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Cellular partitioning of β-1 integrins and their phosphorylated forms is altered after transformation by Rous sarcoma virus or treatment with cytochalasin D

Beatrice Haimovich, Brian J. Aneskievich,* and David Boettiger
Department of Microbiology
University of Pennsylvania
Philadelphia, Pennsylvania 19104-6076

A sequential extraction procedure of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) buffer followed by RIPA or Laemmli sample buffer was developed to define two distinct subpopulations of β-1 integrins in primary chicken embryo fibroblasts. Extraction of cells in culture revealed an association of adhesion plaque-localized integrin with the CHAPS-insoluble fraction. Phosphorylated integrins were found in both fractions, but the specific phosphorylation was 12-fold higher in the CHAPS insoluble fraction. The phosphorylation was evenly distributed between phosphoserine and phosphotyrosine. Transformation by Rous sarcoma virus caused a redistribution of integrin to rosettes and an increase in total integrin phosphorylation. Treatment with cytochalasin D caused a redistribution of the adhesion plaque-associated integrin into lacelike structures and reduced the level of integrin phosphorylation. These treatments also caused an altered distribution of phosphorylated integrin between the CHAPS soluble and insoluble fractions. These results suggest a role for integrin phosphorylation in the assembly and disassembly of cellular adhesion structures.

Introduction

Integrins form a family of transmembrane heterodimers, composed of one α and one β subunit, which serve as surface receptors for extracellular matrix molecules (Buck and Horwitz, 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Cheresh *et al.*, 1989). The cloning of the chick β-1 integrin chain revealed a tyrosine in a consensus sequence for phosphorylation by the *src* and other families of membrane-associated tyrosine kinases (Tamkun *et al.*, 1986). Subsequent studies have demonstrated both increased phosphorylation of integrin β-1 subunit on tyrosine in cells carrying an active v-*src* (Hirst *et al.*, 1986; Duband *et al.*, 1988a; Tapley *et al.*, 1989) and the ability of immunoprecipitated $pp60^{v\text{-}src}$ to phosphorylate immunoaffinity-purified integrin in vitro (Tapley *et al.*, 1989). Hyperphosphorylation of integrin has been implicated in regulation of adhesion using extracts from vanadate-treated cells transformed by Rous sarcoma virus (RSV). Integrin from these extracts exhibited reduced affinity both to fibronectin and to talin in a column-binding assay (Tapley *et al.*, 1989). Phosphorylated integrin has been also isolated from vanadate-treated normal chicken embryo fibroblasts, raising the possibility that phosphorylation of integrin plays a role in normal cell behavior (Duband *et al.*, 1988a).

There are two patterns of integrin distribution in normal chicken embryo fibroblasts (CEF), diffuse and concentrated in adhesion plaques (Neff *et al.*, 1982; Chen *et al.*, 1985; Damsky *et al.*, 1985; Brown and Juliano, 1987). Adhesion plaques are characterized by the codistribution of extracellular ligands such as fibronectin and intracellular components such as talin, vinculin, and actin (Burridge *et al.*, 1988). One presumes that integrin molecules located in adhesion plaques are more likely to be associated with their ligands, and therefore functional, than integrin molecules diffusely distributed outside the plaque. Indeed, Singer *et al.* (1988) have demonstrated that plating cells on fibronectin or vitronectin substrates drives the appropriate integrin receptor into adhesion plaques. The diffusely distributed integrin molecules would therefore appear to represent a reserve pool. The association of integrin with either or both of its intracellular and extracellular ligands is likely to render it less soluble and less mobile. These assumptions have been partially sub-

* Present address: Howard Hughes Medical Institute, Department of Cell Biology, University of Chicago, Chicago, Illinois 60637.

stantiated by Brown and Juliano (1987), who found that, by linking an anti-integrin monoclonal antibody to the substrate, they could drive integrin molecules into a nonionic detergent insoluble pool. Experiments using fluorescence recovery after photobleaching similarly demonstrated decreased mobility of integrin molecules associated with adhesion plaques relative to the mobility of the diffuse population (Duband *et al.*, 1988b).

To establish a regulatory role for phosphorylation in integrin-mediated adhesion, it is critical to discern the levels of integrin phosphorylation characteristic of each pool. Both the extractability of integrin and its level of phosphorylation in different cellular pools may functionally correlate with altered cell morphology and adhesion. To investigate this possibility, we manipulated cells by transforming them with either wild-type RSV (WT) or the partial transforming mutants CU2 and CU12 (Anderson *et al.*, 1981) or by treating them with cytochalasin D (CD). Each of these treatments causes specific changes both in cell morphology and in the distribution of components of the adhesion plaques (Anderson *et al.*, 1981; Rohrschneider and Rosok, 1983; Brett and Godman, 1986; Roman *et al.*, 1989). The partial transformation mutants of *src* produce intermediate transformation phenotypes and carry point mutations that may alter their substrate repertoire (Parsons and Weber, 1989). Treatment of cells with CD separates actin from its association with integrin in the adhesion plaques (Roman *et al.*, 1989). Our results indicate that total levels of integrin phosphorylation and the relative detergent partitioning profile of integrin and its phosphorylated forms are both altered by CD treatment and transformation by *src*. The possible biological relevance of these events is discussed.

Results

Biochemical analysis of total cellular integrin and its phosphorylation

To provide a measure of the total levels of cellular integrin and its phosphorylation in normal, noninfected (NI), WT, CU2 and CU12, and NI+CD chicken embryo fibroblasts, we extracted parallel cultures with 10 mM tris-(hydroxymethyl)aminomethane-HCl (Tris-HCl), pH 7.2, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) (RIPA). The total levels of β-1 integrin were determined by Western blots of the RIPA extracts and the use of a polyclonal-integrin specific antisera (Figure 1A). Transformation by either WT-*src* or CU2 and CU12, or treatment with CD resulted in a 10–20% drop in total β-1 integrin (Table 1). Chen *et al.* (1986) showed a similar decrease for WT compared

