

On the self-Affinity of Heparan Sulfates from Quiescent or Proliferating Normal 3T3 Cells and from SV40-Transformed Cells

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³⁵S-Labelled heparan sulfates derived from the culture medium (extracellular), a trypsinate of the cells (pericellular) and the cell residue (intracellular) of quiescent normal, proliferating normal or SV40-transformed 3T3 cells were analyzed for charge heterogeneity, by ion exchange chromatography and for self-affinity, by chromatography on heparan sulfate-agarose gels. Quiescent normal cells retained most of their heparan sulphate intra- or pericellularly. The surface-exposed material was charge heterogeneous and had a strong affinity for heparan sulfate. In cultures of growing cells and transformed cells most of the heparan sulfate was found in the medium. The heparan sulfate retained on the surface or growing cells had a lower self-affinity than did the corresponding material from normal and transformed cells. Although cell surface heparan sulfates from transformed cells showed affinity for a matrix substituted with the total heparan sulfate pool, the affinity for one particular subtype was much less pronounced or non-existent.

Heparan sulfate, in proteoglycan form, is a common cell surface constituent of many or all eukaryotic cell types (see, *e.g.* Refs. 1–7). Heparan sulfate is released from the cell surface during the premitotic (G₂) stage of the cell cycle.⁸ Accordingly, cell cultures in the growth-phase or transformed cells in culture contain less trypsin releasable heparan sulfate than do normal quiescent cells (see Refs. 9–11). In addition, qualitative changes have been noted. Heparan sulfates from transformed cells or tumour cells have a reduced degree of ester-sulfation^{11–15} and heparan sulfate from transformed cells have altered self-affinity.¹¹ In the latter study it was observed that various sulfate subfractions from transformed cells had no affinity for agarose gels substituted with the corresponding heparan sulfate subspecies. When a broader distribution of heparan sulfates was used in similar experiments¹⁶ it was noted that a crossreaction with non-cognate heparan sulfate could take place. This was more pronounced with heparan sulfates from transformed cells than with heparan sulfates from growing normal cells. The present study was carried out in an attempt to better understand the growth- and/or transformation-dependent changes in the structure and properties of heparan sulfate.

EXPERIMENTAL

Materials. Heparan sulfate was purified from bovine lung as described extensively elsewhere.¹⁷⁻¹⁹ The total pool was subfractionated according to charge density by step-wise precipitation with cetylpyridinium chloride in the presence of decreasing concentrations of NaCl. The following fractions were obtained: HS1 (0.2–0.4 M-NaCl), HS2 (0.4–0.6 M), HS3 (0.6–0.8 M), HS4 (0.8–1.0 M), HS5 (1.0–1.2 M) and HS6 (complexes not soluble in 1.2 M-NaCl). The total pool (HS) as well as the subfraction HS2–HS4 were separated into more or less association-prone variants by gel chromatography²⁰ giving rise to preparations designated HS-A, HS2-A, HS3-A and HS4-A.

Other sources of materials were as follows. Glycosaminoglycan standards from the the NIH collection; Heparin (pig mucosa), Glaxo, Runcorn, U.K.; Chondroitinase-ABC, Miles, Elkhart, U.S.A.; Insta-gel, Packard; Sepharose and Sephadex gels, Pharmacia; microgranular DEAE-cellulose (DE 52), Whatman; other chemicals were of analytical grade. Na₂³⁵SO₄ (carrier-free) was from The Radiochemical Centre.

