In one of the matings, a single intersexual female was recovered. A stock was made from the females that were heterozygous for a balancer chromosome and the irradiated chromosome, and the line was designated as $B^{M2\neq1.36}$. Less than 5% of heterozygous females manifest transformation in sexual characteristics (Figure 1b). $B^{M2\neq1.36}$ males were viable. Sexually dimorphic characters of such males were unaffected, with the exception of one male where the orientation of the sex comb was altered. Cytological examination did not reveal the presence of any visible deletions at the 16A region of the X chromosome. Third instar male larvae of $B^{M2\neq1.36}$ however did not show any puffy X chromosomes when reared at 18°C. This indicated that reversal of the puffy X phenotype had been obtained. Mapping of the mutation in $B^{M2\neq1.36}$ is currently underway. Although there are three ealier reports of perturbation of the sexual phenotype in *In(1)BM2(reinverted)* (Kar and Pal, 1995; Chakraborti *et al.*, 1996; Mukherjee and Basu, 1997), it is not known whether the structural alteration of the X chromosome and the sexual transformation are brought about due to the perturbation in the function of the same gene or not.

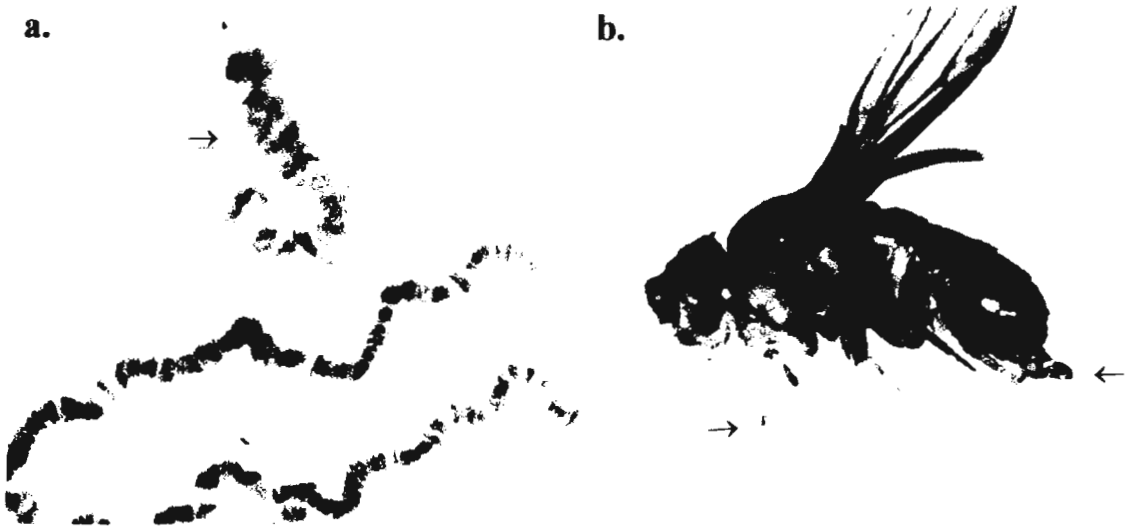