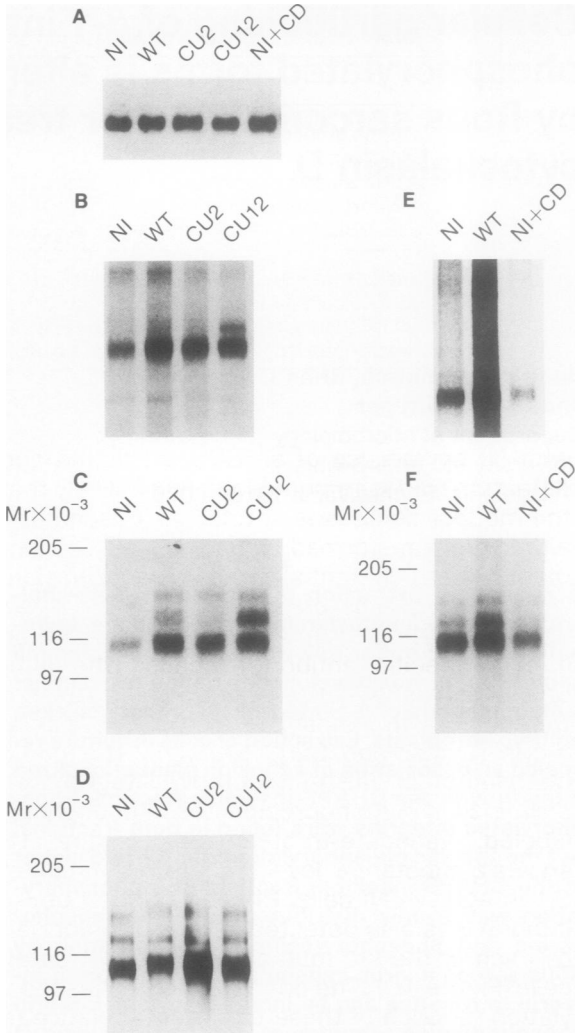

Figure 1. **RIPA extraction of total cellular integrin and its phosphorylated forms.** Noninfected (NI), wild-type RSV-transformed (WT), partially transformed (CU2 and CU12), and cytochalasin D-treated (NI+CD) cells were extracted with RIPA buffer. (A) Equal amounts of proteins were separated on 7% SDS-PAGE gel under nonreducing conditions, blotted onto nitrocellulose, and probed with an integrin-specific antisera followed by ^{125}I-donkey anti-rabbit IgG. (B and E) Cells were labeled for 6 h with [^{32}P]-orthophosphate and lysed in RIPA buffer. Immunoprecipitation from equal amounts of cellular proteins was carried out using a β-1 integrin-specific antisera. Immunoprecipitated proteins were analyzed by electrophoresis through 7% SDS-PAGE under nonreducing conditions. (C and F) The same gels shown in B and E were treated with KOH to increase detectability of phosphotyrosine-containing proteins. (D) Cells were labeled for 6 h with [^{35}S]-methionine. Equal amounts of TCA-precipitable counts were immunoprecipitated with a β-1 integrin-specific antisera and analyzed as described above.

Table 1. RIPA extraction of β-1 integrin and its phosphorylated forms

	NI	WT	CU2	CU12	NI+CD
Integrin amount	1	0.9	0.8	0.8	0.9
[^{32}P]-integrin	1	2.9	2.5	2.6	0.3
KOH-resistant [^{32}P] integrin	1	5.4	4.6	6.5	0.2
[^{32}P]-integrin/total integrin	1	3.2	3.1	3.2	0.3
KOH-resistant [^{32}P]-integrin/total integrin	1	6.0	5.7	8.1	0.2

Total β-1 integrin, total phosphorylated β-1 integrin, and alkali-resistant phosphate in β-1 integrin were calculated from the gels in Figure 1 and normalized to the levels in the NI (non-infected) cells. Other cells were WT, wild-type Rous sarcoma virus-transformed; CU2 and CU12, partially transformed; NI+CD, cytochalasin D treated.

with NI by the use of an SDS extraction and detection by dot immunoblots. It is unlikely that the modest decreases in total β-1 observed in either the transformed cells or after CD treatment have a substantial effect on cell adhesion.

For the analysis of phosphorylation, it was necessary to use immunoprecipitation with an integrin-specific antibody because the anti-phosphotyrosine antisera available appear not to recognize the phosphotyrosine (PTYR) in integrin (Linder and Burr, 1988a,b). The phosphorylation of RIPA-extractable integrin was examined in immunoprecipitates of phosphate-labeled, vanadate-treated cells. Figure 1B shows a substantial level of phosphorylation of β-1 integrin in NI cells. Phosphorylation of β-1 integrin was also detected in cells transformed by each of the src mutants and in CD-treated cells (Figure 1C). The quantitation of phosphorylation for each of these treatments relative to the level of phosphorylation for NI cells is given in Table 1. Cells transformed either by WT-*src* or the CU2 and CU12 mutants exhibited about a threefold increase in phosphorylation, whereas the CD-treated cells exhibited a threefold reduction relative to NI. To increase the sensitivity of detecting PTYR-containing proteins, we hydrolyzed the phosphate-labeled gels with hot alkali (Figure 1, C and F). Because no phosphothreonine (PTHR) was detected in direct phosphoamino acid analysis (see below), the band intensities from the alkali-treated gels (Figure 1, C and F) provide a better estimation of the relative levels of PTYR. When these bands are quantitated by scanning and normalized to NI cells, the cells transformed by each of the *src* viruses exhibit a fivefold increase, whereas CD treatment produced a fivefold decrease in alkali-resistant phosphate (Table 1). Hence the largest increase in phosphorylation in cells transformed by *src* appears to be on tyrosine. This increase parallels the increased tyrosine phosphorylation seen on the other adhesion plaque-related proteins talin (Pasquale *et al.*, 1986; DeClue and Martin, 1987) and vinculin (Sefton *et al.*, 1981). The decrease in kinase activity of CU2 relative to WT-*src* was not considered sufficient to explain its partial transformation phenotype (Parsons and Weber, 1989). Both CU2 and CU12 appeared to give β-1 integrin phosphotyrosine levels similar to WT-*src* (Table 1). Thus, levels of integrin phosphorylation on tyrosine do not correlate with the extent of transformation.

Immunoprecipitations from methionine-labeled cultures were performed to allow identification of the α subunits associated with the β-1 integrin (Figure 1D). Similar immunoprecipitates of integrins have been described previously; the upper two bands (1 and 2) contain the α subunits, whereas the lower band (3) contains the β subunit (Neff *et al.*, 1982; Hynes *et al.*, 1989). There appeared to be a preferential PTYR phosphorylation of band 2 relative to band 1 in most cases (Figure 1, C and F). Phosphorylation of one α subunit in cells transformed by src has been reported (Hirst *et al.*, 1986). The relative increased phosphorylation of band 2 in CU12 and decreased level in CU2 relative to WT-*src* (Figure 1C) suggest that this phosphorylation is not directly related to the extent of morphological transformation.

