Activation of Integrin-based Cell Adhesion in Mouse L Fibroblasts Expressing Stabilized β Catenin in the Nucleus

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Abstract

β catenin is involved not only in the cadherin-based cell adhesion system but also in the *Wnt* signaling pathway. In this pathway, it is supposed that β catenin is phosphorylated by glycogen synthase kinase-3β (GSK-3β) to become susceptible to degradation, that the *Wnt* signaling suppresses GSK-3β to increase the amount of non-phosphorylated, stable β catenin, and that the stable β catenin affects the transcription of various genes in the nucleus. To separate the role of β catenin in intracellular signaling from its role in the cadherin-based cell adhesion, we constructed a mutant β catenin (*mbH*) with amino acid substitutions at its putative GSK-3β phosphorylation site (GSKP sequence), and introduced it into L fibroblasts that lack the cadherin expression. In stable transfectants (LmbH), not only mbH but also endogenous wild type β catenin was stabilized. Green fluorescence protein (GFP) carrying the mutated GSKP sequence also stabilized the endogenous β catenin in L cells, indicating that the mutated GSKP sequence suppresses the degradation machinery for β catenin. Furthermore, by adding the nuclear localization signal to mbH, we obtained L transfectants (LmbHN) expressing the stabilized β catenin predominantly in the nucleus. Under the long-term aggregation conditions, LmbHN formed compact cell aggregates consisting mainly of 2-5 cells. LmbH also showed a similar aggregation activity but to a lesser extent, and parent L cells mostly remained to be dissociated. Close analyses by immunofluorescence microscopy and the cell adhesion inhibition assay with RGD peptides revealed that this aggregation activity is attributed to the integrin-based cell adhesion. Considering that the amount of stabilized β catenin in the nucleus is significantly larger in LmbHN than in LmbH, we speculate that the accumulation of β catenin in the nucleus resulted in the activation of integrin-based cell adhesion. The transfectants established in this study, thus, will provide an advantageous system to selectively analyze the role of β catenin in the

nucleus and in the regulation of integrin activity.

β catenin with a molecular mass of 94kD was originally identified as a component of the E-cadherin immunoprecipitate (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; McCrea and Gumbiner, 1991a). Since cadherins play a central role in the cell-cell adhesion and morphogenesis of various organs, this cadherin-associated protein has been attracting increasing interest (Takeichi, 1995). Evidence has accumulated that the cytoplasmic domain of cadherins directly binds to β catenin, which then binds to another cadherin-associated protein called α catenin (Aberle et al., 1994; Hinck et al., 1994; Jou et al., 1995). Mutant cadherins lacking the β catenin-binding sites show no adhesion activity, indicating that the cadherin/catenin complex formation is indispensable for the cadherin-based cell adhesion system (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990). In addition to the cadherin/α catenin cross-linking activity, β catenin is reportedly involved in the regulation of intercellular motility through modifying the dynamic property of cadherin-based cell adhesion (Nagafuchi et al., 1994).

Sequence analysis revealed that β catenin (McCrea et al., 1991b) shows a similarity to plakoglobin (Franke et al., 1989) and to the product of the *armadillo* gene, a segment polarity gene in *Drosophila* (Riggleman et al., 1989). Genetic evidence in *Drosophila* indicates that armadillo is involved in the *wingless* signaling pathway located downstream of a serine-threonine kinase zw3/shaggy (Siegfried et al., 1994; Noordermeer et al., 1994). In this pathway, the stimulation with the *wingless* product, a secretary protein, not only suppresses the zw3/shaggy kinase but also decreases the phosphorylation level of armadillo with its concomitant stabilization. This stable armadillo regulates the downstream elements of the pathway to determine the polarity of each segment in *Drosophila* (Peifer et al., 1994b). These findings suggest the possible involvement of β catenin in the intercellular *Wnt* (a vertebrate homologue of *wingless*) signaling for morphogenesis in vertebrates. Indeed, overexpression of β catenin in normal *Xenopus* and *zebrafish* embryos reportedly induces a secondary

dorsal axis (Funayama et al., 1995; Kelly et al., 1995), and overexpression of β catenin in UV-irradiated *Xenopus* embryos rescues their dorsalizing activity (Guger and Gumbiner, 1995). Such dorsalizing activity of β catenin is reportedly associated with its nuclear localization (Funayama et al., 1995). Furthermore, expression manipulation of *Xenopus* homologues for *wingless* and *zw3/shaggy* revealed that these molecules are also involved in the formation of *Xenopus* dorso-ventral axis (Cui et al., 1995; Dominguez et al., 1995; He et al., 1995). Moreover, *Xenopus* eggs depleted of maternal β catenin transcripts reportedly do not respond to ectopic *Wnt* signals (Heasman et al., 1994). Taken all together, also in vertebrates, β catenin is now believed to be directly involved in the *Wnt* signaling pathway to determine the early dorso-ventral axis.

A serine/threonine kinase, glycogen synthase kinase-3β (GSK-3β), is a mammalian homologue of zw3/shaggy kinase (Siegfried et al., 1992; Ruel et al., 1993). Considering that the zw3/shaggy kinase was genetically shown to be located just upstream of armadillo, GSK-3β is expected to phosphorylate and unstabilize β catenin. In fact, both β catenin and armadillo contain a potential GSK-3β phosphorylation site (GSKP sequence) at their amino-terminal region. In vertebrates, in addition to GSK-3β, the product of the *APC* gene, a tumor suppresser gene in familial adenomatous polyposis, is also involved in the regulation of the stability of β catenin (Rubinfeld et al., 1993; Su et al., 1993; Munemitsu et al., 1995; Rubinfeld et al., 1996). It is then important in vertebrates to evaluate whether or not GSK-3β phosphorylates and unstabilizes β catenin, and how the APC gene product is involved in this unstabilization mechanism.

β catenin has dual roles, i.e. as a regulator for the cadherin-based cell adhesion and as a transducer in the *Wnt* signaling pathway. The question is then whether or not these two roles are inter-dependent or independent *in vivo*. The amount of β catenin involved in the cadherin/catenin complex is not reportedly regulated by the

Wnt signaling (Orsulic and Peifer, 1996). Both in *Drosophila* armadillo and *Xenopus* β catenin molecules, the domain specific for the *wingless/Wnt* signaling or for the cadherin/catenin complex formation can be identified separately (Funayama et al., 1995; Orsulic and Peifer, 1996). These findings favor the notion that the dual roles of β catenin are independent. However, since most of experiments were performed using cells or tissues expressing cadherin molecules, it is difficult to clearly discriminate these dual roles of β catenin.

In this study, we attempted to analyze the role of β catenin in the intracellular signaling in mouse L fibroblasts lacking the cadherin-based cell adhesion system. We constructed cDNA encoding the mutant β catenin with amino acid substitutions at its putative GSK-3β phosphorylation site and introduced it into L cells. This mutation stabilized not only the mutated β catenin but also the endogenous β catenin in L fibroblasts. When the nuclear localization signal was added to this stabilized mutated β catenin, β catenin was concentrated in the nucleus as a stable form. Interestingly, in the L fibroblasts bearing stabilized β catenin in the nucleus, the integrin-based cell adhesion activity was significantly elevated. Our system allowed us to analyze the intracellular signaling role of β catenin as completely separated from its role in the cadherin-dependent adhesion, and to find out the possible β catenin-dependent regulation of the integrin-based cell adhesion system.

Materials and Methods

Cells and Antibodies

Mouse L cells (Earle, 1943) were grown in DMEM supplemented with 10% FCS. The various stable L transfectants were grown in the same medium containing 50 μg/ml of G418. Mouse anti-β catenin mAb and mouse anti-GSK-3β mAb were purchased from Transduction Laboratories (Lexington, KY). Mouse anti-HA mAb, rabbit anti-HA pAb, mouse anti-T7 mAb, and rabbit anti-GFP pAb were purchased from Boehringer Mannheim (Mannheim, Germany), MBL (Nagoya, Japan), Novagen (Madison, WI) and Clontech (Palo Alto, CA), respectively. Rabbit pAbs against extracellular matrix proteins such as fibronectin, collagen, vitronectin etc. and various types of integrins were purchased from Chemicon International Inc. (Temecula, CA). Rabbit anti-APC pAb was a gift from Dr.T.Akiyama (Osaka University, Osaka, Japan) (Matsumine et al., 1996). A pan-cadherin antibody that recognized classic cadherins has previously been discribed (Takeichi et al., 1991).

Constructs and Transfections

A mouse β catenin cDNA fragment encoding amino acids 104-664 was isolated from mouse F9 embryonal carcinoma total RNAs by RT-PCR. Using this PCR fragment as a probe, we screened a λgt11 library made from mouse F9 poly(A)+ RNAs. Four positive clones were isolated, and the longest one with ~140 bp of 5'UTR, 2343 bp of ORF, and ~700 bp of 3'UTR was subcloned into pBluescript (designated as pBC).

