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Characterization of Lipovitellin 2 as a Tyrosine-phosphorylated Protein in Oocytes, Eggs and Early Embryos of *Xenopus laevis*

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A tyrosine-phosphorylated protein of 33 kDa is shown to be present in the solubilized yolk fraction of *Xenopus laevis* oocytes, eggs, and early embryos. The phosphoprotein is lipovitellin 2 as demonstrated by immunoprecipitation and mass spectrometry studies, and is termed pp33/LV2. Subcellular fractionation and immunoblotting studies demonstrate that pp33/LV2 is stably present in the Triton X-100-resistant and SDS-soluble yolk fractions during oogenesis, oocyte maturation, and early embryogenesis. From after the swimming tadpole stages (stage 39~), however, it becomes partly soluble to Triton X-100-containing buffer and all disappear thereafter (stage 48~49). In vitro enzyme assays with the use of the tyrosine phosphatase LAR and the tyrosine kinase Src demonstrate the reversible nature of the tyrosine phosphorylation of pp33/LV2. Microinjection studies demonstrate that the solubilized yolk fractions, but not those immunodepleted of pp33/LV2 or those pretreated with LAR, inhibit progesterone- or insulin-induced oocyte maturation. A pp33/LV2-like protein seems to present in two *Xenopus* subspecies, one other frog species, and two fish species, but not in other amphibian species, such as newt and salamander. These results suggest that LV2, in its tyrosine-phosphorylated form, serves in a cellular function in a species-specific manner, but the mechanism is still unknown.

Key words: lipovitellin, signal transduction, tyrosine phosphorylation, *Xenopus laevis*, yolk protein

INTRODUCTION

Tyrosine phosphorylation is one of the mechanisms for the posttranslational modification of cellular proteins. The first example of protein-tyrosine phosphorylation was demonstrated in the late 1970's by Hunter and Sefton (1980) who showed that immunoprecipitates containing the product of an oncogene of Rous sarcoma virus, namely viral Src, contained tyrosine-specific kinase activity. Since its discovery, tyrosine phosphorylation has been analyzed extensively, and is now recognized as one of the cellular mechanisms for carcinogenesis and tumorigenesis (Jove and Hanafusa, 1987). Currently, it is well accepted that the constitutive and/or deregulated occurrence of protein-tyrosine phosphorylation, as catalyzed by viral Src or Src-related oncogene products, leads to malignant cell transformation. Non-oncogene (cellular gene) products, e.g. cellular

Src protein and other cellular protein-tyrosine kinases, can also catalyze the tyrosine phosphorylation of cellular proteins (Brown and Cooper, 1996; Thomas and Brugge, 1997). In this case, the occurrence of tyrosine phosphorylation is tightly regulated under several cellular conditions. Such finely tuned and transient protein-tyrosine phosphorylation is now known to be involved in several aspects of physiological and pathological functions of cells; e.g. proliferation, differentiation, motility, immunity, neuronal signaling, development, infection and diseases.

In eggs of *Xenopus laevis*, rapid and transient tyrosine phosphorylation catalyzed by the egg-associated Src protein plays an important role in sperm-induced egg activation at fertilization (Sato et al., 1999; for review see Sato et al., 2004). Previously, we showed that Src-dependent tyrosine phosphorylation of phospholipase C γ is required for a transient increase in the intracellular Ca²⁺ concentration, which is indispensable for sperm-induced egg activation (Sato et al., 2000). Cytoplasmic proteins, such as the adaptor protein Shc (Aoto et al., 1999) and the RNA-binding protein hnRNP K (Iwasaki et al., 2008), were also identified as substrates of Src in fertilized eggs. Uroplakin III, an egg plasma membrane-associated protein, was initially identified as a

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substrate of Src (Sakakibara et al., 2005) and then found to be a target of sperm-derived protease activity that is essential for egg-sperm interaction at fertilization (Mahbub Hasan et al., 2005). Altogether, eggs of *Xenopus laevis* have provided an excellent system for analyzing roles of protein-tyrosine phosphorylation in the fertilization and initiation of development.

In this study, we identified a 33-kDa tyrosine-phosphorylated protein in the SDS-solubilized fraction of eggs and oocytes of *Xenopus laevis*. The 33-kDa tyrosine-phosphorylated protein is also present in early embryos of *Xenopus laevis*. Mass spectrometry demonstrated that the protein is lipovitellin 2, a proteolytic fragment of vitellogenin B1/A2 that is a yolk-associated nutrient resource for oogenesis and early development (Brgink and Wallace, 1974; Wiley and Wallace, 1981). To our knowledge, this is the first report to demonstrate that a yolk protein is stably phosphorylated on tyrosine residues in the course of oogenesis and early development.

MATERIALS AND METHODS

Animals, antibodies, and other materials

African clawed frogs (*Xenopus laevis*, *Xenopus borealis*, and *Xenopus tropicalis*) were obtained from local dealers and maintained in de-chlorinated tap water at ambient room temperature. Unfertilized eggs and/or early embryos of a newt (*Cynops pyrrhogaster*), a salamander (*Hynobius nebulosus*), and other types of frog (*Bufo japonicus* and *Hyla japonica*) (Harada et al., 2007; Iwao, 1987, 1989) were gifts from Dr. Yasuhiro Iwao (Yamaguchi University). Immature oocytes of medaka (*Oryzias latipes*) and unfertilized eggs of zebrafish (*Danio rerio*) (Hanno et al., 2010) were gifts from Dr. Shoji Oda (University of Tokyo). Immature oocytes of sawfly (*Athalia rosae*) (Yamamoto et al., 2008) were gifts from Dr. Masatsugu Hatakeyama (National Institute of Agrobiological Sciences).

A mouse monoclonal antibody against phosphotyrosine, PY99, was purchased from Santa Cruz (CA, USA). A mouse monoclonal antibody against tubulin- β was obtained from Amersham Biosciences (Uppsala, Sweden). Rabbit polyclonal antibodies against mitogen-activated protein kinase (anti-MAPK antibody) and phosphorylated MAPK (anti-pMAPK antibody) were purchased from Biolabs (MA, USA). Rabbit polyclonal antibodies against the extracellular domain of *Xenopus* uroplakin III (anti-xUPIII antibody) (Sakakibara et al., 2005) or the internal sequence of *Xenopus* vitellogenin B1 (residues 1401–1415, NPHEAQAEKLGWQNC) was prepared as described previously (Iwasaki et al., 2008).

Leupeptin was obtained from Peptide Institute (Osaka, Japan), (*p*-Amidinophenyl)methanesulfonyl fluoride dichloride (APMSF) from EMD Chemicals (NJ, USA), protein A-Sepharose was from Amersham Biosciences, L-phosphoamino acids (phosphotyrosine, phosphoserine, and phosphothreonine), progesterone, and insulin from Sigma (MO, USA), and recombinant, catalytic domain of leukocyte-related antigen protein-tyrosine phosphatase (LAR) (Itoh et al., 1992) from EMD Chemicals. Unless otherwise indicated, other chemicals were obtained from Sigma, Wako Pure Chemicals (Osaka, Japan), or Nacalai Tesque (Kyoto, Japan).