Distribution of phosphorylated β-1 integrin

Sequential extractions of NI, WT, CU2, CU12, and CD-treated cells were performed with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS buffer) followed by RIPA and sample buffer (Laemmli, 1970). Extractions were performed in parallel on cultures treated with vanadate for 6 h before extraction for Western blot analysis, methionine labeling, and phosphate labeling. The only differences were the inclusion of label and deletion of the cold form of the molecule from the medium. Western

Table 2. Differential extraction of cellular proteins

	CHAPS	RIPA	Sample buffer
NI	80	12	8
WT	90	7	3
CU2	70	21	9
CU12	79	14	7
NI+CD	73	14	13

Cells were extracted with a small volume of CHAPS buffer. The insoluble pellet was extracted with RIPA buffer, and the resulting pellet was extracted with sample buffer. For each cell type the total protein recovered in all three fractions was normalized to 100%.

blots carried out on non-vanadate-treated cultures gave levels and distribution of integrin similar to the vanadate-treated cultures. The distribution of total cellular protein into the three fractions for each cell type is given in Table 2. Twenty micrograms of protein from each of the extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted for β-1 integrin using polyclonal antisera (Figure 2). These blots were scanned, and the relative amount of the β-1 integrin subunit in each fraction was calculated relative to the NI CHAPS extract (Table 3). These values could then be used to calculate the relative amount of integrin in each fraction for each cell type (Table 4). Both the WT-transformed and the CD-treated cells showed a two- to threefold increase in the proportion of CHAPS-insoluble integrin. Whereas the CHAPS-insoluble proteins in the WT transformants decreased twofold, the actual level of CHAPS-insoluble integrin increased, showing that integrin solubility does not just follow the general protein extraction.

Parallel cultures labeled with [^{32}P]-phosphate in the presence of vanadate were extracted with CHAPS buffer followed by RIPA. To remove potential differential effects of these detergents on the immunocomplexes, we added additional detergent to the CHAPS extract to bring it to the same detergent level as the RIPA before the immunoprecipitation. Equal amounts of protein from each extract were immunoprecipitated with β-1 integrin antiserum, separated by SDS-PAGE, and analyzed by autoradiography (Figure 3, A and B). Table 5 presents the detergent partitioning data for unlabeled integrin (taken from Table 3) and for [^{32}P]-labeled β-1, as resolved by scans of the phosphate-labeled gels (taken from Figure 3). This allows the calculation of specific phosphorylation levels for each of the fractions relative to the CHAPS-extracted NI cells. Transformation by any of the

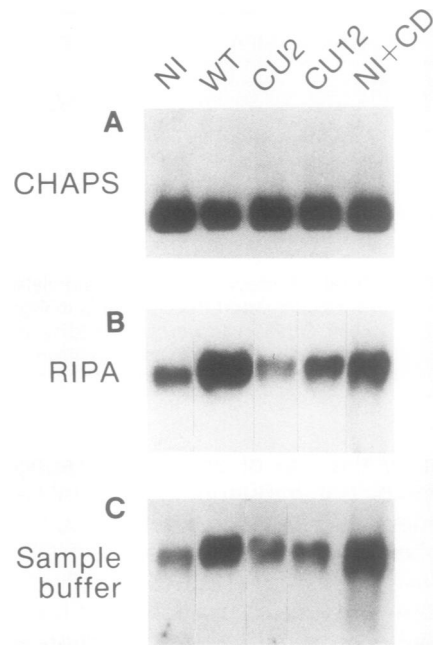

Figure 2. **Differential extraction of cellular β-1 integrin.** Noninfected (NI), wild-type RSV-transformed (WT) partially transformed (CU2 and CU12), and cytochalasin D-treated (NI+CD) cells were scraped from the dishes and extracted in CHAPS buffer. The CHAPS-insoluble pellet was extracted with RIPA, and the RIPA-insoluble pellet was solubilized in nonreducing Laemmli sample buffer. Equal amounts of proteins from each cell type were separated on 7% SDS-PAGE gel under nonreducing conditions, blotted onto nitrocellulose, and probed with an integrin-specific antisera followed by ^{125}I-donkey anti-rabbit IgG.

src viruses induced a 3- to 4-fold increase in total phosphorylated integrin in both the CHAPS and RIPA fractions. In contrast, CD treatment induced a 3- to 4-fold decrease in phosphorylation in the CHAPS-soluble fraction and a 14-fold decrease in the RIPA fraction.

Phosphorylation of integrin may affect its protein associations, which in part determine

Table 3. Differential extraction of cellular β-1 integrin

	CHAPS	RIPA	Sample buffer
NI	1	0.23	0.12
WT	0.76	1.00	0.70
CU2	0.91	0.15	0.14
CU12	0.93	0.29	0.23
NI+CD	1.07	0.42	1.14

Quantitation of levels of β-1 integrin shown in Figure 2. Equal amounts of proteins from each detergent fraction were analyzed by immunoblots using an anti-β-1 integrin antiserum. All the values were normalized relative to the NI CHAPS fraction.

Table 4. Corrected detergent fractionation profile of β-1 integrin

	CHAPS	RIPA	Sample buffer
NI	95	3	2
WT	88	10	2
CU2	93	5	2
CU12	93	5	2
NI+CD	79	6	15

To determine the actual distribution of β-1 integrin in the various detergent fractions, we corrected the values in Table 3 for the distribution of total cellular proteins (Table 2) and normalized them to 100% for each cell sample.

extractability and thus contribute to its partitioning characteristics. In the NI cells, the relative level of integrin phosphorylation in the RIPA fraction was 12-fold higher than that in the CHAPS fraction. This partitioning of phosphorylated β-1 integrin was perturbed by either cell transformation or treatment with CD (Table 5). Both the WT transformants and the CD-treated cells showed a similar change in the partitioning of the phosphorylated integrin, but this was accomplished by different means. Relative to NI there was a large increase in the specific phosphorylation for the WT transformants in the CHAPS fraction. The CD-treated cells exhibited an overall drop in specific phosphorylation, which was greater in the RIPA than in the CHAPS fraction. The partial transformants exhibited similar increases in specific phosphorylation in both the CHAPS and RIPA fractions, resulting in a ratio of CHAPS:RIPA similar to NI.

Phosphoamino acid analysis was performed by two-dimensional electrophoresis of the β-1 integrin bands excised from the gels (Figure 3). Both phosphoserine (PSER) and PTYR but not PTHR were found in each sample. For the NI cells there was proportionally higher PTYR than PSER in the CHAPS relative to the RIPA fraction. In the WT transformants there were higher levels of PTYR relative to PSER in both fractions. These increases are consistent with both the increased levels of tyrosine kinase activity in cells transformed by *src* and with β-1 integrin serving as a direct target for the src kinase. Tyrosine phosphorylation was reduced relative to serine in both the CHAPS and RIPA fractions after CD treatment.

