Analysis of phosphorylation sites on fibronectin

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Fibronectin *Phosphorylation site*

1. INTRODUCTION

Fibronectin, a well characterized adhesive glycoprotein of several cell types, is composed of discrete functional domains that display affinities for and interact with a number of macromolecules. Such interactions of fibronectin with several molecules at the cell surface could account for its reported activities in biological processes such as cellular adhesion, migration, shape formation, embryonic differentiation and opsonization [1–6].

Fibronectin is present on the cell surface in an elaborate fibrillar network and seems to be extensively disulfide bonded to itself and other proteins [7,8]. The disulfide bonds appear to be important in the retention of fibronectin at the surface of normal cells [9] and are located near the COOH terminus linking the subunits to form the fibronectin dimer [10]. In addition, the presence of about two free sulfhydryl groups has been reported whose positions have have been mapped in the COOH-terminal half of fibronectin on fragments containing fibrin-binding and cell-binding domains [11–16].

Fibronectin is phosphorylated [10,17–19]; the phosphorylation of fibronectin seems to be a conserved phenomenon and occurs on a limited number of serine sites [18]. On bovine plasma fibronectin a single site of phosphorylation on the serine residue close to COOH terminus has been reported. This study attempts to localize the phosphorylation sites on secreted fibronectins from human and hamster cells with reference to other reported domains on the molecule.

2. MATERIALS AND METHODS

2.1. *Limited cleavage of labeled fibronectin by trypsin or chymotrypsin*

NIL 8 hamster cells and human skin fibroblasts were grown and labeled as described previously [18,20]. Fibronectin from NIL 8 cells was isolated by immunoprecipitation as described in detail [18]. Fibronectin from the medium of the GM0077 cell line was isolated by affinity chromatography on a gelatin–Sepharose column [21] or by immunoprecipitation with the monoclonal antibody described according to [22]. Fibronectin present in the immune complex was treated with 1 μg/ml trypsin or chymotrypsin [18] for different time periods (see figure legends) and the reaction was stopped by the addition of 50-fold excess of soybean trypsin inhibitor or 2 mM PMSF, respectively. The samples were dissolved by boiling in electrophoresis sample buffer (50 mM Tris HCl, pH 6.7, 2% SDS, 10 mM dithiothreitol, 2 mM PMSF, 2 mM EDTA and 10% glycerol). In some instances the released fragments of proteolytic digestion

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were analyzed by centrifugation of the digestion mixture followed by application of the supernatant directly on gels. Samples were analyzed on 5–20% gradient polyacrylamide gels or on a 20% gel under non-reducing or reducing conditions using the Laemmli buffer system [23]. The gels were dried without staining or fixing as this was extremely important to see the low molecular weight peptides [18]. An intensifying screen was used for detection of ^{32}P-radioactivity by autoradiography.

2.2. *Peptide mapping*

^{32}P-labeled fibronectin was released from the immune complex by raising the pH to 12. The precipitate was centrifuged and the supernatant containing highly purified ^{32}P-labeled intact fibronectin was neutralized. Extensive digestion with trypsin or chymotrypsin was then carried out followed by two-dimensional analysis of the enzyme digests as described in detail [18] with one modification. The oxidation of fibronectin with performic acid was omitted.

Peptide mapping of the 4 kDa phosphopeptides released by limited cleavage with 1 μg/ml of trypsin or chymotrypsin utilized the supernatant removed after 30 min or 10 min of digestion of the second immune complex respectively. The extensive digestion with trypsin or chymotrypsin was then performed as described above for intact fibronectin.

2.3. *Labeling of fibronectin with iodo[^{3}H]acetic acid*

^{32}P-labeled GM 0077 fibronectin was purified by affinity chromatography, peak fractions eluted with 4 M urea were pooled and reacted with iodo-[^{3}H]acetic acid (1 mCi/ml) for 90 min in the dark at 37°C. The unreacted iodo[^{3}H]acetic acid and urea were dialyzed out against several changes of phosphate-buffered saline and the fibronectin was subjected to trypsin digestion as described above.

