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NOVEL CHARACTERISTICS OF A 33KDa PROTEIN (pp33) RAPIDLY PHOSPHORYLATED IN IL3 DEPENDENT CELLS BY STIMULATION WITH IL3

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We report the novel properties of a 33 KDa cellular protein rapidly phosphorylated by stimulation of growth by IL3 in IL3 dependent lines. Although pp33 is readily soluble in SDS, SDS-solubilised pp33 is insoluble in non-ionic detergents and is excluded from electrophoretic analysis (IEF, NEPHGE) employing such detergents. Native pp33 is not extracted by non-ionic detergents with or without cation chelaton. pp33 is concentrated in a cell fraction containing endoplasmic reticulum where it is associated with a specific trypsin-sensitive degredative enzyme, active at 4 degrees. Its unusual characteristics and kinetics of phosphorylation suggest pp33 may be a novel molecule, explain its absence in studies elsewhere where non-ionic detergent extraction has been exclusively used and suggest it is intimately related to the signal transduced by IL3. © 1989 Academic Press, Inc.

Activation of protein kinases is a key event in signal transduction(1-4). However, little is known about haematopoeitic growth factor signaling. Although the CSF-1 receptor (c-fms) possesses tyrosine kinase activity (5), the receptor for the multipoetin Interleukin 3 (IL3) has not yet been characterised. Nevertheless, stimulation of IL3-dependent cells with IL3 or other moeities results in phosphorylation of a number of proteins with MW's ranging from 60-160 KDa (6-13).

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Recently, very rapid IL3-dependent phosphorylation of a 33 KDa protein, pp33, has been demonstrated in two physiologically different IL3 dependent lines (14). Phosphorylation of pp33 is insensitive to protein kinase c stimulation or inhibition, can be mediated by other growth factors (but only those stimulating active growth) and is constitutively phosphorylated in a spontaneous, IL3-independent malignant clone. However, since pp33 has not been described in other studies, various extraction methods have been used to analyse its electrophoretic behaviour. The results show that pp33 is insoluble in non-ionic detergents commonly used to prepare samples for isofocussing or SDS-PAGE, with or without calcium chelation. Further, pp33 solubilised in SDS is precipitated by non-ionic detergents. The close correlation of pp33 phosphorylation with the effects of IL3 on cell metabolism (15, 16) suggest it may be a novel protein forming an integral part of the signal transduced by IL3.

Materials and Methods

Cell lines. The murine dependent lines AC2 (sub clone RB5), 123, FDC-P1 and NFS 60.8 were maintained as previously described (14). Recombinant IL3 was used in experiments.

Labelling with ^{32}P. ()Labelling with phosphorus was as previously described (14). Briefly, (2×10^6 - 10^7 exponential phase cells were washed free of IL3, incubated for specific times in RPMI/5%FCS, followed by 30 min incubation in phosphate-free RPMI/2% FCS (dialysed against 1.4M choline chloride, 0.1M glucose, 0.01M KCl, 0.01M CaCl, 0.01M MgCl, 0.2M HEPES). Cells were resuspended to 5×10^6 to 5×10^7 per ml in phosphate- and serum-free RPMI, 0.5 ml distributed to Eppendorf tubes and the cells pre-labelled for 15 min with 200-400 uCi/ml phosphate at 37 degrees. IL3 was then added (10-100 U/ml), and after known intervals the cells spun down (10 sec), the supernates removed and pellets precipitated by direct addition of 250 uL 10% TCA; or extracted in 100-300 uL detergent extraction buffer and extracted 10 - 60 min at room temperature or 4 degrees with mixing. Control cells received equal volumes of control-vectored cos supernates or medium.

Electrophoresis. TCA precipitates were washed once in PBS and once in distilled water. Aliquots were spun and pellets dissolved in Laemmli sample buffer. Detergent extraction buffers were: for 2D electrophoresis, 20mM Tris HCl pH 7.4/ 50mM NaCl/ 0.5% NP40/ 0.5% sodium deoxycholate/ 4mM iodoacetic acid/ 5mM sodium pyrophosphate/ 5mM NaF and 6M urea; for single-dimension electrophoresis, 0.1 - 10% NP40, Triton x100 or octyl glucoside/ 10mM Tris HCl pH 7.1/ 5mM NaF and 5mM sodium orthovanadate, with or without 10mM EDTA. Detergent extracts were spun 8000g x 30 min and both supernates and

pellets processed for IEF or SDS-PAGE. In some experiments, washed TCA precipitates were solubilised in the non-ionic detergent buffers. Samples for SDS-PAGE were reduced with 2ME with boiling before electrophoresis in reducing SDS-PAGE Laemmli gels. IEF or NEPHAGE first-dimension gels were run in the presence of 6M urea in 1.5mm x 12 cm tube gels, followed by reducing SDS-PAGE in the second dimension.

