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Cytoskeletal Proteins from Human Skin Fibroblasts, Peripheral Blood Leukocytes, and a Lymphoblastoid Cell Line Compared by Two-Dimensional Gel Electrophoresis

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Differences in proteins between cells grown as suspension cultures and those grown as attached cultures were studied by comparing the proteins of detergent-resistant cytoskeletons prepared from peripheral blood leukocytes and a lymphoblastoid cell line (GM607) (both grown as suspension cultures) and those of human skin fibroblasts (grown as attached cultures) by two-dimensional gel electrophoresis. The major cytoskeletal proteins of the leukocytes were also present in the protein pattern of GM607 cytoskeletons. In contrast, the fibroblast cytoskeletal protein pattern contained four groups of proteins that differed from the patterns of the leukocytes and GM607. Three groups ($Cytosk_f$:8–10, :14–16, and :17–18) showed qualitative differences, and the fourth group ($Cytosk_f$:11 and :13) showed quantitative differences. In addition, surface labeling of GM607 and human fibroblasts with ^{125}I demonstrated that substantial amounts of vimentin and actin are exposed at the surface of the attached fibroblasts, but there is little evidence of similar exposure at the surface of the suspension-grown GM607. $Cytosk_f$:11 and :13 in fibroblast preparations were also labeled with the ^{125}I. These results demonstrate some differences in cytoskeletal protein composition between different types of cells could be related to their ability or lack of ability to grow as attached cells in tissue culture.

Additional Keyphrases: *surface vs interior cytoskeletal proteins · commonality of proteins in two sorts of cells · vimentin · actin · differences related to growth predilections · cell adhesion*

The term "cytoskeleton" refers to the filamentous cytoplasmic structure that remains associated with nuclei after extraction of whole cells with detergent solution. Three types of filaments make up the cytoskeleton, two serving as structural components and a third functioning as a contractile apparatus. In the former category are the microtubules, which are the largest of the three filaments (25 nm in diameter), and the intermediate filaments (10 nm in diameter) (*1*). Microtubules appear to be involved with chromosome movement during mitosis, intracellular transport of organelles, the movement of cells during tissue differentiation, and the formation and maintenance of cellular processes. The function of the intermediate-size filaments remains obscure, but some evidence suggests that these structures may also be involved with organelle movements. The smallest of the three cytoskeletal elements, the microfilaments (6 nm in diameter), are directly involved in cytoplasmic streaming, cytokinesis, membrane ruffling, cell locomotion, endocytosis, and exocytosis (*1*). The major protein component of the microtubules is tubulin, a complex of three proteins (α_1, α_2, and β tubulins) with relative molecular masses (M_r) between 55 000 and 60 000 (*2*). The major protein component of the intermediate filaments in nonmuscle cells is vimentin (*3*). Actin is the major protein in the microfilaments (*4*).

The distribution of cytoskeletal filaments within the cell differs between cells grown in suspension and those that grow as attached monolayers (*1*, *5*), but whether there are corresponding differences in the protein composition of those filaments in cells grown as suspensions or monolayers was unknown. This paper compares the two-dimensional gel-electrophoretic patterns of cytoskeletal proteins from three types of human cells. Two of the cell types—peripheral blood leukocytes (primarily lymphocytes and monocytes) and the lymphoblastoid cell line GM607—grow only as suspension cultures. The third cell line—human skin fibroblasts—grows in culture only as an attached monolayer.

The two-dimensional electrophoresis patterns show that the fibroblasts contain several major cytoskeletal proteins that are absent from both of the suspension-grown cell types. In addition, surface labeling with ^{125}I shows that several of the major fibroblast cytoskeletal proteins are exposed at the cell surface, but little such labeling was obtained with the suspended cells. These findings provide a clue as to which unique protein gene products associated with the cytoskeleton may be involved with cell adhesion.

Materials and Methods

Radiolabeling of Cellular Proteins

Human peripheral blood leukocytes were isolated as previously described (*6*). The leukocytes (4×10^6 cells/mL) were cultured for 18–24 h in flat-bottomed multi-well plates containing methionine-free RPMI 1640 medium (GIBCO, Grand Island, NY 14672) with added fetal bovine serum (50 mL/L), 2-mercaptoethanol (4×10^{-5} mol/L), and 60 mCi/L of [^{35}S]-methionine (Amersham, Arlington Heights, IL 60005).