2.4. *Separation of 4 kDa phosphopeptides on HPLC*

50–100 μl of samples of ^{32}P-/iodo[^{3}H]acetic acid-labeled 4 kDa phosphopeptides were injected into a Waters HPLC system. The column utilized for the separation was a Radial Pak C-8 (column diameter 8 mm; pore size, 10 μm). The column was eluted with 0.1 M ammonium carbonate in an increasing gradient (setting no. 6) of acetonitrile (10–75%). The change in acetonitrile was 1%/min. The sample was run at 1 ml/min and 1 ml fractions were collected. The fractions were mixed with Aquasol (NEN) and counted in a Beckman Model LS 700 scintillation counter set to count ^{3}H and ^{32}P simultaneously.

3. RESULTS

3.1. *Digestion of fibronectin with trypsin and chymotrypsin*

The kinetics of trypsin (1 μg/ml) digestion of secreted fibronectin from NIL 8 cells labeled with either [^{35}S]cystine or [^{32}P]orthophosphate is shown in fig.1. Along with several other fragments, a major 40 kDa fragment appeared during the

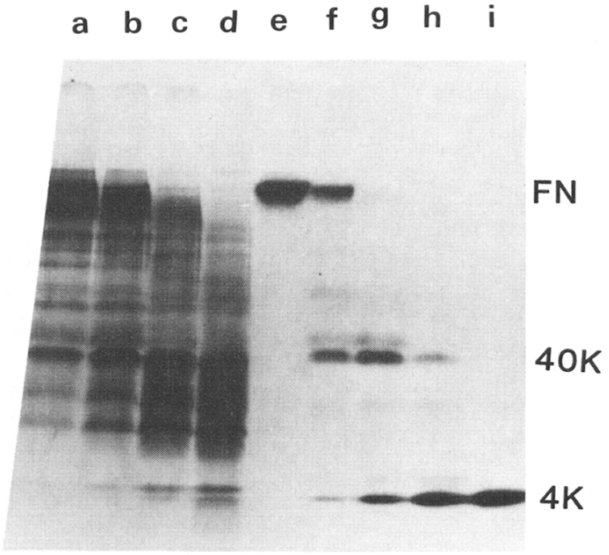

Fig. 1. Kinetics of trypsin digestion of [^{35}S]cystine- or [^{32}P]orthophosphate labeled fibronectin. Fibronectin was isolated from conditioned medium of NIL 8 cells by immunoprecipitation and the immune complex was treated with 1 μg/ml of trypsin for various time periods. Samples were analyzed on a 5–20% SDS–polyacrylamide gel a–d. [^{35}S]Cystine-labeled fibronectin. (a) 30 s, (b) 2 min, (c) 10 min, (d) 30 min. e–i: ^{32}P-labeled fibronectin. (e) intact fibronectin, (f) 30 s, (g) 2 min, (h) 10 min, (i) 30 min. The positions of fibronectin (FN), 40 kDa and 4 kDa fragments are shown. The 40 kDa fragment runs slightly ahead of ovalbumin (M_r = 43 000) and the 40 kDa fragment runs slightly slower than insulin (M_r = 3000).

course of digestion of [^{35}S]cystine-labeled fibronectin (fig.1, lanes b and c). Its level was somewhat decreased after 30 min of treatment with trypsin (fig.1, lane d). There was also an increased accumulation of two small peptides of around 4000 Da with increasing time periods of trypsin digestion (fig.1, lanes b, c and d). Similarly, a major 40 kDa phosphopeptide and a low molecular mass peptide around 4 kDa were generated very early during the digestion (30 s –, fig.1, lane f; and 2 min – fig.1, lane g). The high molecular mass, tryptic digest intermediates lacking the interchain disulfide bonds reported by Ledger and Tanzer [19] were never observed in this study. After 10 min of treatment with trypsin, the 4 kDa phosphopeptide accounted for most of the ^{32}P-label, apparently at the expense of 40 kDa phosphopeptide which was present only at a much reduced level (fig.1, lane h). Treatment of fibronectin for 30 min with 1 μg/ml trypsin released virtually all of the ^{32}P radioactivity in the form of a 4 kDa peptide (fig.1, lane i). However, from this experiment one cannot exclude the presence of more than one phosphopeptide species in the 4 kDa band (see below, fig.2 and 4).