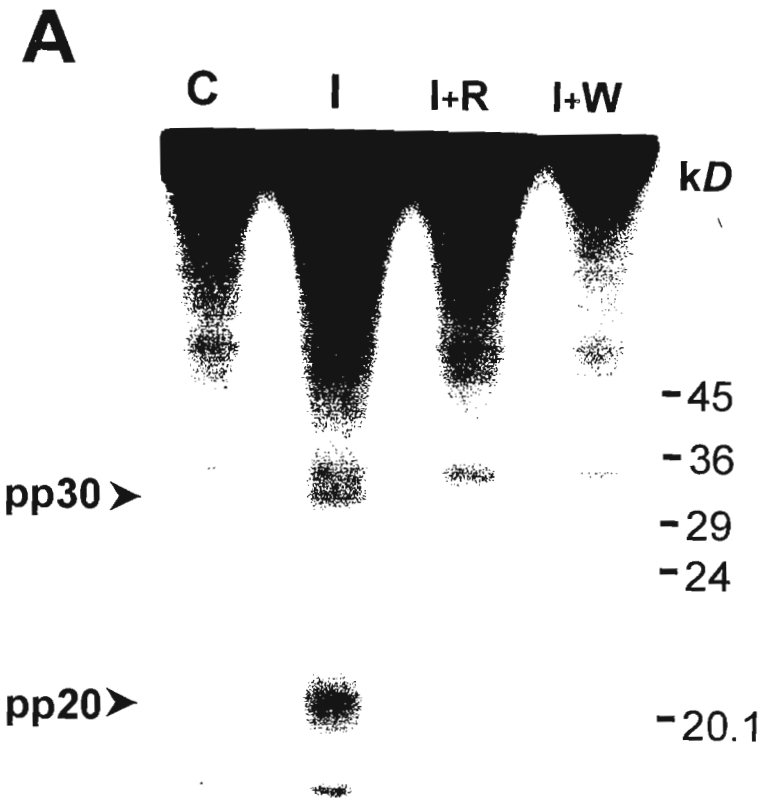
Figure 1. (a), Puffy male X chromosome (→) of the strain $In(1)B^{M2}$ *(reinverted);* (b), Phenotype of intersexual $B^{M2\neq1.36}$ / FM7 female. ← indicates transformed genitalia and → indicates sex combs on prothoracic leg.

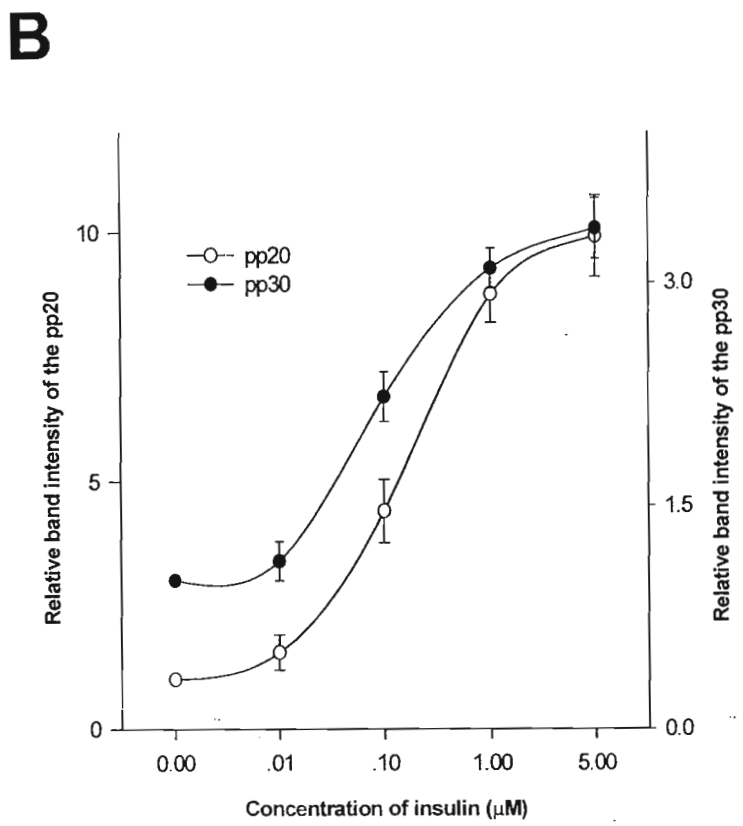
References : Chakraborti, D., *et al.,* 1995, Genome 38: 105-109; Kar, A., and J.K. Pal 1995, J. Genet. 74: 47-59; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster; Academic Press Inc., New York; Majumdar, D., *et al.,* 1978, Cell Chromosome News Lett. 1: 8-12; Mukherjee, A. S., and S. Basu 1997, Indian J. Exp. Biol. 35: 203-211; Zhimulev, I.F., 1995, Adv. Genet. 34: 1-497.

Effects of insulin, wortmannin, LY294002, and rapamycin on protein phosphorylation in the *Drosophila* ovary.

Won, Dong Hwan[1], **Kyoung Sang Cho**[2], **Ki Wha Chung**[3], **Se Jae Kim**[4], **Myung Chan Gye**[5] **and Chung Choo Lee**[1]. Departments of [1]Biology and [2]Molecular Biology, Research Center for Cell Differentiation, Seoul National University, Seoul 151-742, Korea; [3]Department of Biology, Kongju National University, Kongju 314-701, Korea; [4]Department of Biology, Cheju National University, Cheju 690-756, Korea; [5]Department of Biology, Kyonggi University, Suwon 442-760, Korea.