We constructed five types of expression vectors (pEFBCha, pEFMMha, pEFMMGFP, pEFMMt7, and pEFMMnlsha) for wbH, mbH, mG, mbT, and mbHN molecules, respectively (see Fig.1). We used the following plasmids: a vector carrying three HA epitopes in tandem and the termination codon at SpeI in pBluescript (designated as pHAs), a vector carrying four T7 epitopes in tandem and the termination codon

between PstI and SpeI in pBluscript (designated as pTS), phGFP-S65T encoding green fluorescent protein (Clontech, Palo Alto, CA), and pExEF containing human polypeptide chain elongation factor 1α promoter (Mizushima and Nagata, 1990) at EcoRI and a poly(A)adenylation signal at PstI of pUC19. For the production of pEFBCha, the β-catenin ORF, whose termination codon was changed with a restriction site by the PCR methods, was inserted into the amino-terminal end of HA epitopes of pHAs in frame. This tagged β catenin fragment was then transferred to pExEF between the promoter and the poly(A) signal. For the production of pEFMMha and pEFMMt7, mutant β catenin cDNA was generated by PCR with overlapping oligonucleotides to introduce the amino acids substitutions shown in Fig.1. After the termination codon was changed with a restriction site, it was inserted into pHAs or pTS, then to pExEF. For pEFMMnlsha, the synthetic oligonucleotide encoding the nuclear localization signal derived from SV40 large-T antigen (GSTPPKKKRKVEEFGS) (Kalderon et al., 1984) was inserted into pEFMMha between the β catenin and HA epitopes. For pEFMMGFP, the synthetic oligonucleotide encoding the mutated putative GSK-3β phosphorylation site (see Fig.1) was inserted to the amino-terminal region of GFP, which was then transferred to pExEF.

L cells (1 x 10^7 cells) were co-transfected with 3 μg of each expression vector and 0.3 μg of pSTneoB (Katoh et al., 1987) by electroporation using a Bio-Rad Gene Pulser electroporator (960 μF, 200 V). The cells were plated on three 6-cm dishes and cultured in the presence of 400 μg/ml G418 to select stable transfectants. Colonies of G418-resistant cells were isolated, recloned, and subsequently maintained in complete medium with 50 μg/ml of G418. L transfectants co-expressing wbH and mbT were isolated by cotransfection with 3 μg of pEFMMt7 and 0.3 μg of pSV2hph (Kaster et al., 1983) to L transfectants expressing wbH. The cells were selected in the presence of 500 μg/ml hygromycin B. We isolated several independent clones for each transfection experiment. Since LwbH-e1, LmbH-l2,

LmG-f, LmbHN-q2, and LwbHmbT-β subclones expressed a relatively large amount of wbH, mbH, mG, mbHN, and wbH/mbT mRNAs, respectively (see Fig.1), we used them for the demonstrations in this paper.

Northern Blotting

Total RNAs from parent L cells and various transfectants were isolated according to the method described by Maniatis et al. (1989). 10 μg of total RNA was subjected per well to electrophoresis and blotted onto nylon membranes. A 3.2-kb fragment of pBC including the full-length β catenin cDNA and a 0.09-kb fragment of pHAs encoding 3 copies of HA epitopes were labeled with [α-^{32}P]dCTP using *Bca*BEST Labeling Kit (Takara, Kyoto, Japan), and were used as probes. Prehybridization and hybridization were carried out at high stringency [50 mM phosphate buffer (pH 6.5), 5x SSC, 5x Denhart's solution, 1% SDS, 100 μg/ml boiled salmon sperm DNA, 100 μg/ml yeast tRNA, and 50% formamide]. Hybridized filters were washed twice by 1st washing solution (2x SSC, 0.1% SDS) for 15 min at room temperature, and then twice by 2nd solution (0.1x SSC, 0.1% SDS) for 15 min at 65℃.

SDS-PAGE and Immunoblotting

SDS-PAGE was based on the discontinuous Tris-glycine system of Laemmli (1970). Cultured cells were washed in Hepes-buffered saline [HBS; 25 mM Hepes-NaOH (pH 7.05), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM Dextrose] supplemented with 1 mM $CaCl_2$, then homogenized and boiled in SDS-sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 0.1% bromophenol blue, and 10% glycerol]. The lysate containing 10 μg or 30 μg of total protein was separated by SDS-PAGE and transferred to nitrocellulose sheets electrophoretically. After blocking with 2% skim milk in TBS, nitrocellulose transfers were incubated with each antibody. Antibody detection was performed using the biotin-streptavidin system with

biotinylated second antibodies and the NBT-BCIP system (Blotting Detection Kit; Amersham, Arlington Heights, IL).

Immunofluorescence Microscopy

Cells cultured on cover slips were fixed with 3.5% formaldehyde in PBS for 15 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 15 min. The fixed cells were blocked with 1% BSA in PBS for 10 min, then incubated with each antibody diluted with PBS containing 1% BSA for 60 min. After extensive washing with PBS containing 1% BSA, the specimens were incubated with fluorescence-labeled second antibodies diluted with PBS containing 1% BSA for 30 min. After washing thoroughly with PBS, samples were mounted in 90% glycerol-PBS containing 0.1% para-phenylendiamine and 1% n-propyl gallate., and examined with a Zeiss Axiophot photomicroscope. Cell aggregates were washed with HBS containing 1 mM $CaCl_2$, attached to polyethylenediamine-coated cover slips for 15 min at 37℃, then processed for immunostaining as described above.

Long-term Cell Aggregation Assay

Semi-confluent cultured cells (~1 x 10^7 cells per 10-cm dishes) were washed with HBS containing 1 mM $CaCl_2$, incubated with 0.1% trypsin in the same saline for 10 min, then dissociated by pipetting to obtain single cells. 2.5 x 10^5 cells were suspended in 1 ml DMEM containing 10% FCS and placed on an agar-coated well of a 12-well plate dish. The dish was gently rotated at 80 rpm for 3 h at 37℃. In some experiments, during rotation, 1 mg/ml Fibronectin Related Peptides SP001 (GRGDSP) or SP002 (GRGESP) (Takara, Kyoto, Japan) were added to the medium. After the incubation, the cells were pipetted vigorously, placed on new dishes, and photographed. For quantitation, more than 600 particles (single cells and cell aggregates) were randomly chosen for each experiment, and the number of cells in

each particle was counted (see Fig.8).

Results

Stable Expression of Mutant β Catenin with Amino Acid Substitutions at Putative GSK-3β Phosphorylation Site in L Fibroblasts

β catenin contains a consensus sequence for the GSK-3β phosphorylation (S/T XXX S/T XXX S/T XXX S/T) at the amino-terminal region (Fig.1) (Fiol et al., 1987). This is also found in the amino-terminal corresponding region of the *armadillo* product (Peifer et al., 1994b). We generated cDNA encoding mutant β catenin with amino acid substitutions at the putative GSK-3β phosphorylation site (GSKP sequence). In this mutant, one threonine and three serine residues were substituted with four alanine residues as shown in Fig.1. To detect this mutant, HA or T7 epitope was tagged to the carboxyl-terminal end, which is called mbH or mbT, respectively. As a control construct, HA epitope was tagged to the carboxyl-terminus of wild type β catenin (designated as wbH).

In mouse L fibroblasts, a significant amount of β catenin mRNA and only a trace amount of β catenin protein is detectable, indicating that the endogenous β catenin protein is fairly unstable (Fig.2). To test whether the GSKP sequence is involved in the β catenin instability in L fibroblasts, we introduced the expression vector for wbH or mbH into L cells. After G418 selection, we isolated several stable transfectants expressing mRNAs of wbH or mbH (Fig.2). In L transfectants expressing wbH (LwbH), its mRNA was easily detected by Northern blotting, whereas its protein was hardly detected by Western blotting with anti-HA mAb. By contrast, in L transfectants expressing mbH (LmbH), its protein appeared to be stabilized enough to be detected with anti-HA mAb. The mRNA level of mbH in LmbH was compatible with that of wbH in LwbH. Considering that the GSKP sequence is mutated in mbH, these findings suggest that in L fibroblasts the GSK-3β -dependent phosphorylation

unstabilizes the β catenin protein.