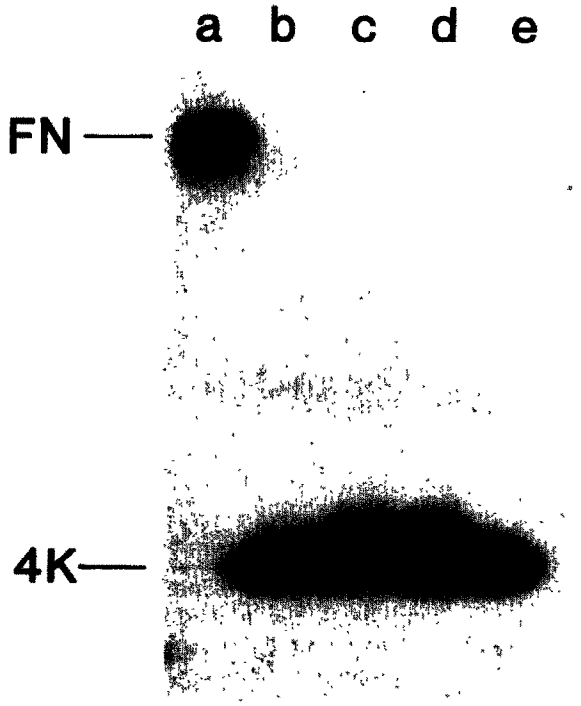

Fig. 2. Limited trypsin and chymotrypsin digestion of ^{32}P-labeled fibronectin. Fibronectin was isolated from the conditioned medium of NIL 8 cells by immunoprecipitation and the immune complex was treated with 1 μg/ml trypsin for 30 min (b,e) or with 1 μg/ml chymotrypsin for 10 min (c,d). Samples were analyzed on a 5–20% SDS–polyacrylamide gel. (a) intact fibronectin; (b,c) samples were boiled in electrophoresis sample buffer as usual; (d,e) supernatants of the digestion mixtures were directly applied on gel (see Experimental Procedures).

In a second experiment fibronectin was treated with trypsin (1 μg/ml) for 30 min or with chymotrypsin (1 μg/ml) for 10 min. Fig.2, lane b shows that treatment with trypsin gave rise to a band at the 4000 Da position, as in the previous experiment. Chymotryptic digestion generated a closely spaced doublet (fig.2, lane c). It is difficult to assign precise molecular mass in the high percentage region of the gradient gel, therefore, the two closely migrating bands were both designated as 4 kDa phosphopeptides. In the course of the trypsin digestion of ^{32}P-labeled fibronectin from NIL 8 cells immunoprecipitated with polyclonal antibodies it was also noticed that the 40 kDa phosphopeptide remained attached to the immune complex (data not shown), whereas the 4 kDa phosphopeptides generated by both chymotrypsin and trypsin digestion were released from the immune complex (fig.2, lanes d and e).

3.2. *Tryptic and chymotryptic peptide mapping of [^{32}P]orthophosphate-labeled fibronectin and 4 kDa phosphopeptides*

Fig.3 shows the two dimensional tryptic and chymotryptic maps of intact ^{32}P-labeled fibronectin and the 4 kDa phosphopeptides released by limited trypsin and chymotrypsin digestions, respectively. The maps generated by trypsin digestion of both the 4 kDa phosphopeptides and intact fibronectin had four major spots and were identical (fig.3A and B).

The chymotryptic maps of intact ^{32}P-labeled fibronectin and the 4 kDa phosphopeptides are shown in fig.3C and D. Each map had the same five spots. Spot 2 was always the major spot, whereas the intensity of spot 1 varied in the two maps.

It is clear from the 2D maps that all the ^{32}P-radioactivity was present in the form of 4 kDa phosphopeptides and none was lost as very low molecular mass peptides.

