BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **228,** 115–121 (1996)
ARTICLE NO. 1625

Detection of a Secreted MUC1/SEC Protein by MUC1 Isoform Specific Monoclonal Antibodies

Nechama Smorodinsky, Mordechai Weiss, Mor-Li Hartmann, Amos Baruch, Ella Harness, Margalit Yaakobovitz, Iafa Keydar, and Daniel H. Wreschner[1]

Department of Cell Research and Immunology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel

Received September 5, 1996

Although MUC1 proteins are known to be secreted by breast cancer cells, the mechanism of their release from the cell is still obscure. Our previously reported MUC1 cDNA sequences suggested the existence of a secreted MUC1 isoform, MUC1/SEC, that includes a sequence of intron 2, and terminates prematurely at a stop codon within this intron. It is thus devoid of a transmembrane domain. As no formal evidence for MUC1/SEC expression at the protein level had been provided, we generated monoclonal antibodies (mAbs) against a peptide sequence (sec peptide) that is unique for the MUC1/SEC protein. Two anti-sec peptide mAbs were obtained which reacted strongly with (a) the immunizing peptide, (b) recombinant MUC1/SEC protein, and (c) MUC1 proteins secreted from breast cancer cells. The immunoreactivity of the anti-sec peptide mAbs with MUC1 proteins secreted by breast cancer cells was specifically inhibited by the sec peptide—it was completely unaffected by a peptide sequence that represents a MUC1 repeat motif. Significantly, the anti-sec peptide mAbs also detected MUC1/SEC protein in sera of breast cancer patients. We have established here that these mAbs recognize the MUC1/SEC isoform via a peptide sequence which is unique for the MUC1/SEC protein. Our studies thus demonstrate that the MUC1/SEC protein is a bona-fide MUC1 isoform and that its expression may contribute to the secretion of MUC1 proteins by secretory epithelial cells in general and breast cancer cells in particular.

The *MUC1* gene, is expressed by secretory epithelial cells and at especially high levels in human breast cancer tissue (1-4)- it has therefore generated much interest. Although the MUC1 proteins are readily detected in human body fluids (5-7), the mechanisms whereby they are secreted from the cell remain uncharacterized. A molecular analysis of the mRNAs coding for MUC1 proteins (previously referred to as episialin, H23Ag, ETA-epithelial tumor antigen, PEM-polymorphic epithelial mucin, EMA-epithelial membrane antigen, CA15-3, MCA-mammary carcinoma antigen etc.) revealed the existence of several different MUC1 isoforms (8-11). A major MUC1 gene product (designated (MUC1/REP/TM) is a polymorphic type 1 transmembrane protein that consists of a large heavily glycosylated extracellular domain, a transmembrane domain and a 72 amino acid (aa) cytoplasmic tail (Fig. 1). The polymorphism derives principally from variations in the numbers of a 20 aa tandem repeat unit present in the extracellular domain.

We previously reported a second MUC1 cDNA sequence, designated MUC1/SEC, which downstream to the tandem repeat array is, in contrast to MUC1/REP/TM, colinear with the genomic sequence (9). The predicted MUC1/SEC protein thereby formed (Fig. 1) does not contain a transmembrane domain and is expected to be secreted from the cell. However, formal evidence demonstrating the actual expression of the MUC1/SEC protein was lacking. We report here the generation of mAbs specific for a peptide sequence that is unique for the MUC1/SEC isoform and which recognize both bacterial and animal recombinant MUC1/SEC

[1] To whom correspondence should be addressed. Fax: 972-3-6422046.

0006-291X/96 $18.00

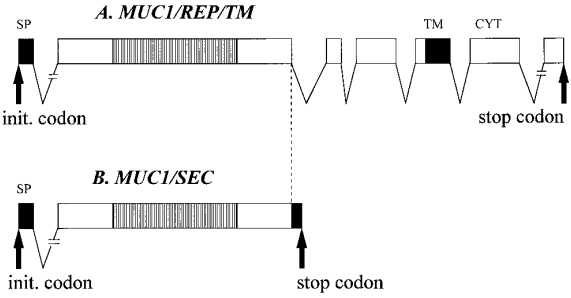

FIG. 1. Scheme of the MUC1/REP/TM and MUC1/SEC isoforms. MUC1 cDNA forms. The cDNAs are depicted from their 5′ termini at the left of the figure. The tandem repeat array is depicted by the barred region- the regions coding for the signal peptide, transmembrane domain and cytoplasmic domain are indicated by SP, TM and CYT respectively and have the same shadings in the various forms.

protein. Using these MUC1/SEC specific mAbs, we unequivocally demonstrate that the MUC1/SEC protein is indeed expressed by breast cancer cells grown in-vitro, and that it is also present in the sera of breast cancer patients.