Radiolabelled glycosaminoglycans. ³⁵S-labelled glycosaminoglycans were obtained from a Balb-3T3 cell line and an SV40-transformed 3T3 cell line essentially as described previously.^{10,16,21} The non-transformed cells were given the isotope both at the time of plating and after reaching confluency. In the former case cells were exposed to radiosulfate until confluent (usually 2–3 d). In the latter case incorporation of ³⁵SO₄²⁻ was carried out for 48 h without changing the medium. Transformed cells were given the isotope after 2–3 d in culture. After these periods of time all pools of heparan sulfate should have been equilibrated in terms of radiosulfate. In all cases, medium and cells were collected separately. The cells were digested briefly with trypsin and material released by this treatment (pericellular material) as well as the remaining material (intracellular) was recovered. In one case, cells were treated with heparin (500 µg/ml of growth medium for 3h) to yield heparin-removable material. The various fractions were processed as follows. Glycosaminoglycans were released as free chains by digestion with pronase in 50 mM Tris-HCl, pH 7.4/10 mM Na₂EDTA/10 mM β-mercaptoethanol at 37 °C (3 additions of 0.1 mg/ml of enzyme during 36 h). Non-digested proteins and the pronase were then precipitated by the addition of 3 vols. of saturated (NH₄)₂SO₄-solution, pH 10. The mixture was incubated at 65–70 °C for 2 h to allow protein coagulation. After centrifugation the supernatants were dialyzed against 10 mM NH₄OH, pH 11 followed by 1 mM NH₄OH and distilled water (3 changes), and finally freeze-dried. The yields of ³⁵S-glycosaminoglycans as 10⁻⁶ × ³⁵S-radioactivity (d.p.m.)/2 × 10⁷ cells were as follows (for the intra-, peri- and extracellular sources, respectively): normal quiescent cells, 2.23, 1.31, and 0.26; for normal proliferating cells, 1.87, 1.46, and 4.66; and for SV40-transformed cells, 5.23, 3.11, and 9.94. Heparin-treatment of normal quiescent cells released approx. 40 % of the total pericellular pool (trypsin-removable) of ³⁵S-glycosaminoglycans.

Preparation of ³⁵S-heparan sulfate. The ³⁵S-glycosaminoglycan samples were dissolved in 1 ml 0.5 M Tris-acetate, pH 8.0, containing 0.5 mg of carrier heparan sulfate (HS-A) and contaminating galactosaminoglycans were digested with 50 mU of chondroitinase-ABC at 37 °C overnight. The heparan sulfates were isolated by gel chromatography on Sephadex G-50 (void volume fraction), recovered, freeze-dried and dissolved in 0.5 ml 0.15 M NaCl. The purity was checked by deaminative cleavage using the pH 1.5 HNO₂ method.²²

Affinity matrices. The procedure for immobilising heparan sulfates on Sepharose 4B was outlined previously.²⁰ In brief, the agarose gel is activated with CNBr and adipic acid dihydrazide is introduced as a spacer group. Heparan sulfate preparations (HS-A, HS2-A, HS3-A or HS4-A) are oxidised briefly (15 min) with periodate (5–10 % destruction of GlcA) at 4 °C, dialysed, recovered and mixed with the gel. The aldimins formed between the –NH–NH₂ group of the spacers and the dialdehyde structures of the partially oxidised heparan sulfates are finally stabilized by reduction with BH₄⁻. The amount of immobilized chains was 3–4 mg/ml gel.

Chromatographic methods. Affinity chromatography of [³⁵S]heparan sulfate was conducted on agarose gels substituted with various heparan sulfate species. The columns (6 × 140 mm) were equilibrated with 0.15 M NaCl and samples were applied (containing 1 mg of carrier/ml) in the same solvent. Elution was performed with a linear gradient of 0.15 M NaCl–1.5 M guanidinium chloride (total volume, 100 ml) at a rate of 3 ml/h. The shape of

the gradient was checked by conductivity measurements. The effluent was collected in 1.2–1.3 ml fractions with the aid of an LKB Redirac equipped with a drop counter and analysed for radioactivity in a Packard 2650 liquid scintillation counter with automatic quench correction using Instagel (0.5 ml of sample and 5 ml of liquid) as scintillator.

Ion exchange chromatography was carried out on columns (6×140 mm) of DE-52 DEAE cellulose that were equilibrated with 0.1 M sodium acetate, pH 5.0. Elution was performed with a linear gradient of 0.1–2.5 M-sodium acetate, pH 5.0 as described above.

Gel chromatography was performed on columns of Sephadex G-50, Superfine, which were eluted with 0.5 M NH_4HCO_3 at a rate of 6 ml/h. The effluents were analyzed as described above. For further details, see legends to appropriate Figures.