Stabilization of Wild Type β Catenin by Mutant β Catenin

As shown in Fig.2B, when the whole cell lysate of LmbH was immunoblotted with anti-β catenin mAb, two closely-migrating bands were detected. The electrophoretic mobilities of the upper and lower bands were identical to those of mbH and wild type β catenin, respectively, and only the upper band was detected by anti-HA mAb, suggesting that the lower band corresponds to the endogenous β catenin. Considering that endogenous β catenin was hardly detected in parent L cells, we suspected that in LmbH, the endogenous β catenin as well as mbH are stabilized. It is still, however, possible that the lower band is attributed to the degraded product of stabilized mbH, in which only the HA tag was cut off. We then obtained L transfectants co-expressing wbH and mbT (LwbHmbT). As shown in Fig.3, when wbH was singly expressed in L cells (LwbH), the protein expression level of wbH as well as endogenous β catenin was negligible. In sharp contrast, in LwbHmbT, not only mbT but also wbH were remarkably stabilized at the protein level, which were respectively detected with anti-T7 and anti-HA mAbs. Furthermore, in LwbHmbT, we detected another β catenin-positive, T7/HA-negative band, which may correspond to the stabilized endogenous β catenin. Thus, we conclude that the expression of mutant β catenin proteins such as mbH and mbT in L cells significantly stabilizes the wild type β catenins such as endogenous β catenin and exogenous wbH at the protein level.

To examine whether or not the mutated GSKP sequence is sufficient to stabilize the wild type β catenin protein, we constructed cDNA encoding green fluorescence protein (GFP) with the mutated GSKP sequence (*mG*; see Fig.1) and introduced its expression vector into L cells. Several stable transfectants expressing mG (LmG) were obtained, and their protein expression level of endogenous β catenin was examined.

As shown in Fig.4, as compared to the parent L cells, endogenous β catenin was significantly stabilized in LmG. These data indicate that the mutated GSKP sequence is sufficient to stabilize of the endogenous β catenin.

Mutant β Catenin Carrying the Nuclear Localization Signal

We succeeded in stabilizing wild as well as mutant types of β catenin in L fibroblasts lacking the cadherin cell adhesion system. These mutant L transfectants may provide an advantageous system in which we can examine the role of β catenin in the intracellular signaling completely separated from its role in the cadherin-dependent cell adhesion. Before intensively analyzing the phenotypic changes of these transfectants, we further constructed cDNA encoding mutant β catenin carrying the nuclear localization signal, with the expectation that the nucleus-localizing stabilized β catenin would induce more marked phenotypic changes. As shown in Fig.1, the nuclear localization signal was inserted into mbH between the carboxyl-terminus of β catenin protein and HA tag. When this construct (mbHN) was expressed in L cells (LmbHN), mbHN itself as well as endogenous β catenin was again stabilized at the protein level (Fig.5).

Next we immunofluorescently stained LmbH and LmbHN cells with anti-HA mAb (Fig.6). In LmbH, stabilized mbH was distributed both in the cytoplasm and the nucleus, whereas in LmbHN most of the stabilized mbHN was found in the nucleus. Although endogenous wild type β catenin could not be selectively stained in these cells, immunostaining with anti-β catenin mAb revealed the superimposed distribution of mutant and wild types of β catenin. Also in such total β catenin staining, β catenin was predominantly localized in the nucleus in LmbHN, in sharp contrast to LmbH where β catenin was evenly distributed in the cytoplasm and the nucleus.

Activation of Integrin-based Adhesion in LmbH and LmbHN

In response to the *Wnt* signal, β catenin is thought to be stabilized, be translocated into the nucleus and determine the cell fate (Funayama et al., 1995; Molenaar et al., 1996; Behrens et al., 1996; Orsulic and Peifer, 1996). Our knowledge of the function of β catenin in the nucleus is still limited, but β catenin has recently been reported to modulate the E-cadherin transcription (Huber et al., 1996). We then examined the cadherin expression and the cadherin-based cell adhesion activity of LmbH and LmbHN. However, pan-cadherin pAb which recognizes various cadherin subclasses did not detect any cadherin expression in these transfectants. Cell dissociation and aggregation assays could not at all detect the cell adhesion activity mediated by cadherin molecules in these transfectants (data not shown).

During the course of the cell aggregation assay with these cells, we noticed their strange cell adhesion activity which was not dependent on cadherins. When single dissociated cells were allowed to aggregate for 3 h in the normal culture medium, L cells expressing stabilized β catenin such as LmbH and LmbHN formed a large number of small cell aggregates as shown Fig.7. This type of cell aggregation was rare in parent L cells. Fig.8 summarizes the quantitative results of the long-term cell aggregation assay, in which we counted the number of cells in each particle. In the parent L cells, about 70% of the total cells remained dissociated. In LmbH and LmbHN, 40% and 20% were not aggregated, respectively, and the other cells formed aggregates mainly consisting of 2-5 cells. In L, LmbH, and LmbHN, 2%, 4%, and 18% of the total cells formed aggregates consisting of 5 cells, respectively. The cell aggregation activity was in the order of LmbHN>LmbH>L.

Although neither LmbH nor LmbHN showed any cadherin-dependent cell adhesion activity, their cell aggregates were compact (see Fig.7D) and resisted dissociation by vigorous pipetting. This type of aggregation required the presence of serum in the culture medium during the long-term aggregation assay. We then

suspected that some extracellular matrix protein mediates the cell adhesion, and searched for it by immunofluorescence microscopy. As shown in Fig.9, fibronectin but not collagen or vitronectin was clearly concentrated at cell-cell contact sites in aggregates of LmbH and LmbHN. In good agreement, among the various isotypes of integrin α subunits, $\alpha 5$ subunit was also highly concentrated at cell-cell contact sites. Furthermore, the RGD peptide but not the RGE peptide suppressed the cell aggregation activity of LmbHN (Fig.10). We therefore conclude that in LmbHN (also in LmbH to a lesser extent) the integrin-based cell adhesion is up-regulated as compared to parent L cells. Actually, we confirmed that LmbH and LmbHN adhere to serum-covered slide glass more rapidly than parent L cells (data not shown). As far as we could examine by Northern blotting or immunoblotting, however, no significant change in the expression level of each isotype of integrin subunits has been detected in LmbH or LmbHN.

Discussion

Advantage of L Transfectants Expressing β Catenin with Mutations at Putative GSK-3β Phosphorylation Site in the Study of β Catenin Signaling

In the study of the possible involvement of β catenin in the *Wnt* signaling pathway, we face two types of technical difficulties. First, judging from the genetical analyses of *wingless/armadillo* signaling in *Drosophila*, β catenin is expected to be phosphorylated by GSK-3β in the absence of the *Wnt* signal, and this phophorylated form is highly susceptible to degradation (Peifer et al., 1994ab). This means that the phosphorylation level of β catenin is important in terms of the *Wnt* signaling, but the phosphorylated type is difficult to detect experimentally. Secondly, not only the *Wnt* signaling but also the cadherin/catenin complex formation stabilizes β catenin at the protein level, making it difficult to selectively examine the *Wnt*-dependent stabilization of β catenin. In this study, we were able to circumvent these difficulties by the following ways. We stabilized β catenin by introducing mutations at the putative GSK-3β phosphorylation site (GSKP sequence). Since this mutant β catenin was expected not to be phosphorylated by GSK-3β, it would work as a constitutively-activated factor in the *Wnt* signaling pathway. Furthermore, we introduced this construct into mouse L fibroblasts. Since L cells lack the expression of cadherins, we discriminated the *Wnt*-dependent stabilization of β catenin from the cadherin-dependent stabilization.

At the beginning of this study, there were two assumptions which would obscure the physiological relevance of the β catenin stabilization in mouse L fibroblasts. There was no direct evidence to show that β catenin is phosphorylated by GSK-3β and that this phosphorylation is involved in the *Wnt* signaling in vertebrate systems. While this study was being conducted, however, Yost et al. (1996) adopted a similar

experimental strategy for the β catenin stabilization in the *Xenopus* system. They showed that the putative GSK-3β phosphorylation site of β catenin is actually phosphorylated *in vitro* by GSK-3β, and that β catenin with the mutated putative GSK-3β phosphorylation site behaved as a dominant-active construct in the dorsal-ventral axis formation, which is supposed to be regulated by *Wnt* signaling.

Ectopic expression of Wnt protein in L cells causes the accumulation of β catenin protein (Takada and Takeichi, personal communication), and significant amount of GSK-3β was detected in the cytoplasm of L cells (data not shown). Taking all these results into consideration, we concluded that in L cells the stability of β catenin is regulated by *Wnt* signaling through the GSK-3β-dependent phosphorylation, leading us to speculate that some functions of L cells are physiologically regulated by *Wnt* signaling.