Oocytes, eggs, and embryos

To obtain oocytes of *Xenopus laevis*, we performed manual dissection of ovarian tissues from female frogs anesthetized in tap water containing 2 mg/ml of 3-aminobenzoic acid ethyl ester methanesulfonate salt (Sigma). Dissected ovarian tissues were cut into small pieces containing ~100 oocytes, washed several times with OR-2 buffer containing 82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, and 5 mM HEPES-NaOH, pH 7.8, and

treated with OR-2 buffer supplemented with 0.5 mg/ml of collagenase (Sigma), to remove the follicle cell layer from the oocytes, with gentle rocking for 2–3 h at 21°C. After the treatment, oocytes were washed extensively with OR-2 buffer, manually subdivided into three groups by oogenesis stage (stages I and II, III and IV, and V and VI), packed into 1.5-ml microtubes (5–20 oocytes per tube), removed of excess OR-2 buffer, frozen in liquid nitrogen, and stored at –30°C prior to use. We applied a similar approach to the preparation of oocytes from *Xenopus borealis* and *Xenopus tropicalis*, except that only the largest oocytes were selected and stored.

Unfertilized eggs and early embryos of *Xenopus laevis* were prepared as described previously (Sato et al., 1996, 2000). Early developmental stages of *Xenopus laevis* were verified according to Nieuwkoop and Faber (1994, www.xenbase.org/anatomy/alldev.do). Jelly coats of eggs or embryos were removed by treatment with DeBoer's solution (110 mM NaCl, 1.3 mM KCl, and 0.33 mM CaCl₂, pH 7.2) containing 2% cysteine for 3–8 min and then washed extensively with DeBoer's solution. The dejellied egg/embryo samples were frozen in liquid nitrogen, and stored at –30°C.

Extraction and fractionation

All manipulations were carried out at 4°C or on ice. Cell samples (oocytes, eggs, or embryos) were mixed and homogenized with a 5–10 x cell volume of buffer A containing 20 mM Tris-HCl, H 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β -mercaptoethanol, 1 mM sodium orthovanadate, 10 μ g/ml leupeptin, and 20 μ M APMSF. The homogenates were centrifuged at 12,000 g for 10 min. The supernatants were collected as cytosolic fractions. The pellets were mixed and homogenized with a 2.5–5 x cell volume of buffer A supplemented with 1% Triton X-100. The homogenates were centrifuged at 12,000 g for 10 min. The supernatants were collected as Triton X-100-soluble fractions, which contained most of the membranous parts of the cells. The pellets were mixed and homogenized with a 2.5–5 x cell volume of buffer A supplemented with 1% Triton X-100 and 0.1% SDS. The homogenates were centrifuged at 12,000 g for 10 min. The supernatants were collected as SDS-soluble fractions, which contained cytoskeletal parts and yolk materials. When analyzing the developmental expression of pp33/LV2 in *Xenopus laevis*, cell samples were initially mixed and homogenized with buffer A supplemented with 1% Triton X-100 to obtain a mixture of the cytosol and the Triton X-100-soluble fractions. When microinjection of pp33/LV2 into *Xenopus laevis* oocytes was performed (see below), yolk-containing pellets were solubilized with buffer A supplemented with 1 M NaCl, instead of 1% Triton X-100 and 0.1% SDS. All protein samples were used within 3 h of extraction.

SDS-polyacrylamide gel electrophoresis

Protein samples, as normalized by the cell equivalent amount (e.g. one oocyte) of protein, were mixed with SDS sample buffer (Laemmli, 1970) and treated at 95°C for 5 min. SDS-denatured protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels. After the electrophoresis, gels were subjected to immunoblotting (see below) or protein staining with either Coomassie Brilliant Blue R-250 (Nacalai) or silver (Silver stain kit, Nacalai).

Immunoprecipitation and immunoblotting

In some experiments, protein samples were immunoprecipitated with 3–5 μ g of the anti-phosphotyrosine antibody PY99 for 3–6 h at 4°C. Immune complexes were adsorbed onto 10 μ l of protein A-Sepharose beads by gentle agitation for 30 min at 4°C. The beads were then washed three times with buffer A supplemented with 1% Triton X-100 and 0.1% SDS. The washed beads were treated with Laemmli's SDS sample buffer and subjected to SDS-PAGE as described above. After SDS-PAGE, immunoblotting of protein samples or the immunoprecipitated samples was done as

described previously (Sato et al., 1999, 2000). Briefly, an electro-transfer of proteins from SDS-gels to polyvinylidene difluoride membranes was done with the use of a semi-dry blotting apparatus (Bio-Rad, CA, USA). After the transfer, membranes were blocked with 3 mg/ml of bovine serum albumin in T-TBS buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20. After the blocking treatments, membranes were sequentially treated with primary antibodies specified in the text, secondary antibodies labeled with biotin, and streptavidin-biotin complex conjugated with horseradish peroxidase. Detection of the immune complexes on the membranes was achieved with the addition of a developing solution containing 50 mM Tris-HCl, pH 7.5, 0.2 mg/ml diaminobenzidine, and 0.005% hydrogen peroxide (6.7 μ l of 30% solution per 40 ml).

Mass spectrometry

A 33-kDa tyrosine-phosphorylated protein, designated pp33, was concentrated by immunoprecipitation of the SDS-soluble fractions of *Xenopus laevis* immature oocytes and separated by SDS-PAGE. Proteins were visualized using the Nacalai Tesque silver stain kit. In-gel digestion of the pp33-containing SDS gels with trypsin, liquid chromatography mass spectrometry and the database search were done by Apro Science (Tokushima, Japan).

Dephosphorylation and rephosphorylation of pp33

To perform dephosphorylation of pp33/LV2, Triton X-100-insoluble yolk-containing pellets prepared from *Xenopus laevis* immature oocytes (see above) were solubilized in sodium orthovanadate-free buffer A containing 1% Triton X-100 and 0.1% SDS. The solubilized materials were clarified by centrifugation at 12,000 g for 10 min. The resulting supernatants (0.02–0.2 oocyte-equivalent amount of protein per assay) were treated in the absence or presence of LAR, a recombinant human protein-tyrosine phosphatase, for 30 min at 30°C.