Distribution of integrin subunits

Because integrin exists on the cell surface as an α-β heterodimer (Hynes 1987), defining the distribution of the specific integrin receptors involves identification of both the α and β subunits. The detergent partitioning and immunoprecipitation experiments described above were repeated using [^{35}S]-methionine-labeled cultures and α-chain-specific antisera. Antisera raised to a peptide representing the internal sequences for α-3 and α-5, which recognize the chicken isoforms (Hynes *et al.*, 1989), and the monoclonal antibody LM609, which recognizes the chicken αvβ3, were used (Cheresh and Spiro, 1987; Hynes *et al.*, 1989). Figure 4 shows

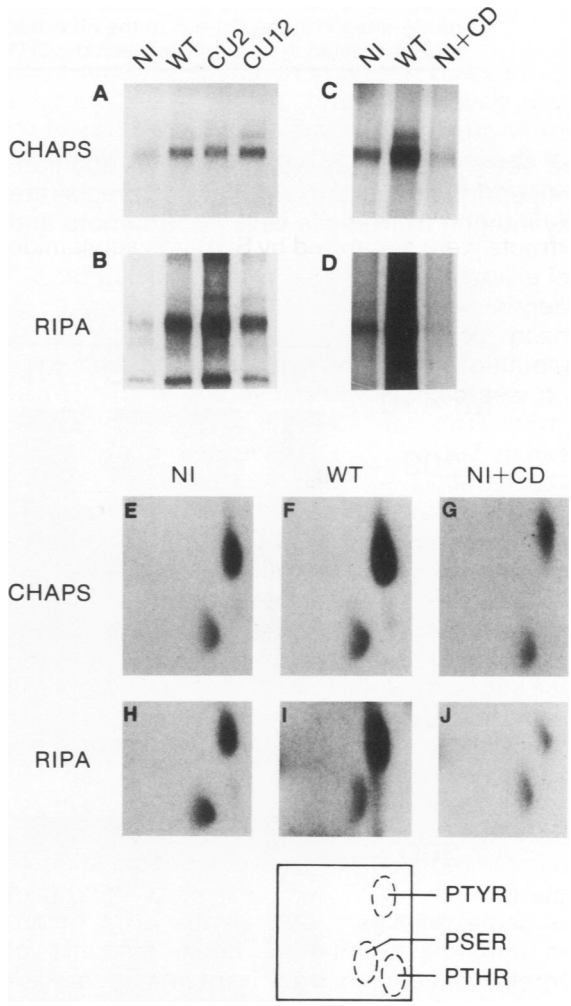

Figure 3. **Distribution of phosphorylated integrin.** Non-infected (NI), wild-type RSV-transformed (WT), partially transformed (CU2 and CU12), and cytochalasin D-treated (NI+CD) cells were labeled for 6 h with [^{32}P]-orthophosphate. Sequential CHAPS (A and C) and RIPA (B and D) extracts were prepared from the labeled cultures. An equal amount of protein from each extract was immunoprecipitated with anti-β-1 integrin antiserum, separated by SDS-PAGE, and analyzed by autoradiography. (E–J) Phosphoamino acid analysis was performed by two-dimensional electrophoresis of the integrin β-1 bands excised from the gels.

Table 5. Partitioning of β-1 integrin and its phosphorylated forms

	CHAPS			RIPA			C:R ratio
	I	^{32}P	S.P.	I	^{32}P	S.P.	
NI	1.00	1.0	1.0	0.23	2.8	12.2	1:12.2
WT	0.76	3.6	4.7	1.00	11.5	11.5	1:2.4
CU2	0.91	3.3	3.6	0.15	10.4	69.3	1:19.2
CU12	0.93	3.3	3.5	0.29	9.1	31.4	1:8.9
NI+CD	1.07	0.3	0.3	0.42	0.2	0.48	1:1.6

I represents the relative levels of β-1 integrin protein, taken from Table 3. ^{32}P represents the labeled phosphate present in each β-1 integrin fraction, calculated from the gels shown in Figure 3. Values for integrin protein and integrin protein phosphorylation were normalized to NI CHAPS. S.P. is a calculation of the relative specific phosphorylation obtained by phosphate levels by total integrin protein levels. C:R ratios given in the last column represent the ratios of specific phosphorylation on β-1 integrin in the CHAPS to the RIPA fraction. This represents the relative partitioning of the phosphorylated β-1 integrin between the CHAPS- and RIPA-soluble fractions.

the detection of α-3, α-5, and α-v; in addition, antipeptide antisera to α-2 did not precipitate any integrin from these cells (M. Enomoto and D. Boettiger, unpublished data). The antisera to α-3 and α-5 coprecipitate β-1, indicating the presence of heterodimers between these αs

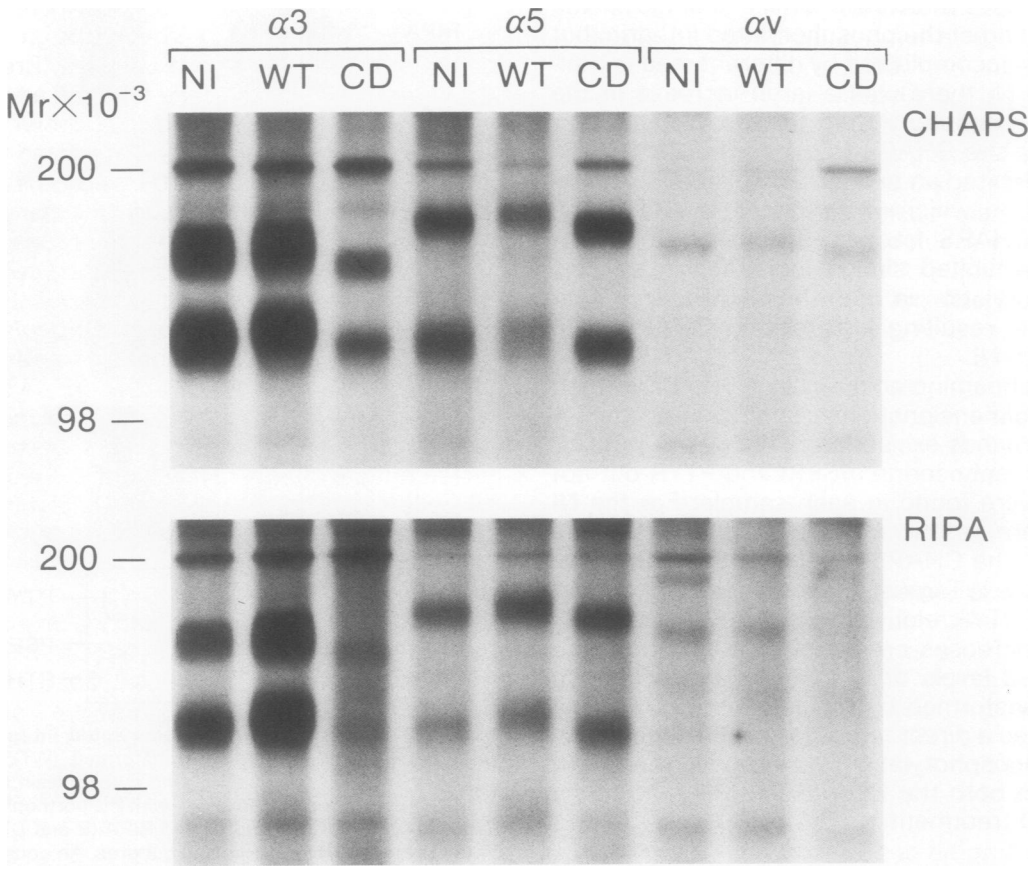

Figure 4. **Cellular partitioning of α integrin subunits.** Noninfected (NI), wild-type RSV-transformed (WT), and cytochalasin D-treated (CD) cells were labeled for 20 hours with [^{35}S]-methionine scraped from the dish and extracted with CHAPS buffer and the insoluble pellet extracted with RIPA buffer. Aliquots of each extract containing equal levels of protein were immunoprecipitated with α integrin-specific antisera and analyzed by SDS-PAGE.