Molecular Mechanism for the β Catenin Stabilization

When β catenin with mutations at the GSKP sequence was expressed in L cells, it was significantly stabilized at the protein level. Interestingly, in these L transfectants (LmbH), the endogenous β catenin was also stabilized remarkably. Furthermore, overexpression of GFP carrying the mutated GSKP sequence at its amino-terminal end also stabilized the endogenous β catenin. These findings suggest that not only the wild-type but also the mutated GSKP sequence can bind to the putative degradation machinery for β catenin. Only when the GSKP sequence is phosphorylated, the associated machinery may digest the β catenin molecule. When the mutated GSKP sequence is overexpressed, it may occupy the degradation machinery without being degraded, resulting in the stabilization of the endogenous wild-type. Of course, this is one of the possible mechanisms to explain the stabilization of the endogenous β catenin in LmbH, but what is the physiological relevance of this mechanism in normal L cells ? In L cells, it would work as a positive

feedback system in the *Wnt* signaling. When GSK-3β is initiated to be down-regulated by the *Wnt* signaling, the increase of non-phosphorylated β catenin itself occupies and suppresses the degradation machinery, accelerating the β catenin stabilization. It is tempting to speculate that, when the stabilization of proteins is utilized in the intracellular signaling pathway in general, this type of positive feedback system is advantageous to amplify the signal.

The degradation machinery for β catenin itself remains elusive. The APC gene product is reportedly involved in the degradation of β catenin (Munemitsu et al., 1995; Rubinfeld et al., 1996; Papkoff et al., 1996; Munemitsu et al., 1996). Since the normal size of APC is expressed in L cells (data not shown), it may be directly or indirectly included in the machinery. Various proteases constituting proteasomes are also probably involved in the β catenin degradation. To identify the degradation machinery for β catenin, the normal as well as mutated GSKP sequences will be utilized as useful probes.

Activation of Integrin-based Cell Adhesion by β Catenin Signaling

A large amount of evidence obtained in *Drosophila* and *Xenopus* indicates that *armadillo* as well as vertebrate β catenin functions as a signal transducer to determine the cell fate, which is independent from their role in the cadherin-based cell adhesion (Funayama et al., 1995; Orsulic and Peifer, 1996). Furthermore, both armadillo and β catenin are reportedly localized in the nucleus, when they appear to be involved in the *wingless/Wnt* signaling pathway (Funayama et al., 1995; Orsulic and Peifer, 1996). In this connection, β catenin was found to bind directly to a transcription factor, LEF-1, to co-translocate from the cytoplasm to the nucleus (Molenaar et al., 1996; Behrens et al., 1996; Huber et al., 1996). Also in mammalian cells, β catenin is suggested to have independent dual roles, which are namely the regulation of cell growth and the

cadherin-based cell adhesion (Whitehead et al., 1995). In this study, we succeeded in expressing the stabilized β catenin predominantly in the nucleus of L cells by introducing β catenin bearing the mutated GSKP sequence and the nuclear localization signal. This transfectant (LmbHN) provides us with an advantageous system to experimentally analyze the functions of β catenin in the nucleus.

Overexpression of β catenin reportedly transforms mouse NIH3T3 cells (Whitehead et al., 1995). However, since parent L cells grow in an anchorage-independent manner, LmbHN was not appropriate to examine the transformation activity of the nuclear β catenin. The β catenin/LEF-1 complex reportedly modulates the transcription level of E-cadherin (Huber et al., 1996), but LmbHN showed no detectable cadherin-dependent cell adhesion activity. Further analyses of cell aggregation activity of LmbHN as well as LmbH, however, led us to find unexpected phenotypic changes in these cells. Under the long-term aggregation condition, LmbHN (and LmbH to a lesser extent) formed compact cell aggregates consisting mainly of 2-5 cells. Detailed analyses by immunofluorescence microscopy and the cell adhesion inhibition assay with RGD peptides revealed that the integrin/fibronectin-dependent cell adhesion is activated in LmbH and LmbHN. This activation was detected more prominently in LmbHN than in LmbH. Considering that the amount of the nuclear β catenin is lager in LmbHN than in LmbH, we speculate that the nuclear rather than the cytoplasmic β catenin up-regulates the integrin-based cell adhesion in these cells.

The molecular mechanism of the activation of integrin-based cell adhesion by the nuclear β catenin remains elusive. Most simply, it is possible that the nuclear β catenin directly regulates the transcription level of various integrin subunits, but as far as we have been able to examine by Northern blotting and immunoblotting, no elevated production of integrins was detected in LmbHN or LmbH cells as compared to parent L cells (data not shown). Since β catenin is believed to lead to changes in

gene expression during embryogenesis (Miller and Moon, 1996), it is likely that the nuclear β catenin induces the transcription of some proteins which up-regulate the integrin-based cell adhesion. Subtraction screening of cDNA between L and LmbHN cells may be a promising way to identify such a key protein.

So far the relationship between the *Wnt*/β catenin signaling and the integrin-based cell adhesion has not been given much attention. In vertebrates, the *Wnt*/β catenin signaling is one of the important morphogenic regulation mechanisms, and the integrin-based cell adhesion is also one of the key systems in morphogenesis (Hynes, 1992). It is noteworthy to further analyze this relationship at the molecular level. Furthermore, considering that β catenin reportedly regulates the transcription of E-cadherin (Huber et al., 1996), studies along this line will also lead us to a better understanding how the integrin-based and cadherin-based cell adhesion systems are coordinated in various types of organogenesis.

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Figure 1. Structure of wild-type and mutant β catenin polypeptides. HA epitope was tagged to the carboxyl-terminus of wild type β catenin (*wbH*). In mutant β catenins (*mbH*, *mbT*, and *mbHN*), one threonine and three serine residues in the putative GSK-3β phosphorylation site (GSKP sequence; 33-45 a.a. in wild-type β catenin) were substituted with four alanine residues, and HA or T7 epitope was tagged to their carboxyl end. In mbHN, the nuclear localization signal was inserted between β catenin and the HA epitope. A fusion protein between the GSKP sequence and the green fluorescence protein was also produced (*mG*).

Figure 2. Stable L transfectants expressing HA-tagged wild-type (wbH) or mutated β catenin (mbH). In parent L cells (*L*) and L transfectants expressing wbH (*LwbH*) or mbH (*LmbH*), the expression level of endogenous and exogenous β catenins were examined both at mRNA and protein levels by Northern blotting (*A*) and Western blotting (*B*), respectively. In Northern blotting, respective cDNA fragments encoding the HA epitope and the full length β catenin were used for the detection of exogenous and total β catenin mRNA. In Western blotting, exogenous and total β catenin proteins were detected by anti-HA epitope and anti-β catenin mAbs, respectively. In both parent L cells and LwbH, neither exogenous nor endogenous wild type β catenin protein could be detected significantly. By contrast, in LmbH, exogenous β catenin (*mbH*) appeared to be stable enough to be detected with anti-HA or anti-β catenin mAb. In LmbH, in addition to the mbH band, another rapidly-migrating β catenin-positive, HA-negative band was reproducibly detected (*arrow*).

Figure 3. Stable L transfectant co-expressing HA-tagged wild type (wbH) and T7-tagged mutated β catenin (mbT). This transfectant was designated as LwbHmbT (*LwbHmbT*). In LwbHmbT and L transfectant singly expressing wbH (*LwbH*), the protein expression levels of mbT and wbH were compared by Western blotting with anti-T7 epitope and anti-HA epitope mAbs, respectively. Total β catenin proteins including endogenous (*endo*) and exogenous β catenin (*mbT* and *wbH*) were detected with anti-β catenin mAb in parent L cells (*L*) as well as in LwbH and LwbHmb*T* cells. These β catenin proteins migrated differently in SDS-PAGE depending on the epitope added. In LwbHmbT, not only mbT but also wbH were stabilized. Furthermore, endogenous β catenin (*endo*) also appeared to be stabilized.

Figure 4. Stable L transfectant (*LmG*) expressing a fusion protein (*mG*) between the mutated putative GSK-3β phosphorylation sequence and green fluorescence protein (*GFP*). As compared to parent L cells (*L*), LmG expressed stabilized endogenous β catenin that was detected by Western blotting with anti-β catenin mAb (*endo*). The expression of mG in LmG was confirmed by Western blotting with anti-GFP pAb (*mG*).

Figure 5. Stable L transfectant expressing a mutant β catenin carrying the nuclear localization signal and HA epitope (mbHN). In parent L cells (*L*) and L transfectants expressing a mutant β catenin tagged with HA epitope (*LmbH*) or mbHN (*LmbHN*), the exogenous and total β catenin were detected by Western blotting with anti-HA and anti-β catenin mAbs, respectively. Similar to LmbH, in LmbHN both exogenous (*mbHN*) and endogenous β catenins (*endo*) were stabilized.

