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Biochemical Characterization of the *Drosophila dpp* Protein, a Member of the Transforming Growth Factor β Family of Growth Factors

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The decapentaplegic (*dpp*) gene of *Drosophila melanogaster* is required for pattern formation in the embryo and for viability of the epithelial cells in the imaginal disks. The *dpp* protein product predicted from the DNA sequence is similar to members of a family of growth factors that includes transforming growth factor β (TGF-β). We have produced polyclonal antibodies to a recombinant *dpp* protein made in bacteria and used a metallothionein promoter to express a *dpp* cDNA in *Drosophila* S2 cells. Similar to other proteins in the TGF-β family, the *dpp* protein produced by the *Drosophila* cells was proteolytically cleaved, and both portions of the protein were secreted from the cells. The amino-terminal 47-kilodalton (kDa) peptide was found in the medium and in the proteins adhering to the plastic petri dish. The carboxy-terminal peptide, the region with sequence similarity to the active ligand portion of TGF-β, was found extracellularly as a 30-kDa homodimer. Most of the 30-kDa homodimer was in the S2 cell protein adsorbed onto the surface of the plastic dish. The *dpp* protein could be released into solution by increased salt concentration and nonionic detergent. Under these conditions, the amino-terminal and carboxy-terminal portions of *dpp* were not associated in a stable complex.

Analysis of mutant alleles in the *Drosophila* decapentaplegic (*dpp*) gene indicates that the *dpp* gene product is required for the proper development of the embryonic dorsal hypoderm (18), for viability of larvae (42), and for cell viability of the epithelial cells in the imaginal disks (5, 45). Molecular isolation of the *dpp* DNA and mapping of mutant lesions onto the molecular map indicates that the gene spans over 50 kilobases (kb) of DNA (R. D. St. Johnston, F. M. Hoffmann, R. K. Blackman, D. Segal, R. Grimaila, R. W. Padgett, H. A. Irick, and W. M. Gelbart, Genes Dev., in press). The two protein-coding exons are located near the center of this region, and their expression is driven from at least five promoters distributed across 20 kb of 5′ DNA. *dpp* expression in the larval imaginal disks requires *cis*-regulatory elements distributed across 25 kb of DNA 3′ to the coding exons.

The two exons that are common to all of the transcriptional units contain an open reading frame whose predicted protein product is 588 amino acids in length; the carboxy-terminal 100 amino acids have sequence similarity to proteins in the transforming growth factor β (TGF-β) superfamily, which at this time includes five TGF-βs, Mullerian inhibiting substance (MIS), inhibins, the *Xenopus* protein Vg-1, the mouse protein Vgr-1, and three human bone morphogens (BMPs) (7, 10, 24, 25, 35, 47, 50, 52). Sequence similarities to this 100-amino-acid region are highest between *dpp* and human bone morphogenetic proteins (75%) or murine Vgr-1 protein (77%) and range between 23 and 57% for the other members of the TGF-β family (25, 52); the conserved amino acids include all seven cysteine residues in *dpp*. All of the vertebrate proteins examined to date are secreted proteins in which the carboxy-terminal domain is proteolytically cleaved from the full-length protein (10, 24, 28, 46). The active ligands for the TGF-βs and inhibin/activin are homo- or heterodimers of the carboxy-terminal regions linked by disulfide bonds (8, 10, 17, 24, 28, 46). TGF-β is secreted as a latent complex that must be activated to release the active carboxy-terminal dimer (30, 49).

We are interested in the role of growth factors in *Drosophila* development (15, 31a) and are studying the regulation and functions of the *dpp* growth factor. To obtain *dpp* protein in sufficient quantities to characterize its properties, processing, and biological effects, we have produced *dpp* in Schneider-2 (S2) cells by using an inducible promoter fused to a *dpp* cDNA. Further, we have raised antibodies to a *dpp* protein made in bacteria. These antibodies specifically detect *dpp* protein produced by the S2 cells. In this report, we demonstrate that the *dpp* protein is both cleaved and secreted and that quantities of *dpp* protein can be recovered from the S2 cell expression system.