Cells from the human lymphoblastoid cell line GM607 (Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ) were labeled with [^{35}S]-methionine in an identical manner.

Human skin fibroblasts (cell line 1494 from Meloy Laboratories, Springfield, VA; National Cancer Institute Contract N01-CP91000) were cultured (5×10^4 cells/well) for 24 h in flat-bottomed multi-well plates containing Eagle Minimal Essential Medium (MEM; GIBCO) with 100 mL of added fetal bovine serum per liter, in order to obtain semiconfluent cultures. The medium was then removed, replaced with methionine-free MEM containing fetal bovine serum (50 mL/L) and [^{35}S]methionine (60 mCi/L), and the cells were cultured for an additional 18–24 h.

Cell-surface proteins were labeled with ^{125}I by using the glucose oxidase–lactoperoxidase reaction as described by Hynes (*7*). Human fibroblasts were iodinated while they were attached to the tissue-culture plates, but GM607 cells were iodinated as a cell suspension.

Preparation of Cytoskeletons

Cytoskeletons were prepared from peripheral blood leu-

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kocytes and GM607 by resuspending washed cell pellets (approximately 8×10^6 cells) in 5 mL of phosphate-buffered isotonic saline (without calcium and magnesium; GIBCO) containing, per liter, 180 mL of glycerol, 5 mL of Nonidet P40 detergent (NP40; Particle Data Laboratory Ltd., Elmhurst, IL 60126), 1 mmol of phenylmethylsulfonyl fluoride, and 875 μmol of pepstatin (a hexapeptide carboxyprotease inhibitor). After incubation at room temperature for 10 min, the samples were centrifuged for 15 min at $700 \times g$ (JS 3.0 rotor, Beckman J-6B centrifuge). The supernates were discarded and the pellets were solubilized as described below.

Fibroblast cytoskeletons were prepared as described previously (*8*).

Sample Preparation for Electrophoresis

Labeled cells and cytoskeleton preparations were harvested for electrophoresis with 50 μL/well of a solution containing, per liter, 9 mol of urea, 50 mL of NP40, 20 g of LKB ampholyte (pH range 3.5–10; LKB Instruments, Inc., Rockville, MD 20852), 50 mL of 2-mercaptoethanol, and 1.0 mmol of phenylmethylsulfonyl fluoride. Samples were centrifuged for 30 s in a Beckman Microfuge B to remove insoluble material, and 20 μL of the resulting supernates was analyzed by two-dimensional gel electrophoresis.

Two-Dimensional Gel Electrophoresis

First-dimension isoelectric focusing was done by using the ISO apparatus (*9*) and LKB ampholytes (10% pH range, 2.5–4; 90% pH range, 3.5–10). The proteins were focused for 14 000 V · h at room temperature. The second-dimension separation (sodium dodecyl sulfate electrophoresis) was done on linear 10–20% polyacrylamide gradient gels as described (*10*). Gel pouring, electrophoresis, staining, and destaining were all as described previously (*11*). Gels of whole-cell proteins were autoradiographed (*12*); gels of cytoskeletal proteins were fluorographed (*13*).

Results

Figure 1 shows the two-dimensional electrophoresis patterns of [^{35}S]methionine-labeled proteins from the three human cell types studied. The intermediate filament protein, vimentin, is located in an identical position in all three patterns, as is actin. The IgM heavy-chain and light-chain proteins, distinctive markers of the lymphoblastoid cell line GM607, are prominent only in the GM607 pattern. The areas outlined in the three patterns of Figure 1 are proteins known to be associated with the detergent-resistant cytoskeletons of all three cell types. Although the protein patterns of the leukocytes (*A*) and GM607 (*B*) are identical in the regions outlined, the protein pattern of the fibroblasts (*C*) has very different constellations of spots in three of the four areas outlined.