Intracellular distribution of pp33. Labelled cells were immediately diluted by addition of an equal volume of ice-cold Tris HCl pH 7.4 containing 1mM NaF/1mM sodium orthovanadate and 10ug/ml PMSF. Suspensions were sonicated on ice with 6 x 1 sec pulses of ultrasound, checked for breakage and spun at various speeds at 4 degrees. Pellets and samples of supernates from each spin were precipitated with TCA or solubilised directly in Laemmli sample buffer for SDS-PAGE analysis. For trypsin treatment, the 100,000 g x 1 hr pellets were suspended in 100-300 uL 10mM Tris HCl buffer pH 7.6 or PBS containing various concentrations of trypsin and incubated on ice for the times shown. Trypsin inhibitor was added in 10-fold excess and the suspensions respun at 100,000g x 1 hr. Pellets were solubilised in Laemmli buffer, supernates either TCA precipitated or mixed with 10x concentrated Laemmli buffer for direct electrophoresis.

Phospho-amino-acid analysis. Bands identified from autoradiographs were excised from the gels, extracted with 100-300 uL 50 mM ammonium carbonate/ 0.1% SDS for 15 min2-mecapto-ethanol added to 5% and the gel fragment boiled 10 min, followed by extraction at 37 for 24 hr. Gel fragments were removed, the tubes dried under vacuum and residues washed 3 times with 100-300 uL ether-alcohol 1:3 and dried. Samples were hydrolysed with 6N HCl for 1 to 1½ hr at 110 degrees, dried, resolubilsed in water and electrophoresed on cellulose acetate plates in pyridine:acetic-acid:water 1:10:100. Phospho-amino-acids were identified by incorporation of known standards in the samples identified by ninhydrin and autoradiography.

Chemicals and growth factors. Recombinant IL3 (2 x 10^{5}U/ml in serum-free cos supernates) was a kind gift of Dr. T. Yokota, DNAX Laboratories, Palo Alto, California. Purified IL3 (2 x 10^{5} U/ml) was a kind gift of Drs. Keller and Ruscetti, NCI, Frederick. Alcohol- and carrier-free isotopes were obtained from Amersham International. Ampholines were obtained from LKB-Pharmacia.

Results

Early changes in pp33 on IL3 starvation. Pure and recombinant IL3 stimulated the same pp33 phosphorylation identified in TCA precipitates, (fig.1) and in direct SDS extracts (not shown). To determine the rapidity of phosphate exchange on pp33, cells were washed in phosphate-free conditions as quickly as possible (3 washes over 15 min), prelabelled for 10 min and then stimulated with IL3 (total starvation time of 25 min); controls were starved of IL3 throughout. Samples were

