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Subcellular Distribution of *ras* in Human and Murine Fibroblasts

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Ras proteins play a significant role in signal transduction in response to growth factors and in cell transformation. To be active, *ras* has to be translocated to the cell membrane. Since subcellular distribution has been mainly studied in vector-transformed cells which highly express *ras* proteins, and it has been difficult to detect *ras* in cells expressing the protein at physiological levels, we studied subcellular distribution in human and murine fibroblasts. Here we show for the first time that a significant amount of *ras* is associated with the membrane skeleton and the cytoskeleton.

The three cellular *ras* genes (H-, N-, and K-*ras*) encode related 21-kDa guanine nucleotide-binding proteins (1). *Ras* normally functions to relay mitogenic and developmental signals initiated by cell-surface receptors into the cytoplasm and nucleus (2). Beside this physiological function of normal *ras* which is still not fully understood, *ras*, in combination with an additional oncogene, can malignantly transform cells, and activating mutations of *ras* are found in nearly one-third of all human cancers (3). To exert its function, *ras* has to be located to the inner surface of the plasma membrane (4) which requires a complex series of posttranslational modifications. The *ras* protein is synthesized in the cytosol as an inactive precursor (pro-*ras*) and converted into an intermediate form (c-*ras*) by removal of the three C-terminal amino acids and subsequent carboxy-methylation resulting in increased hydrophobicity. In a next step, which includes farnesylation at Cys186 and palmitoylation (Cys180, 181, or 184), c-*ras* is converted into the mature, membrane-bound form of m-*ras* (5,6,7). Farnesylation and membrane-association has been studied extensively by using cell systems which express high levels of *ras* proteins either by an inducible promoter (5,6) or by transient expression in COS-1 cells (7). In non-*ras* transfected cells, detection of *ras* proteins has remained difficult (8). We therefore studied subcellular distribution of *ras* in normal and transformed human and murine lung fibroblasts. We here show for the first time, that *ras* is not only bound to the plasma membrane, but is associated in proliferating cells with the membrane- and cytoskeleton in significant amounts.

MATERIAL AND METHODS

Cell culture. FH109 human embryonal lung fibroblasts (9), C3H10T1/2 cells, SV40-transformed WI38 cells (WI38SV40), and methyl-cholanthrene transformed C3H10T1/2 cells (C3H10T1/2 $_{trans.}$, (10)) were cultured for three days in 6-well plates in DMEM (PAA) supplemented with 10 % FCS (Gibco).

Triton X-114 separation (three phase system). Cell proteins were fractionated according to (11). Briefly, cells were trypsinized, pelleted, and washed with phosphate-buffered saline. The sample was diluted in 200 μl of ice-cold Hepes-buffer (10 mM Hepes/NaOH pH 7.4, protease inhibitors as mentioned) and Triton X-114 was added to a final concentration of 2 %. Immediately, the sample was vortexed and placed on ice for 5 min after which the sample was centrifuged at 8,800 *g* for 10 min at 4°C. The detergent insoluble protein pellet (DIP) was solubilized in 200 μl of SDS-sample buffer (12) and the supernatant was layered on top of 300 μl of a sucrose cushion (10 mM Tris /HCl pH 7.4, 150 mM NaCl, 6 % (w/v) sucrose, 0.06 % (v/v) Triton X-114). After incubation for 3 min at 30°C, followed

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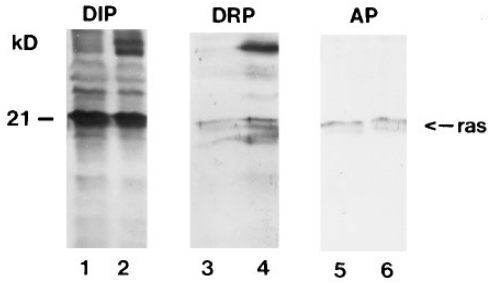

FIG. 1. Western blot analysis of *ras* after differential extraction and temperature-induced three phase separation. WI38SV40 (lanes 1,3, and 5) and C3H10T1/2 $_{trans.}$(lanes 2,4, and 6) cells were solubilized with Triton X-114 and the fractions separated into a detergent-insoluble phase (DIP), a detergent-rich phase (DRP), and an aqueous phase (AP). The lanes were loaded with 5 μg of protein. The position of the molecular size marker in kilodaltons is shown on the left.