MATERIALS AND METHODS

Monoclonal antibodies. The anti-MUC1/SEC mAbs 7H10/5 and 7H8/2 were generated by immunizing mice with the MUC1/SEC specific peptide ''VSIGLSFMLP''. The culture supernatants of the resulting hybridomas were screened in parallel by solid phase ELISA using 96 well plates that had been coated either with the immunizing peptide or with a partially purified preparation of MUC1 proteins secreted from T47D breast cancer cells (see below). The mAbs H23 (12) and 1D1 were prepared by immunizing mice with proteins secreted from T47D breast cancer cells. These mAbs recognize a peptide epitope which resides within the 20 amino acid tandem repeat motif of the MUC1 proteins.

Antigen preparation. A partially purified sample of MUC1 proteins secreted by T47D breast cancer cells was prepared by growing T47D cells in suspension culture on a rotary shaker set at 100 rotations/minute. The breast cancer cells were grown in serum-free DMEM supplemented with progesterone and estrogen. Conditioned media (CM/T47D) was collected daily for 5 consecutive days, spun at 2,000 rpm and the secreted proteins present in the supernatant precipitated with 40% (final concentration) ammonium sulfate. The precipitated proteins were collected by centrifugation and resuspended in a minimum volume of PBS followed by extensive dialysis against PBS. Aliquots of the T47D secreted proteins were stored at −20°C.

Generation of recombinant bacterial MUC1/SEC protein. Recombinant bacterial MUC1/SEC protein was prepared by inserting the MUC1/SEC cDNA into the pUC12N vector a derivative of pUC12 which contains an *Nco*1 site in the *lacZ* initiator ATG. A MUC1/SEC cDNA fragment extending from the *Ban1* site at nucleotide #67 till the *Ban1* site at nucleotide #1414 (nucleotide numbering as in the MUC1/SEC sequence appearing in 8) was obtained following a partial *Ban1* digest of the full length cDNA coding for MUC1/SEC. This fragment was Klenow ''filled-in'' and ligated to the expression vector pUC12N, previously digested with *EcoR1* and then Klenow ''filled-in''. The recombinant plasmid obtained retains the first 5 amino acids of the *lacZ* gene (Met Val Thr Asn Cys) followed, in the correct reading frame, by the 5th amino acid (Thr) of the MUC1/SEC protein. This plasmid was introduced into competent DH5 cells which were then screened by DNA/DNA hybridization for the presence of the tandem repeat array. Positive colonies were selected and further analyzed for expression of the MUC1/SEC protein by screening with the H23 mAb which recognizes an epitope contained within the tandem repeat array.

RESULTS

mAbs generated against the sec peptide. To specifically test for expression of the secreted MUC1/SEC isoform (Fig. 1), mAbs were generated against a dodecamer peptide which is unique for the MUC1/SEC isoform (sec peptide ''VSIGLSFPMLP''). The mAbs obtained were screened, in parallel, for immunoreactivity with (a) the sec peptide and (b) proteins secreted by human breast cancer cells grown in-vitro. This screening procedure resulted in a number of mAbs that reacted well with the immobilized sec peptide. A subset of these mAbs displayed immunoreactivity both with MUC1 proteins secreted from breast cancer cells and with the sec peptide. Two