RESULTS

Sulfated glycosaminoglycans in quiescent, proliferating or transformed cells. The total pools of [^{35}S]glycosaminoglycans were digested with chondroitinase-ABC followed by gel chromatography on Sephadex G-50 to separate heparan sulfate (void volume fractions) from degradation products of galactosaminoglycans (dermatan sulfate and chondroitin sulfate). The heparan sulfate-containing fractions were all susceptible to HNO_2 (results not shown). The quiescent normal 3T3 cells contained relatively large amounts of heparan sulfate in the intra- and pericellular pools, whereas the medium had almost equal proportions of galactosaminoglycans and heparan sulfate (results not shown). Heparin treatment of the cells exclusively displaced heparan sulfate (40 % of total cell-surface heparan sulfate). Growing 3T3 cells contained less heparan sulfate (relative to galactosaminoglycans) in all cell fractions. Similar results were obtained with SV40-transformed cells, except that the pericellular pool contained very little galactosaminoglycan.

The heterogeneity in charge-density of the [^{35}S]heparan sulfates isolated from the different sources was investigated by ion exchange chromatography. In quiescent normal cells, the heparan sulfate of the pericellular pool was heterogeneous and included HS2-, HS3- and HS4-like components in almost equal proportions (Fig. 1a). In the medium of the same cells the more highly charged components (HS3 and HS4) were more prominent (Fig. 1b). In the trypsinase of proliferating normal cells the distribution of heparan sulfate species was very similar to that seen in the medium of quiescent cells (*cf.* Figs. 1b and 1c). Growing cells extruded into the medium a relatively homogeneous HS3-like heparan sulfate (Fig. 1d). Transformed cells retained in the pericellular pool a population of chains that resembled those of normal cells (*cf.* Figs. 1a and 1e). The heparan sulfate from the medium of transformed cells contained both an HS3-like component, as in the case of proliferating normal cells (*cf.* Figs. 1d and 1f), and a more highly charged HS4-like component, as in the case of quiescent normal cells (*cf.* Figs. 1b and 1f).

Self-affinity of heparan sulfates in quiescent, proliferating or transformed cells. The heparan sulfate chain-chain association was assayed by affinity chromatography experiments. In the first series of experiments the various [^{35}S]heparan sulfate preparations were tested against an affinity gel that contained, as immobilised ligands, the total population of association-prone heparan sulfates (HS-A) from bovine lung. The heparan sulfate from the pericellular pool of quiescent normal cells showed considerable affinity for the heparan sulfate-agarose (Fig. 2a). The heparin-removable chains showed essentially the same degree of binding as did the heparin-non-removable chains (Fig. 2b). Both the intracellular pool and the medium of quiescent cells contained heparan sulfate with poor affinity for this matrix (Figs. 2c and 2d). In the case of proliferating normal cells (Figs. 2e–g) a considerable proportion of all the heparan sulfate chains was not bound to the matrix. In particular,