MATERIALS AND METHODS

Cell culture and generation of clonal cell lines. *Drosophila* S2 cells (41) were maintained in M3 medium (43) supplemented with 12.5% fetal calf serum (GIBCO Laboratories). Transfected cells were maintained on the same medium with 2×10^{-7} M methotrexate (MTX) at cell densities of 10^5 to 10^7/ml and fed two to three times per week. S2 cells (2.0×10^6 total) were transfected with 10 μg of pMTDPP (Fig. 1a) and 10 μg of pHGCO DNA by means of calcium phosphate-DNA coprecipitation (R. E. Moss, Ph.D. thesis, Harvard University, Cambridge, Mass., 1985). The pHGCO vector contains a dihydrofolate reductase gene which confers MTX resistance on transformed cells (2). On day 3 after transfection, transfected cells were placed on M3-MTX. On day 7, 4.0×10^6 cells from each transfection, together with 1.0×10^6 gamma-irradiated S2 feeder cells, were plated into 2 ml of 0.3% Noble agar.

On day 34, 20 clones were picked from the agar and transferred to 96-well dishes containing 0.2 ml of M3-MTX per well. On day 48, the clones were transferred to 24-well dishes containing 1 ml of medium per well. On day 60, 4 of the 20 clones picked were replated into 0.3% agar and allowed to grow for 14 days. On day 74, 20 clones were

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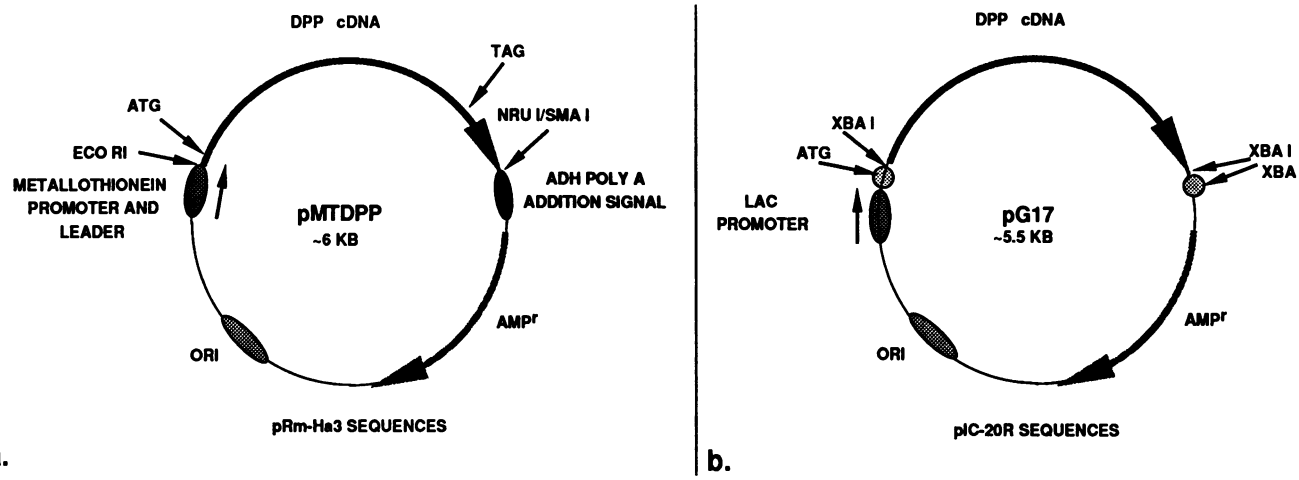