by centrifugation at 3,000 *g* for 3 min, the upper aqueous phase was transferred to a clean cap and Triton X-114 was added to a final concentration of 0.5 %. After mixing and incubation on ice for 5 min, this phase was overlayed on the same sucrose cushion, incubated for 3 min at 30°C and then centrifuged at 3,000 *g* for 3 min. The resulting upper aqueous phase was again transferred to a clean cap and fresh Triton X-114 was added to a final concentration of 2 %. After incubation on ice for 5 min and at 30°C for 3 min, the sample was centrifuged at 3,000 *g* for 3 min. Proteins of this aqueous phase (AP) were precipitated according to (13) and solubilized in SDS-sample buffer. The sucrose cushion was then aspirated from the remaining detergent-rich phase (DRP) and proteins were precipitated and solubilized as described. Protein determination of each fraction was performed according to (14).

Subcellular fractionation. Cells were lysed at 4°C for 15 min in 1,000 μl of 2 % Triton X-100 in TBS-buffer (100 mM Tris/HCl pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.3 μM aprotinin, 0.1 μM soybean trypsin inhibitor, 60 μM phenylmethylsulfonyl fluoride, 0.4 mM iodoacetamid, 5 μM pepstatin, 4 μM leupeptin) and harvested with a rubber policeman. The cytoskeletal fraction was separated by centrifugation at 10,000 *g* for 15 min at 4°C and solubilized in 200 μl of SDS-sample buffer (12). The supernatant was centrifuged at 100,000 *g* for 2 h at 4°C and the pelleted membranes solubilized in 100 μl of SDS-sample buffer. Proteins of the supernatant were precipitated (13) and solubilized in SDS-sample buffer.

Western blotting. Proteins were separated by SDS-polyacrylamide gel electrophoresis (15%), transferred overnight to Immobilon membrane (Millipore) and blocked for 1 h with 5 % FCS in TBS (50 mM Tris / HCl pH 7.4, 150 mM NaCl, 0.05 % Tween 20). For immunodetection, membranes were incubated for 3 h with *anti*-ras antibodies (1:1000, Paesel & Lorey) in blocking buffer. After three short and three 5-min washing steps with TBS, the membranes were incubated for 1.5 h with biotin-streptavidin conjugated *anti*-sheep antibodies (1:1000, Dianova) in blocking buffer and again washed as described, followed by incubation with streptavidin-biotinylated horseradish peroxidase complex (1: 100, Amersham) for 20 min. The membranes were washed with three short and three 5-min washing steps and once with phosphate-buffered saline. The blots were developed using 4-chloro-1-naphthol (Sigma) and 3,3′-diaminobenzidine (Sigma).

RESULTS AND DISCUSSION

Subcellular distribution of *ras* was examined by using two different fractionation methods with the non-ionic detergents Triton X-100 and Triton X-114 in non-transformed human and murine fibroblasts (FH109 and C3H10T1/2), in virally transformed human fibroblasts (WI38SV40), and in chemically transformed murine fibroblasts (C3H10T1/2 $_{trans.}$).

We first used differential solubilization and temperature-induced three-phase-separation with Triton X-114 according to Hooper and Bashir (11) in transformed fibroblasts: Hydrophilic proteins are found in the aqueous phase whereas transmembrane polypeptide anchored proteins are recovered in the detergent-rich phase; proteins tightly bound to cytoskeletal structures remain in the detergent-insoluble pellet.