Preparation of detergent-resistant cytoskeletons from cell suspensions requires more manipulation than does similar preparation from attached cells. Fibroblast cytoskeletons remain attached to the plastic tissue-culture dish after detergent extraction and can be easily removed with the NP40/urea solubilization mixture described above. However, cytoskeletons from peripheral blood leukocytes and GM607, which grow as suspension cultures, must be pelleted by centrifugation after detergent extraction. Because the cytoskeleton is still associated with the nucleus under the extraction conditions described, a short low-speed centrifugation suffices to collect the cytoskeletons. Figure 2 shows that this technique yields cytoskeletons of peripheral blood leukocytes (*A*) that are as pure as those prepared from attached fibroblasts. The GM607 cytoskeleton preparations (*B*) consistently contain more protein components than do the fresh leukocyte preparations, despite their similar growth conditions. Comparison with Figure 1*B*, however, shows that even the GM607 cytoskeleton preparations are enriched in specific proteins. The extra proteins seen in the GM607 cytoskeletal preparations may be actual cytoskeletal components peculiar to the long-term culture conditions of this cell line, or they may indicate that the lymphoblastoid cells are more resistant to detergent extraction, requiring longer extraction times or higher concentrations of detergent. The appearance of the IgM heavy-chain and light-chain proteins with the cytoskeletal proteins indicates that some membrane components are still associated with the cytoskeleton in these GM607 preparations.

As mentioned, intact nuclei are associated with the cytoskeletal filaments after detergent extraction. Two major nuclear proteins (identified by co-migration with proteins from purified nuclei) are visible in the patterns of cytoskeletal proteins from peripheral blood leukocytes and GM607. Comparable proteins have not as yet been identified in the fibroblast pattern. The heat-shock protein (*14*) is found associated to some extent with the cytoskeletons of all three cell types. Vimentin and actin are present with almost equal abundance in cytoskeleton preparations from all three types of cells, although a bit more vimentin appears to be associated with fibroblast cytoskeletons than with those of either of the suspension-cell types. The presumed degradation products of vimentin (Cytosk:1 and :4–7) are not visible in the GM607 pattern, a finding specific to this cell line.

The major cytoskeletal proteins that have not yet been identified are designated by numbers (*8*, *15*). Of these, Cytosk$_f$:11 and :13 (subscript f signifying fibroblast proteins) are believed to be identical to Cytosk$_l$:11 and :13 (subscript l signifying leukocyte proteins) because they co-migrate electrophoretically. However, the leukocyte and lymphoblastoid cytoskeleton preparations always show a greater abundance of Cytosk:13 than Cytosk:11, while the reverse is true of fibroblast cytoskeleton preparations. Cytosk$_f$:11, but not :13, has been found to copurify with nonmuscle tropomyosin (*8*). In contrast, preliminary data show that both Cytosk$_l$:11 and :13 copurify with nonmuscle tropomyosin, although Cytosk$_l$:11 is more abundant (i.e., more enriched) in such preparations than Cytosk$_l$:13.

The fibroblast cytoskeletal proteins Cytosk$_f$:8–10 have no counterparts in the leukocyte or lymphoblastoid cytoskeleton preparations. Cytosk$_l$:8, thought to be a possible candidate for a protein analogous to Cytosk$_f$:8, has a completely different peptide pattern after partial proteolysis with chymotrypsin (data not shown). Therefore, Cytosk$_f$:8–10 appear to be unique to fibroblasts, and possibly to attached cells [with the exception of peripheral blood monocytes (*16*)], while Cytosk$_l$:8 is unique to leukocytes, or possibly to cells that grow as suspension cultures.

The fibroblast proteins Cytosk$_f$:14–18 are enriched, together with actin, in nonmuscle myosin preparations done according to Burridge and Bray (data not shown) (*17*). In the whole-cell protein pattern (Figure 1*C*), Cytosk$_f$:14, :15, and :16 each have two "satellite" spots slightly above and to the left of (i.e., acidic to) the major protein spot. These "satellite" spots are phosphorylated forms of Cytosk$_f$:14, :15, and :16, analogous to the phosphorylated myosin light chains of muscle (*18*). The peripheral blood leukocyte and lymphoblastoid cytoskeletons show only three proteins in positions similar to the myosin light chains of fibroblasts, and these have been numbered to correspond to the fibroblast proteins based on their co-migration characteristics—i.e., Cytosk$_l$:15, :16, and :18. Cytosk$_l$:15 and :16 each have phosphorylated forms that can be seen in the whole-cell patterns (Figure 1,*A* and *B*), suggesting that they too may be myosin light chains modified by phosphorylation.