and β-1. In contrast, anti-α-v antisera do not precipitate β-1, hence α-v β-1 complexes were not detected. It is likely that it is complexed with β-3 and does not show well because of the poor labeling of β-3 with methionine (Hynes *et al.*, 1989). Both α-3 and α-5 were detected in the CHAPS-soluble and -insoluble pools with a distribution similar to that of β-1 (Table 6). There is an apparent increase in the CHAPS-insoluble fraction for α-5 in the WT-transformed cells relative to NI controls, which is counter to the trend for β-1; however, this is compensated for by twofold decrease in the total level of α-5 synthesis, leaving the total amount of α-5 in the CHAPS insoluble fraction little changed. A similar drop in level of αs has been previously reported for transformed mammalian cells (Plantefaber and Hynes, 1989). α-v shows a distinctly different distribution, with relatively high proportions in the CHAPS-insoluble fractions for both the NI controls and the WT-transformed cells but an undetectable level in the CHAPS-insoluble fraction for the CD-treated cells.

The scanning of these gels and calculation of the molecular mobilities of the bands gives values of 171 kDa for α-5, 158 kDa for α-3, and 162 kDa for α-v. A comparison of similar scans for the phosphate-labeled gels (Figure 3) shows phosphate-labeled alpha bands of 170 kDa and 142 kDa. This suggests that α-5 is phosphorylated whereas α-3 and α-v are not.

Hence integrin complexes in both the CHAPS-soluble and -insoluble fractions contain both α-3 β-1 and α-5 β-1 heterodimers. In addition, there is at least one more β-1 heterodimer that is not either α-2 or α-v. Because α-1 is expected to be considerably larger (210 kDa in human [Hemmler *et al.*, 1987]), and phosphorylation of α-6 has been reported (Shaw *et al.*, 1990), it is likely to be α-6.

Localization of the CHAPS-insoluble integrin fractions

The previous experiments described a distribution of integrin into CHAPS-soluble and -insoluble (RIPA- and sample buffer-soluble) fractions. A similar CHAPS detergent extraction was done on coverslips in an attempt to correlate the CHAPS-insoluble fraction with a subcellular localization. The CHAPS-insoluble material was completely removed by RIPA extraction.

In unextracted control cultures, integrin was localized using a β-1-specific antiserum and exhibited its typical streaklike adhesion plaque distribution in NI cells (Figure 5A), as previously described (Damsky *et al.*, 1985; Chen *et al.*, 1986). Transformation by WT-*src* induced a redistribution of integrin, with some associated in the rosettes and the majority diffusely distributed (Figure 6B). Similar results have been previously reported for transformed cells (Tarone *et al.*, 1985; Chen *et al.*, 1986). At the concentration of CD used for these experiments (2 μM), two different morphologies were present. First, there were spread cells, which were irregular in shape and contained regions of high optical density, particularly at the cell tips (Brett and Godman, 1986). These cells retained a few very long and thin actin filaments (not shown) along which integrin was primarily distributed (Figure 5C). Second, there were arborized cells (Croop and Holtzer, 1975; Brett and Godman, 1986) with long, branching dendritic processes. In these cells the actin had completely collapsed (not shown) and integrin had redistributed into a characteristic lacelike pattern particularly apparent along the cell processes (Figure 5D).

After extraction with CHAPS on coverslips, the residual cell material was examined. No identifiable cellular material was seen under phase optics. Integrin staining of the extracted NI cells revealed residual cell footprints containing streaklike patterns reminiscent of the adhesion plaques seen in the intact cells (Figure 6A). These integrin prints showed a distribution that appeared to reveal the outlines of individual cells and were present at a density expected from the original cell population. Costaining the same coverslips for actin revealed a complete absence of actin in the integrin cell footprints for both uninfected and WT transformants (data not shown). In the transformed cells, integrin was localized in groups of small aggregates, reminiscent of its distribution in the rosettes before extraction (Figure 6B). The integrin staining of the CD-treated CHAPS-extracted cells was even more dramatic. Spread cells with fine integrin streaks preserved their character-

Table 6. Corrected detergent fractionation profile of integrin α subunits

	NI		WT		NI+CD	
	CHAPS	RIPA	CHAPS	RIPA	CHAPS	RIPA
α-3	92	8	88	12	90	10
α-5	89	11	80	20	86	14
α-v	59	41	72	28	(100)	N.D.

Quantitation of the detergent partitioning of α subunits shown in Figure 4. The final values were corrected for the distribution of total cellular proteins and normalized to 100% for each sample. N.D., not detected.