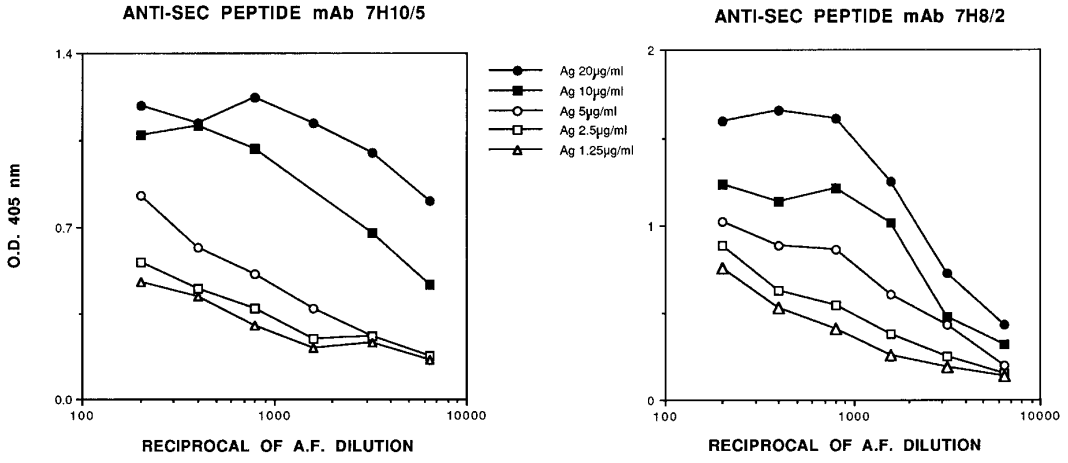

FIG. 2. Titration of immunoreactivity of anti-sec-peptide mAbs with MUC1 proteins secreted from T47D breast cancer cells. A partially purified preparation of MUC1 proteins secreted into serum-free media by T47D breast cancer cells was coated onto 96 well microtiter plates- antigen concentrations ranged from 20 μg/ml down to 1.5 μg/ml. Double dilutions of the anti-sec peptide mAbs 7H10/5 and 7H8/2 were then added to the wells, incubated overnight followed by extensive washing with PBS and addition of alkaline phosphatase conjugated anti-mouse antibodies. Substrate was added to the plates followed by a reading of optical density at 405nm.

of these mAbs (7H8/2 and 7H10/5) revealed a high and reproducible immunoreactivity and were chosen for further study. A titration of their immunoreactivity towards proteins secreted from the T47D breast cancer cells showed that the 7H8/2 and 7H10/5 mAbs retained considerable activity even following a several thousand fold dilution (Fig. 2).

Recognition of recombinant MUC1/SEC protein by the anti-MUC1/SEC antibodies. To verify that the anti-sec peptide mAbs are indeed recognizing the MUC1/SEC protein, we tested their reactivity against recombinant bacterial MUC1/SEC protein. In this ''sandwich'' ELISA assay, the capture antibody immobilized on resin beads was a mAb, 1D5, that recognizes the tandem repeat array motif- the detecting antibody was either the anti-sec peptide mAb 7H10/5 conjugated to alkaline phosphatase or the anti-repeat array H23 mAbs, similarly conjugated. The recombinant bacterial MUC1/SEC protein was readily detected by the anti-sec mAbs which demonstrated an even higher sensitivity than the anti-repeat (H23) mAbs (rows 2 and 3, Table 1). Supernatants from control bacteria transformed with a non-relevant plasmid revealed only background levels (row 1, Table 1). Using this ELISA assay, the anti-sec peptide mAbs also detected MUC1/SEC protein present in the conditioned medium of T47D breast cancer cells (row 4, Table 1). Similarly recombinant MUC1/SEC protein produced by MUC1/SEC cDNA transfected rat fibroblasts and present in the conditioned media of these cells was also detected using the anti-sec peptide mAbs- no immunoreactive protein could be detected in the conditioned media of control rat fibroblast transfectants (data not shown).

Specificity of anti-sec mAbs for the MUC1/SEC isoform. Preincubation of the mAbs 7H8/2 and 7H10/5 with the sec peptide strongly inhibited their binding to immobilized secreted T47D proteins- this binding was not at all inhibited by RPM-6, the tandem repeat array peptide (Fig. 3). Conversely, preincubation of mAb H23, that recognizes an epitope contained within the tandem repeat array, with RPM-6 severely compromised its immunoreactivity- this reactivity was completely unaffected by the sec peptide (Fig. 3). These experiments thus established anti-MUC1/SEC mAb specificity towards a protein that contains the sec peptide sequence and is secreted by breast cancer cells.