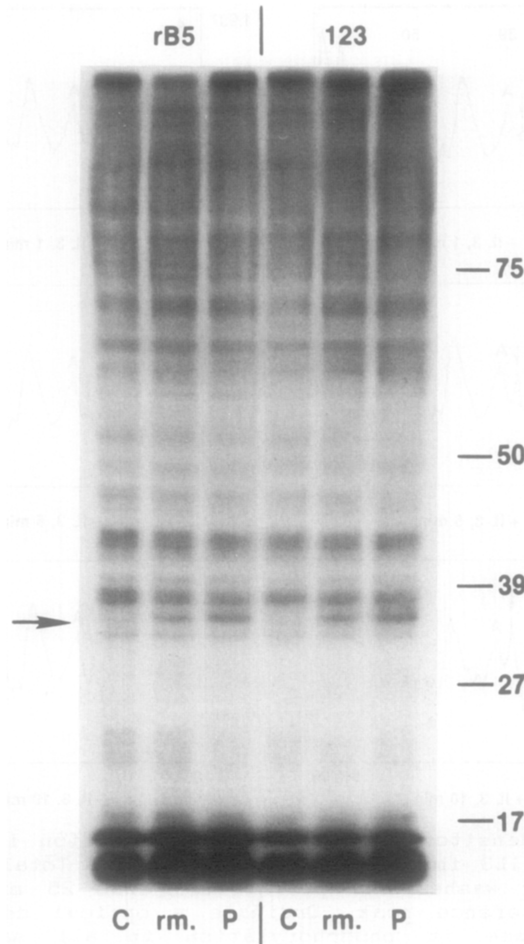

Fig. 1. Stimulation of pp33 by purified and recombinant IL3. Cells were starved for 1 hr, and stimulated for 15 min. Controls received an amount of PBS equal to the volume of pure IL3. Prestained MW standards. rm = recombinant IL3; P = pure IL3. Arrow marks pp33. TCA precipitation/SDS-PAGE.

removed at 1, 5 and 10 min. for SDS-PAGE. Fig. 2 shows that in cells supplemented with IL3 the pp33 phosphorylation significantly increased within 5 min and five-fold over 10 min; in control cells the ratio remained equal to the initial value. pp33 phosphorylation was unaffected by the presence of cycloheximide (50ug/ml) (data not shown). Since pp33 is virtually absent from cells depleted of IL3 for longer periods (see fig 1), changes in phosphorylation appear to occur on a relatively stable protein immediately IL3 is withdrawn. Acid hydrolysis of excised pp33 demonstrated that all incorporated label was on serine. No labelled phosphotyrosine was detected.

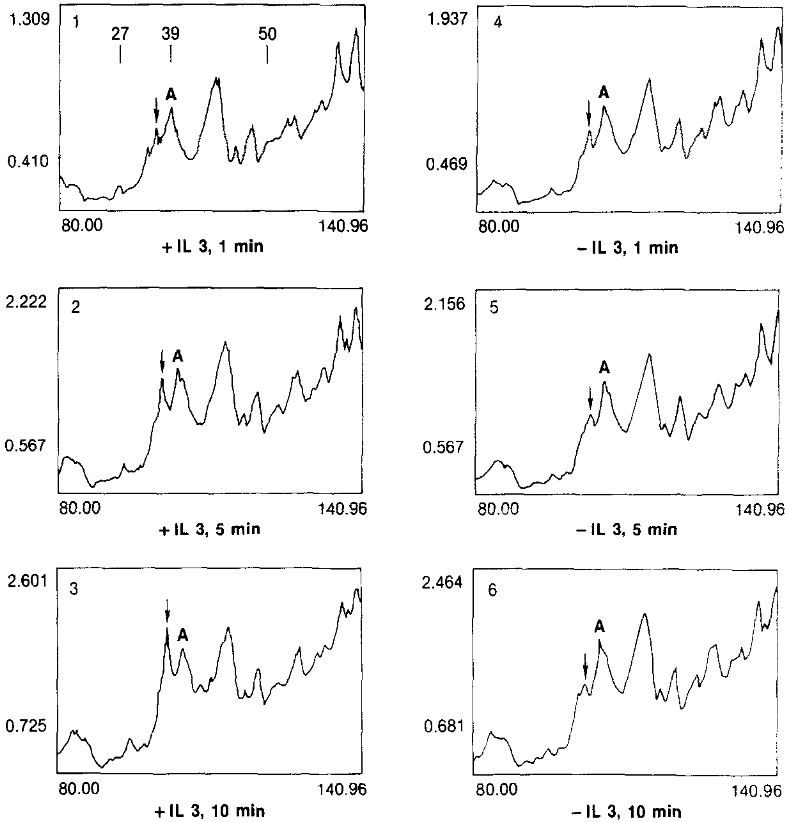

Fig. 2. Microdensitomtry of p33 phosphorylation in RB5 cells stimulated by IL3 immediately after washing. Total starvation time including washes and prelabelling was 25 min. Arrow = pp33; A = reference peak. Ordinate = optical density. Note overall increase in phosphorylation in all samples, and relative increase in pp33 in stimulated cells. Similar results were recorded for 123 cells. TCA precipitation/SDS-PAGE.