Abstract: Although *Drosophila* insulin-like peptide and insulin receptor have been isolated and characterized, the downstream signal of insulin has not been described well in *Drosophila*. To examine the regulatory mechanism of insulin in the ovary, we investigated the protein phosphorylation induced by insulin. Two proteins (appropriate *M*r-20,000 protein and a *M*r-30,000 protein) were identified as insulin-induced phosphoproteins at low molecular weight range (70,000 – 14,000 Dalton). As in vertebrates, these insulin-





induced protein phosphorylations were inhibited by wortmannin, LY294002, and rapamycin which are known to be inhibitors of elongation initiation factor 4E binding protein (4E-BP) and ribosomal protein S6 phosphorylation. These results imply that the downstream signal of insulin might be well-conserved in *Drosophila.*

Introduction:

Recent studies in vertebrates have suggested that insulin is essential for normal development including oocyte maturation as well as for proper signal pathways (James and John, 1981; Chung *et al.*, 1992; Jefferies *et al.*, 1994). Insulin has been proposed to be a *Drosophila* hormone, although it is originally identified as a pancreatic hormone of vertebrates. Primary embryonic cells treated with high concentration of insulin were induced to differentiate (Seecof and Dewhurst, 1974). Moreover, mutations in the *Drosophila* insulin receptors result in abnormal development of both neurons and glia (Fern<ndez *et al.*, 1995).

The use of specific inhibitors wortmannin, LY294002, and rapamycin facilitated the studies on the insulin pathways. The rapamycin

Figure 1. Effects of insulin, rapamycin, and wortmannin on the phosphorylation of the pp20 and the pp30 (A) C, control; I, insulin (1 μM); R, insulin (1 μM) after treatment of rapamycin (1 μM); W, insulin (1 μM) after treatment of wortmannin (1 μM). (B) The results represent relative band intensity of the pp20 (open circle) and the pp30 (closed circle). Its band intensity was measured as described under "Materials and Methods". The values are means ± S.E. (n = 5).

is an immunosuppressant that specifically inhibits activity of the mammalian target of rapamycin (Chung *et al.*, 1992; Price *et al.*, 1992). Treatment of mammalian cells with rapamycin results in blocking of the phosphorylation of elongation initiation factor 4E binding protein (4E-BP) (von Manteuffel *et al.*, 1996; Brunn *et al.*, 1997) and ribosomal protein S6, by blocking stimulation of p70 S6 kinase (Chung *et al.*, 1992). Wortmannin and LY294002, PI3-kinase inhitors, also exert the same effects on these molecules (Chung *et al.*, 1994; Vlahos *et al.*, 1994; Brunn *et al.*, 1996). These inhibitions repress translation of mRNAs having the polypyrimidine tract motif (Jefferies *et al.*, 1994).

The insulin has been well described as a potential mechanism for regulation of cellular function in the ovary of vertebrates; however, insulin signal has been rarely explored in the *Drosophila* ovary. To investigate the downstream of insulin pathway in the ovary of *Drosophila*, we examined the effects of insulin, rapamycin, wortmannin, and LY294002 on ovary protein phosphorylation.

Table 1. Effects of LY294002 on the insulin-induced phosphorylation of the pp20 and the pp30. The results represent relative band intensity of the pp20 and the pp30. The values obtained were normalized to that obtained in the absence of LY294002 (1) and are means ± S.E. (n =3).

	Insulin (1 μM)	LY294002 (5 μM) + Insulin (1 μM)	LY294002 (10 μM) + Insulin (1 μM)
pp20	1	0.93 ± 0.03	0.19 ± 0.02
pp30	1	0.65 ± 0.12	0.34 ± 0.12

Materials and Methods:

Materials: Porcine insulin and LY294002 were purchased from Sigma Chemical Co. Rapamycin and wortmannin were from Research Biochemicals International Co. Ortho-$^{32}PO_4$ (10 mCi/ml) was from Amersham Co.