To reconstitute the tyrosine phosphorylation of pp33, the

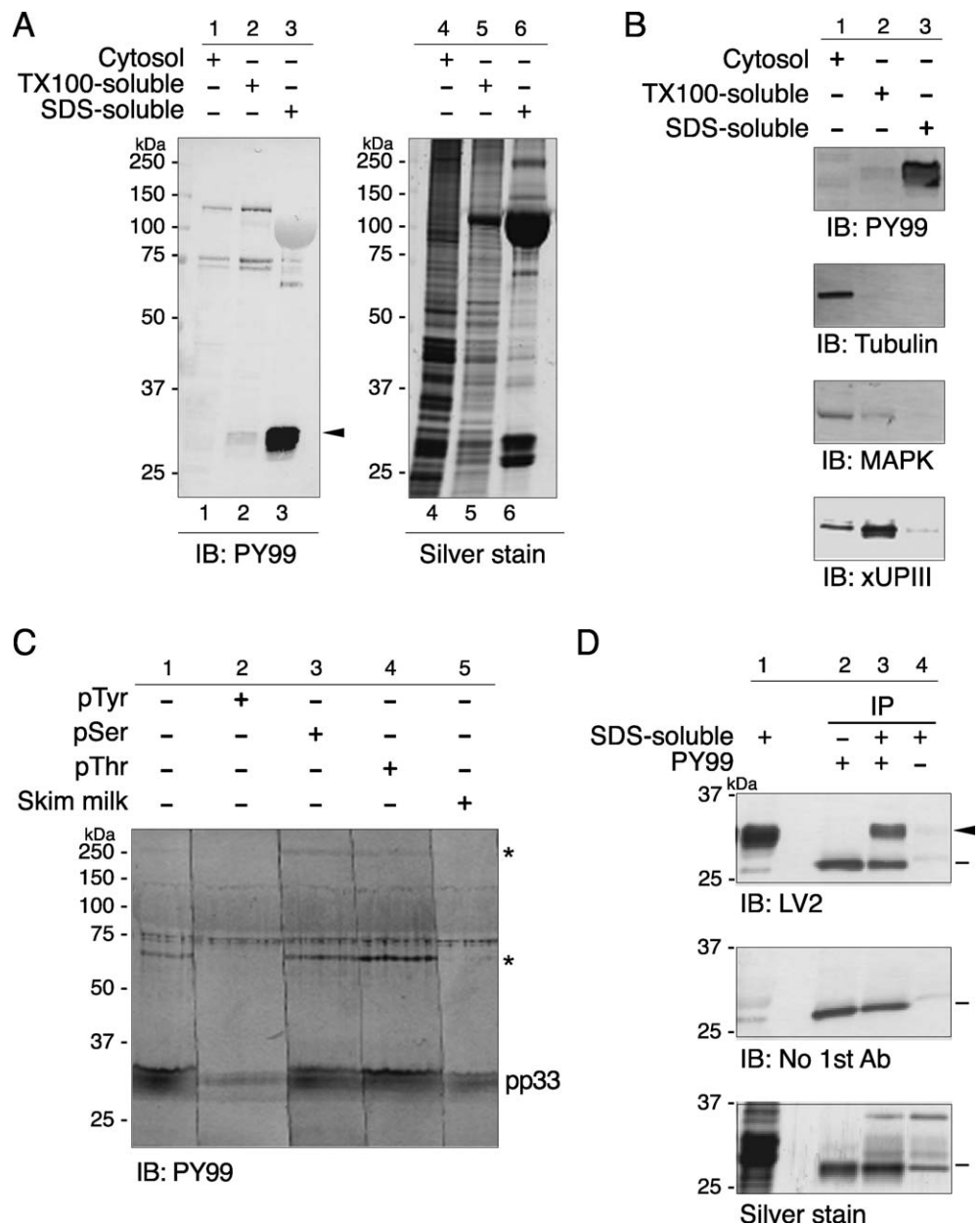


Fig. 1. Identification of a 33-kDa yolk-associated tyrosine-phosphorylated protein in *Xenopus* oocytes. **(A)** *Xenopus* immature oocytes were fractionated into cytosolic, Triton X-100-soluble, and SDS-soluble fractions. These fractions containing one oocyte-equivalent proteins were analyzed by immunoblotting with the anti-phosphotyrosine antibody PY99 (IB: PY99, lanes 1–3) or silver stain (lanes 4–6). An arrowhead indicates the position of a 33-kDa tyrosine-phosphorylated protein in the SDS-soluble fraction. **(B)** Fractions obtained as in (A) were analyzed by immunoblotting with the indicated antibodies. **(C)** SDS-soluble fractions were analyzed by immunoblotting with PY99 in the absence (lane 1) or presence of 1 mM phosphotyrosine (lane 2), 1 mM phosphoserine (lane 3), 1 mM phosphothreonine (lane 4), or 1% skim milk (lane 5). The positions of pp33 and other phosphotyrosine-containing proteins (asterisks) are indicated. **(D)** SDS-soluble fractions (ten oocyte-equivalent of protein per lane) were immunoprecipitated with PY99 (lane 3). As a control, we also performed immunoprecipitation with no protein samples (lane 2) or no PY99 (lane 4). The immunoprecipitates and the SDS-soluble fractions (one oocyte-equivalent of protein per lane, lane 1) were analyzed by immunoblotting with either anti-lipovitellin 2 antibody (top panel) or no primary antibody (middle panel), or silver stain (bottom panel). An arrowhead indicates the position of a tyrosine-phosphorylated protein at 33 kDa. Solid lines indicate the positions of the light chains of immunoglobulin G. In panels (A), (C), and (D), positions of molecular size markers (25–250 kDa) are also indicated.

LAR-treated samples were added with sodium orthovanadate at a final concentration of 1 mM. The samples were further supplemented with ATP and MgCl₂ at final concentrations of 1 mM and 5

mM, respectively. The mixtures were then treated in the absence or presence of the tyrosine kinase Src purified from bovine brain, for 30 min at 30°C. Both LAR and Src treatments were stopped by the

A

VgB1

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1 MRGILLALLL ALAGSEKSYQ EPPFSESKTY VVNYEGIILN GIPENGLARS
51 GIKLNCKAEI SGYAQRSYML KIRDPEIKEF NGLWPKDPPT RATKLSQILA
101 EHLTRFVKFE YRNGQVGNIF AAEDVSETAL NIQRGILNLL QLTIKKSNV
151 YDLQEDSISG VCHTKYTIQE DKKAERLTVM KSRDFDRCES RVEKKIGVAY
201 LETCTQCCKK VQHLRGSATF TYKLQNKDQG ALIVEASAKQ VYRYAPYYEL
251 YGAAVMEARQ ILLTAEATKSN RVSESQVQLT KRGGILNYHFV QEEHQMPFHL
301 LKTKNAESQV VETLQHLVQN NQELVHGEST SKFFELVQLL RTTNQENIQA
351 IWKQFSDRPO HRHWLWNAIP MAGSVDALKF IKQMIHNDQM THQEAATAIL
401 MAMHYTRASQ RAVEIAADLV TDSRVKNAPS LYKSALLAYG SLVYKYCVDS
451 QSCPEAALQP LHEFAEAAN NAHQEETILA LKAIGNAGQP ASIKRIQKFL
501 PGFSSGASRL FVRLQTDVAM ALRNIAIKDP RKVQEILLQL FMDRDVHPEV
551 RMITCVALFE TNPGLAIVTA VANAVLRESK DNMQLASPTY SHIKSLSKSM
601 MPEFYNLASA CRVALNIMNP VFDQLSFRYS KAFHVDTFNY PMMGGLALNV
651 VFINSNPNTAF PVFALIKVRE CSPGASTDVF EVALRAEGLQ EVIRKQNIQF
701 SEFMSHKKIV KILKALMAYK EAPANVPLVS ASIKLLGQEV NFNQATRDIT
751 QNAMKLLNEP AERHTMVYKI LNKLLNGVTG QWSHPMLVAE ARHIVPTCVG
801 LPLEIATHSA AVANAALNVD MKVSPSPNSD FSLSQLLESN IQLHTDMSPS
851 VLIHLVNTMG VNTPLIQSGL EFGHKVRIQT PAKFTAQLDM KDKNFKIETP
901 PCQREVELVT VRHQMFAVTR NVGQDSEKR MLVMPKQHGQ STMEOHFESK
951 GRTSAESASM MEVSSSENGR NKNQAQHQLL NNVEYRYCF RLSKLNINAC
1001 LQSKLNTVQG DRRSFLYENI GEHEIKFLLK PTGNEPAIEK LQVEITAGPR
1051 ASSKIIALAE VEEKEGENLD ESAVQKRLKI ILGINEEYS ARNSSLVRKA
1101 SKKRKVKKTK AERHTMDVLEA ERKMQSSSS SSSSSSPSSS SSSSSSSSSS
1151 SSSSSSSSSK SSSSSSSSPF QRNSRNKGIK VDVLGRVSKR QQEKNKDTHG
1201 QTGRNKHRSR SSSSSSSSSS SSSSSSSSES SSSSSSSSES SSSSSRRHGK
1251 SGKQQQGDRE QOREKQTHQN KQKQKHRSR HPQDPSQMPF QLYKLFQRF
1301 YKQYKGMNSG QSESSSSSSS SSSSSSSSES ARSPHKKKFL GDKKSPVTVI
1351 TAQAVRNDNV KQGYQVILYT DDTTRPKMQA YLVDITGKSR WRACAEAFVR
1401 NPHEAQAELK WQCNCKQYKM EFIAQTGIFG NQPALRLRVN WPRIPSKLSK
1451 VGEVGEFLP GAAYMGLLFP KFORNPSKQA KFIFLSLSPR TNNAVIQVPW
1501 ATCYQAINL PFAYTAAYHL HSSDIQAPTW NIFANSYSSM VDRLSGECTM
1551 SSDQIKTFNE VKFNNTLFGS CFHVLAQDCS TELKFLVLAK KSPESSELRG
1601 INIKLADYDI DMTYSSEERL LKVNGLVVPK EKLPIYIASN PIVEIKMEEG
1651 ALVLTAPVFG IEQLYFNGKL TKLRTAVWMR GGTGCGICGRH DDETERBYQM
1701 PDGYAARDEN SFTHSWILPE EPCSCTGCKLQ HTSVKLEKTI HIDDQKSKCQ
1751 SVRPVLRVCK GCSPSTVTPV TVGFHCLPFD ASTDMLESQN IMGKTEDLVE
1801 TVDAHTACSC EALKCAA