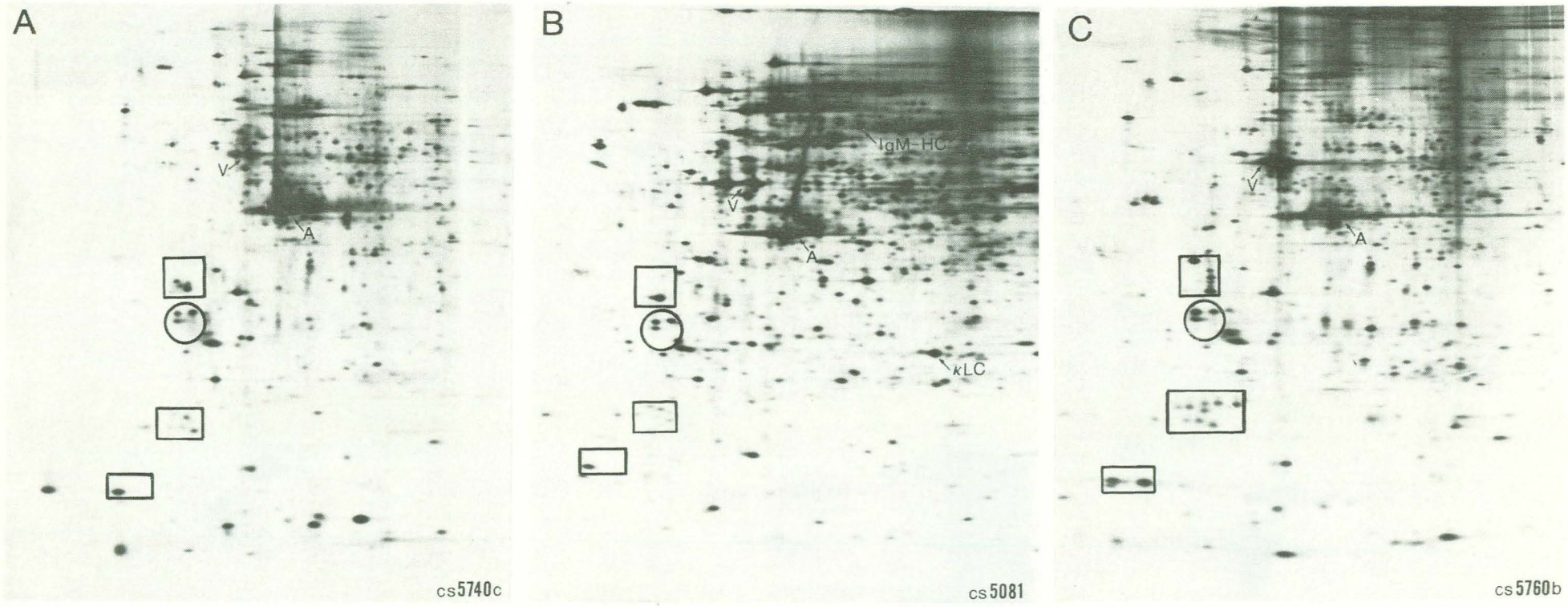

Fig. 1. Two-dimensional electrophoresis patterns of total cellular proteins from cells grown as suspension cultures (*A* and *B*) or as attached monolayers (*C*)

A, peripheral blood leukocytes; *B*, lymphoblastoid cell line GM607; *C*, human skin fibroblast line 1494. Abbreviations: *V*, vimentin; *A*, actin, *IgM-HC*, IgM heavy chain; κLC, kappa light chain. Spot constellations inside *rectangles* differ between cytoskeleton preparations from suspension culture cells and attached monolayer fibroblasts. Spot constellation within the *circles* is found in cytoskeletons from all three types of cells. Patterns are oriented with the acidic side to the left, basic side to the right

