Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 166-173

IDENTIFICATION OF THE MAJOR PHOSPHOPROTEIN SECRETED BY MANY RODENT CELL LINES AS 2AR/OSTEOPONTIN: ENHANCED EXPRESSION IN H-RAS-TRANSFORMED 3T3 CELLS

Ann Marie Craig*, Mohamed Nemir¶, Barid B. Mukherjee¶, Ann F. Chambers§, and David T. Denhardt*

*Cancer Research Laboratory and Department of Biochemistry, University of Western Ontario, London, Ontario N6A 5B7, Canada

¶Department of Biology and Centre for Human Genetics, McGill University, Montreal, Quebec H3A 1B1, Canada

§London Regional Cancer Centre, and Department of Oncology, University of Western Ontario, London, Ontario N6A 4G5, Canada

Received October 6, 1988

2ar, a tumor promoter-inducible protein secreted by mouse JB6 epidermal cells, is the murine homolog of rat osteopontin, or 44 kD bone phosphoprotein. We report here that 2ar is also related to pp69, a major phosphoprotein secreted by normal rat kidney cells. Antisera raised against pp69 and against ß-galactosidase-2ar fusion proteins are able to immunoprecipitate the same major phosphoproteins, of apparent Mr 55-69 kD, secreted by several rat and mouse cell lines. The levels of secreted protein and cytoplasmic mRNA are dramatically elevated in NIH 3T3 cells transformed with the human bladder cancer T24 (H-ras) oncogene. These results and the work of Senger and colleagues (Cancer Res., **45**, 5818-5823, 1985) imply that enhanced secretion of 2ar/pp69/osteopontin by transformation of a wide variety of mammalian fibroblasts and epithelial cells is often correlated with tumorigenicity.

Genes induced by tumor promoters and growth factors are of interest both as models for studying the regulation of gene expression and as genes contributing to cellular proliferation and tumorigenesis (for reviews, see 1,2,3). One gene of this type, provisionally called 2ar, was cloned on the basis of its induction by 12-O-tetradecanoylphorbol-13-acetate (TPA) in JB6 mouse epidermal cells (4). Induction appears to be mediated by protein kinase C (Smith and Denhardt, submitted). We have shown (2; Craig et al., submitted) that mouse 2ar is homologous to rat osteopontin, a secreted, highly acidic, glycosylated 44 kD bone phosphoprotein (5,6). Although the physiological role of this protein is not known, it has a functional RGDS cell binding site (5,7), binds strongly to hydroxyapatite (bone matrix), and is present in

Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate; nt, nucleotide.

0006-291X/88 $1.50

developing bone as well as in kidney, neurons and neuroepithelial cells in the inner ear, and granulated metrial cells in the deciduum and placenta (8,9,10).

We were intrigued by the observation that 2ar is the major phosphoprotein secreted by JB6 cells. Protein phosphorylation and dephosphorylation is a well-known mechanism of regulating cellular functions, including the response to growth factors and transforming agents (reviewed in 11), but there are very few phosphoproteins that act extracellularly. These include fibronectin, which is synthesized in lesser amounts but in a more highly phosphorylated form by transformed cells than by untransformed cells (12), and pp69, the major phosphoprotein secreted by normal and transformed rat kidney cells (13,14), which is apparently the same as the "transformation-associated phosphoprotein" of Mr 62,000 secreted by a number of tumorigenic mammalian cells (15,16). We show here that 2ar and pp69 are immunologically related, and that transformation of NIH 3T3 cells with H-ras results in a large increase in 2ar/pp69 protein and mRNA expression.

MATERIALS AND METHODS

Cell Culture- The rat cell lines used in this work were NRK (normal rat kidney cells; 17), the fibroblastic subclone NRK-49F (18), and several transformed NRK derivatives: KNRK (19), transformed with Kirsten MSV; B77-NRK (20), transformed with B77-C Bratislava strain of RSV; and LA23-NRK (20), transformed with LA23, a class T ts mutant of Prague strain RSV, subgroup A. Mouse cell lines used were: NIH 3T3 (21); PAP2 (22), derived from pooled clones of NIH 3T3 cells transformed with the human bladder cancer (T24) H-ras oncogene; and KA31 (19), a Kirsten MSV-transformed derivative of Balb/c 3T3 cells. All cells were grown at 37°C in Dulbecco modified Eagles medium (DMEM) supplemented with 10% calf or fetal bovine serum (Gibco). Where indicated, NRK and LA23-NRK cells were grown at 36°C or 39°C for 36-48 h prior to and during metabolic radiolabeling and RNA isolation.