2-dimensional electrophoresis. No pp33 was identified in 2D IEF of cells extracted directly in IEF sample buffer. TCA precipitates were therefore analysed in parallel by SDS-PAGE and 2D IEF and NEPHAGE. Although pp33 was conspicuous in SDS-PAGE, it was not detected in pH 3-10 or 4-7 IEF or in NEPHAGE gels. However, NEPHAGE second-dimension gels showed that pp33 remained in the first-dimension gels (fig. 3), indicating that pp33 was excluded from NEPHAGE gels even in the presence of triton/urea.

Solubility of pp33 in non-ionic detergents. pp33 demonstrated by TCA-SDS was absent from both the soluble and insoluble phases of the same cells extracted in non-ionic detergent (triton) (fig. 4). Similar results were obtained

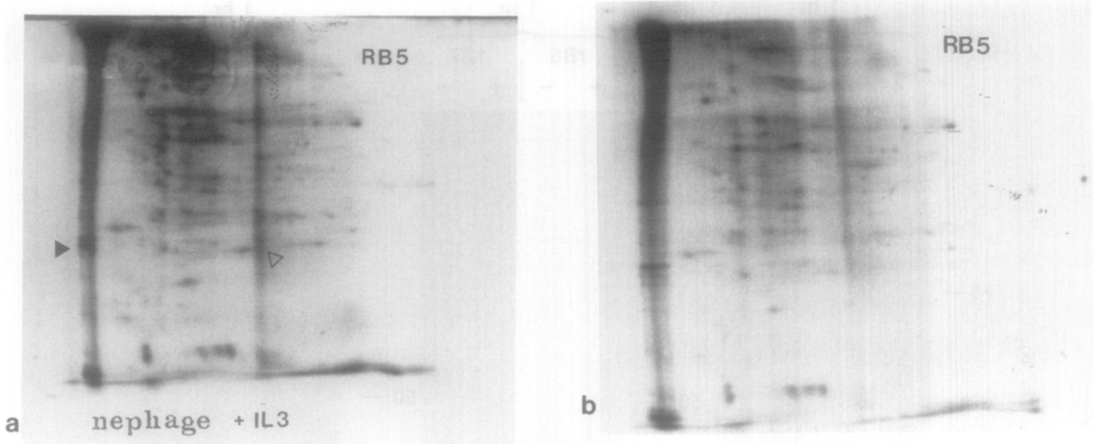

Fig. 3. NEPHAGE electrophoresis of TCA precipitates from RB5 cells starved 1 hr. and stimulated 15 min. (3a) or unstimulated (3b). Second dimension SDS/PAGE. A small amount of pp33 appears as a streak (open arrow), but most remained in the sample application region of the first dimension NEPHAGE gel (solid arrow).

with NP40 and octyl glucoside at three different concentrations (0.1%, 1% and 10%) with or without EDTA. A partial explanation for the complete absence of pp33 is provided by fig. 5; pp33 solubilised in SDS is in fact precipitated by addition of an excess of non-ionic detergent.

Distribution of pp33 and its association with a degredative enzyme. Nearly all pp33 appeared in a 100,000 g x 1 hr pellet, containing endoplasmic reticulum and cytoskeletal fragments. Mild trypsin digestion (50 ug/ml and 4 degrees) of high-speed pellets failed to release pp33, but whereas pp33 persisted in tubes containing trypsin, it was specifically absent from control tubes incubated in buffer on ice (fig. 6). Further experiments showed that loss of pp33 is protected by low concentrations of trypsin. Thus pp33 in situ appears associated with a highly active, trypsin-sensitive degredative enzyme (phosphatase or hydrolase) active at low temperatures.