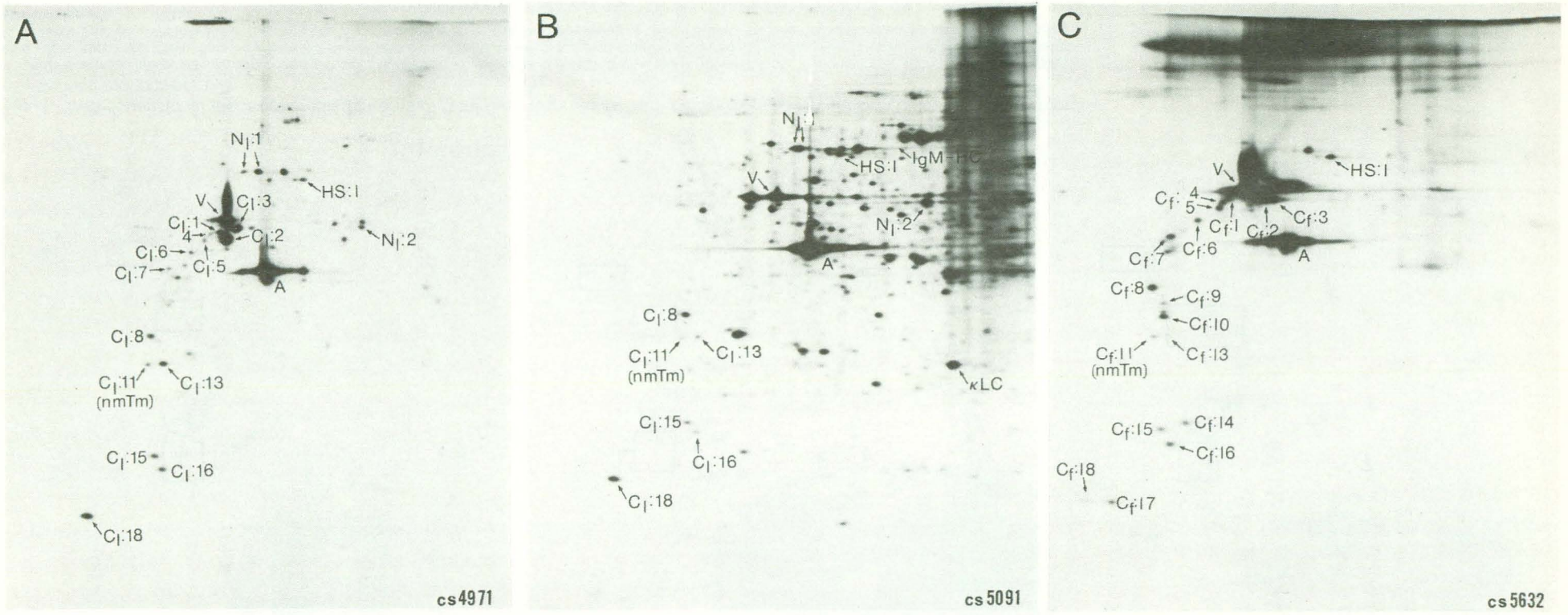

Fig. 2. Two-dimensional electrophoresis patterns of cytoskeletal proteins from cells grown as suspension cultures or as attached monolayers

A, peripheral blood leukocytes; *B*, lymphoblastoid cell line GM607; *C*, human skin fibroblast line 1494. Abbreviations as in Figure 1, with these additions: *N*, nuclear protein; *HS*, heat-shock protein; *C*, cytoskeletal protein, *nmTm*, nonmuscle tropomyosin. Subscripts (*l* or *f*) refer to proteins found in leukocytes (including GM607) or fibroblasts, respectively. Patterns oriented as in Figure 1