Metabolic Radiolabeling- Subconfluent, actively growing cells were labeled 24-48 h after passaging. For labeling of phosphoproteins, cells were washed twice and starved for 0.5 h in serum- and phosphate-free DMEM (Flow Laboratories), followed by incubation for 4 h in fresh serum-free medium containing 400 μCi/ml of carrier-free [^{32}P]orthophosphate (Amersham). Cells were labeled for 4 h with [^{35}S]methionine (300 μCi/ml of Tran^{35}S-label; ICN Radiochemicals) in methionine-free DMEM (Gibco) containing 3% dialyzed serum, after a 1 h starvation for methionine and serum. Conditioned media were adjusted to 5 mM phenylmethylsulfonylfluoride and centrifuged at 2,500g for 5 min then 10,000g for 10 min to remove detached cells and cell debris.

Immunoprecipitation, Gel Electrophoresis and Autoradiography- Portions of conditioned media containing equal amounts of trichloroacetic acid-precipitable radioactivity were incubated with either anti-pp69 serum (14) or anti-Cro-ß-galactosidase-2ar serum (Craig et al., submitted). The resulting immune complexes were collected with an excess of protein A-Sepharose (Pharmacia) and washed as reported previously (14,23). For immunoprecipitation of [^{35}S]-methionine-labeled proteins, an initial pre-adsorption step with pre-immune serum and protein A-Sepharose was included. Proteins were analyzed on 10 or 12.5% polyacrylamide gels containing SDS using the Laemmli buffer system (24). Molecular weight markers used were phosphorylase B (92,500), bovine serum albumin (69,000), ovalbumin (46,000) and carbonic anhydrase (30,000) ([methyl-

^{14}C] methylated; New England Nuclear). Gels containing ^{35}S were impregnated with 2,5-diphenyloxazole according to Bonner and Laskey (25) and fluorographed at -70°C using pre-flashed Kodak XAR film.

RNA Isolation and Northern Blot Analysis- Cytoplasmic RNA was prepared from subconfluent, actively growing cells as described (26). RNA (10 µg/lane) was electrophoresed on 1.1% agarose gels containing formaldehyde (27) and blotted onto either nitrocellulose (Bio-Rad) or GeneScreen Plus (New England Nuclear). Probes were prepared to ≈10^8 cpm/µg by nick translation with [^{32}P]dNTPs (ICN Radiochemicals; 27). The specific clones used were: the 2ar cDNA (4), a plasmid carrying the non-muscle ß-actin cDNA (28; obtained from Margaret Buckingham), and a BamHI-EcoRI fragment of Harvey murine sarcoma virus containing the v-H-ras oncogene (ATCC#41013). The protocols for hybridization and autoradiography have been described (23,26,27). Hybridization was quantified by scanning autoradiograms with an LKB UltroScan XL densitometer.

RESULTS

Immunological Relationship of 2ar and pp69- Conditioned media containing [^{32}P]orthophosphate-labeled proteins from late passage rat NRK-49F cells, KNRK cells, and mouse KA31 cells were immunoprecipitated with antiserum raised against gel-purified pp69 (14) or antiserum raised against a Cro-ß-galactosidase-2ar fusion protein (Craig et al., submitted). As shown in Fig. 1, the anti-pp69 and anti-2ar sera recognized the same species secreted by every one of these cell lines. A second precipitation of the supernatant from the first immunoprecipitation with both antisera ruled out the possibility of two unrelated phosphoproteins with the same electrophoretic mobility (not shown). Normal rabbit serum, pre-immune serum, and anti-Cro-ß-galactosidase antiserum were all ineffective in precipitating this protein. For reasons we do not understand, under only certain culture conditions (compare Fig.1 and Fig.2B) NRK cells and derivatives secreted a second major phosphoprotein, pp62, which is related to pp69 both immunologically (as shown here) and by peptide mapping (13,14). The anti-Cro-ß-galactosidase-2ar antiserum used here was raised against a bacterial fusion protein containing the predicted 2ar C-terminal 80 amino acids. Antiserum against a similar fusion protein containing a non-overlapping 142 amino acid internal sequence in the 2ar coding region also recognized pp69 secreted by NRK-49F cells (not shown). Recognition of pp69 by anti-2ar antisera is specific, as it was the only protein immunoprecipitated from [^{35}S]methionine-labeled conditioned media (see Fig. 2).