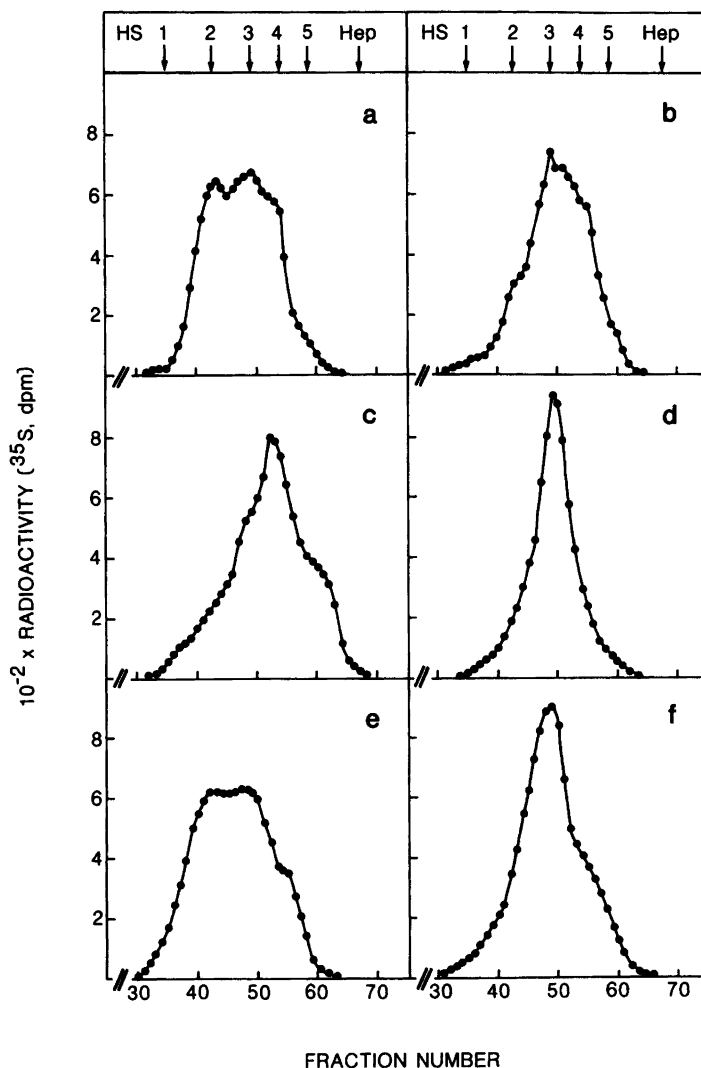


Fig. 1. Ion exchange chromatography of [^{35}S]heparan sulfates from the trypsinates (a,c,e) and the media (b,d,f) of quiescent normal (a–b), proliferating normal (c–d) and SV40-transformed 3T3 cells (e–f). Column: DE52 DEAE-cellulose (6 \times 140 mm) eluted with a linear acetate gradient. The elution positions of the heparan sulfate standards HS1, HS2, HS3, HS4 and HS5 as well as heparin (Hep) are shown in the upper panel.

heparan sulfate associated with the cell surface of growing cells interacted poorly (Fig. 2e) compared with corresponding material from quiescent normal cells (Fig. 2a). The heparan sulfate from the pericellular pool of transformed cells had an affinity for HS–A-agarose that was similar to that of corresponding material from quiescent normal cells (*cf.* Figs. 2a and 2h). The intracellular pool of transformed cells also contained heparan sulfates with affinity for this matrix, albeit of variable strength (Fig. 2i). This pattern was different from that seen in the case of quiescent (Fig. 2c) or in the case of proliferating (Fig. 2f) normal cells. The