Discussion Our unusual finding is that pp33 is rendered insoluble from SDS solution by non-ionic detergents commonly used to prepare samples for electrophoresis. Further, it may be enzymically degraded at low temperatures in these extraction buffers; thus the absence of pp33 in studies using

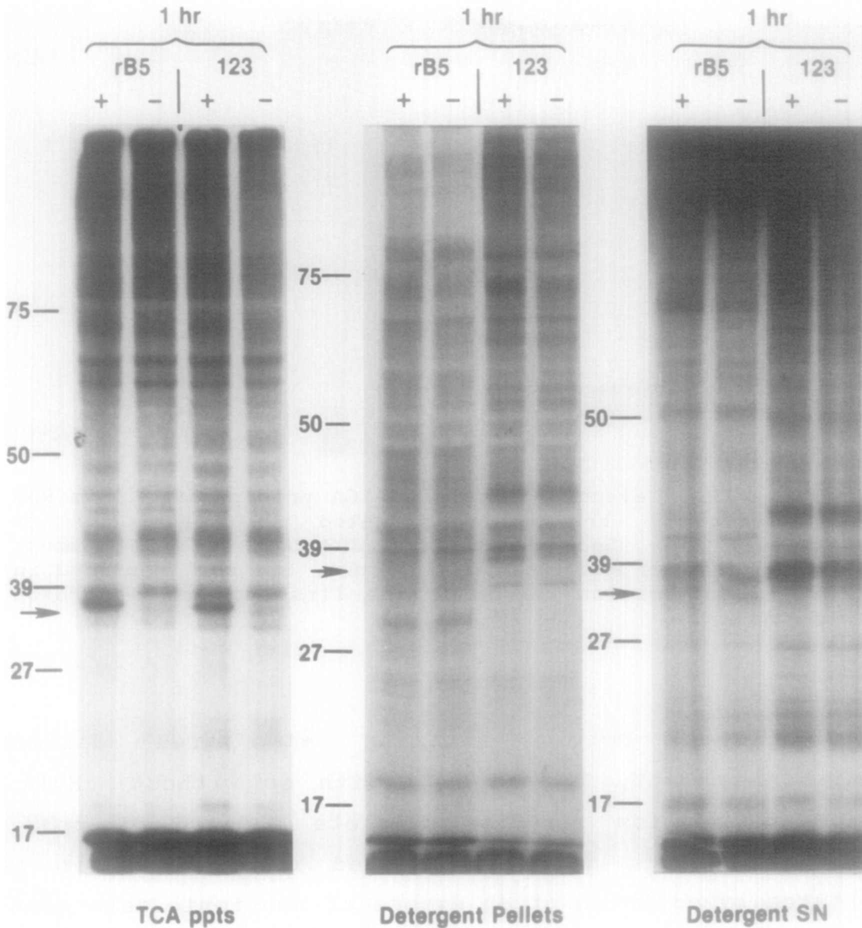

Fig. 4. Distribution of pp33 in TCA and detergent extracts. Cells were starved for 1 hr and stimulated 15 min with IL3. Arrow = pp33. + = with IL3, - = without IL3. pp33 is present in the TCA precipitates of both RB5 and 123 cells stimulated with IL3. Some pp33 is present in the detergent pellet from 123 cells but absent from RB5 cells. No pp33 appears in any supernates.

non-ionic detergent extractions may be explained. Finally, phosphate exchange on pp33 is extremely rapid since changes can be detected immediately IL3 is removed.

Possible candidates for pp33 such as detergent-soluble proteins cyclin (MW 36KDa) (17), the 32 KDa cell cycle control protein cdc-2 (18) (preliminary studies show that cdc-2 is not rapidly phosphorylated by IL3), calpains (19) and histones (20,21) may be reasonably excluded. Although serine phosphorylation of ribosomal protein S6 (30-32 KDa) is stimulated by growth factors and is regulated by both specific kinases and a phosphatase (22-26), S6 would be released into the detergent-soluble phase (27). Lipocortins are membrane-bound calcium and actin-binding proteins of MW

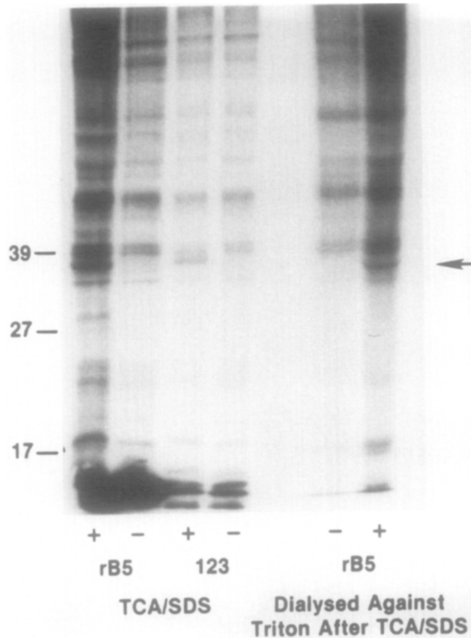

Fig. 5. Precipitation of pp33 by triton X100 after solubilisation in SDS (see text). Arrow = pp33. pp33 appears in the original TCA/SDS solution (left) and in the precipitate formed after addition of excess triton to SDS-solubilised pp33 (right). No pp33 was detected in Triton-soluble supernates (not shown). + = with IL3, – = without IL3.