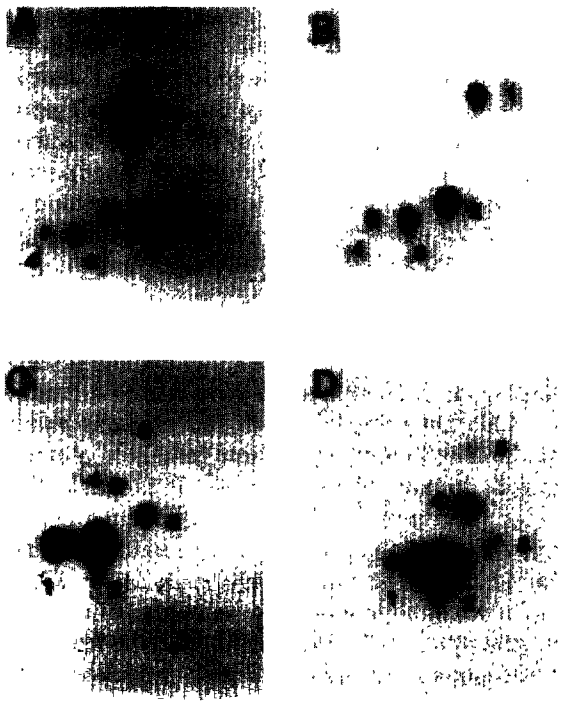

Fig. 3. Two-dimensional tryptic and chymotryptic maps of 4 kDa phosphopeptides and intact fibronectin: Tryptic digests of 4 kDa phosphopeptides (A) and ^{32}P-labeled intact fibronectin (B), and chymotryptic digests of 4 kDa phosphopeptide (C) and intact fibronectin (D) were analyzed by electrophoresis in 30% formic acid toward the cathode in the first dimension and chromatography in butyl alcohol/acetic acid/water/pyridine (60:12:48:40) in the second dimension.

3.3. *Gelatin-binding activity of phosphopeptides*

The 40 kDa and 4 kDa fragments obtained as major phosphopeptides following the trypsin (1 μg/ml) digestion of ^{32}P-labeled secreted fibronectin from NIL 8 cells for 2 min and 30 min respectively were passed through gelatin Sepharose affinity columns. Fig.4 shows that both fragments did not bind to gelatin and were recovered in the eluate.

3.4. *Presence of free sulfhydryl and/or disulfide groups on the 4 kDa phosphopeptide*

Incorporation of [^{35}S]cystine in the 40 kDa and 4 kDa fragments of fibronectin indicated the presence of free sulfhydryl and/or disulfide groups. The following experiments were designed to examine these possibilities.

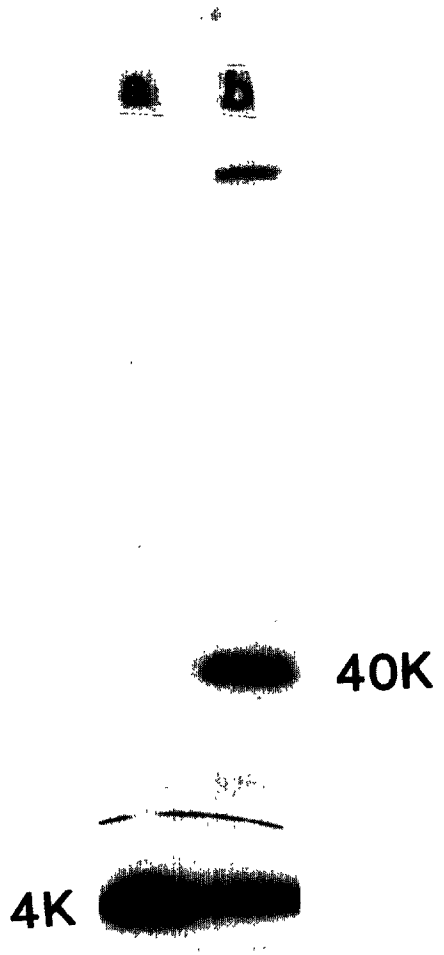

Fig. 4. Gelatin binding activity of phosphopeptides of fibronectin from NIL 8 cells. Eluates from gelatin-Sepharose affinity column were run on a 5-20% gradient gel. (a) 4 kDa phosphopeptide isolated from the supernatant of 30 min trypsin digest (see section 2). (b) 40 kDa phosphopeptide was released from the immunoprecipitate after digestion with 1 μg/ml trypsin for two minutes by raising the pH to 12. It was neutralized and passed through gelatin-Sepharose column.