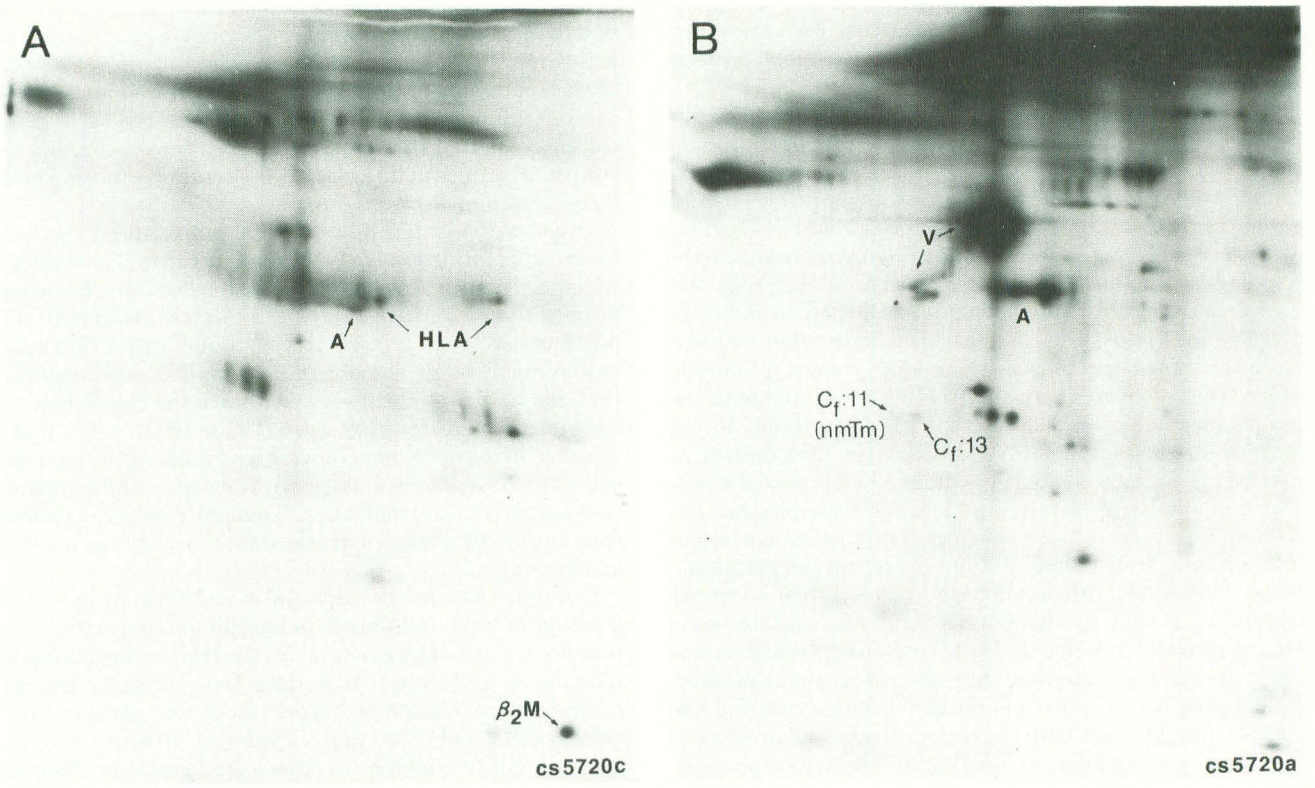

Fig. 3. Two-dimensional electrophoresis patterns of ^{125}I-labeled proteins from whole cells

A, GM607; *B*, human fibroblasts. Abbreviations as in Figures 1 and 2, with these additions: *HLA*, human histocompatibility antigen HL-A; β_2M, β_2-microglobulin. Patterns oriented as in Figure 1

Reportedly, nonmuscle contractile proteins known to be components of the cytoskeleton are sometimes found on the surface of cells (*19*, *20*). To compare the possible surface expression of cytoskeletal proteins in cells grown as suspensions versus those grown as monolayers, we labeled whole GM607 and human fibroblasts with ^{125}I by the glucose oxidase–lactoperoxidase reaction (*7*). Heavy labeling of actin and vimentin was detected in the two-dimensional electrophoresis patterns of the iodinated fibroblast proteins, and Cytosk$_f$:11 and :13 were faintly visible (Figure 3). In contrast, actin could barely be seen in the pattern from iodinated GM607 proteins (Figure 3*A*), and it was difficult to identify which, if any, of the smudges above and to the left of actin might be vimentin. This difference in labeling efficiency of actin and vimentin in the two cell lines shows that the iodination was restricted to the surface of the cells, because labeling of internal proteins would have resulted in equal labeling of actin and vimentin in both cell types. The labeling of the HL-A antigen and associated β_2-microglobulin in GM607 preparations further demonstrated the surface specificity of the label. These data suggest that the microfilaments and intermediate filaments are more exposed at the cell surface of attached cells than is the case for cells in suspension cultures.