The anti-pp69 and anti-2ar sera recognized the secreted phosphoprotein from rat cells with approximately equal efficiency. However, anti-pp69(rat) antiserum was less efficient at precipitating the protein secreted from mouse KA31 cells (Fig. 1), Swiss 3T3 fibroblasts and JB6 Cl22 epidermal cells (not shown). Furthermore, the anti-pp69 serum did not recognize the Cro-ß-galactosidase-2ar fusion proteins by Western blot analysis. These data suggest that the anti-pp69 serum mainly recognizes epitopes containing post-translational

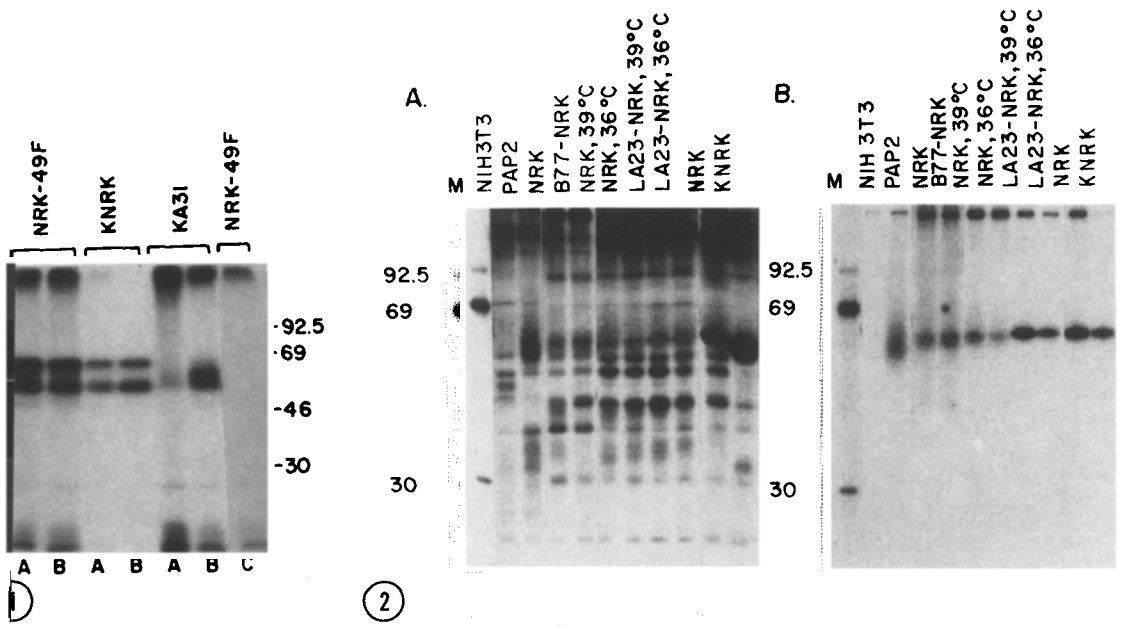

Fig. 1. **Immunoprecipitation of [^{32}P]proteins from conditioned media with anti-pp69 or anti-2ar sera.** Media from the indicated cell lines labeled with [^{32}P]orthophosphate at high passage number were immunoprecipitated with the following antisera: **A:** anti-pp69; **B:** anti-Cro-ß-galactosidase-2ar; **C:** normal rabbit serum. The precipitate was subjected to SDS-PAGE and autoradiography. We have retained the designation pp69 for the higher Mr species, and pp62 for the lower Mr species, secreted by NRK-49F cells (13).