3.4.1. Electrophoretic mobility of phosphopeptides on SDS gels under reducing and non-reducing conditions

The 4 kDa phosphopeptide released from the immune complex of NIL 8 cells after 10 min of

partial trypsin digestion was resolved as a closely spaced doublet on a 20% gel under reducing conditions (fig.5a). However, the supernatant of the partial tryptic digestion of fibronectin from the GM0077 human fibroblast cell line, immunoprecipitated with the monoclonal antibody 4B2 directed against a single antigenic determinant near the gelatin-binding site, contained the 4 kDa doublet as well as bands in the 40 kDa region and the undigested fibronectin (fig.5b), both of which remain attached to polyclonal antibodies in the immune complex of fibronectin from NIL 8 cells. In repeated experiments multiple bands of M_r = 40 000 were observed in the partial trypsin digest of human cellular fibronectin suggesting multiple trypsin sensitive sites in that region. Under non-reducing conditions the 4 kDa bands migrated more slowly and the radioactivity in the 40 kda band migrated faster than under reducing conditions (fig.5c and d).

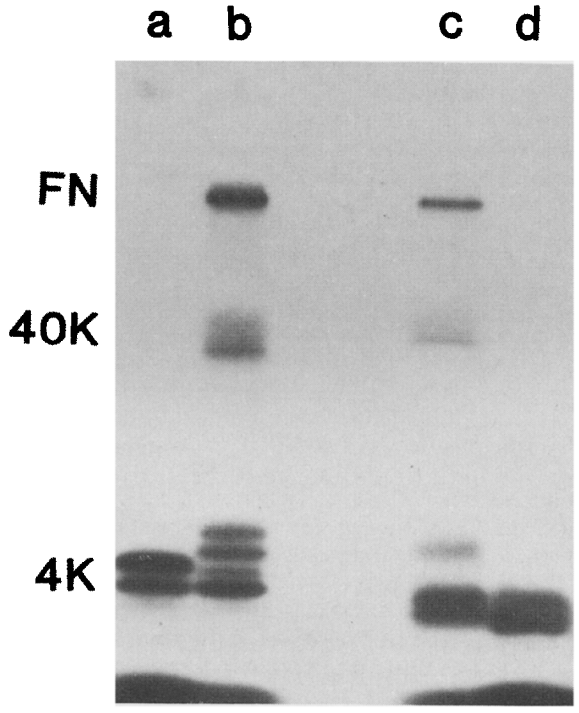

Fig. 5. Electrophoretic mobilities of partial trypsin digest of ^{32}P-labeled fibronectin under reducing and nonreducing conditions on a 20% SDS-polyacrylamide gel. The positions of FN, 40 kDa and 4 kDa fragments are marked. (a) NIL 8 cells non-reduced. (b) GM0077 cells non reduced. (c) GM0077 cells reduced. (d) NIH 8 cells reduced.

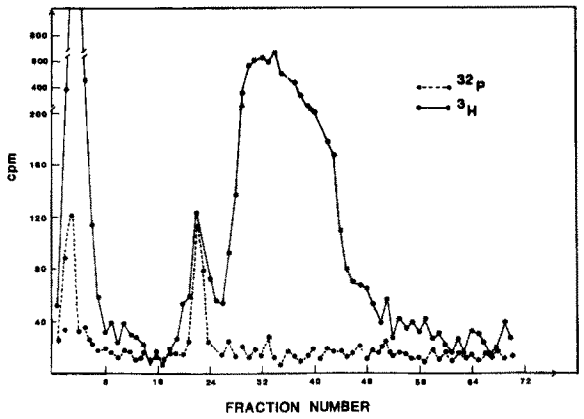

Fig. 6. Separation of the trypsin digest of [^{32}P]orthophosphate- and iodo[^{3}H]acetic acid-labeled fibronectin from GM0077 human fibroblasts on an HPLC column.

3.4.2. Resolution of [^{32}P]orthophosphate and iodo[^{3}H]acetic acid radioactivity on an HPLC column

^{32}P-labeled secreted fibronectin from GM0077 fibroblast was purified on gelatin-Sepharose column, eluted with 4 M urea and reacted with iodo[^{3}H]acetic acid. The free iodo[^{3}H]acetic acid and urea were then dialyzed out and the labeled protein was subjected to digestion with trypsin (1 μg/ml) for 30 min, a treatment that released most of the ^{32}P-label in the form of 4 kDa peptides. Upon injection of the trypsin digest into the HPLC column, ^{32}P and ^{3}H counts were eluted in corresponding peaks (fig.6). The broad peak of ^{3}H radioactivity represented undialyzed free iodo-[^{3}H]acetic acid.