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Start - End	Observed	Mr (expt)	Mr (calc)
1344 - 1356	685.41	1368.81	1368.81
1345 - 1356	621.35	1240.69	1240.71
1345 - 1356	621.35	1240.69	1240.71
1362 - 1377	949.49	1896.96	1896.96
1378 - 1388	627.82	1253.62	1253.63
1401 - 1410	568.78	1135.55	1135.56
1420 - 1436	946.99	1891.96	1891.96
1450 - 1471	767.36	2299.06	2299.09
1450 - 1471	1150.54	2299.07	2299.09
1450 - 1471	772.69	2315.05	2315.08
1482 - 1490	527.28	1052.54	1052.57

VgA2

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1 MKGIVLALLL ALAGSERTHI EPPFSESKIS VVNYEAVILN GPFESGLSRA
51 GIKINCKVEI SAYAQRSYFL KIQSPKIEKY NGVWPKDPPT RSSKLTQALA
101 EQLTKPARFE YNNGRVGDIF VADDVSDTVA NIYRGLNLL QVTIKKSDV
151 YDLQESSVGG ICHTRYVIOE DKRGDQIRII KSTDFFNQCQ KVSKTIGLEL
201 AEFCHSCKQL NRVIQGAATY TYKLKGRDQG TVIMEVTARQ VLQVTPFAER
251 HGAATMESRQ VLAWVGSKSG QLTPPQIQLK NRGNLHYQFA SELHQMPIHL
301 MKTKSPEAQA VEVLQHLVQN NQELVHGEST SKFFELVQLL RTTNQENIQA
351 LWKQFAQRTQ YRRCLLDALP MAGTVDCLKF IKQLIHNEEL TTQEAAVLIT
401 FAMRSARPGQ RNFQISADLV QDSKVQKYST VHKAAILAYG TMVRYCDQL
451 SSCPEHALEP LHELAEEAAN KGHYEDIALA LKALGNAGQP ESIKRIQKFL
501 PGFSSSADQL FVRIQTDVAM ALRNIAKEDP RKVQEILLQI FMDRDVHPEV
551 RMMACLALFE TRPGLATVTA IANVAARESK TMLQLASFTY SQMKALSASS
601 VPHLEPLAAA CSVALKILNP SLDNLGGRYS KVMRVDTFKY NLMAGAAAKV
651 FIMNSANTMF PVFILAKFRE YTSLVNDDI EIGIRGEGIE EFLRQNIQF
701 ANFPMRKKIS QIVKSLGFKP GLPSQVPLIS GYIKLFGQEI AFTELNKEVI
751 QNTIQALNQP AERHTMIRNV LNKLLNGVTG QYARRMWTWE YRHIIPTTVG
801 LPAEILSYQS AIVHAAVNSD VKVKPTPSGD FSAQQLLESQ IQLNGEVKPS
851 VLVHTVATMG INSPFLQAGI EFGHKVHAHL PAKFTAFLDM KDRNFKIETP
901 PFQGNHLEVE IRAQTFAPTR NIADLDSARK TLVVPVRRNEQ NILKHFETT
951 GRTSAEAGSM MEDSSEMGPK KYSAEPGHHQ YAPNINSYDA CTKFSKAGVH
1001 LCIQCKTHNA ASRRNTIFYQ AVGEHDFKLT MKPAHTEGAI EKLQLEITAG
1051 PKAASKIMGL VEVEGTGEP MDETAIVPKRL KMILGIDESR KDTNETALYR
1101 SKQKKNKIKH NRRLDAEVVE ARKQSSSLSS SSSSSSSSSS SSSSSSSSSS
1151 SSSSSSSSSS SVSKRSKRRR HNPHHQRESS SSSSQQNKK RNLQENRKHG
1201 QKGMSSSSSS SSSSSSSSSS SSSSSSSSSS SSSSENRPHKN RQHDNKQAKM
1251 QSNHQKQKKN KFSSESSSSS SSSSSSEMWNK KKHRRNFYDL NFRFARTKG
1301 TEHRGSRLLS SSSSSSSSES SBYRHKAKFL GDKEPPVLV TFKAVRNDNT
1351 KQGYQMVVYQ EYHSSKQIQI AYVMDISKTR WAACFDVAVV NPHEAQAELK
1401 WGNQCDYKI NMAETGTFG NQPALRVAN WPKIPSKKWS TGKVVGEVLP
1451 GAMYMMGFQO EYKRNRSQV KLVFALSPPR TCDVIRIIPR LTVYVRLALR
1501 PVPVPGVHHA KENVLQPTW NIFAEAPKLI MDSIQGECKV AQQITTFYDI
1551 VDLASALPEN CYNVLAQDCS PEMKFMVLMR NKSSEPNHND INVKLEBYDI
1601 DMYSADAFK LKVNLEVSE EHLPIKKNFN PVEIKKKN GNLSASEYEG
1651 IDSLDYDGLT FKFRPTIWMK GKTGICGHN DDESEKELQM PDGSAKDKM
1701 RFIHSWILPA ESCSEGNLKL HTLVKLEKAI ATDGAKACKY SVQVLRCAK
1751 GCSPVKTVEV STGFHCLPFD VSLDLPEGQI RLEKSEDFSE KVEAHTACSC
1801 ETSPECAA

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Start - End	Observed	Mr (expt)	Mr (calc)
1414 - 1426	687.84	1373.67	1373.67
1414 - 1426	687.86	1373.70	1373.67

B

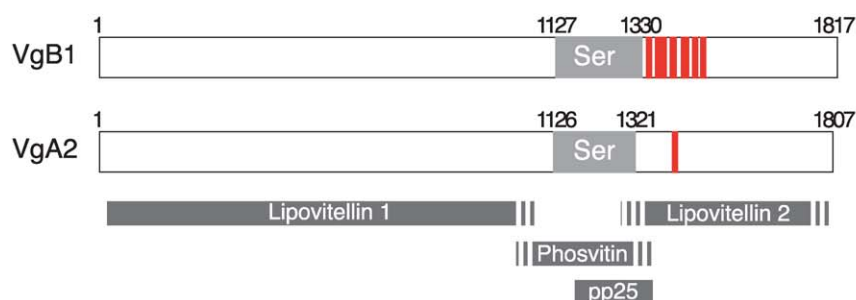


Fig. 2. Mass spectrometric analysis of the 33-kDa tyrosine-phosphorylated protein. **(A)** The whole amino acid sequences of *Xenopus* vitellogenin B1 (VgB1, 1817 amino acids) and A2 (VgA2, 1807 amino acids) are shown. Amino acid sequences that matched those of known partial sequences in the mass spectrometric (MS) analysis are shown in red. Shown in squares are the amino acid sequences that correspond to phosvitin, a serine-rich phosphoprotein. Shown below is the MS identification of thirteen peptide fragments found to be part of *Xenopus* vitellogenin B1 or A2. Mass values of these peptide fragments were obtained from the 33-kDa tyrosine-phosphorylated protein digested with trypsin (see "Materials and Methods" for details) and were found to be part of the *Xenopus* vitellogenin B1/A2 using the MASCOT database search algorithm. Amino acid numbers (start-end) and expected (expt) as well as calculated (calc) mass values for each peptide fragment are also shown. **(B)** Schematic structure of *Xenopus* VgB1 and VgA2. Positions of serine-rich sequences and four major subunits in *Xenopus* VgB1 and VgA2 (lipovitellin 1, lipovitellin 2, phosvitin, and pp25) and the number of amino acid residues are indicated. Indicated by red areas are the amino acid sequences matching those in the MS analysis (see panel A).