Figure 6. Immunofluorescence localization of total (*A,C,E*) and HA-tagged exogenous β catenin (*B,D,F*) in parent L cells (*A,B*) and L transfectants expressing mbH (LmbH;*C,D*) or mbHN (LmbHN;*E,F*). As compared to parent L cells, in LmbH both exogenous (mbH) and endogenous β catenins were stabilized and distributed both in the cytoplasm and the nucleus. In LmbHN, exogenous β catenin (mbHN) was stabilized and localized in the nucleus (*F*). Interestingly, endogenous β catenin also appeared to be concentrated in the nucleus (*E*). Bar, 40 μm.

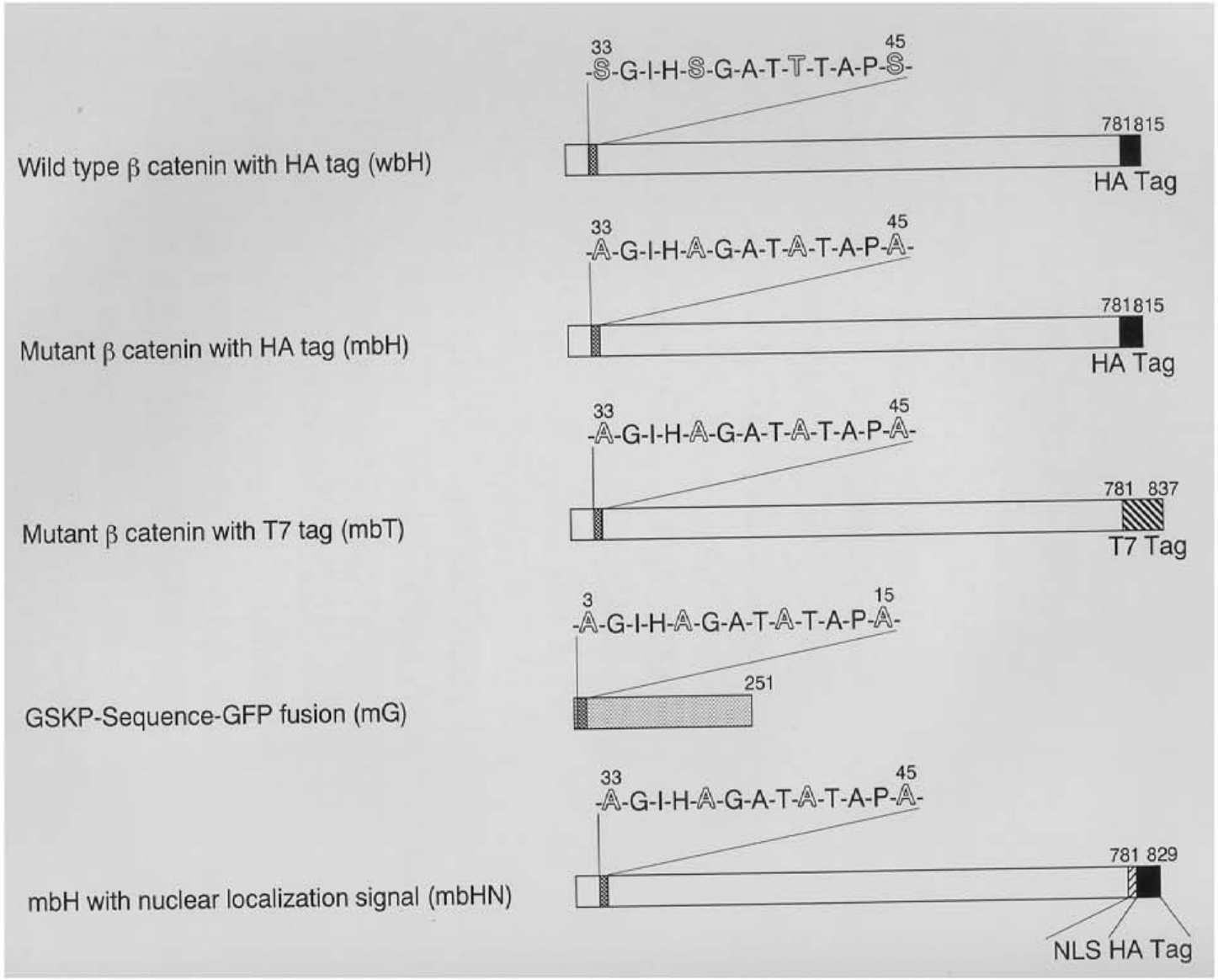
Figure 7. Long-term cell aggregation assay of parent L cells (*A*) and L transfectants expressing mbH (LmbH;*B*) or mbHN (LmbHN;*C,D*). Semi-confluent cultured cells were incubated with 0.1% trypsin and dissociated by pipetting. Dissociated cells were allowed to aggregate by gentle rotation for 3 h at 37℃, and were observed by phase contrast microscopy. Note that LmbHN (and LmbH to a lesser extent) formed a large number of small cell aggregates, which are enlarged in *D*. Bars, 200 μm in *A-C* and 20 μm in *D*.

Figure 8. The degree of aggregate formation of parent L cells (*L*) and L transfectants expressing mbH (*LmbH*) or mbHN (*LmbHN*). Semi-confluent cultured LmbHN cells were incubated with 0.1% trypsin and dissociated by pipetting. Dissociated cells were allowed to aggregate by gentle rotation for 3 h at 37℃. More than 600 particles (single cells and cell aggregates) were randomly chosen for each type of cell, and the number of cells in each particle was counted.

Figure 9. Concentration of fibronectin and integrin α5 at the cell-cell border of cell aggregates of L transfectants expressing mbHN. Semi-confluent cultured LmbHN cells were incubated with 0.1% trypsin and dissociated by pipetting. Dissociated cells were allowed to aggregate by gentle rotation for 3h at 37℃. Cell aggregates were then immunofluorescently stained with anti-fibronectin pAb (*A*) or anti-integrin α5 pAb (*B*). Same aggregates were also observed by phase contrast microscopy (*C,D*). Bar, 25 μm.

Figure 10. Inhibition of cell aggregation of L transfectants expressing mbHN (LmbHN) by RGD peptide. Semi-confluent cultured LmbHN cells were incubated with 0.1% trypsin and dissociated by pipetting. Dissociated cells were allowed to aggregate by gentle rotation in the absence of peptides (*A*) or in the presence of GRGDSP peptides (*B*) or in the presence of GRGESP peptides (*C*) for 3 h at 37℃. The GRGDSP peptide specifically suppressed the aggregation activity of LmbHN. Bar, 200 μm.

Fig 1


Wild type β catenin with HA tag (wbH)
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45
781815
HA Tag
Mutant β catenin with HA tag (mbH)
33
-A-G-I-H-A-G-A-T-A-T-A-P-A-
45
781815
HA Tag
Mutant β catenin with T7 tag (mbT)
33
-A-G-I-H-A-G-A-T-A-T-A-P-A-
45
781 837
T7 Tag
GSKP-Sequence-GFP fusion (mG)
3
-A-G-I-H-A-G-A-T-A-T-A-P-A-
15
251
mbH with nuclear localization signal (mbHN)
33
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45
781 829
NLS HA Tag