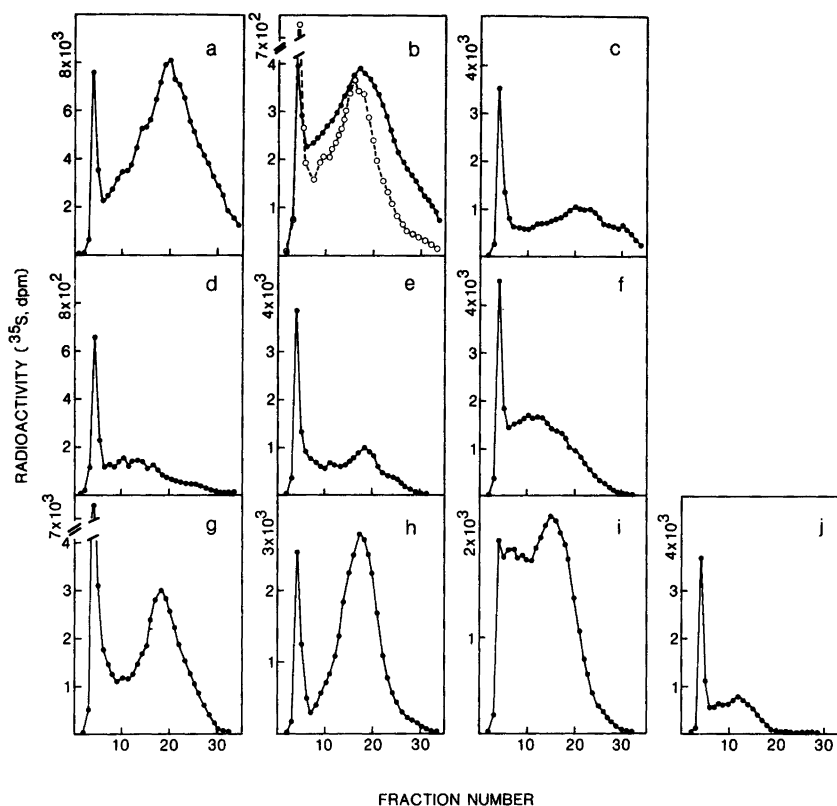


Fig. 2. Affinity chromatography on HS-A-agarose of [^{35}S]heparan sulfates from (a) the trypsinase of quiescent normal 3T3 cells, (b) a heparin-removable (\bullet) and a heparin-non-removable (\circ) population of heparan sulfates from quiescent cells, (c) the cell fraction of the same cells, (d) the medium of the same cells, (e) the trypsinase of proliferating normal 3T3 cells, (f) the cell fraction of the same cells, (g) the medium of the same cells, (h) the trypsinase of SV40-transformed 3T3 cells, (i) the cell fraction of the same cells and (j) the medium of the same cells. Column size: 6×140 mm; elution, linear guanidine gradient.

culture medium of the various cells generally contained heparan sulfates with little affinity (Figs. 2d, 2g and 2j). However, normal proliferating cells (Fig. 2g) appeared to secrete a component of high affinity.

In the second series of experiments, the various [^{35}S]heparan sulfates were chromatographed on affinity columns substituted with either of the three different subtypes of heparan sulfate, *i.e.* HS2-A, HS3-A or HS4-A. Results obtained after affinity chromatography on HS2-A-agarose are shown in Fig. 3. A portion of the [^{35}S]heparan sulfate from the pericellular pool of quiescent normal cells had affinity for this matrix (Fig. 3a). However, the proportion that was bound was much less than in experiments using the matrix substituted with the total heparan sulfate pool (*cf.* Figs. 2a and 3a). The heparin-removable heparan sulfate (approx. 40 % of the total) showed both a higher degree of binding and a higher strength of binding to HS2-A-agarose than did the non-removable material (Figs. 3b and 3c). The heparan sulfates from the pericellular pools of proliferating normal and transformed cells showed little, if any, affinity for this matrix (Figs. 3d and 3e). When similar

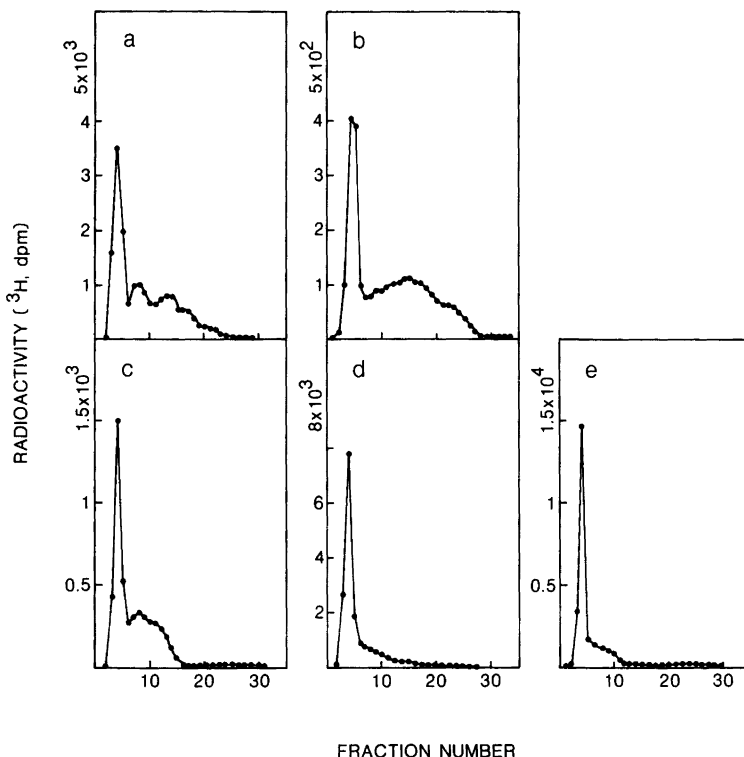


Fig. 3. Affinity chromatography on HS2-A-agarose of [^{35}S]heparan sulfate from (a) the trypsinates of quiescent normal 3T3 cells, (b) a heparin-removable and (c) a heparin-non-removable population of heparan sulfates from quiescent cells, as well as the trypsinates of (d) proliferating normal and (e) SV40-transformed cells. The column was eluted as in Fig. 2.

experiments were carried out on HS3-A- or HS4-A-agaroses, no binding was observed in any case (results not shown).