addition of Laemmli's SDS sample buffer followed by heat treatment for 5 min at 98°C. Dephosphorylation and re-phosphorylation of pp33/LV2 were verified by SDS-PAGE and immunoblotting with the anti-phosphotyrosine antibody PY99.

Immunodepletion

Protein samples (yolk fractions solubilized with high NaCl, see above) containing pp33/LV2 were either untreated or immunoprecipitated with anti-phosphotyrosine antibody PY99 as described above (see *Immunoprecipitation and Immunoblotting*). After the treatments, the samples were incubated with 10 µl of protein A-Sepharose beads by gentle agitation for 30 min at 4°C and subjected to centrifugation at 2,000 rpm for 1 min. After centrifugation, the unbound, supernatant materials were collected and used as "immunodepleted" samples.

Microinjection and oocyte maturation

Quantitative injection of pp33/LV2-containing *Xenopus laevis* oocyte fractions (solubilized with high NaCl-containing buffer and either left intact, enzyme-treated, or immunodepleted) into fully-grown stage VI oocytes was made under micro-

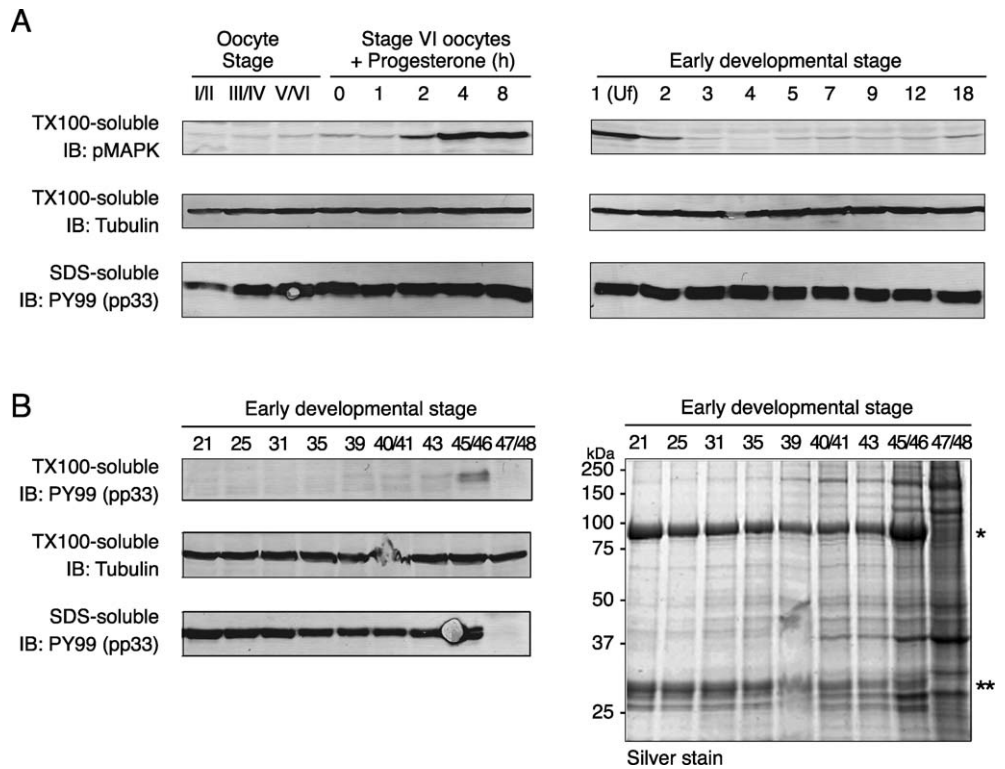


Fig. 3. Developmental expression of pp33/LV2. (A) Oocytes at the indicated oogenesis stage (I–VI), the stage VI oocytes at the indicated time points after progesterone treatment (0–8 h), and early embryos at the indicated Nieuwkoop-Faber's developmental stages (1–18) were fractionated into Triton X-100-soluble fractions and SDS-soluble fractions. Protein samples (20 oocytes for stage I/II, 5 oocytes for stage III/IV, one oocyte for stage V/VI and thereafter one embryo-equivalent of protein per lane) were analyzed by immunoblotting with the indicated antibodies: anti-phosphorylated MAPK (pMAPK), anti-tubulin, and PY99. (B) Embryos at the indicated Nieuwkoop-Faber's developmental stages (21–47/48) were fractionated and analyzed (one embryo-equivalent of protein per lane) by immunoblotting with the indicated antibodies (left panels) or silver stain (right panel). Asterisks in the silver stain data indicate the positions of LV1 (~100 kDa) and LV2 (~33 kDa).

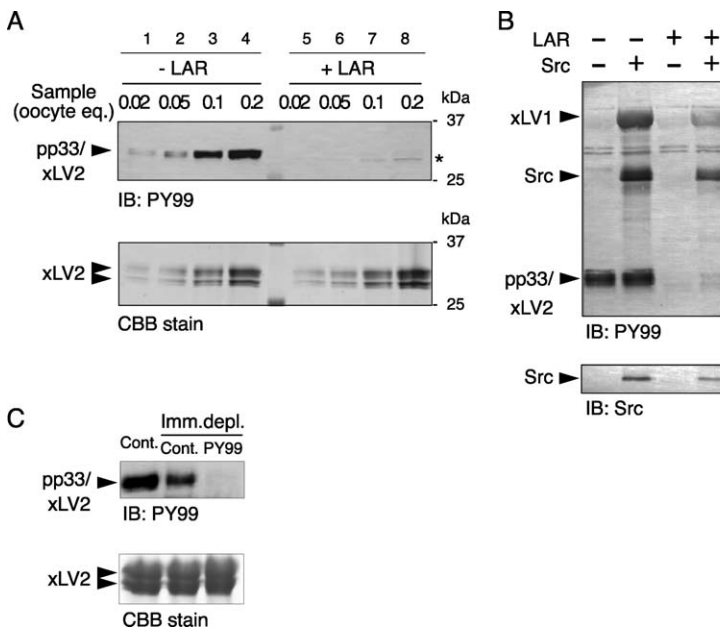


Fig. 4. In vitro dephosphorylation/re-phosphorylation and immunodepletion of pp33/LV2. (A) SDS-soluble fractions (0.02–0.2 oocyte-equivalent of protein per lane) prepared from *Xenopus* immature oocytes were treated in the absence or presence of protein-tyrosine phosphatase LAR as described in "Materials and Methods". After the treatments, protein samples were analyzed by immunoblotting with PY99 (upper panel) or CBB stain (lower panel). The positions of an intact pp33/LV2, and a hypo-phosphorylated form of pp33/LV2 (asterisk) are indicated. (B) The LAR-treated SDS-soluble fractions (LAR +) were subjected to an in vitro kinase assay in the absence or presence of the tyrosine kinase Src as described in "Materials and Methods". As control experiments, the SDS-soluble fractions were directly subjected to in vitro kinase assay in the absence or the presence of Src. After the treatments, the reaction mixtures were analyzed by immunoblotting with PY99 (upper panel) or anti-Src antibody (IB: Src). The positions of LV1, Src and pp33/LV2 are indicated. (C) High NaCl-solubilized fractions (Cont., one oocyte-equivalent of protein per lane) prepared from *Xenopus* immature oocytes were either untreated (Imm.depl., cont.) or immunoprecipitated with PY99 (Imm.depl., PY99). The samples were then incubated with protein A-Sepharose as described in "Materials and Methods" and the resulting unbound materials were collected by a brief centrifugation. The presence of pp33/LV2 was evaluated by immunoblotting with PY99 and CBB protein staining.