Discussion

In this preliminary study, the cells used for analysis by two-dimensional electrophoresis were from different individuals and were grown under different culture conditions. In addition, the growth characteristics of the cells used—i.e., growth as attached or suspension cultures—necessitated the use of two different methods of cytoskeleton preparation. Could any or all of these differences in experimental conditions explain the cytoskeletal protein variations observed when the two-dimensional electrophoresis patterns of human leukocytes, GM607, and skin fibroblasts were compared? We have analyzed skin fibroblasts from over 100 different humans and in every case have found all of the major cytoskeletal proteins discussed in this paper to be located in positions identical to those shown in Figure 2*C*, whether the cells were grown in RPMI 1640 (*8*) or MEM. In cases where variants of cytoskeletal proteins have been found, the variant protein has been present in addition to those normally seen in fibroblast patterns (*8*). In addition, analysis of leukocyte samples (both normal and leukemic) from over 100 different humans and ten different lymphoblastoid cell lines has demonstrated that the proteins described as fibroblast-specific cytoskeletal proteins (e.g., Cytosk$_f$:8) are never found in leukocytes, and the leukocyte-specific cytoskeletal proteins (e.g., Cytosk$_l$:8) are never found in fibroblasts. Therefore, the possibility that the differences shown in Figures 1 and 2 are due to individual polymorphisms or culture conditions is unlikely. In experiments where we attached leukocytes or GM607 to culture dishes by using concanavalin A and subsequently prepared cytoskeletons according to the methods described for fibroblasts, two-dimensional electrophoresis patterns of the cytoskeletal proteins were identical to those shown in Figure 2*A* and *B* with the addition of some cell-surface proteins probably involved with binding to the concanavalin A. None of the human fibroblast-specific proteins were found. Therefore, the preparation of cytoskeletons from cells that are in suspension or attached has no effect on the protein population that is recovered. As discussed below, the comparison of proteins present in suspension and attached cells will be made more complete by the future analysis of a cell line that can be stimulated to convert from one type of culture to the other (*21*). However, the protein differences reported here are true variations among the three cell types analyzed and should be considered as candidate proteins for involvement with cell attachment.

The appearance of differences in cytoskeletal protein composition between cells grown as suspension cultures and those grown as attached monolayers is not surprising, because

the cytoskeletal filaments are thought to be responsible for cell shape and, perhaps, attachment sites. The interesting problem is to determine in which of the cytoskeletal proteins the expected heterogeneity is found.

Our comparison of two types of human cells grown as suspension cultures showed that all of the cytoskeletal proteins found in peripheral blood leukocytes are also found in lymphoblastoid cells, except for those proteins believed to be vimentin degradation products (Cytosk$_l$:1 and :4–7). This difference suggests the absence of a proteolytic enzyme in the lymphoblastoid line that is present in all other cell types analyzed thus far, both attached and unattached, or indicates a difference in the GM607 vimentin structure that makes it more resistant to proteolysis than is the vimentin found in other types of cells. There are proteins associated with the lymphoblastoid cytoskeletons that are not present in the pattern for the peripheral blood leukocyte. These proteins may be cytoskeletal components unique to lymphocytic cells grown in long-term cultures, because the same proteins can be found in the two-dimensional electrophoresis pattern of total proteins from GM607 but not the pattern of peripheral blood leukocytes (predominantly lymphocytes). Another possibility is that the lymphoblastoid cells contain many noncytoskeletal proteins that adhere to the cytoskeletons as prepared for these studies. Only the cytoskeletal proteins common to both the peripheral blood leukocytes and the GM607, but different from the major cytoskeletal proteins of the fibroblast, can be considered candidates for suspension-cell-specific proteins.