DISCUSSION

Previous studies from this laboratory have shown that certain heparan sulfate chains are able to self-associate.^{17,18,23,24} Variants that form aggregates in solution (as determined by gel filtration or light scattering) also bind to affinity matrices substituted with heparan sulfate. Results have been obtained which suggest that the interaction may be specific, in as much as certain subtypes preferentially bind to gels substituted with the same or cognate chains. Heparan sulfates from transformed cells show considerable charge heterogeneity and are unable to bind to affinity matrices substituted with anyone of the various subtypes of heparan sulfate.^{11,15}

The results of the present work confirm and extend previous observations. The heparan sulfates were isolated after 48 h or more of radiosulfate administration. Therefore, the materials analyzed are representative of the steady-state situation in each case. When the ability to aggregate was assayed using an affinity matrix containing the total pool of beef

lung heparan sulfates, certain cell-surface heparan sulfates were strongly association-prone. These included heparan sulfate of quiescent normal 3T3 cells and transformed cells (Figs. 1a and 2h). These heparan sulfates were also heterogeneous (Figs. 1a and 1e). When normal cells were in the proliferating state they were shedding a large part of their heparan sulfate into the medium. The latter chains represented a relatively homogeneous population (Fig. 1d) a portion of which showed strong aggregation (Fig. 2g). The heparan sulfate remaining on the surface of growing cells was less heterogeneous (Fig. 1c) and of lower aggregation (Fig. 2e). As shown by Kraemer and Tobey (Ref. 8), cells may release a large part of their glycocalyx (including proteoglycan sulfate) into the medium before entering mitosis. The results obtained in the present study suggest that a certain subtype of heparan sulfate is preferentially produced and shed during the growth-phase. Even in the quiescent state cell surface heparan sulfate has a rapid turn-over rate; values between 5 and 8 h have been reported.²⁶⁻²⁸ Therefore, cell surface heparan sulfate is probably continually degraded. We propose that normal fibroblasts in the quiescent state produce association-prone heparan sulfate species that are retained in the pericellular region. During growth the heparan sulfates produced appear less prone to associate.

Transformed cells at high density release a large part of their heparan sulfate into the medium like normal growing cells. However, the heparan sulfate remaining on the surface of transformed cells was similar to corresponding material in normal confluent cells both with regard to heterogeneity (Figs. 1a and 1e) and tendency to aggregate (Figs. 2a and 2h). Further experiments showed that heparan sulfate from transformed cells was only bound to the total pool of heparan sulfate in affinity chromatography experiments (Fig. 2h) but not to any of the subtypes that are part of this pool (see Fig. 3e and Ref. 11). Hence, previous and present findings show that cell-surface associated heparan sulfates from primary cultures of fibroblasts are able to bind to a high degree to a particular subtype of heparan sulfate,^{11,29} corresponding heparan sulfate from confluent cultures of an established cell-line (3T3) bind to a smaller extent (Figs. 3a-c) and heparan sulfates from growing or transformed 3T3-cells do not bind at all (Figs. 3d and 3e). One possible explanation of the latter finding is that heparan sulfate of transformed cells interacts partially with all subtypes of bovine lung heparan sulfate but not in a sufficiently strong way to be retained on an affinity matrix substituted with only one particular subtype. Co-operative interaction with many forms of heparan sulfate could provide sufficient strength of binding. Growing normal 3T3-cells and transformed ones differ in one respect. Heparan sulfate from the surface of the former cells is considerably less prone to associate (Fig. 2e) than is the corresponding material from transformed ones (Fig. 2). The latter cells also differ from quiescent normal cells by retaining large proportions of associative heparan sulfates intracellularly (cf. Figs. 2c and 2i). The biological significance of these findings is difficult to ascertain but may be related to the postulated growth-control functions for cell surface heparan sulfate.

The heparan sulfate of the pericellular pool of 3T3 cells was partly liberated by treatment with heparin. The heparin-removable material had a higher affinity for heparan sulfate-agarose than did the remaining material. This is consistent with the observation that heparin efficiently displaces [3H]heparan sulfate from a cognate heparan sulfate-affinity matrix.²⁴

Acknowledgements. This work was supported by grants from the Swedish Medical Research Council (567), *Kocks Stiftelser*, *Gustaf V:s 80-års fond*, *Alfred Österlunds Stiftelse*, the Medical Faculty, University of Lund and the University of Florence.