Fig. 2. **Comparative analysis of 2ar protein secretion by transformed and untransformed cell lines.** Proteins secreted by the indicated cell lines were metabolically labeled with [^{35}S]methionine. **A. Total Protein:** Portions of conditioned media containing equal amounts of trichloroacetic acid-precipitable radioactivity were electrophoresed on SDS-polyacrylamide gels and fluorographed. **B. Immunoprecipitated Protein:** Portions of media normalized to total incorporation were immunoprecipitated with anti-Cro-ß-galactosidase-2ar antiserum prior to SDS-PAGE and fluorography. The protein immunoprecipitated from NRK cells and derivatives corresponds to pp69 (the higher Mr species in Fig.1); this was determined by electrophoresis of samples shown in Fig.1 and Fig.2 on a single gel. The mobility of osteopontin/2ar is known to depend on electrophoretic conditions (6).

modifications on pp69, such as glycosyl and phosphoryl groups, and that these modifications are different in the homologous proteins secreted by mouse cells (and presumably absent in the Cro-ß-galactosidase-2ar fusion proteins isolated from E. coli). The anti-2ar serum was thus used for the following studies.

Effect of Transformation on 2ar/pp69 Expression- We determined the level of expression of 2ar/pp69 in four pairs of transformed and parental untransformed cell lines. Fig. 2A shows total [^{35}S]methionine-labeled secreted proteins, while Fig. 2B shows immunoprecipitates made with the anti-2ar serum. PAP2 cells, derived from pooled clones of NIH 3T3 cells transformed with the human bladder cancer (T24) H-ras oncogene (22), secreted more 2ar than the parental NIH 3T3 cells; densitometric analysis indicated a greater than 10-fold difference in the level of labeled immunoprecipitated protein. In

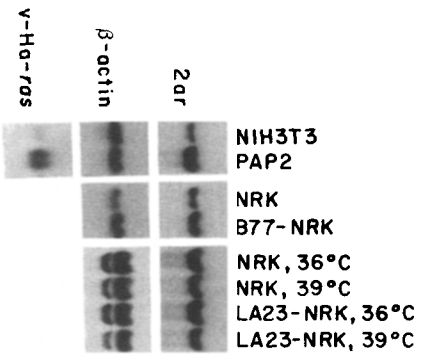

Fig. 3. **Northern blot analysis of 2ar mRNA levels in transformed and untransformed cell lines.** Cytoplasmic RNA was electrophoresed (10 μg/lane), blotted and probed successively with ^{32}P-labeled 2ar, ß-actin and,where shown, viral H-ras. The results of two separate experiments are shown. In the blot hybridized to the ß-actin probe, the lower Mr species in the four right lanes is a residual signal from the 2ar probe. The sizes of the messages determined by reference to [^{32}P]lambda HindIII-EcoRI markers were ≈1600 nt for 2ar, ≈2200 nt for ß-actin, and ≈1300 nt for ras.

contrast, NRK cells transformed with viral K-ras (KNRK) or viral src (B77-NRK) showed no obvious change in the level of expression of 2ar. This result was confirmed with LA23-NRK, a cell line that expresses a ts src oncogene and thus is transformed at 36°C but not at 39°C (20).

To determine at what level the induction of 2ar is occurring, cytoplasmic RNA was isolated from subconfluent cells and subjected to Northern blot analysis (Fig. 3). A non-muscle ß-actin probe was used to control for loading differences. Densitometry of the hybridization signal obtained with the 2ar cDNA probe normalized to the actin signal showed no difference in 2ar mRNA steady state level due to transformation of NRK cells with viral src. This was the case both for B77-NRK versus NRK, and for LA23-NRK at 36°C versus 39°C, in agreement with the protein data. In PAP2 versus NIH 3T3 cells, there was a ≈3.5-fold increase in H-ras mRNA and a ≈3-fold increase in 2ar mRNA.

DISCUSSION

An immunological relationship has been demonstrated here between 2ar/ osteopontin, a tumor promoter-inducible protein secreted by mouse epidermal and rat osteosarcoma cells, and pp69, a major phosphoprotein secreted by normal rat kidney cells. We have only detected one hybridizing mRNA species in mouse and rat cells (as shown here). We have also demonstrated that 2ar is encoded by a unique single-copy gene (Craig et al., submitted). The simplest interpretation of these results is that pp69 and osteopontin are encoded by a single rat gene and mRNA, and 2ar by the mouse homolog. In accordance with this interpretation, it is most likely that the various forms of this protein

(2ar/pp69/osteopontin) differ only in post-translational modifications and, of course, species differences. Osteopontin isolated from rat bone was reported to contain 16.6% carbohydrate, likely present as 1 N-linked and 5-6 O-linked oligosaccharides, and about 12 phosphoserine and 1 phosphothreonine residues per molecule (6). Differences in glycosylation and/or phosphorylation could account for the variation in electrophoretic mobility of the protein secreted by different cell lines, and even the apparent microheterogeneity within a single cell line (PAP2).