4. DISCUSSION

Fibronectin is involved in some of the basic cellular functions such as adhesion and locomotion ([24–27]; also see reviews [1–6]). Considering this, it is not surprising that fibronectin is a highly conserved protein in structure [28]. Phosphorylation of fibronectin, which occurs only on serine residues, also seems to be a conserved phenomenon as the phosphopeptides of fibronectin are shared by different species [18].

The similarity of the two-dimensional maps of fibronectin with those from 4 kDa phosphopeptides demonstrate that fibronectin is phosphorylated within discrete, protease sensitive, low

molecular mass domains. The migration of tryptic and chymotryptic phosphopeptides as doublets on SDS–polyacrylamide gels (figs.2 and 4) indicate that fibronectin from hamster and human cells might be phosphorylated at least on two serine residues located on two different fragments. Furthermore, the resolution of the phosphopeptides of hamster cellular fibronectin in four to five spots in two dimensional maps (fig.3) strongly suggests multiple phosphorylation sites and also microheterogeneity in fibronectin molecules due to post-translational modifications. A single serine residue near the COOH terminus has been reported to be partially phosphorylated in bovine plasma fibronectin [10]. That one of the sites of phosphorylation of hamster and human cellular fibronectin might also be near the COOH terminus is suggested by two observations. First, the 4 kDa phosphopeptide is produced in less than a minute of treatment with very low concentrations of trypsin. It has been demonstrated that a small COOH-terminal region of fibronectin (between 3 kDa and 10 kDa depending on the enzyme and source of fibronectin) containing the intermolecular disulfide bonds is readily susceptible to proteolysis [16,29–32]. Second, in the absence of reducing agents at least one of the 4 kDa phosphopeptides of fibronectins from both hamster and human cells migrate on a 20% SDS–polyacrylamide gel with a higher apparent molecular mass (fig.4).

The coelution of ^{32}P and ^{3}H radioactivities of 4 kDa phosphopeptides from the fibronectin of human fibroblasts after passing through an HPLC column (fig.5) shows that a cysteine residue available for reaction with iodo[^{3}H]acetic acid is located close to the phosphorylation site(s). Several studies based upon limited protease digestions, cyanide cleavage experiments and recognition of sulfhydryl-containing fragments by hybridoma antibodies propose the location of 1–2 moles of free sulfhydryl groups/mole of fibronectin monomer about 30–70 kDa from the COOH terminus [12,13,16,33,34]. The data presented here demonstrate that the 40 kDa phosphopeptide which appears as a major kinetic intermediate during the tryptic digestion of fibronectin displays a slower electrophoretic mobility in its reduced state than under nonreducing conditions. This indicates that the 40 kDa phosphopeptide is constrained by intramolecular disulfide bonds. A slower migration under reducing conditions has been reported for two fragments of fibronectin, a 25 kDa fragment near the NH_2 terminus [32] and a 31 kDa fragment near the COOH terminus [34]. This is consistent with the sequence studies of bovine plasma fibronectin demonstrating the presence of the disulfide loops in a COOH terminal fragment [10,35]. The 40 kDa phosphopeptide must originate from the COOH-terminal region of fibronectin containing the disulfide loops as it is not located near the gelatin-binding region close to the NH_2-terminus and does not bind to gelatin.

The other free sulfhydryl group of fibronectin is located on a domain that contains 40% of the carbohydrate and the cell spreading activity [16]. The results presented here are suggestive of more than one phosphorylation site on fibronectin molecule. The likely candidates are serine residues (a) near the COOH-terminus and probably within the disulfide-bonded fragment, and (b) near the free sulfhydryl group located in the fibrin-binding domain and/or in the fragment containing the cell attachment site.

It is tempting to speculate that yet another potential site of phosphorylation exists on the serine residue of the tetrapeptide Arg-Gly-Asp-Ser that carries the cell-attachment promoting activity of fibronectin [36]. It would be interesting to investigate the effect of phosphorylation of serine residue on the activity of this tetrapeptide, especially in the light of findings that phosphorylation occurs to a much greater extent on fibronectins from transformed and rapidly growing cells [18,20]. More definite information on the precise location and function of the phosphorylation sites on fibronectin will come from sequence studies and by use of defined hybridoma antibodies.

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