Detection of MUC1/SEC protein in human body fluids. We thus demonstrated: (a) that

TABLE 1
Recognition of Recombinant MUC1/SEC Protein by the Anti-sec-Peptide 7H10/5 mAbs

	Sample assayed	Detecting antibody: 7H10/5-AP [anti-sec peptide mAbs]	Detecting antibody: H23-AP [anti-repeat mAbs]
1	supernatant of control bacteria	0	0.049
2	supernatant of MUC1/SEC bacteria	1.394	0.949
3	supernatant of MUC1/SEC bacteria-diluted 1:10	1.130	0.342
4	concentrated media from T47D cells	1.376	1.912

A mAb (1D1, N.S. unpublished) that recognizes an epitope contained within the MUC1 tandem repeats was covalently bound to Eupergit beads as previously described. Culture supernatants (200 μl) from control pSV2neo transformed bacteria (row 1), MUC1/SEC expressing bacteria transformed with the pUC12N//MUC1/SEC plasmid (rows 2 and 3), and concentrated serum-free conditioned media from T47D breast cancer cells (row 4), were incubated with the anti-repeat mAb Eupergit beads for 2 hr. at room temperature followed by 4 washes of the beads with PBS + 0.1% gelatin. The bound MUC1/SEC protein was detected either with alkaline phosphatase conjugated anti-sec-peptide mAbs (7H10/5-AP) or AP conjugate anti-repeat motif mAbs (H23-AP) and, following addition of the substrate, the plates were read at 405 nm.

the anti-sec peptide mAbs recognize proteins secreted from breast cancer cells; (b) that this immunoreactivity can be specifically inhibited by preincubating the anti-sec peptide mAbs with the MUC1/isoform specific peptide and (c) that the anti-sec peptide mAbs recognize recombinant MUC1/SEC protein produced both by bacteria and animal cells. We next investigated whether the anti-sec peptide mAbs could be used to detect the MUC1/SEC isoform in serum samples obtained from breast cancer patients. Two variant ''sandwich'' ELISA assays

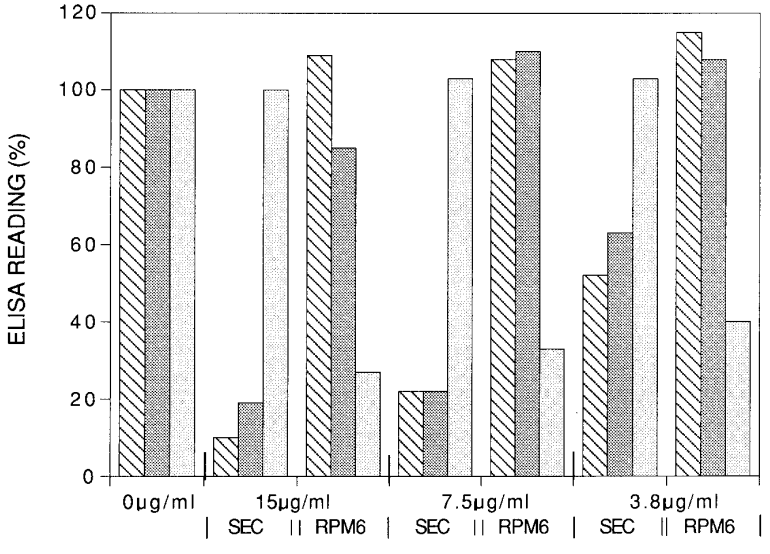

FIG. 3. Inhibition by sec peptide [''VSIGLSFPMLP''] of anti-sec-peptide mAb binding to secreted MUC1/SEC proteins. 96 well plates were coated with a partially purified preparation of MUC1 proteins secreted from T47D breast cancer cells. The anti-sec-peptide mAbs 7H10/5 and 7H8/2 (cross hatched and heavily stippled columns respectively) as well as the anti-repeat domain H23 mAbs (lightly stippled column) were mixed with the indicated concentrations of either the sec peptide (SEC) or the repeat peptide (RPM6) and incubated with the antigen coated wells for 60 min. Bound mAbs were detected as in the legend to Fig. 2.

TABLE 2
Detection of MUC1/SEC Protein in Human Sera

coating with mAb	7H10/5	H23	7H10/5	H23
reciprocal dilution of coating mAb	serum B165 (diluted 1:10)		serum B337 (diluted 1:10)	
100	1.130	0.959	0.102	.027
200	1.287	0.759	0.097	.027
400	1.171	0.434	0.079	.037
800	1.066	0.219	0.068	.034
1600	0.983	0.129	0.059	.035
3200	0.943	0.073	0.044	.036
6400	0.731	0.037	0.035	.036

96 well plates were coated with reciprocal dilutions (ranging from 100 to 6,400 fold) of either the anti-MUC1/SEC 7H10/5 mAb or the anti-repeat H23 mAb. Two different human sera (B165 and B337) were diluted 10 fold and 200 microliters added to each well. Bound MUC1 proteins were detected with alkaline phosphatase conjugated anti-repeat mAbs and, following addition of the substrate, the plates were read at 405 nm.