REFERENCES

1. Kraemer, P.M. *Biochemistry* 10 (1971) 1437.
2. Augusti-Tocco, G. and Chiarugi, V.P. *Cell Differ.* 5 (1976) 161.
3. Dietrich, C.P., Sampaio, L.O., Toledo, O.M.S. and Cassaro, C.M.E. *Biochem. Biophys. Res. Commun.* 75 (1977) 329.
4. Oldberg, Å., Höök, M., Öbrink, B., Pertoft, H. and Rubin, K. *Biochem. J.* 164 (1977) 75.
5. Sjöberg, I. and Fransson, L.-Å. *Biochem. J.* 167 (1977) 383.
6. Glimelius, B., Norling, B., Westermark, B. and Wasteson, Å. *Biochem. J.* 172 (1978) 443.
7. Gill, P.J., Adler, J., Silbert, C.K. and Silbert, J.E. *Biochem. J.* 194 (1981) 299.
8. Kraemer, P.M. and Tobey, R.A. *J. Cell. Biol.* 55 (1972) 713.
9. Nicolson, G.L. *Biochim. Biophys. Acta* 458 (1976) 1.
10. Vannucchi, S. and Chiarugi, V.P. *J. Cell Physiol.* 90 (1977) 503.
11. Fransson, L.-Å., Sjöberg, I. and Chiarugi, V.P. *J. Biol. Chem.* 256 (1981) 13044.
12. Keller, K.L., Keller, J.M. and Moy, J.M. *Biochemistry* 19 (1980) 2529.
13. Chiarugi, V.P., Cappelletti, R., Vannucchi, S., Fibbi, G., Pasquali, F., del Rosso, M. and Urbano, P. *Caryologia* 33 (1980) 441.
14. Nakamura, N. and Kojima, J. *Cancer Res.* 41 (1981) 278.
15. Winterbourne, D.J. and Mora, P.T. *J. Biol. Chem.* 256 (1981) 4310.
16. Del Rosso, M., Cappelletti, R. and Pasquali, F. *Ital. J. Biochem.* 31 (1982) 227.
17. Fransson, L.-Å., Nieduszynski, I.A. and Sheehan, J.K. *Biochim. Biophys. Acta* 630 (1980) 287.
18. Fransson, L.-Å., Havsmark, B., Nieduszynski, I.A. and Huckerby, T.N. *Biochim. Biophys. Acta* 633 (1980) 95.
19. Fransson, L.-Å., Sjöberg, I. and Havsmark, B. *Eur. J. Biochem.* 106 (1980) 59.
20. Fransson, L.-Å., Havsmark, B. and Sheehan, J.K. *J. Biol. Chem.* 256 (1981) 13039.
21. DelRosso, M., Cappelletti R., Vannucchi, S., Romagnani, S. and Chiarugi, V.P. *Biochim. Biophys. Acta* 586 (1979) 512.
22. Shively, J.E. and Conrad, H.E. *Biochemistry* 15 (1976) 3932.
23. Fransson, L.-Å. *Eur. J. Biochem.* 120 (1981) 251.
24. Fransson, L.-Å. and Havsmark, B. *Carbohydr. Res.* 110 (1982) 135.
25. Fransson, L.-Å., Havsmark, B. and Chiarugi, V.P. *Biochem. J.* 201 (1982) 233.
26. Sjöberg, I., Carlstedt, I., Cöster, L., Malmström, A. and Fransson, L.-Å. *Biochem. J.* 178 (1979) 257.
27. Vogel, K.G. and Kendall, V.F. *J. Cell. Physiol.* 103 (1980) 475.
28. Dietrich, C.P., Armelin, H.A., Nogueira, Y.L., Nader, H.B. and Michelacci, Y.M. *Biochim. Biophys. Acta* 717 (1982) 387.
29. Fransson, L.-Å., Carlstedt, I., Cöster, L. and Malmström, A. *J. Biol. Chem.* 258 (1983) 14342.

Received May 17, 1984.