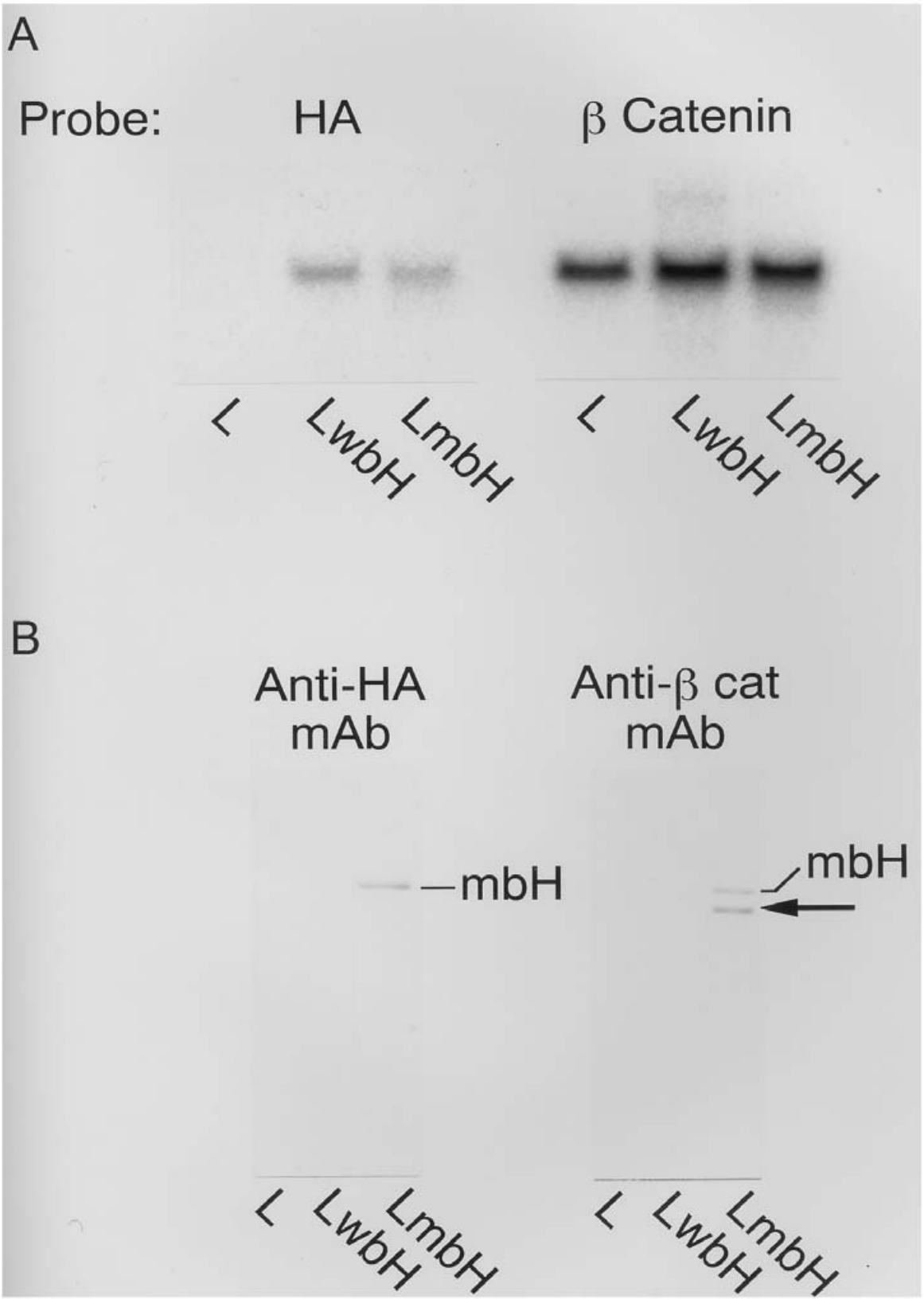

A
Probe:
HA
β Catenin
L
LwbH
LmbH
L
LwbH
LmbH
B
Anti-HA
mAb
Anti-β cat
mAb
—mbH
mbH
L
LwbH
LmbH
L
LwbH
LmbH

Fig 2

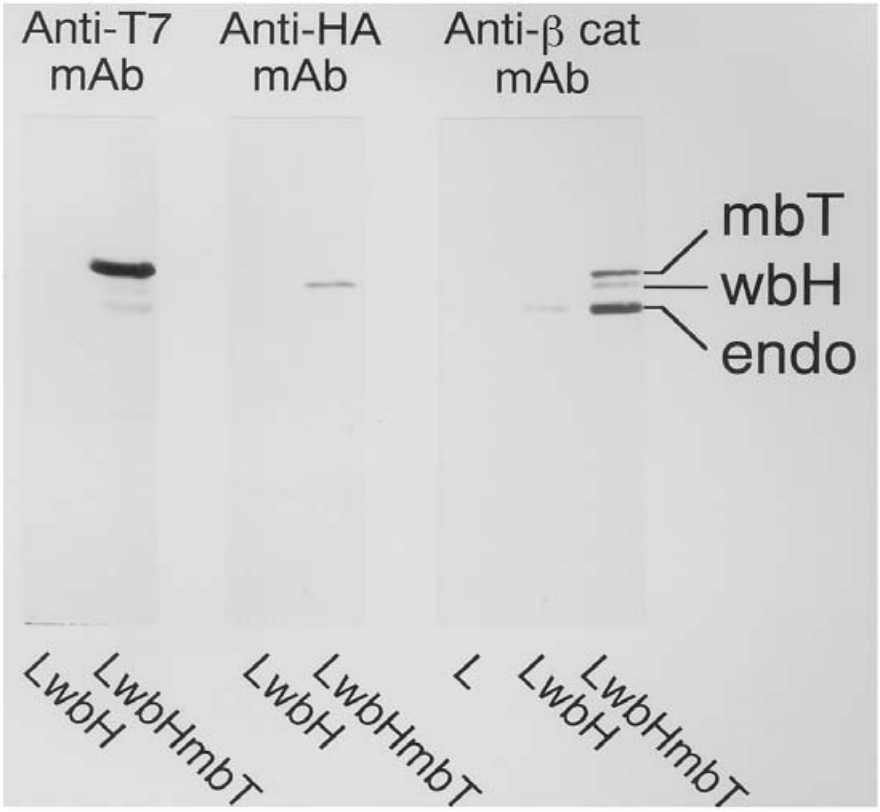

Anti-T7 mAb
Anti-HA mAb
Anti-β cat mAb
mbT
wbH
endo
LwbH
LwbHmbT
LwbH
LwbHmbT
L
LwbH
LwbHmbT