Phosphorylation of ovary proteins: A pair of ovary was dissected from adult fly (5-day old) and incubated in phosphate-free Grace's medium for 15 min. The sample was incubated for another 15 min in the absence or the presence of rapamycin, wortmannin, or LY294002 and then porcine insulin was added. After 15 min incubation, ortho-$^{32}PO_4$ (0.5 mCi/ml) was added to the media. After 90 min labeling, the tissue was sonicated, and the extract was separated by SDS-PAGE (15%). The gel was dried and autoradiographed. In order to determine the level of protein phosphorylation, the autoradiograph was scanned and analyzed with SigmaGel (ver 1.0).

Results and Discussion:

From phosphorylation assay with intact ovaries, at least two prominent proteins (one is a *Mr*-30,000 protein and the other is about *Mr*-20,000 protein) were found to be phosphorylated by insulin. These were referred to as phosphoprotein 20 (pp20) and phosphoprotein 30 (pp30), respectively (Figure 1). Insulin significantly increased the levels of the phosphorylation with half-maximal phosphorylation at 0.21 μM (pp20) and 0.09 μM (pp30) (Figures 2A and B). At concentrations above 1 μM, incubation of the ovary with insulin resulted in about 10-fold (pp20) and 3-fold (pp30) increases. From their size and effects of specific inhibitors described below, these proteins can be inferred as the *Drosophila* homologue of 4E-BP (apparently molecular mass of rat counterpart is 22 kDa; Diggle *et al.*, 1995) and *Drosophila* ribosomal protein S6 (31 kDa; Spencer and Mackie, 1993). It was reported that 4E-BP from most mammalian cells appears as several bands in SDS-PAGE (Lin *et al.* 1994), because of decreased electrophoretic mobility following the phosphorylation levels, and of the existence of isoforms. Consistent with this, pp20 showed a broad band pattern, and appeared as 3 bands sometimes (data not shown).

As in vertebrates, rapamycin, a mTOR inhibitor, attenuates the effect of insulin in *Drosophila*. The insulin-stimulated protein phosphorylations were blocked by rapamycin in a dose-dependent manner and with half-maximal inhibition (I_{50}) at each 0.5 μM (pp20) and 0.8 μM (pp30), respectively (Figure 3A). Wortmannin, a PI3-kinase inhibitor, also inhibits the phosphorylation of the proteins. I_{50} of wortmannin on the pp20 and the pp30 were 0.05 and 0.06 μM, respectively (Figure 3B). LY294002, another PI3-kinase inhibitor that is more specific than wortmannin (Vlahos *et al.*, 1994), inhibited the insulin-induced phosphorylation on the concentration of 10 μM (Table 1). These results suggest that the pp20 and the pp30 might be the components of the pathway regulated by PI3-kinase and mTOR.

So far, there is no evidence for the *Drosophila* homologue of mTOR (DTOR). However, evidence for the existence of the substrates of the DTOR has been accumulated. It was reported that the S6 phosphorylation and *Drosophila* p70 S6K activity are sensitive to rapamycin (Fern<ndez *et al.*, 1995; Stewart *et al.*, 1996).