The membrane-bound form of *ras*, i.e. the farnesylated and palmitoylated form, is detergent-sensitive and can be extracted by Triton X-100 and Triton X-114 (6,15). In accordance, we could partly solubilize *ras* with Triton X-114 (Fig.1, lanes 3 and 4). A small amount of *ras*

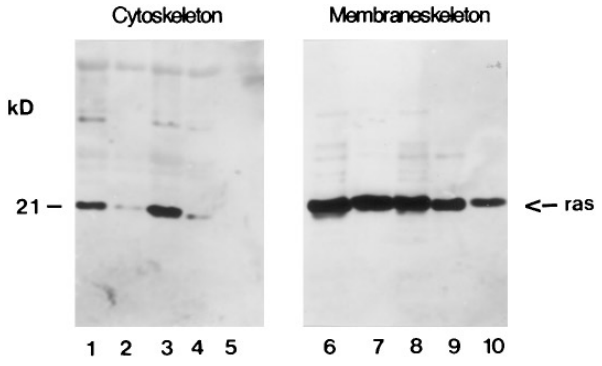

FIG. 2. Western blot analysis of *ras* after subcellular fractionation. C3H10T1/2 $_{trans.}$ (lanes 1, and 6), C3H10T1/2 (lanes 2, and 7), WI38SV40 cells (lanes 3, and 8), proliferating (lanes 4, and 9), and contact-inhibited (lanes 5, and 10) FH109 cells were solubilized with Triton X-100 and separated into a cytoskeletal (10 μg protein / lane), a membraneskeletal (3 μg protein / lane), and a detergent-soluble fraction (data not shown). The position of the molecular size marker in kilodaltons is shown on the left.

was found in the aqueous phase (lanes 5 and 6), which represents the cytosolic form of *ras.* Unexpectedly, a significant amount of *ras* was discovered in the detergent-insoluble pellet which gave a hint that *ras* might be associated with cytoskeletal structures. We therefore used next differential extraction with Triton X-100 as described in the method section in order to separate the cytoskeletal, the membrane-skeletal and the detergent-sensitive / cytosolic fractions.

Fig. 2 shows that a significant amount of *ras* in transformed (lanes 6 and 8) and non-transformed proliferating (lanes 7 and 9) and quiescent cells (lane 10) is Triton X-100-insensitive and is discovered in the membrane skeletal fraction. Only a small amount can be solubilized with Triton X-100 (data not shown). Interestingly, we found that, in transformed fibroblasts, a high amount is also bound to the cytoskeleton (Fig. 2, lanes 1 and 3). In proliferating, non-transformed fibroblasts (lanes 2 and 4), a small amount is bound to the cytoskeleton whereas in contact-inhibited, quiescent fibroblasts (lane 5), we failed to detect *ras* in the cytoskeletal fraction.

CONCLUSIONS

In our study, we revealed for the first time that a significant amount of *ras* is bound to cytoskeletal and membrane skeletal structures. Since we only detected cytoskeletal-associated *ras* in proliferating cells and not in contact-inhibited, quiescent cells, we suggest that the cytoskeletal fraction represents newly synthesized mature *ras* and that the cytoskeleton might be important for the translocation of *ras* from the endoplasmatic reticulum to the membrane hence serving as a transport system.

Although there is no doubt about the transforming properties of *ras* and its important role in cancer (3), the biological function of *ras* is still not fully understood. *Ras* proteins have been described to play an important role in the fast response of growth factor receptor activation, but the long term responses are still unclear (2). The fact that *ras* was predominantly detected in the detergent-resistant membrane skeletal fraction points out that association with additional subcellular structures might be important for *ras* activation and signal transduction. Possibly, efficient signal transduction requires concentration of *ras* and its effector proteins on specific locations. For instance, such a positive relationship between transformation capacity and cytoskeleton association has been demonstrated for $p60^{v\text{-}src}$ (16) and Bcr-Abl oncogene (17).

Accordingly, $p60^{c\text{-}src}$ becomes activated and associated with the cytoskeleton in response to growth factor stimulation (18).

Vice versa, growth factors are known to induce drastic reorganisation in cytoskeletal architecture (19, 20). The *ras*-related protein *rho,* for instance, regulates the assembly of focal adhesions and actin stress fibers in response to growth factors such as platelet-derived growth factor (20), and the *ras*-related *rac* is important for growth-factor induced membrane ruffling (21). The results of our study demonstrating that a significant amount of *ras* is associated with the membrane skeleton, might give a hint that *ras* itself also plays a role in regulating cytoskeletal architecture.

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