scopic observation with a pulse-directed injector system Nanoject (Drummond, PA, USA) as described previously (Sato et al., 1999, 2000; Tokmakov et al., 2005). A protein sample (0.8–1.0 mg/ml) or buffer alone was loaded into an oil-filled glass microcapillary needles with a tip 25 μm in diameter. Up to 25 ng of protein was injected per oocyte in a total volume of 25 nl. Samples of oocytes were then subjected to treatment with either 10 μM progesterone or 5 μM insulin as described previously (García de Herreros et al., 1991). Successful oocyte maturation was monitored by occurrence of germinal vesicle breakdown (GVBD), as determined by dissection of oocytes fixed in 10% trichloroacetic acid and microscopic observations.

RESULTS

We prepared subcellular fractions of *Xenopus* immature oocytes by successive extraction with detergent-free buffer (cytosolic fraction), Triton X-100-containing buffer (TX100-soluble fraction), and TX100 plus SDS-containing buffer (SDS-soluble fraction). Immunoblotting of these fractions with the anti-phosphotyrosine antibody PY99 demonstrated that a 33-kDa tyrosine-phosphorylated protein (pp33) was present in the SDS-soluble fraction (Fig. 1A, lane 3; Fig. 1B, lane 3). Silver staining of the SDS-PAGE gel (Fig. 1A, right panel) and immunoblotting studies (Fig. 1B) showed that pp33 was present predominantly in the SDS-solubilized fractions where yolk-associated proteins such as vitellogenin fragments and phosvitin (90–110 kDa and 25–35 kDa) were enriched, but not in the other fractions where cytosolic markers tubulin and mitogen-activated protein kinase (MAPK) or a membrane protein marker uroplakin III (UPIII) were enriched. The presence of phosphotyrosine or skim milk, but not phosphoserine or phosphothreonine, reduced the immunoreactivity of PY99 to pp33 and some other SDS-solubilized proteins (Fig. 1C).

As we expected, PY99 immunoprecipitated pp33 effectively (data not shown), allowing us to perform immunopurification of pp33. Moreover, an antibody that was raised against lipovitellin 2, the ~35 kDa fragment of vitellogenin was found to recognize pp33 immunoprecipitated with PY99 (Fig. 1D, lane 3), suggesting that pp33 is identical to lipovitellin 2. This idea is supported by Mass spectrometry and database search of the immuno-purified pp33 (Fig. 2). In *Xenopus* oocytes, at least two similar but distinct types of lipovitellin 2 are expressed; one from vitellogenin B1 (VgB1) and the other from vitellogenin A2 (VgA2). Of the nine peptides annotated to be part of lipovitellin 2, eight corresponded to parts of VgB1 and one corresponded to parts of VgA2

(Fig. 2). The amino acid sequence of lipovitellin 2 contains several tyrosine residues; some of which were included in the annotated sequences in the mass spectrometric analysis. However, we failed to obtain data that showed peptide fragment(s) containing phosphate group(s). We hereafter retermed the tyrosine-phosphorylated protein pp33/LV2 (lipovitellin 2).

We next examined the presence of pp33/LV2 during the growth and maturation of oocytes, fertilization, and early embryogenesis. Immunoblot analyses demonstrated that pp33/LV2 was present at the beginning (stages I and II) as well as subsequent stages (stages III–VI) of oocyte growth (Fig. 3A). Progesterone-induced oocyte maturation, fertilization, and early embryogenesis up to stages 45/46 did not alter the amount or extent of the tyrosine phosphorylation of pp33/LV2 (Fig. 3A, B). However, the protein signal suddenly

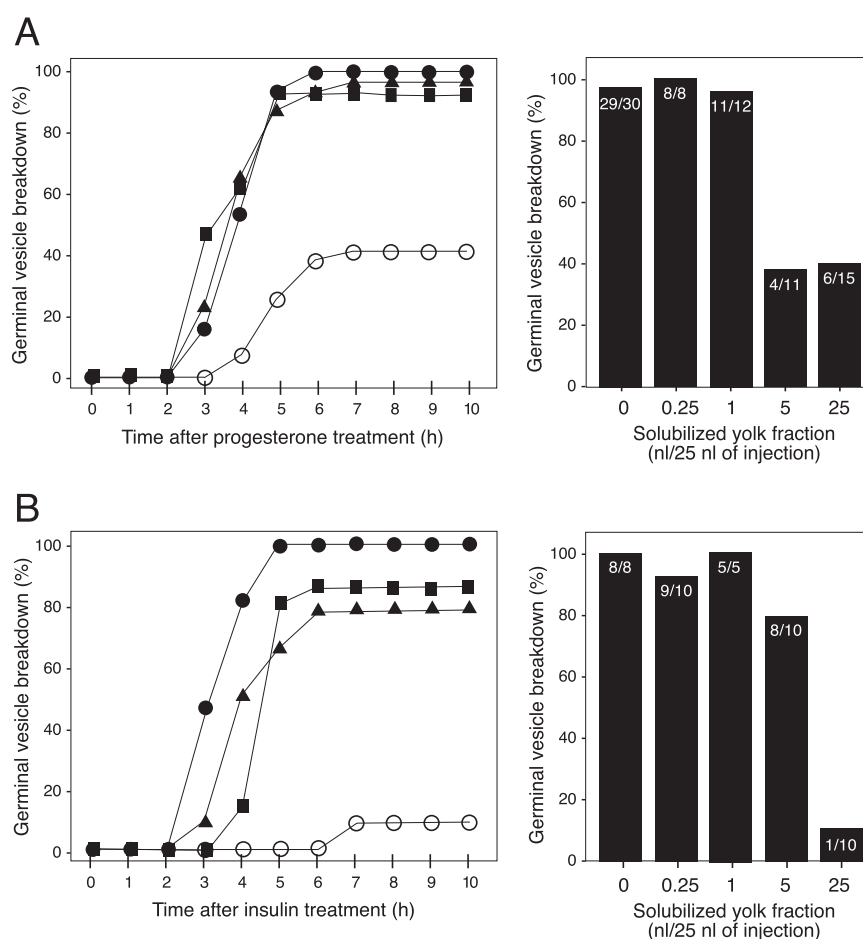


Fig. 5. Effect of microinjection of the solubilized yolk fractions on hormone-induced oocyte maturation. Shown in left are the time course data of germinal vesicle breakdown after the addition of (A) 2 μM progesterone or (B) 10 ng/mL insulin to oocytes. In each experiment, the following four kinds of microinjection were examined: control injections (closed circles), high NaCl-solubilized yolk fraction (open circles), high NaCl-solubilized and LAR-treated yolk fraction (closed triangles), and high NaCl-solubilized and PY99-immunodepleted yolk fraction (closed squares). Twenty-five ng of protein (in 25 nL) was used for all of the injections except for the control injections. Results of a representative experiment are shown. Shown in right are graphs representing the effect of the NaCl-solubilized yolk fraction on (A) progesterone- or (B) insulin-induced oocyte maturation as a function of the amounts of the fractions (0–25 ng protein per microinjection). The number of oocytes showing germinal vesicle breakdown versus the number of oocytes examined is plotted.

disappeared at developmental stage 47/48, when the disappearance of both lipovitellin 1 and 2 was taking place (Fig. 3B, silver stain). Shortly before the disappearance of pp33/LV2 in the SDS-soluble fractions, a PY99-immunoreactive band of 33 kDa became detectable in the TX100-soluble fractions (stages 39–41 and 43, Fig. 3B), suggesting that pp33/LV2 becomes a soluble component at these embryonic stages.