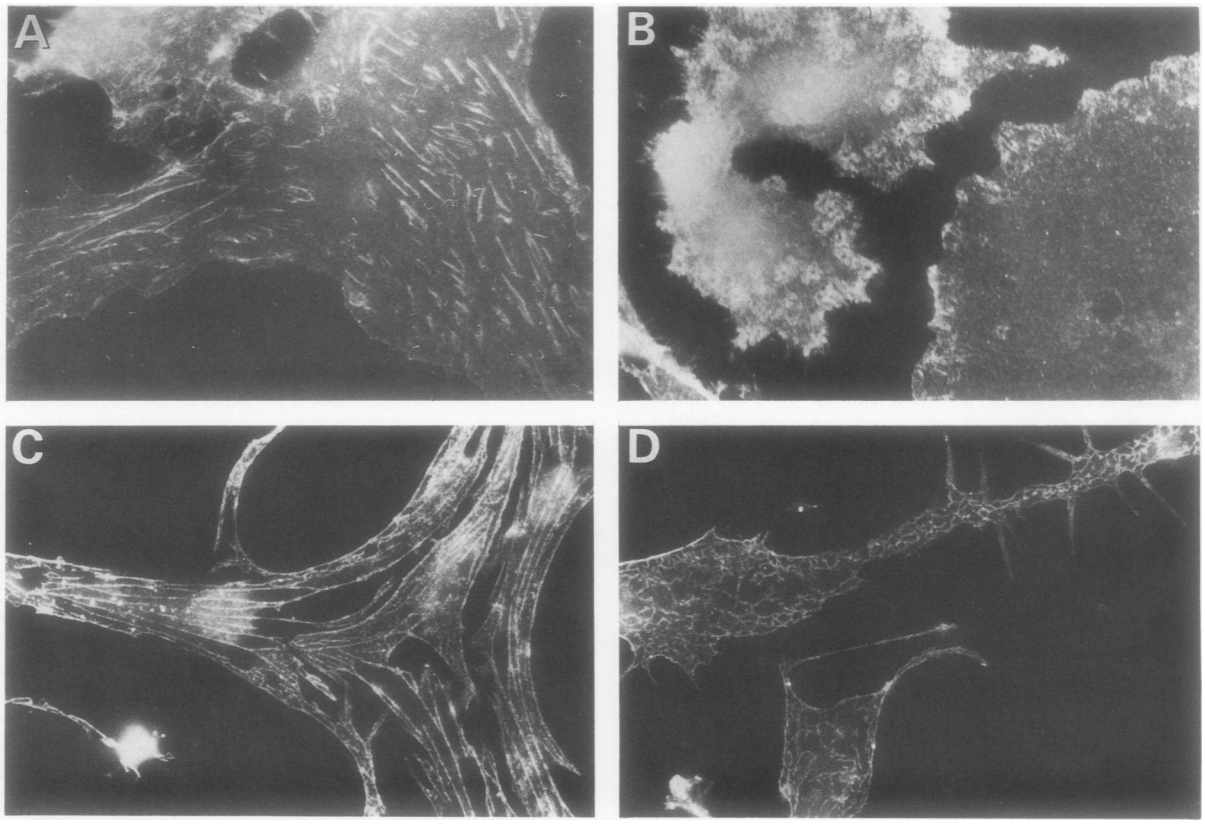

Figure 5. **Localization of β-1 integrin.** Immunofluorescence localization of integrin in (A) noninfected (NI), (B) wild-type RSV-transformed (WT), and (C and D) cytochalasin D-treated (NI+CD) cells. The cells shown in C and D are typical of the concentration of CD used through this study (2 μM CD). Integrin was localized using β-1-specific antisera followed by rhodamine-conjugated goat anti-rabbit IgG.

istic streak patterns after extraction (Figure 6C); the arborized cells retained their lacelike integrin distribution (Figure 6D). There was residual actin staining in the CD-treated cells, but it was present as amorphous deposits (not shown). These data indicate that the CHAPS extraction, unlike similar experiments done with NP-40 or Triton X-100, removes the cytoskeleton. The residual cytoskeleton in the CD-treated cells probably is the result of the formation of large amorphous actin aggregates in these cells (Brett and Godman, 1986). In these experiments, the integrin-insoluble fraction from NI and CEF transformed by src remained attached to the coverslips even though the actin cytoskeleton was removed. Because the fibronectin matrix was not affected by CHAPS (not shown), it is likely that integrin remained associated with it. The characteristic integrin distribution pattern was strikingly retained after CHAPS extractions in four distinct morphological cell types. The implication of these localization experiments is that the integrin present in the CHAPS-insoluble pool is topographically found at adhesion plaques. Because these structures are removed by RIPA, we take the RIPA fraction to be highly enriched in adhesion plaque integrin.

Discussion

Detergent partitioning of integrin

Adhesion plaque components are involved in spatially discrete macromolecular associations. The assembly of these macromolecular complexes involves lateral associations between homologous or heterologous molecules and transmembrane associations between extracellular and intracellular compartments, such as that mediated by integrins. In addition to their location in the plaque, integrin and other adhesion plaque molecules are also diffusely distributed in the cell. Both the nature of the immediate chemical environment (e.g., protein concentrations and surrounding lipids) and the characteristic protein-protein and protein-lipid associations are important factors in determining the protein-specific detergent extractability

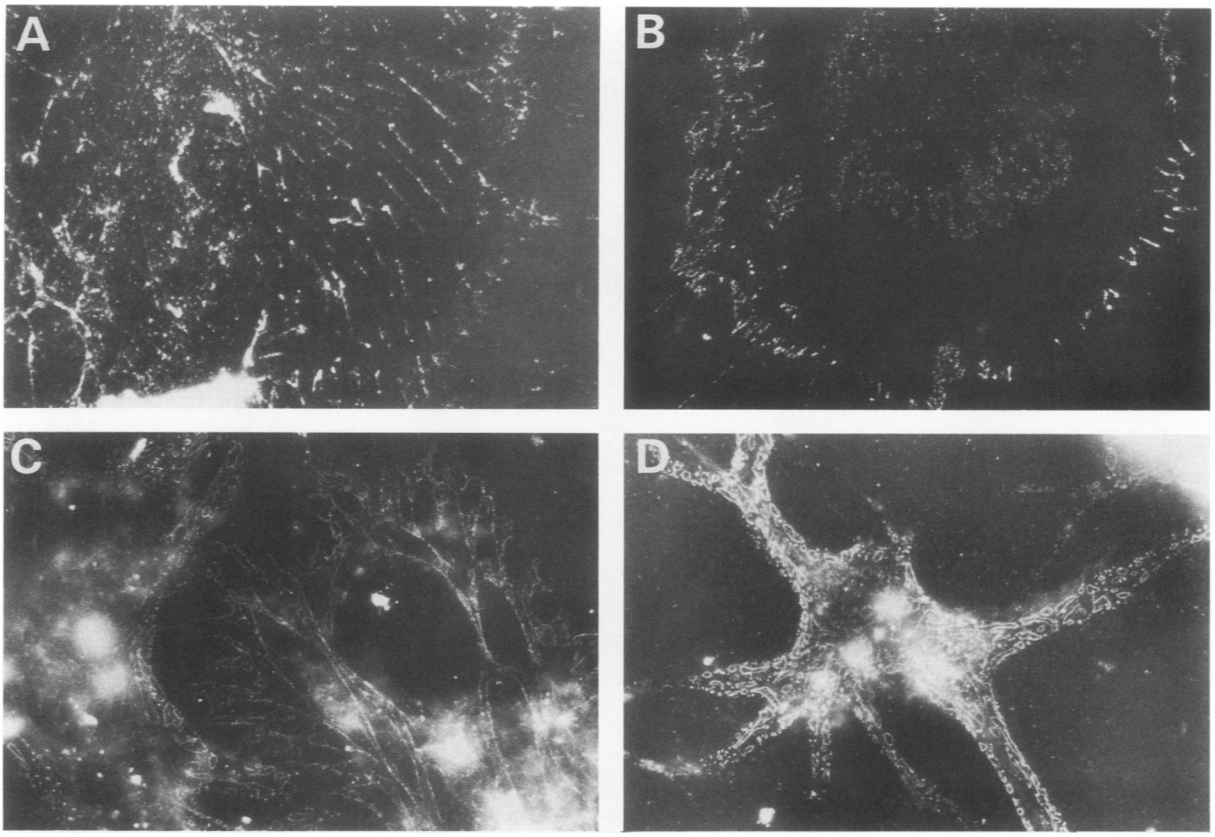

Figure 6. **Localization of the CHAPS-insoluble β-1 integrin fraction.** Immunofluorescence localization of integrin in (A) noninfected (NI), (B) wild-type RSV-transformed (WT), and (C and D) cytochalasin D-treated (NI+CD) cells. Cultures parallel to those shown in Figure 5 were extracted with CHAPS buffer for 3 min before their fixation. Integrin was localized using β-1-specific antisera followed by rhodamine-conjugated goat anti-rabbit IgG.