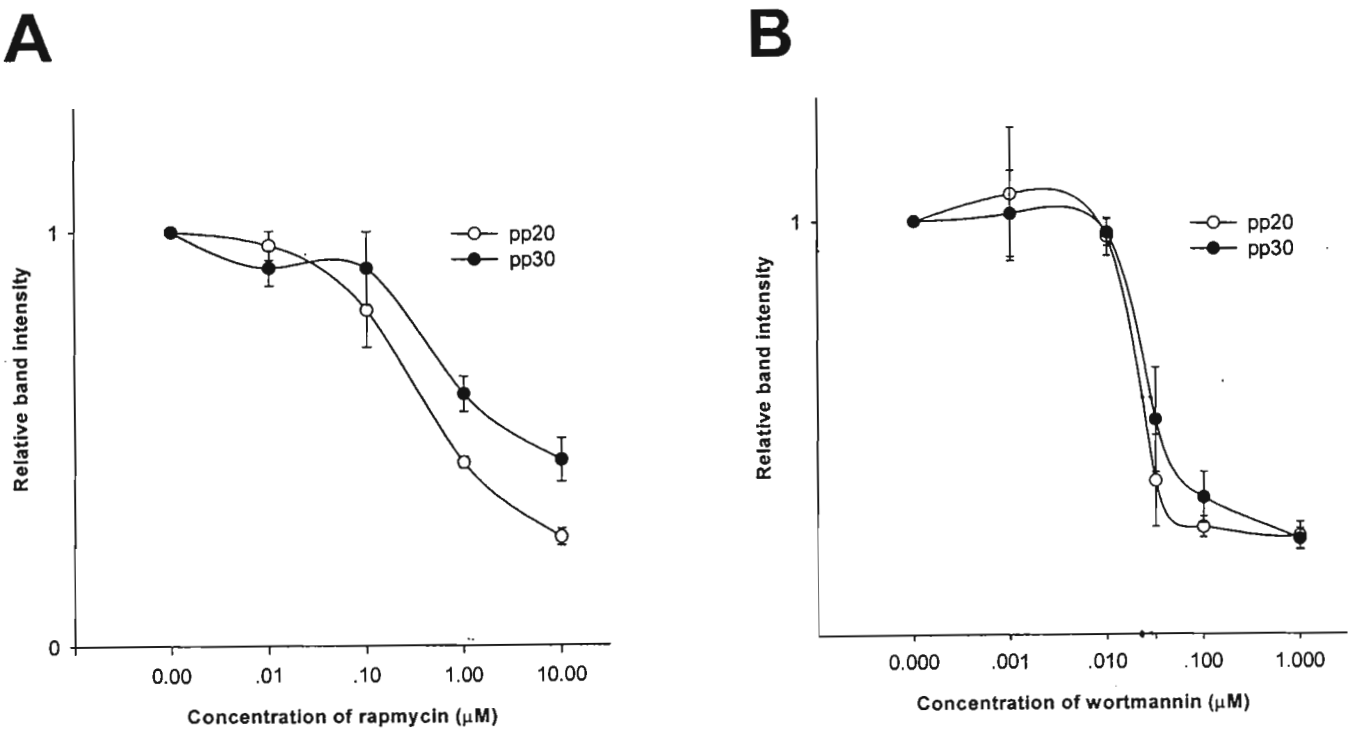


Figure 2. Effects of rapamycin and wortmannin on the insulin-induced phosphorylation of the pp20 and the pp30. The results represent relative band intensity of the pp20 (open circles) and the pp30 (closed circles). The values obtained were normalized to that obtained in the absence of rapamycin and wortmannin (1) and are means ± S.E. (n = 5). (A) Effects of rapamycin on the insulin-induced phosphorylations. (B) Effects of wortmannin on the insulin-induced phosphorylations.

Another potential substrate of the DTOR, *Drosophila* homologue of 4E-BP, has recently been cloned and characterized (Miron *et al.*, 1997). Taken together, our results suggest that the insulin pathway might be well conserved in *Drosophila*, and that the insulin might play an important role in the cellular process of the *Drosophila* ovary.

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References: Brunn, G.J., *et al.*, 1996, EMBO J. 15: 5256-5267; Brunn, G.J., *et al.*, 1997, Science 277: 99-101; Chung, J., *et al.*, 1992, Cell 69: 1227-1236; Chung, J., *et al.*, 1994, Nature 370: 71-75; Diggle, T.A., *et al.*, 1995, Biochem J. 306: 135-139; Fern<ndez, A.R., *et al.*, 1995, EMBO J. 14: 3373-3384; James, L.M., and W.K. John 1981, Dev Biol. 85: 309-316; Jefferies, H.B., *et al.*, 1994, Proc. Natl Acad. Sci. USA 91: 4441-4445; Lin, T.A., *et al.*, 1994, Science 266: 653-656; Miron, M., *et al.*, 1997, A. Conf. Dros. Res. 38: 282B; Price, D.J., *et al.*, 1992, Science 257: 973-977; Seecof, R.L., and S. Dewhurst 1974, Cell Differ. 3: 63-70; Spencer, T.A., and G.A. Mackie 1993, Biochim Biophys Acta 1172: 332-334; Stewart, M.J., *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93: 10791-10896; Vlahos, C.J., *et al.*, 1994, J. Biol. Chem. 269: 5241-5248; von Manteuffel, S.R., *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93: 4076-4080.