The difference in level of 2ar secretion by various cell lines may reflect their tissue of origin. Expression is high in normal rat kidney and ROS 17/2.8 rat osteosarcoma cells (29) but barely detectable in mouse NIH 3T3 fibroblasts and JB6 Cl22 epidermal cells not exposed to TPA (Craig et al., submitted). This is consistent with the high level *in vivo* expression of 2ar in kidney and bone but not epidermis.

2ar/pp69/osteopontin is regulated in various cell lines by TPA and other tumor promoters, serum, epidermal growth factor, platelet derived growth factor, basic fibroblast growth factor, embryonal carcinoma-derived growth factor, transforming growth factor-ß, 1,25-dihydroxyvitamin D_3, dexamethasone, sodium butyrate, and parathyroid hormone (4,10,14,29,30; Hofbauer, personal communication; Noda and Rodan, personal communication). The induction of 2ar by tumor promoters and growth factors suggested that its regulation may be under the control of specific oncogenes. As shown here, increased expression of H-*ras* in NIH 3T3 cells did enhance 2ar expression. Chemical transformation of mouse Balb/c 3T3 fibroblasts, which poorly express 2ar, with 9,10-dimethyl-1,2-benzanthracene also resulted in a large increase in 2ar expression (Parfett, personal communication). Transformation of NRK cells with viral *src* or K-*ras* did not affect the level of expression of 2ar, which was already quite high, perhaps due to kidney-specific factors, as suggested above. The H-*ras* transformation-induced increase in steady-state 2ar mRNA may result from changes in transcription, processing, transport and/or stability of the message. Increased transcription is likely involved, since the 2ar promoter region contains a consensus recognition sequence for AP-1/c-Jun, which has been shown to mediate transcriptional activation due to elevated levels of the H-*ras* oncogene (Craig, unpublished).

These results prompt speculation that a high level of 2ar may actively contribute to tumorigenesis, particularly in a cell type in which it is not normally expressed. This speculation is strengthened by the work of Senger and colleagues (15,16,31,32). They have described a "transformation-specific phosphoprotein" which is secreted by a wide variety of rodent and human tumor cells of fibroblastic or epithelial origin at levels at least 10-fold greater than the corresponding nontumorigenic cells. This protein has an apparent Mr of 58,000-69,000, is the major phosphoprotein secreted by these cells, and

displays microheterogeneity in the mouse cell lines. This description could be applied to the results we report here for the PAP2 and NIH 3T3 cells. In fact, for all of the cell lines analyzed for 2ar/pp69/osteopontin secretion, this protein is the major secreted phosphoprotein. One of the cell lines reported to synthesize this transformation-specific phosphoprotein is SR Rat, which is RR1022 (an epithelial-like RSV-transformed rat cell line) passaged three times in vivo, and the major phosphoprotein secreted by RR1022 is recognized by anti-pp69 antiserum (13,14). We conclude, therefore, that this transformation-specific phosphoprotein is 2ar/pp69/osteopontin.

NOTE- After submission of this manuscript, the sequence of the N-terminal nine amino acids of the rat "transformation-specific phosphoprotein" was published [Senger, D.R., Perruzzi, C.A., Gracey, C.F., Papadopoulos, A., and Tenen, D.G. (1988) Cancer Res. **48**, 5770-5774]; this sequence is identical to the predicted N-terminus of mature rat osteopontin, corresponding to residues 17 to 25 of the unprocessed polypeptide deduced from the cDNA sequence (5).

ACKNOWLEDGEMENTS- This research was supported by the National Cancer Institute and the Medical Research Council of Canada. A.M.C. was a recipient of an MRC studentship and M.N. was a recipient of an Algerian M.E.S. scholarship. We thank B. Orphan for artistic contributions, M. McLeod and S. Wilson for excellent technical assistance, and J. Kowalski and R. Hofbauer for critically reviewing the manuscript.