properties. Contrary to what might be expected from the compact macromolecular organization at adhesion plaques, the majority of the cellular integrin, vinculin, and talin can be extracted by nonionic detergents, suggesting that they are held by lipid bilayers or by low-affinity interactions. This is supported by the low-affinity interactions between nonionic detergent-solubilized integrin and either fibronectin or talin, as measured by in vitro column-binding assays (Horwitz *et al.,* 1985, 1986). However, proteins involved in more stable complexes are likely to be less accessible to solubilization by mild nonionic detergents. Although this insoluble fraction might represent only a small proportion of the total amount of any given protein, this fraction is likely to be of greater significance to cell adhesion because of its association in the more stable complex. Fluorescent recovery after photobleaching has demonstrated that the fibronectin, integrin, actin, and α-actinin in adhesion plaques have a decreased mobility compared with these molecules outside the adhesion plaque (Schlessinger *et al.,* 1977; Kreis *et al.,* 1982; Stickel and Wang, 1987; Duband *et al.,* 1988b). This is consistent with more stable associations of the molecules within adhesion plaques. In complementary experiments, the nonionic detergent extractability of integrin was reduced by integrin-specific antibodies cross-linked to the cell substrate (Brown and Juliano, 1987).

Biological significance of CHAPS-soluble and -insoluble integrin

There are four arguments that support the biological significance of the CHAPS fractionation. First, the presence of a CHAPS-insoluble pool of integrin in each of the cell types examined suggests that these integrin molecules are involved in higher affinity interactions than integrin molecules in the CHAPS-soluble pool and in distinct intermolecular complexes. Second, the phosphorylation of the integrin molecules in the two fractions is distributed in a highly asymmetric manner, implying that two distinct populations of integrin are identified by this

partitioning. Third, the distribution of integrin and its phosphorylated forms between the fractions is strongly affected by treatments such as CD or transformation, which alter both cell adhesion and integrin distribution. Fourth, CHAPS extraction of four cell types generated either by cell transformation or by CD treatment resulted in distinct integrin staining patterns that reflected their plaque distribution before extraction. These arguments make it likely that the integrin in the CHAPS-insoluble fraction is highly enriched for adhesion plaque-associated integrin.

Role of phosphorylation in assembly and function of integrin

The fractionation of cellular integrin into adhesion plaque-associated and plasma membrane pool provides a new window for investigation of the role of phosphorylation in function of integrin. The β-1 integrin subunit contains tyrosine, serine, and threonine residues in the cytoplasmic domain. Tyrosine 788 occurs in a consensus sequence for phosphorylation by src family tyrosine kinases and can be phosphorylated by v-*src* in vitro (Tamkun *et al.,* 1986; Tapley *et al.,* 1989). Only a single serine residue is present at position 790. The phosphoamino acid analysis shown here, and previously (Hirst *et al.,* 1986), implies that both of these residues can be phosphorylated in both normal and transformed cells. We found no evidence for threonine phosphorylation in chicken embryo fibroblasts. The detergent fractionation studies indicate that integrin molecules in both fractions may be phosphorylated on serine and tyrosine. The phosphorylation of two distinct sites on β-1 integrin suggests that more than one kinase may be involved in the regulation of integrin function.

The pool of β-1 integrin associated with adhesion plaques has a higher specific phosphorylation than the diffuse (non-adhesion plaque) pool. This suggests that the primary site of integrin phosphorylation in normal cells is within the adhesion plaque. This is supported by the staining of adhesion plaques in normal cells by PTYR antibodies (Maher *et al.,* 1985). The transformed cells show an increase in phosphorylation, whereas the CD-treated cells show a decrease in the total level of integrin phosphorylation. These differences may result from the redistribution of the adhesion plaque-associated proteins (David-Pfeuty and Singer, 1980; Tarone *et al.,* 1985; Brett and Godman, 1986), including the kinases responsible for the phosphorylation of integrin. A decrease in integrin phosphorylation has been noted in migratory cells, which, like the CD-treated cells, have fewer actin filaments and lack typical focal contacts (Duband *et al.,* 1988a). The altered phosphorylation of integrin pools in these cells that lack normal focal contacts supports the hypothesis that the kinases involved in the phosphorylation of integrin are associated with the adhesion plaque.

Both RSV and CD reduce cell adhesion (Domnina *et al.,* 1982; Lotz *et al.,* 1989; Parsons and Weber, 1989) and increase the dependence of cell adhesion on integrin (Haimovich *et al.,* as-yet-unpublished data). In contrast with their opposite effects on the total level of integrin phosphorylation, both treatments resulted in a more symmetric partitioning of phosphorylated integrin. This loss of the normal asymmetric distribution of phosphorylated integrin between CHAPS-soluble and -insoluble fractions is an indication of differences in the macromolecular assemblies (or adhesion structures) in which integrin is involved and correlates with the altered morphologies of the integrin structures in the treated cells. These results suggest that multiple mechanisms might exist for regulating the ratio of phosphorylated to nonphosphorylated forms of integrin and hence for control of cell adhesion.

Integrin phosphorylation might have both lateral (packing of integrin molecules within the plaque) and linear (ligand binding at the membrane) consequences on protein-protein interactions and thus differentially affect adhesion. Phosphorylation may alter the binding of integrin to other constituents within the adhesion plaque complex, which could serve either to strengthen or to weaken the complex. Binding assays have been used to demonstrate that hyperphosphorylated CHAPS-soluble integrin isolated from vanadate-treated, RSV-transformed chicken embryo fibroblasts had a lower binding affinity for talin and fibronectin than integrin isolated from normal or src-transformed cells in the absence of vanadate (Tapley *et al.,* 1989). These results suggested that phosphorylation of integrin would decrease its binding to both intracellular and extracellular ligands and thus reduce cell adhesion. However, the adhesion plaque-associated integrin (CHAPS insoluble) in normal cells had a 12-fold higher specific phosphorylation than the diffuse fraction, the opposite of the result that would be predicted by the previous study. Although increased integrin phosphorylation may have a negative effect on its affinity for fibronectin and talin, it might allow for more intimate association with its lateral neighbors, these being either integrin

molecules or other known or yet-unidentified adhesion plaque proteins at the membrane. A stabilization of adhesion plaques by integrin phosphorylation has been proposed by Duband *et al.* (1988a,b) to account for the lack of integrin phosphorylation in motile compared with phosphorylated integrin isolated from stationary cells.