Fig 3

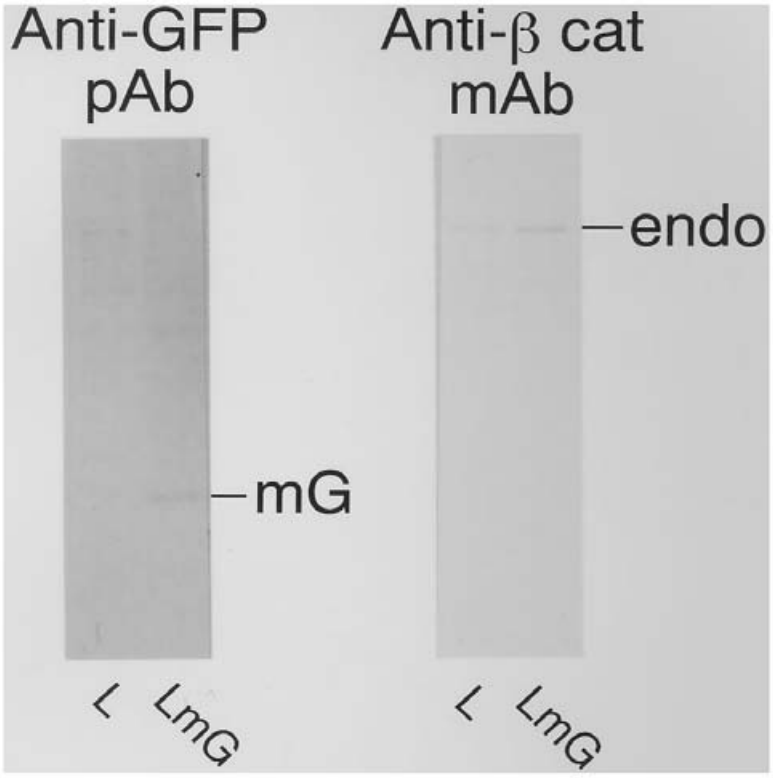


Fig 4

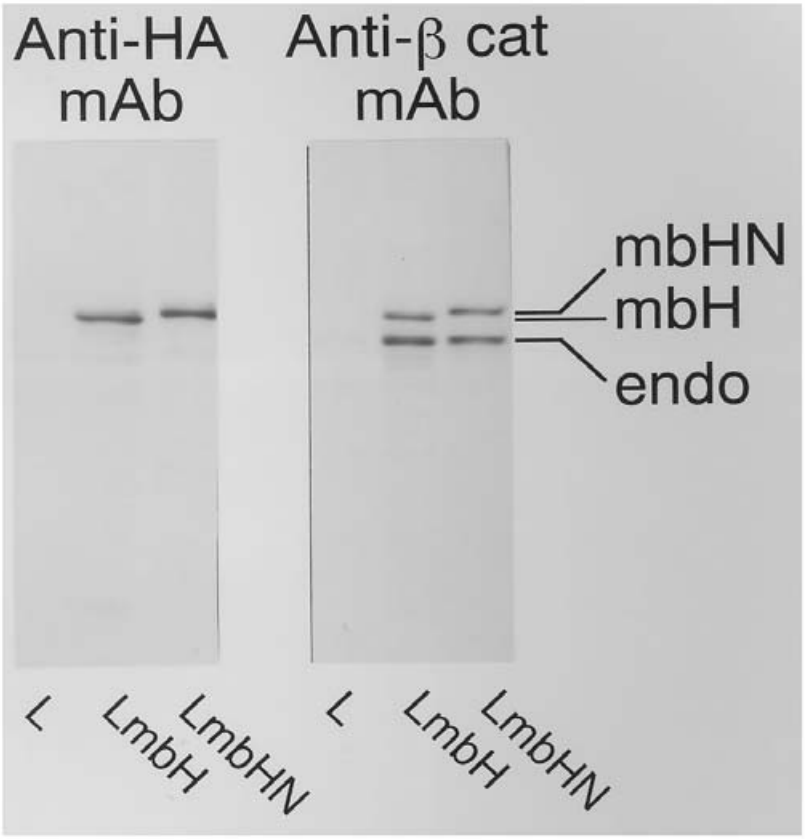

Anti-HA
mAb
Anti-β cat
mAb
mbHN
mbH
endo
L
LmbH
LmbHN
L
LmbH
LmbHN

Fig 5

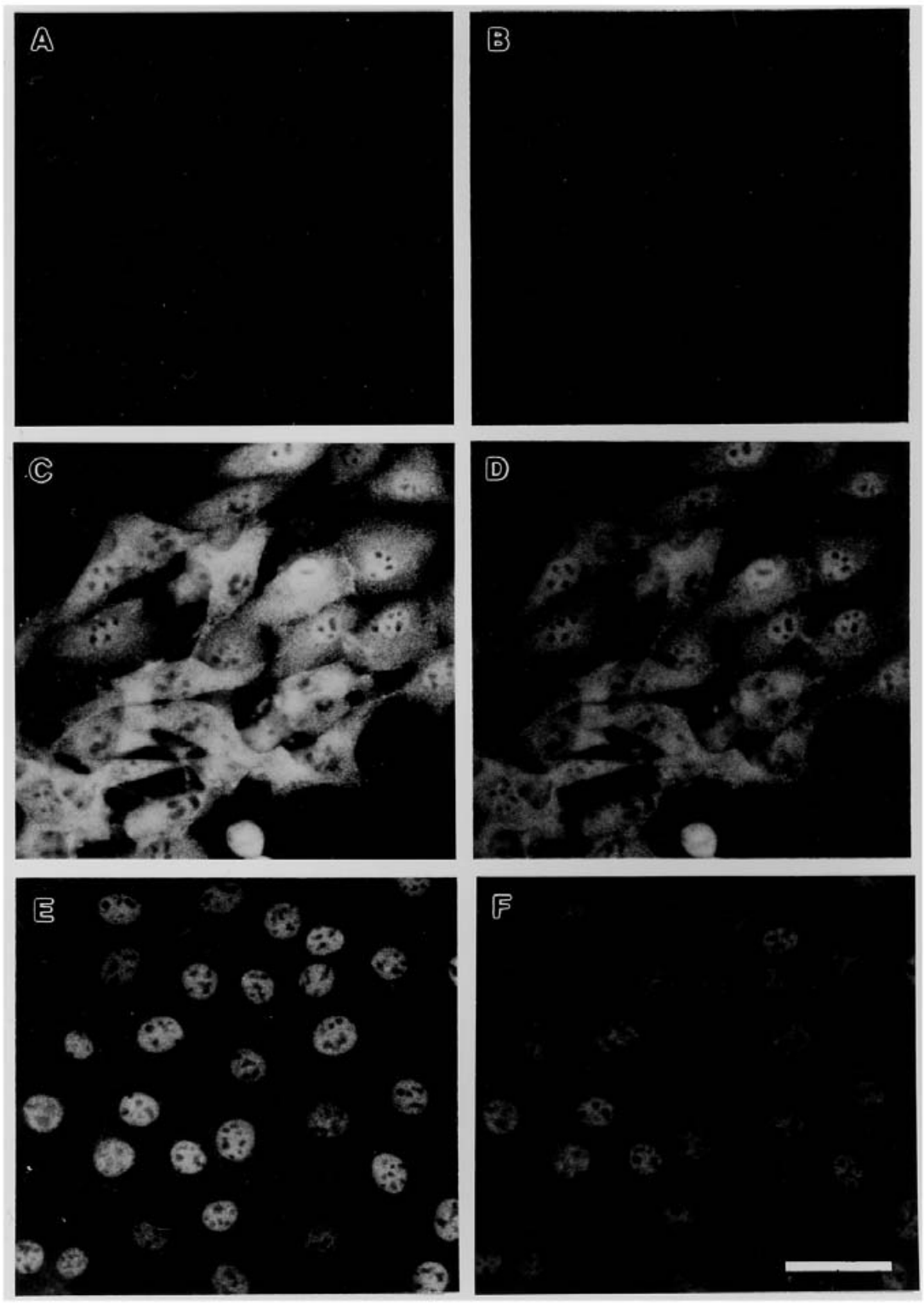


Fig 6

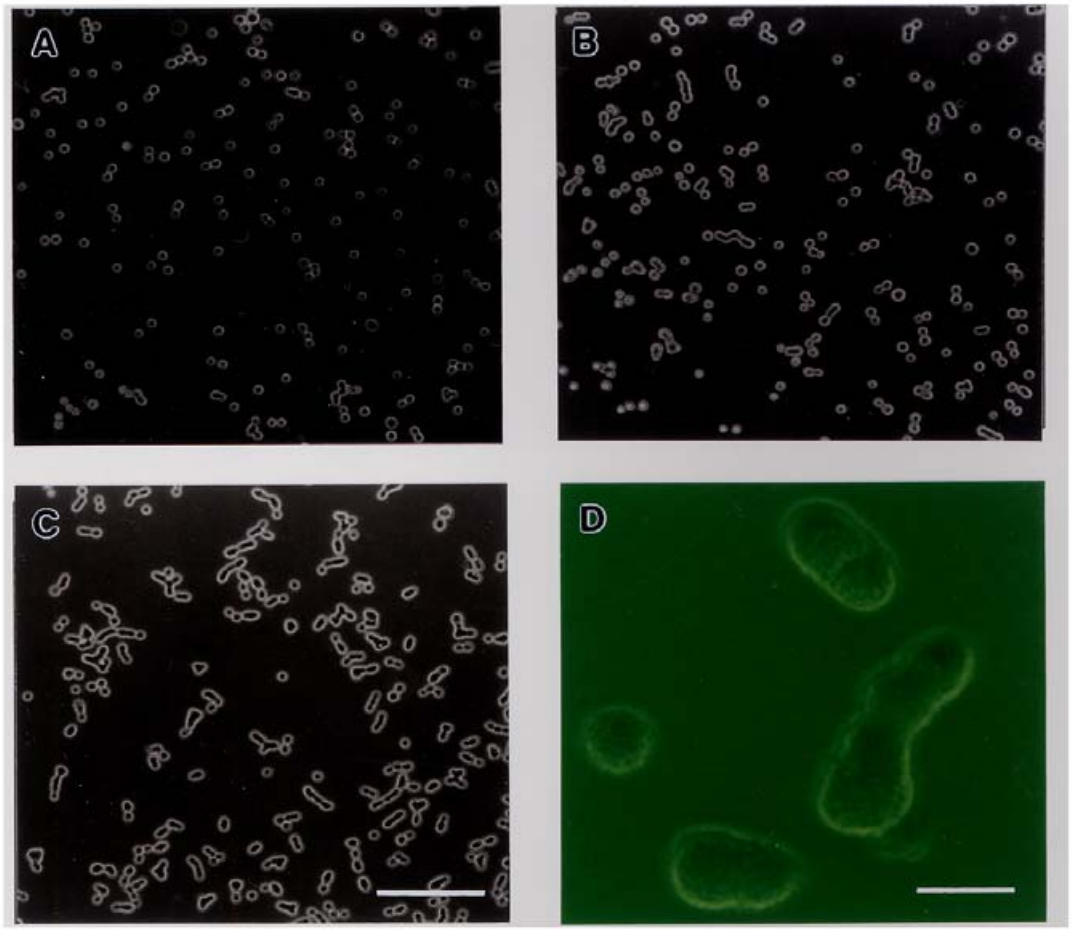
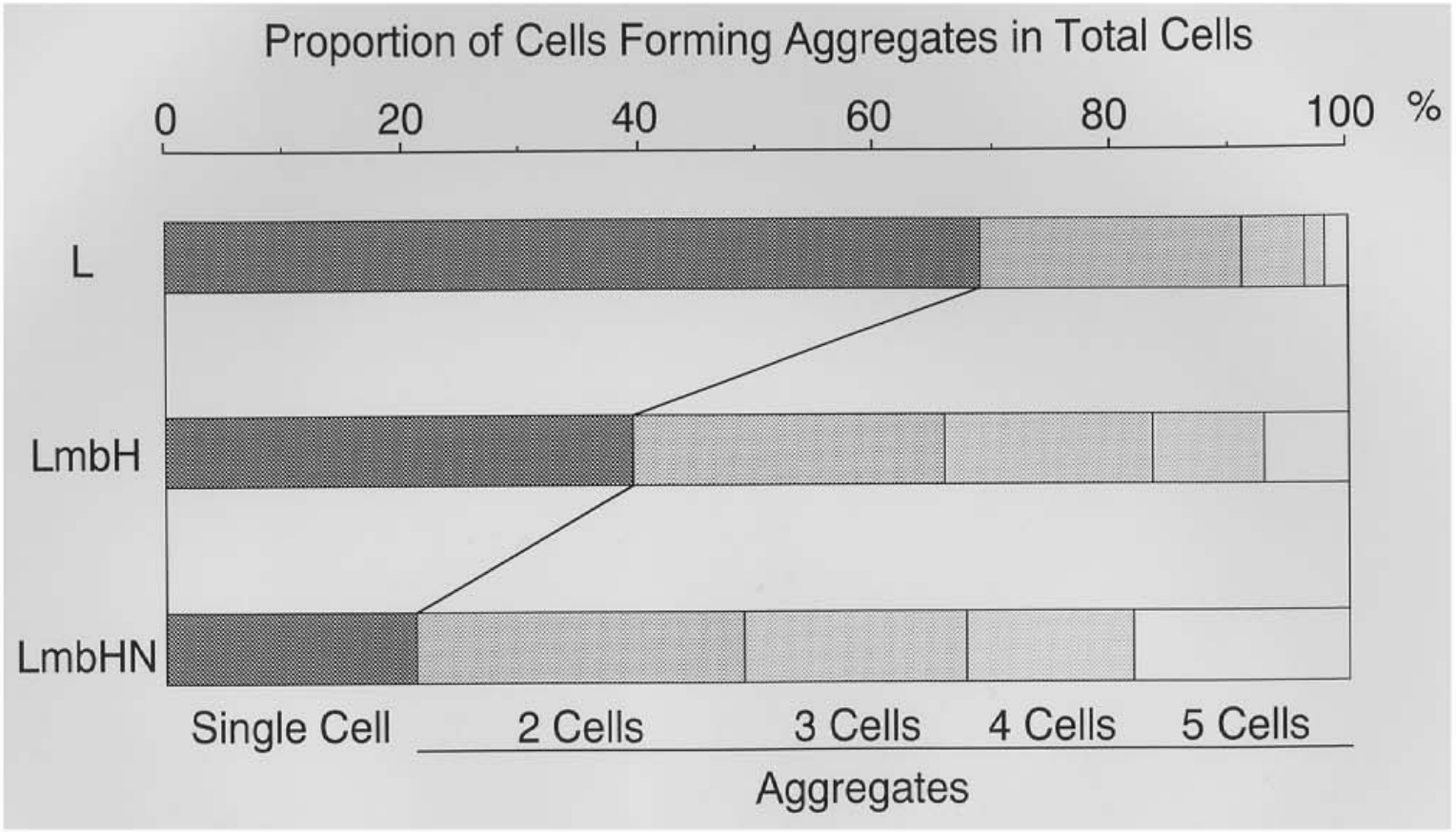