Comparison of the cytoskeletal protein patterns from leukocyte and GM607 with patterns from similar preparations of human fibroblasts showed several groups of proteins that were unique to the fibroblast. The differences seen between the myosin light-chain regions (Cytosk$_f$:14–18) of the human fibroblast and suspension-culture patterns, however, have also been found in a comparison of proteins from the fibroblasts of different animals. For example, cytoskeletons from fibroblasts of mink and dog lack Cytosk$_f$:14, as do human leukocytes and GM607 (data not shown). Therefore, the protein heterogeneity found in this region of the patterns reflects some functional difference between cells other than simply the characteristic of attachment or nonattachment in tissue culture. In contrast, the expression of Cytosk$_f$:8–10, although the number of proteins in this set varies among animals, has been seen only in attached cell cultures, suggesting that those proteins are required for some aspect of fibroblast structure or function. ^{125}I-surface labeling of fibroblasts showed no evidence of either the Cytosk$_f$:8–10 or Cytosk$_f$:14–18 spot constellations, indicating that these proteins are not externally involved with cell attachment.

Of the groups of cytoskeletal proteins that differ between human fibroblasts and leukocytes (including GM607), the Cytosk:11 and :13 pair is the most well-characterized. Both proteins are present in all three cell lines studied, but the ratio of Cytosk:11 to :13 appears to be related in some way to cell attachment or nonattachment. An interspecies comparison of fibroblast cytoskeletal proteins has shown that Cytosk$_f$:11 is always more abundant that Cytosk$_f$:13 in fibroblasts from species as diverse as cows and dolphins (data not shown). Both the human peripheral blood leukocytes and GM607, however, contain more Cytosk$_l$:13 than Cytosk$_l$:11. Similar results have been obtained from the analysis of purified populations of human granulocytes and lymphocytes from peripheral blood (*16*). In each case, Cytosk$_l$:13 is more abundant than Cytosk$_l$:11. However, monocytes, after several days in culture, will grow as attached cells and have almost equal amounts of Cytosk$_l$:11 and :13 (*16*). This variation in abundance and the copurification of both Cytosk$_l$:11 and :13 with nonmuscle tropomyosin from leukocytes and GM607 but only Cytosk$_f$:11 from fibroblasts poses interesting questions as to the number of tropomyosin genes present in the human genome and the regulation of their expression in different cell types. For study of the expression of proteins involved with cell attachment, cytoskeletal proteins from a cell line such as HL60 (*21*), which can be converted from a suspension culture to an attached culture at various points in the cell-alteration process, should be isolated and electrophoresed.

Other proteins in the cytoskeletal preparations that thus far are unidentified may also be found to have significance in the comparison of attached with nonattached cells. These may be additional nuclear proteins, proteins associated with polyribosomes (*22*), proteins serving regulatory functions such as the microtubule-associated proteins (*23*), or membrane proteins that are tightly associated with the cytoskeleton in a manner similar to the 5′-nucleotidase (EC 3.1.3.5) that is attached to the detergent-resistant matrix of murine lymphoid cells (*24*). The microtubule proteins remain to be compared, because tubulin and many associated proteins were extracted from the cytoskeletons under the conditions we used for these experiments (*23*).

Our data demonstrate that the cytoskeletal protein composition of cells is tailored to specific cell structures and functions. Such data, essential for the Human Protein Index (*25*), allow us to begin to catalog those proteins that are common to all human cell types, those that are specific to certain cells, and those that vary during different stages of normal cell differentiation or pathological processes. Recently, Bachvaroff et al. used two-dimensional electrophoresis to show that the cytoskeletal proteins actin and tubulin could be detected on the surface of human leukemia cells and of human lymphocytes transformed by mitogens or Epstein–Barr virus, but not on normal resting lymphocytes (*18*). The identification of other cytoskeletal proteins, such as nonmuscle tropomyosin and myosin, on two-dimensional electrophoresis patterns of human cells and knowledge of their normal occurrence could extend such studies and perhaps provide specific clinical markers for alterations in cell structure that correlate with diseases such as leukemia.

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