REFERENCES

1. Denhardt, D.T., Edwards, D.R., and Parfett, C.L.J. (1986) Biochim. Biophys. Acta **865**, 83-125.
2. Denhardt, D.T., Craig, A.M., and Smith, J.H. (1988) In Genes and Signal Transduction in Multistage Carcinogenesis, (N. Colburn, ed.) pp.167-189, Alan R. Liss, New York.
3. Chambers, A.F., and Tuck, A.B. (1988) Anticancer Res., in press.
4. Smith, J.H., and Denhardt, D.T. (1987) J. Cell. Biochem. **34**, 13-22.
5. Oldberg, A., Franzén, A., and Heinegård, D. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8819-8823.
6. Prince, C.W., Oosawa, T., Butler, W.T., Tomana, M., Bhown, A.S., Bhown, M., and Schrohenloher, R.E. (1987) J. Biol. Chem. **262**, 2900-2907.
7. Somerman, M.J., Prince, C.W., Sauk, J.J., Foster, R.A., and Butler, W.T. (1987) J. Bone Min. Res. **2**, 259-265.
8. Mark, M.P., Prince, C.W., Oosawa, T., Gay, S., Bronckers, A.L.J.J., and Butler, W.T. (1987) J. Histochem. Cytochem. **35**, 707-715.
9. Mark, M.P., Prince, C.W., Gay, S., Austin, R.L., and Butler, W.T. (1988) Cell Tissue Res. **251**, 23-30.
10. Nomura, S., Wills, A.J., Edwards, D.R., Heath, J.K., and Hogan, B.L.M. (1988) J. Cell Biol. **106**, 441-450.
11. Krebs, E.G. (1986) The Enzymes **17**, 3-18.
12. Ali, I.U., and Hunter, T. (1981) J. Biol. Chem. **256**, 7671-7677.
13. Chackalaparampil, I., Banerjee, D., Poirier, Y., and Mukherjee, B.B. (1985) J. Virol. **53**, 841-850.
14. Laverdure, G.R., Banerjee, D., Chackalaparampil, I., and Mukherjee, B.B. (1987) FEBS Lett. **222**, 261-265.

15. Senger, D.R., Wirth, D.F., and Hynes, R.O. (1979) Cell **16**, 885-893.
16. Senger, D.R., and Perruzzi, C.A. (1985) Cancer Res. **45**, 5818-5823.
17. Duc-Nguyen, H., Rosenblum, E.N., and Zeigel, R.F. (1966) J. Bacteriol. **92**, 1133-1140.
18. DeLarco, J.E., and Todaro, G.J. (1978) J. Cell. Physiol. **94**, 335-342.
19. Aaronson, S.A., and Weaver, C.A. (1971) J. Gen. Virol. **13**, 245-252.
20. Chen, Y.C., Hayman, M.J., and Vogt, P.K. (1977) Cell **11**, 513-521.
21. Jainchill, J.L., Aaronson, S.A., and Todaro, G.J. (1969) J. Virol. **4**, 549-553.
22. Hill, S.A., Wilson, S., and Chambers, A.F. (1988) J. Natl. Cancer Inst. **80**, 484-490.
23. Denhardt, D.T., Greenberg, A.H., Egan, S.E., Hamilton, R.T., and Wright, J.A. (1987) Oncogene **2**, 55-59.
24. Laemmli, U.K. (1970) Nature **227**, 680-685.
25. Bonner, W.M., and Laskey, R.A. (1974) Eur. J. Biochem. **46**, 83-88.
26. Edwards, D.R., and Denhardt, D.T. (1985) Exp. Cell. Res. **157**, 127-143.
27. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
28. Minty, A.J., Alonso, S., Guénet, J.L., and Buckingham, M.E. (1983) J. Mol. Biol. **167**, 77-101.
29. Prince, C.W., and Butler, W.T. (1987) Collagen Rel. Res. **7**, 305-313.
30. Yoon, K., Buenaga, R., and Rodan, G.A. (1987) Biochem. Biophys. Res. Comm. **148**, 1129-1136.
31. Senger, D.R., Wirth, D.F., and Hynes, R.O. (1980) Nature **286**, 619-621.
32. Senger, D.R., Asch, B.B., Smith, B.D., Peruzzi, C.A., and Dvorak, H.F. (1983) Nature **302**, 714-715.