Fig 7

Fig 8


Proportion of Cells Forming Aggregates in Total Cells
0
20
40
60
80
100
%
L
LmbH
LmbHN
Single Cell
2 Cells
3 Cells
4 Cells
5 Cells
Aggregates

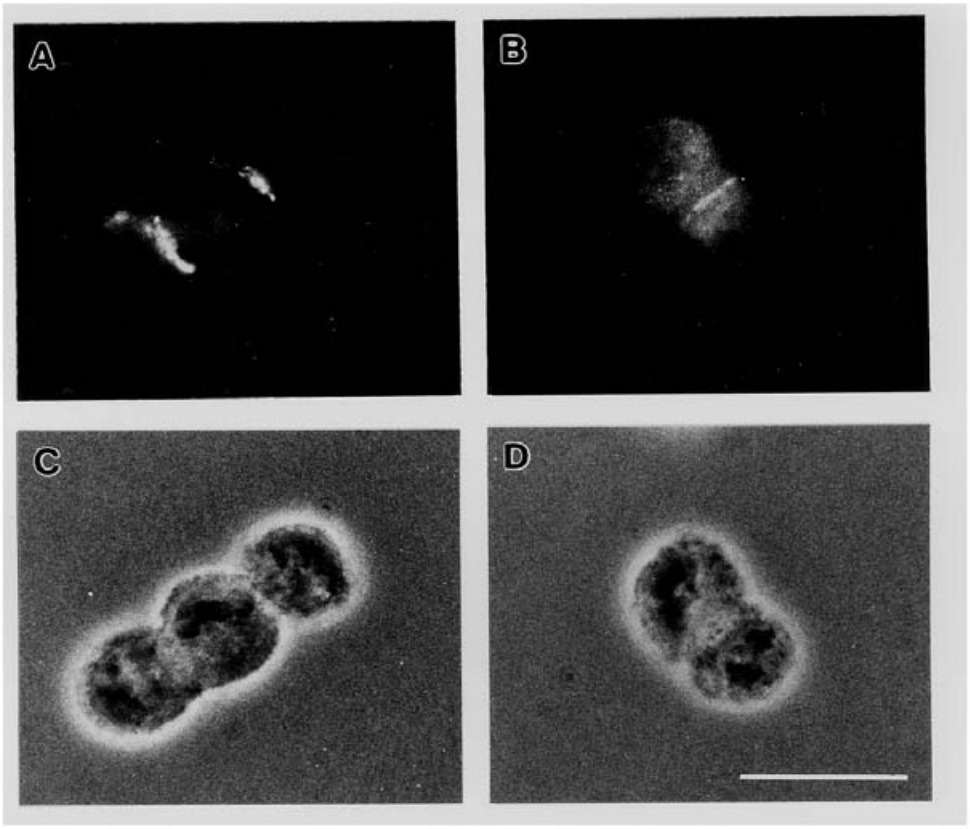


Fig 9

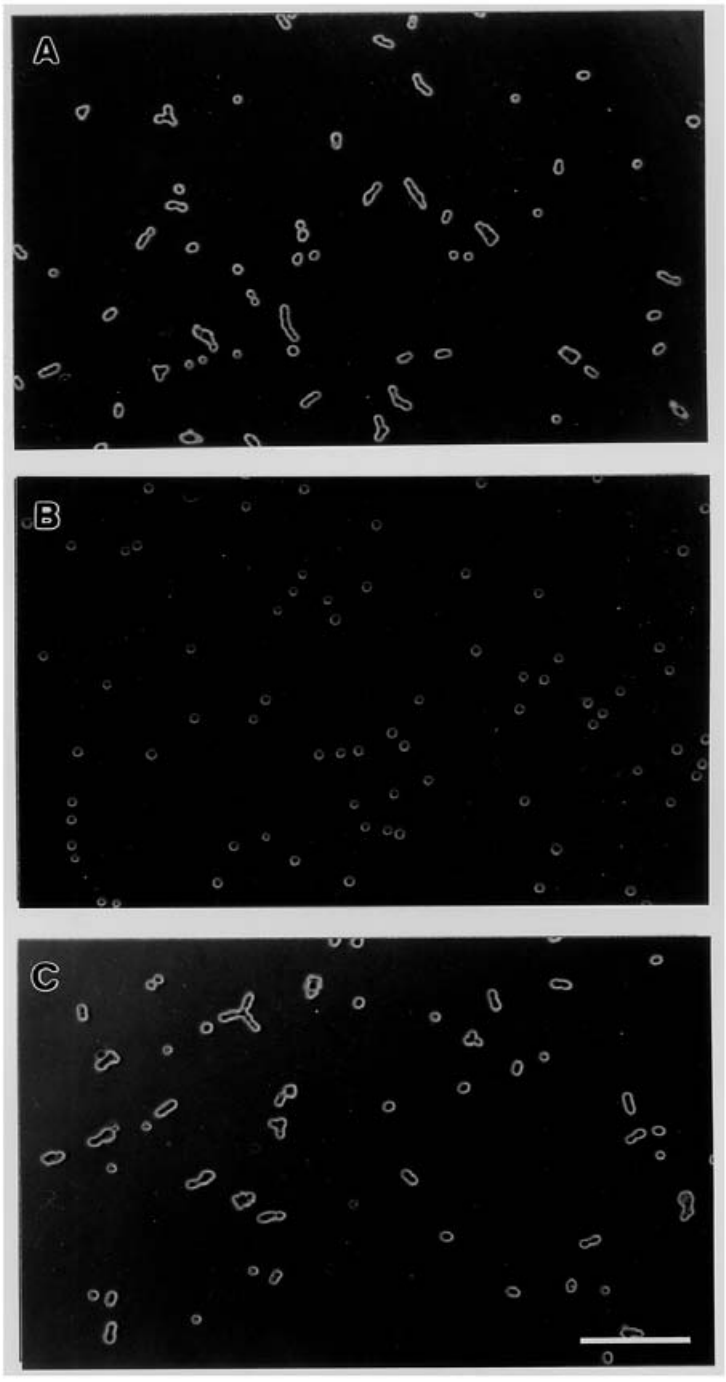


Fig 10