system, the impairment of lateral mobility of receptors in interferon-treated cells is apparently not due to an absence of microfilaments but is instead associated

with an excess of microfilaments in the cortical region. Failure of receptor movement required for cap formation could be due to abnormally increased or structured membrane association of the abundant microfilaments or to an abnormal organization of the microfilaments themselves. It does appear that lateral mobility of cell surface components depends on an optimal association of the microfilaments with the plasma membrane.

The molecular mechanism for the increased assembly and organization of microfilaments in the interferon-treated cells remains an interesting and unsolved question. Interferon treatment of HeLa-S_3 cells causes a biphasic increase in the rigidity of the plasma membrane lipid bilayer (11). The second phase is concurrent with the decrease in cell proliferation. The more rigid membrane might serve as a better substrate for the initiation of actin assembly through nucleation. At the same time increased protein–lipid interaction might contribute to the rigidity of the lipid bilayer.

Recently, several crosslinking proteins have been characterized that are associated with actin filaments in nonmuscle cells (31, 32). Treatment of cells with interferon may initiate a cascade of molecular changes creating a microenvironment in the immediate vicinity of the plasma membrane that favors the assembly, organization, and membrane association of actin. The membrane-associated cytoskeletal proteins vinculin, α-actinin, and gelsolin have been implicated, among others, in the regulation of the formation of microfilament bundles in cultured cells (31–35). Investigation of these proteins in interferon-treated cells should lead to a better understanding of the mechanism of interferon action on the membrane–cytoskeletal system.

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