Treatment of the SDS-soluble oocyte fractions with recombinant LAR, a tyrosine-specific protein phosphatase, effectively diminished the binding of the anti-phosphotyrosine antibody to pp33/LV2 (Fig. 4A). We next examined whether the LAR-treated, i.e. dephosphorylated, pp33/LV2 could be re-phosphorylated on tyrosine residues by using the purified Src protein. As shown in Fig. 4B, pp33/LV2 was re-phosphorylated in a Src-dependent manner, but with low efficiency. On the other hand, LV1, another vitellogenin-derived yolk protein that migrated at 100–150 kDa on the SDS-PAGE gel, is tyrosine-phosphorylated at relatively higher efficiency by Src (Fig. 4B). We also performed immunodepletion experiments. As shown in Fig. 4C, the solubilized yolk fraction immunodepleted with PY99 contained no detectable amount of pp33/LV2, while the same sample still contained LV2, whose amount was highly similar to the non-immunodepleted sample, as judged by CBB staining. The results indicate that only a limited fraction of LV2 is phosphorylated on tyrosine residues in oocytes.

Next, we microinjected the oocyte fractions containing pp33/LV2 into live oocytes. To this end, we extracted the TX100-insoluble yolk fractions with buffer containing 1 M NaCl. Under this condition, the tyrosine-phosphorylated pp33/LV2 was effectively solubilized as in the case of its extraction with SDS (data not shown). We then performed a quantitative microinjection of the NaCl-solubilized yolk fractions into fully-grown, immature oocytes. The injected oocytes did not show any change in cytological morphology (data not shown), indicating that they were not damaged or impaired. However, these oocytes showed a reduced response to hormone (progesterone or insulin)-induced oocyte maturation, as judged by the occurrence of germinal vesicle breakdown (Fig. 5A and 5B, left panels) and phosphorylation of p42 MAPK (data not shown). Such an inhibitory effect of the NaCl-solubilized yolk fraction on progesterone-induced oocyte maturation was dose-dependent (Fig. 5A and 5B, right panels) and was not seen when the yolk fractions were treated with the tyrosine phosphatase LAR (Fig. 5A and 5B, closed triangles) or immunodepleted with PY99 (Fig. 5A and 5B, closed squares). Co-injection of the yolk fraction and the heat-inactivated LAR, or injection of the yolk fraction immunodepleted with no antibody did not inhibit oocyte maturation by progesterone (data not shown). Together, it is suggested that pp33/LV2, under solubilized conditions, is capable of inhibiting hormone-induced oocyte maturation in a phosphotyrosine-dependent manner.

As in *Xenopus laevis*, oocytes and early embryos of all known amphibian species and many other vertebrates and invertebrates possess yolk materials, major components of which are structurally and/or functionally similar to lipovitellins. Therefore we were interested in examining whether yolk-associated proteins are phosphorylated at tyrosine residues in the other species. Immunoblotting experiments

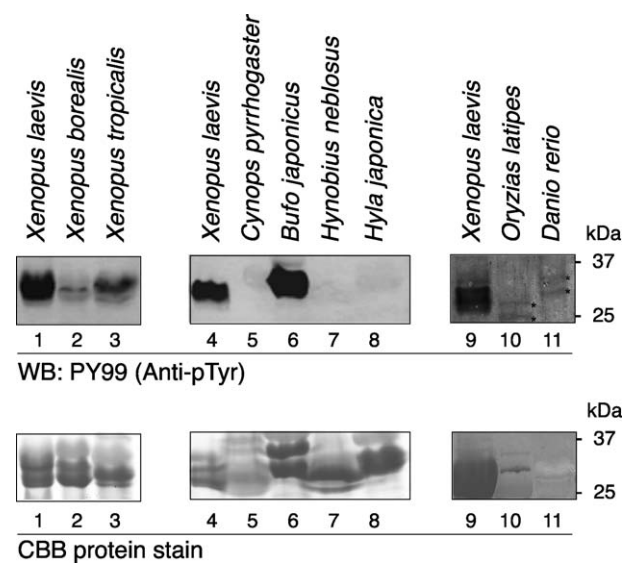


Fig. 6. Presence of a pp33/LV2-like protein in *Xenopus* subspecies, some other anuran and urodele amphibians, and fish species. SDS-solubilized yolk fractions were prepared from immature oocytes or early embryos of *Xenopus laevis* (lanes 1, 4, and 9, one oocyte-equivalent of protein per lane), *Xenopus borealis* (lane 2, two oocyte-equivalents of protein per lane), *Xenopus tropicalis* (lane 3, four oocyte-equivalents of protein per lane), *Cynops pyrrhogaster* (lane 5, 0.33 oocyte-equivalents of protein per lane), *Bufo japonicus* (lane 6, 0.25 blastula embryo-equivalents of protein per lane), *Hynobius nebulosus* (lane 7, one oocyte-equivalent of protein per lane), and *Hyla japonica* (lane 8, one oocyte-equivalent of protein per lane), *Oryzias latipes* (lane 10, one egg-equivalent of protein per lane), and *Danio rerio* (lane 11, one egg-equivalent of protein per lane), and analyzed by immunoblotting with PY99 (upper panels) or by CBB stain (lower panels).

demonstrated that *Xenopus borealis* and *Xenopus tropicalis*, which are subspecies of *Xenopus laevis*, possess phosphotyrosine-containing yolk-associated proteins of a similar molecular size to pp33/LV2 (Fig. 6, lanes 2 and 3). A frog species, *Bufo japonicus* (the bull frog), also showed the presence of prominently tyrosine-phosphorylated, yolk-associated proteins at around 30–35 kDa (Fig. 6, lane 6). Two fish species, *Danio rerio* (zebrafish) and *Oryzias latipes* (medaka), seemed to possess tyrosine-phosphorylated proteins at 30–35 kDa in the solubilized yolk fractions (Fig. 6, lanes 10 and 11). Contrastingly, *Hyla japonica* (frog), *Cynops pyrrhogaster* (newt) and *Hynobius nebulosus* (salamander), were shown to contain no tyrosine-phosphorylated proteins in the yolk fractions in the similar positions (Fig. 6, lanes 5, 7, and 8). Oocytes of *Athalia rosae* (sawfly), employed as an invertebrate species, did not contain such yolk-associated tyrosine-phosphorylated proteins either (data not shown).

DISCUSSION

In multicellular organisms, protein-tyrosine phosphorylation is known to play a crucial role in several kinds of signal transduction. In a physiological context, protein-tyrosine phosphorylation is, in most cases, transient and unstable. However, it is sometimes persistent and stable in cells under pathological conditions (e.g. constitutive phosphorylation of

cellular proteins in viral Src-transformed cells). Therefore, it is generally accepted that the fine-tuned 'on-off' switching of protein-tyrosine phosphorylation is important for normal cellular processes, while its deregulation may lead to pathological features in cells. In this study, we observed that a 33-kDa protein initially identified in the SDS-solubilized yolk fractions of immature oocytes of *Xenopus laevis*, is stably phosphorylated at tyrosine residues during oocyte growth, oocyte maturation, fertilization, and early embryogenesis.