36-40 KDa, which appear to regulate phospholipase A_2 (28) and are substrates for various kinases including the EGF receptor and sarc kinases (29,30). Although they are insoluble in non-ionic detergents (31-35), they are characteristically released by calcium chelation (36), particularly lipocortin I (33 KDa) (37). Lipocortin II (35 KDa) however exists in two forms, only one of which is released by calcium chelation (38). However, preliminary studies using antisera to conserved sequences show that releasable lipocortin is not a significant substrate for IL3 mediated phosphorylation. Mahadevan et al have described serine phosphorylation of a detergent-insoluble 33 KDa protein in the nuclei of fibroblasts stimulated by various ligands (39), but it does not correspond to the topographical location of pp33.

pp33 fulfills certain criteria for a protein intimately involved in signal transduction. Thus, it is present in growing cells before starvation (a necessary criterion for involvement); the rapidity of changes correlate well with known rapid effects of IL3 withdrawal on cell metabolism,

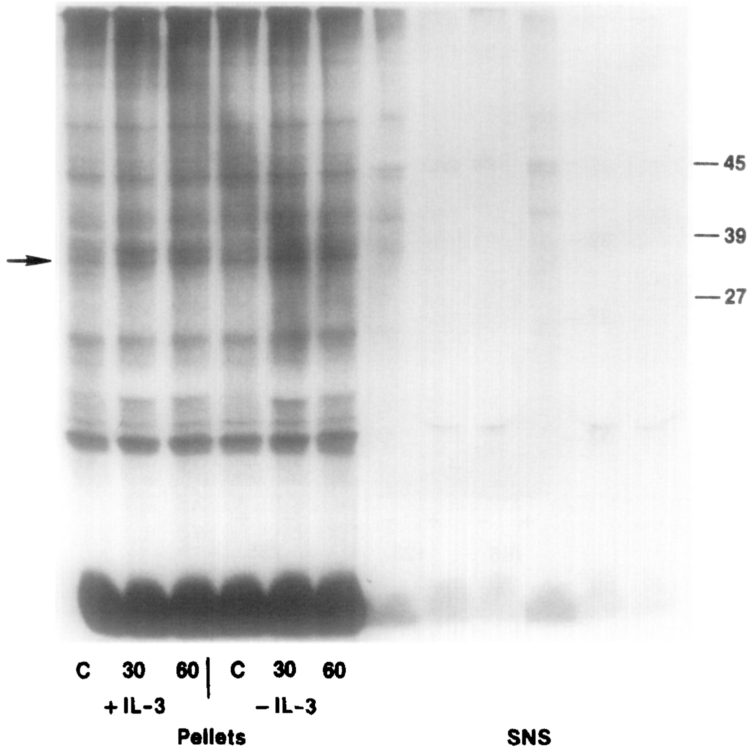

Fig. 6. Effect of trypsin (50 ug/ml) on stability of pp33 in 100,000g x 1 hr pellets from stimulated RB5 cell sonicates. Control pellets (C) were held on ice for 1 hr. Arrow = pp33. pp33 is absent in both control unstimulated and stimulated pellets. pp33 is present in trypsin-treated pellets from stimulated cells.

which in 123 and RB5 cells does not involve glucose transport (15,40,41); only ligands which stimulate growth stimulate pp33; and pp33 phosphorylation becomes constitutive as IL3 dependency is lost. Unlike other ligands which stimulate entry of a resting cell into a division cycle, IL3 dependent cells have insufficient time to arrest at a particular phase of the cycle when IL3 is withdrawn before cell death ensues (20). This suggests that IL3 controls the rapid dynamic equilibrium of a critical but unusual biochemical event. pp33 has the kinetics, location and unusual properties to be a candidate for such a control molecule.

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