The approach described in this study should provide the means for further examination of the role of integrin phosphorylation into biological processes such as cell migration and differentiation.

Materials and methods

Cell culture and viruses

Primary cultures of chicken embryo fibroblasts were prepared from 11-d-old embryos (SPAFAS, Norwich, CT). Growth media was Dulbecco's modified Eagle's medium (DME) supplemented with 1% fetal bovine serum (FBS), 1% chicken serum, and 10% tryptose phosphate broth with glutamine, vitamins, penicillin, and streptomycin. RSV-transformed cells were obtained by infecting primary chicken embryo fibroblasts with WT Schmidt-Ruppin virus or the mutants CU2 and CU12 (Anderson *et al.*, 1981).

For experiments using CD, the cells were plated in DME plus 1% chicken serum, 1% FBS, 10% tryptose phosphate, and 2 μM CD (Aldrich Chemical, Milwaukee, WI) and incubated for 3 d before analysis.

Labeling and cell extraction

For immunoblots and for immunoprecipitations, cells were incubated for 6 h in methionine-free DME containing 200 μCi/ml [^{35}S]-methionine (Translabel, ICN Biochemicals, Irvine, CA) or in phosphate-free DME containing 1 mCi/ml [^{32}P]-orthophosphate (carrier free, ICN), each supplemented with 2% dialyzed FBS and sodium vanadate at 50 μM (Swarup *et al.*, 1982; Piwnica-Worms *et al.*, 1987). At the end of the labeling period, cultures were rinsed with ice-cold TD (25 mM Tris-HCl pH 7.4, 140 mM NaCl, 5 mM KCl, 0.7 mM Na_2PO_4, and 5 mM glucose) containing 100 μM vanadate and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Cells were quickly scraped on ice and pelleted in a small volume at 4°C. Pellets were immediately frozen in a dry ice bath and stored at −80°C until extracted. All buffers used subsequent to this point included 100 μM sodium vanadate and 0.2 mM PMSF. Cell pellets were extracted in CHAPS immunoprecipitation buffer (10 mM Tris-HCl [pH 7.6], 100 mM NaCl, 2 mM EDTA, 0.5% CHAPS) (Hirst *et al.*, 1986) on ice for 1 h with occasional vortexing. Extracts were cleared by centrifugation. CHAPS-insoluble pellets were reextracted under similar conditions with RIPA buffer and again cleared by centrifugation. Cleared extracts were quantitated for either trichloroacetic acid (TCA)-precipitable radioactivity ([^{35}S]-methionine-labeled cells) or total solubilized protein ([^{32}P]-orthophosphate-labeled cells) using BCA reagents (Pierce Chemical, Rockford, IL) with bovine serum albumin (BSA) as a standard.

Immunoprecipitation

Aliquots of extracts containing either equal amounts of TCA-precipitable cpm or protein were reacted with polyclonal anti-integrin antibody overnight at 4°C. The anti-integrin β-1 antibody was prepared in rabbits immunized with chicken integrin purified on an affinity column using the CSAT monoclonal antibody (Hayashi *et al.*, 1990); anti-α-3 was prepared using a 14-amino-acid peptide from the C-terminus of α-3 (Hynes *et al.*, 1989); anti-α-5 antisera was prepared using a 23-amino-acid peptide from the C-terminus of α-5 (Hynes *et al.*, 1989); LM609 is a monoclonal antibody prepared against human vitronectin receptor (Cheresh and Spiro, 1987). Antigen-antibody complexes were collected by adsorption for 4 h at 4°C onto protein A-sepharose beads (Pharmacia LKB, Piscataway, NJ), which had been preincubated with RIPA-extracted CEF protein and 1% BSA to reduce nonspecific binding. Adsorbed complexes were washed three times each in ice-cold RIPA buffer, high-salt buffer (10 mM Tris-HCl [pH 7.5], 1 M $MgCl_2$), and then in NP-40 buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.5% NP-40). Immunoprecipitates were recovered in non-reducing Laemmli sample buffer (Laemmli, 1970) and then analyzed by nonreducing SDS-PAGE through 7% acrylamide gels. For fluorography, gels were treated with Amplify (Amersham, Arlington Heights, IL). For preferential detection of PTYR-containing proteins, gels were treated with 1 M KOH at 55°C for 2 h (Cooper *et al.*, 1983). [^{32}P]-orthophosphate-labeled proteins were detected by autoradiography and the use of an intensifying screen. All film exposures were performed at −80°C. Multiple scans of the same lane were done with the use of a soft laser LKB densitometer (LKB Instruments, Gaithersburg, MD) and computer analyzed using the Hoefer GS365 electrophoresis data reduction system program (Hoefer Scientific Instruments, San Francisco, CA).

Immunoblots

Twenty micrograms protein from each fraction were analyzed by nonreducing SDS-PAGE through 7% acrylamide gels. The proteins were electrophoretically transferred to nitrocellulose and visualized by the use of ^{125}I-donkey anti-rabbit Ig (1 μCi/ml) (Amersham) and autoradiography.

Phosphoamino acid analysis

Phosphoamino acid analysis was performed essentially as described by Cooper *et al.* (1983). [^{32}P]-labeled proteins were located on the dried gel, excised, rehydrated in water, and then crushed into extraction buffer containing 50 mM ammonium bicarbonate, 0.1% SDS and 1% 2-mercaptoethanol followed by incubation with rocking overnight at 37°C. Extracted proteins were TCA precipitated with 25 μg Pentex BSA (ICN) and resulting pellets washed with ethanol-ether (1:1, vol/vol) at −20°C. Pellets were lyophilized and subjected to acid hydrolysis with 6 N HCl (Pierce Chemical) at 110°C for 90 min, lyophilized, and then subjected to separation by two-dimensional electrophoresis at pH 3.5 and then pH 1.9 on thin-layer cellulose plates. Phosphoamino acid standards (Sigma, St. Louis, MO) were visualized by ninhydrin staining (Sigma), and autoradiography was performed as described above.

Immunofluorescence

Cells were grown on coverslips. Cultures were fixed at room temperature for 20 min with 2% paraformaldehyde, rinsed several times with 0.15 M glycine in phosphate-buffered saline (PBS) to block reactive groups, permeabilized for 5 min with 1% NP-40 in PBS, and incubated for 1 h in PBS with 5% goat serum (Cappel, Malvern, PA) to reduce nonspecific binding. For CHAPS extraction, cells were extracted with 200 μl CHAPS buffer for 3 min before fixation with paraformaldehyde. Polyclonal antibodies to chicken integrin were generously provided by A.F. Horwitz (University of Illinois) (Hayashi *et al.*, 1990). After 1 h incubation with the

primary antibodies, the cells were rinsed for 20 min in PBS and then incubated with fluorescein-conjugated goat anti-rabbit IgG (Cappel). Cells were rinsed overnight in PBS and mounted in elvanol.

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