were performed. Either anti-sec peptide mAbs or anti-repeat H23 mAbs, were immobilized onto wells followed by incubation with body fluid samples- detection of bound MUC1 proteins was performed with alkaline phosphatase conjugated anti-repeat H23 mAbs. These two assays are designated [(anti-sec)/(sample)/(anti-repeat AP)] and [(anti-repeat)/(sample)/(anti-repeat AP)] respectively. Using the conventional [(anti-repeat)/(sample)/(anti-repeat AP)] assay (that detects all repeat array containing MUC1 proteins), one serum sample (B165) displayed high levels of MUC1 protein and a second serum sample (B337) contained only very low levels (Table 2). The [(anti-sec)/(sample)/(anti-repeat AP)] which detects only the MUC1/SEC isoform, revealed high MUC1/SEC levels in the B165 sample and only very low levels in the B337 sample, thus correlating well with the results obtained in the [(anti-repeat)/(sample)/(anti-repeat AP)] assay (Table 2).

DISCUSSION

Although it is well known that repeat array containing MUC1 proteins are released into the circulation by malignant epithelial cells, the secretory mechanism remains obscure. A molecular analysis of MUC1 cDNA showed that a major MUC1 protein isoform is a type 1 transmembrane protein containing a large extracellular domain (8-12). This membrane MUC1 protein undergoes proteolytic cleavage at a site that is approximately 45-60 aa N-terminal to the transmembrane domain, generating a heterodimer MUC1/REP protein in which the repeat array-containing extracellular domain is bound in a non-covalent SDS-sensitive linkage to the remaining C-terminal cleavage products (13). Dissociation from the C-terminal cleavage products will result in ''shedding'' of the N-terminal extracellular domain. In fact, similar protein secretion following proteolysis of membrane bound proteins have been previously described. For example, angiotensin-converting enzyme is secreted following cleavage (14), and furin, a membrane bound endopeptidase, is subjected to proteolysis that release a soluble enzyme (15).

We previously suggested the existence of an additional repeat array containing MUC1 protein (9), MUC1/SEC, which is devoid of a transmembrane domain and is thus expected to be secreted from the cell. This observation was supported by the recent report (16) of the isolation of a MUC/SEC mRNA species. In order to demonstrate MUC1/SEC expression at

the protein level, we have generated mAbs which specifically recognize a peptide sequence, that is unique to the predicted MUC1/SEC carboxyl terminus and is not found in the MUC1/REP/TM isoform. Using these antibodies, we have unequivocally demonstrated the presence of the MUC1/SEC isoform in proteins secreted by breast cancer cells as well as in body fluids obtained from breast cancer patients. Generation of protein isoforms by means of intron inclusion thereby introducing a stop codon is unusual. Some previously described examples are the 1.4 kb c-erbB3 mRNA coding for the secreted c-erbB3 protein in MKN45 cells that differs from the 6.2 kb transcript that codes for the membrane-bound protein (17), and the soluble human thyrotropin binding protein that is in fact a truncated form of the membrane-anchored receptor at an intron sequence included following exon 8 (18). The third mechanism described for secretion of membrane-bound protein is alternative splicing of mRNA that generates isoforms devoid of the membrane anchoring sequence (19, 20).

MUC1 proteins released by breast cancer cells are thus likely to represent a mixture of both secreted MUC1/SEC and ''shed'' MUC1/REP/TM proteins. These findings suggest that the relative levels in body fluids of shed MUC1/REP/TM and MUC1/SEC proteins, may vary amongst breast cancer patients- we do not as yet know whether this ratio is of a prognostic or/and diagnostic significance for breast cancer and a larger study will be required to resolve this issue.

The cell-associated MUC1/REP/TM protein has been implicated as an anti-cell-adhesion molecule (21, 22). On the other hand, the functional significance of soluble secreted MUC1/SEC and ''shed'' MUC1 proteins is unknown. It is however notable that proteins with mucin-like features have been recently shown to act as ligands for selectin molecules (23, 24). Research is in progress to test whether the secreted mucinous-like MUC1 proteins may, in a similar manner, function as ligands for as yet unidentified receptor molecules.

ACKNOWLEDGMENTS

Parts of this work were supported by grants from the Israel Academy of Sciences, the Israel Cancer Research Foundation to DHW and Frederico Fund to IK.