Immunoprecipitation and mass spectrometry studies revealed that pp33 is LV2, a fragment of vitellogenin. There have been some reports on phosphorylated yolk-associated or related proteins in *Xenopus* oocytes. Phosvitin, a proteolytic fragment of vitellogenins that has a similar molecular size to pp33/LV2, is a heavily serine-phosphorylated protein (Gerber-Huber et al., 1987; Ohlendorf et al., 1977). It is believed that the high content of phosphoserine residues in phosvitin contributes to a supply of energy in early embryonic development (Sparks et al., 1988; Wahli et al., 1976, 1981; for review see Wahli, 1988). A phosphoprotein of 25-kDa, named pp25, whose amino acid sequence partially overlaps that of phosvitin and of LV2, has been identified in the cytoplasmic fractions of *Xenopus* oocytes and early embryos (Nakamura et al., 2007; Sugimoto and Hashimoto, 2006). Because of its distinct subcellular distribution, it has been suggested that pp25 plays different roles than those of yolk-associated proteins.

Browaeys-Poly et al. (2007) reported that LV1, a larger fragment of vitellogenins, is partly cytoplasmic in *Xenopus* oocytes and that the cytoplasmic LV1 is phosphorylated at tyrosine residues and can serve as a scaffold for a signaling complex containing the adaptor protein Grb2, the guanine nucleotide exchanging factor Sos, and the phospholipase C γ . The present study demonstrated that LV1 solubilized from the yolk materials of *Xenopus* oocytes can be effectively phosphorylated by purified Src. In vitro Src phosphorylation of the LAR-treated pp33/LV2, however, was not as efficient as in the case of LV1. The results suggest that the phosphorylation of LV2 by Src requires a co-factor(s), which is missing in the in vitro kinase assay. Another possibility is that some unknown kinase(s) in oocytes or liver, where vitellogenin is synthesized, catalyzes the tyrosine phosphorylation of pp33/LV2. In support of this idea, we observed that the plasma fractions prepared from *Xenopus* blood contained a significant amount of tyrosine-phosphorylated vitellogenin (data not shown). Thus, we assume that in *Xenopus*, vitellogenin is synthesized and tyrosine-phosphorylated in the liver; transported by blood to the female gonads; internalized into the oocytes via a specific receptor for vitellogenin; and broken down into LV1, pp33/LV2, phosvitin and pp25.

In normal conditions, pp33/LV2 and other yolk-associated materials are present in a crystallized and thus insoluble form in oocytes. So we were interested in examining the possible cellular functions elicited by the presence of the solubilized pp33/LV2 in oocytes. Microinjection studies demonstrated that the solubilized yolk fractions inhibited germinal vesicle breakdown of *Xenopus* oocytes induced by progesterone or insulin (Fig. 5). The inhibitory effect was pp33/LV2-dependent because the yolk fractions treated with LAR or immunodepleted of pp33/LV2 did not show such an

effect. Progesterone- or insulin-induced oocyte maturation in *Xenopus* involves phosphorylation and/or dephosphorylation of proteins at tyrosine residues: they include p34^{cdk1}, a component of maturation-promoting factor that undergoes tyrosine dephosphorylation during oocyte maturation; MAPK (Ferrell et al., 1991; Gotoh et al., 1991) and Xp95/Alix (in the case of progesterone) (Che et al., 1999; DeJournett et al., 2007), both of which become tyrosine-phosphorylated upon oocyte maturation. Thus, we suggest that pp33/LV2, if it is present in a soluble form in oocytes, can interfere with the phosphotyrosine-dependent signal transduction for oocyte maturation.

Then, what is the physiological function of the tyrosine-phosphorylated forms of pp33/LV2 in *Xenopus*? We have shown that only a small fraction of LV2 is the tyrosine-phosphorylated form (Fig. 4C). Immunoblotting data demonstrates that the level of tyrosine phosphorylation of LV2 is unchanged until the developmental stages 45/46 (Fig. 3B, SDS-soluble, IB: PY99). On the other hand, silver stain data of SDS-PAGE gels demonstrate that the amount of total LV2 is decreasing as developmental stages progress (Fig. 3B, silver stain). The data suggest that the tyrosine-phosphorylated forms of LV2 are more stable than the non-phosphorylated forms of LV2 during this developmental period. In addition, at the stages 45/46, Triton X-100-soluble forms of the tyrosine-phosphorylated LV2 become detectable (Fig. 3B, TX100-soluble, IB: PY99). Thereafter (at the stages 47/48), both the tyrosine-phosphorylated forms of LV2 and the non-phosphorylated forms of LV2 disappear from embryo fractions (Fig. 3B, TX100-soluble, IB: PY99; SDS-soluble, IB: PY99; silver stain). From these data, it is suggested that the initial timing of de-crystallization from yolk platelets and/or proteolytic digestion of the tyrosine-phosphorylated LV2 is different from that of the non-phosphorylated LV2, while the disappearance of these LV2 fractions from embryos takes place very synchronously. Physiological meaning of the presence of the tyrosine-phosphorylated LV2 as a yolk platelet-associated form (until stages 45/46) and as a Triton X-100-soluble form (only in the stages 45/46) will be of interest for further study.

As we presented data and discussed, pp33/LV2 appeared in the cytoplasmic fraction at the stages 45/46, but embryonic development progressed normally. This raises questions as to why these soluble forms of pp33/LV2 do not inhibit the developmental processes as they do in the progress of hormone-induced oocyte maturation. We think that the regulated appearance of the pp33/LV2 in the cytoplasmic fraction at the embryonic stage 46/47 may have some physiological role in the progress of development, which we don't know yet the molecular details. On the other hand, it seems that the abnormal presence of the pp33/LV2 (due to its injection) in the oocyte has an inhibitory effect on the progress of oocyte maturation.

We found that a tyrosine-phosphorylated LV2-like protein(s) is present in some anuran amphibians and possibly fish. On the other hand, urodele amphibians and *Hyla japonica*, a frog species that is thought to be phylogenetically distant from *Xenopus*, possess LV2-like proteins with no phosphotyrosine. These results suggest that tyrosine phosphorylation of LV2 (-like proteins) is an evolutionarily transient phenomenon at least in amphibian species. In this

connection, it is interesting to note that Tick oocytes are shown to possess a tyrosine-phosphorylated vitellin, which will be dephosphorylated during egg development (Silveira et al., 2006), and that the present study showed that sawfly oocytes do not possess tyrosine-phosphorylated yolk proteins. These results indicate that insect species also show evolutionarily branching for the presence of tyrosine-phosphorylated LV2-like proteins.

In summary, we report here the identification, purification, and characterization of a 33-kDa *Xenopus* lipovitellin 2 as a tyrosine-phosphorylated protein. This protein, named pp33/LV2, is stably tyrosine phosphorylated during oogenesis, oocyte maturation, fertilization, and early embryogenesis. In vitro enzyme assays demonstrate that pp33/LV2 can be dephosphorylated and re-phosphorylated by recombinant LAR and Src, respectively. Data obtained in microinjection experiments suggest that pp33/LV2 has a role in phosphotyrosine-dependent signal transduction. pp33/LV2-like phosphotyrosine-containing yolk proteins are also present in some other anuran amphibian species and fish, but not in urodele amphibian species and insect species tested so far. These features suggest that tyrosine phosphorylation of pp33/LV2 has been a matter of evolutionary selection, whose biological implications await further investigation.

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