REFERENCES

1. Ceriani, R. L., Thompson, K. E., Peterson, J. A., and Abrahams, S. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74,** 582–587.
2. Kufe, D. W., Nadler, L., Sargent, L., Shapiro, P., Hand, P., Austin, F., Colcher, D., and Schlom, J. (1983) *Cancer Res.* **43,** 851–858.
3. Hilkens, J., Buijs, F., Hilgers, J., Hageman, P., Calafat, J., Sonnenberg, A., and van dar Valk, M. (1984) *Cancer* **34,** 197–204.
4. Burchell, J., Gendler, S., Taylor-Papadimitriou, J., Girling, A., Lewis, A., Millis, R., and Lamport, D. (1987) *Cancer Res.* **47,** 5476–5482.
5. Price, M. R., Edwards, S., Owaintai, A., Bullock, J. E., Ferry, B., Robins, R. A., and Baldwin, R. W. (1985) *Int. J. Cancer* **36,** 567–578.
6. Schechter, R. L., Major, P. P., Kovac, P. E., Ishida, M., Kovalik, E. C., Dion, A. S., Langleben, A., Boileau, G., Boos, G., Panasci, L., and Margolese, R. (1988) *Br. J. Cancer* **58,** 362–371.
7. Tjandra, J. J., and McKnzie, I. F. C. (1988) *Br. J. Surg.* **75,** 1067–1075.
8. Zrihan-Licht, S., Vos, H. L., Baruch, A., Elroy-Stein, O., Sagiv, D., Keydar, I., Hilkens, J., and Wreschner, D. H. (1994) *Eur. J. Biochem.* **224,** 787–795.
9. Wreschner, D. H., Hareuveni, M., Tsarfaty, I., Smorodinsky, N., Horev, J., Zaretsky, J., Kotkes, P., Weiss, M., Lathe, R., Dion, A. S., and Keydar, I. (1990) *Eur. J. Biochem.* **189,** 463–473.
10. Lightenberg, M. J. L., Vos, H. L., Genissen, A. M. C., and Hilkens, J. (1990) *J. Biol. Chem.* **265,** 15573–15578.
11. Gendler, S. J., Lancaster, C. A., Taylor-Papadimitriou, J., Duhig, T., Peat, N., Burcheli, J., Pemberton, L., Lalani, E-N., and Wilson, D. (1990) *J. Biol. Chem.* **265,** 15286–15293.
12. Keydar, I., Chou, C. S., Hareuveni, M., Tsarfati, I., Sahar, E., Seltzer, G., Chaitchik, S., and Hizi, A. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86,** 1362–1366.
13. Lightenberg, M. J. L., Kruijshaar, L., Buijs, F., Van Meijer, M., Litvinov, S. V., and Hilkens, J. (1992) *J. Biol. Chem.* **267,** 6171–6177.

14. Ramchandran, R., Sen, G. C., Misono, R. R., and Sen, I. (1994) *J. Biol. Chem.* **269,** 2125–2130.
15. Vidricaire, G., Denault, J. B., and Leduc, R. (1993) *Biochem. Biophys. Res. Comm.* **195,** 1011–1018.
16. Aplin, J. D., and Hey, N. A. (1995) *Biochem. Soc. Trans.* **2,** 826–81.
17. Katoh, M., Yazaki, Y., Sugimura, T., and Terada, M. (1993) *Biochem. Biophys. Res. Comm.* **192,** 1189–1197.
18. Takeshita, A., Nagayama, Y., Fugiyama, K., Yokoyama, N., Namba, H., Yamashita, S., Izumi, M., and Nagataki, S. (1992) *Biochem. Biophys. Res. Comm.* **188,** 1214–1219.
19. Aveskogh, M., and Hellman, L. (1995) *Scand. J. Immunol.* **42,** 535–539.
20. Liu, C., Cheng, J., and Mountz, J. D. (1995) **310,** 957–963.
21. Hilkens, J., Ligtenberg, M. J. L., Vos, H. L., and Litvinov, S. V. (1992) *Trends Biochem. Sci.* **17,** 359–363.
22. Wesseling, J., van der Valk, S. W., Vos, H. L., Sonnenberg, A., and Hilkens, J. (1995) *J. Cell Biol.* **129,** 255–265.
23. Lasky, L. A. (1995) *Annu. Rev. Biochem.* **64,** 113–139.
24. Puri, K. D., Finger, E. B., Gaudernack, G., and Springer, T. A. (1995) **131,** 261–270.