FIG. 1. Maps of expression constructs. (a) Structure of plasmid pMTDPP. A 2.4-kb *Eco*RI-to-*Nru*I fragment from the *dpp* cDNA E55 (35) was ligated into the *Eco*RI and *Sma*I sites of pRM-Ha3 (6). This plasmid contains both coding exons of *dpp* in their entirety as well as 10 base pairs of 5′ untranslated leader sequences. Additional 5′ untranslated sequences and a cap site are derived from the metallothionein gene, and the polyadenylation signal is derived from the alcohol dehydrogenase gene. (b) Structure of plasmid pG17. A 2.6-kb *Xba*I fragment from *dpp* cDNA E55 was ligated into an *Xba*I site of pIC-20R (27). This plasmid encodes the 533 carboxy-terminal amino acids of *dpp* under control of an IPTG-inducible *lac* promoter. A start codon and eight additional amino acids are encoded by the vector. Restriction digests, ligations, and transformation of bacteria were performed as described previously (26).

picked from each of the four plates and transferred to 96-well dishes, where they were allowed to grow for 9 days. Fourteen of them, representing multiple second clones from each of the original four, were then transferred to 24-well dishes, where they remained for 8 to 10 days before being transferred to small tissue culture flasks containing 3 ml of media.

RNA isolation and analysis. Twenty-four hours before RNA isolation, 2.0×10^7 cells from pMTDPP-transfected clonal lines and control cell lines were resuspended in 10 ml of M3-MTX medium and either left untreated or induced with 500 μM $CuSO_4$. RNA was isolated by a modification of a published procedure (9). Briefly, cells were harvested, suspended in 10 ml of 5 M guanidinium isothiocyanate–10 mM EDTA–50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4)–5% β-mercaptoethanol and disrupted by a Polytron at high speed for 30 s. These cell lysates were layered on top of 2 ml of diethyl pyrocarbonate-treated 5.7 M cesium chloride in an ultracentrifuge tube. Samples were spun at 36,000 rpm for 20 h. Most of the guanidinium buffer was removed from the centrifuge tube by using an RNase-free pipette. The bottom quarter of the tube was then cut with a sterile razor blade and quickly inverted to drain the remaining cesium and guanidinium.

The RNA pellet was resuspended in 200 μl of 0.3 M sodium acetate and precipitated with 500 μl of 100% ethanol three times. After the last precipitation, the samples were dried in a Speed Vac (Savant) and suspended in diethyl pyrocarbonate-treated deionized water. A 5-μg amount of each sample was run on a formaldehyde gel exactly as previously described (12). The RNA was electrotransferred onto a Zeta Probe membrane in 0.5× TAE (20 mM Tris, 10 mM sodium acetate, 0.5 mM EDTA [pH 7.4]) at 100 to 150 mA for 18 to 20 h. The blot was probed with randomly primed (primer pDN6; Pharmacia) ^{32}P-labeled DNA at 10^8 to 10^9 cpm/μg and 10^6 to 10^7 cpm/ml. Hybridizations were performed overnight at 42°C in 50% deionized formamide–5× Denhardt solution–4× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate (SDS). Washes were done twice at room temperature with 2× SSC–1% SDS and four times at 65°C with 1× SSC–1% SDS.

Generation of *dpp* antibodies. The bacterial *dpp* protein was synthesized by using plasmid pG17 (Fig. 1). Host bacteria [CSH 26 Str^r Δ(*pro-lac*) *recA56* Tet^s F′ *pro lacZ*VU8*I*^{q} (J. Yin, personal communication)] harboring pG17 were induced at an A_{600} of 0.7 for 1.5 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were harvested by centrifugation at 5,000 × *g*, and inclusion bodies were prepared as described previously (32).

To generate polyclonal antibodies to *dpp*, rabbits were immunized 5 to 10 times on a monthly basis with 500 μg to 1 mg of *dpp* inclusion bodies (32). Subsequent boosts were with 200 to 500 μg of inclusion body *dpp* protein that had been purified by SDS-polyacrylamide gel electrophoresis (PAGE) (23), followed by electroelution in an Elutrap (Schleicher & Schuell, Inc.) at 200 V for 8 to 12 h. All injections were in MPL+TDM adjuvant (RIBI Immunochem).

Antisera were passed over Affigel-protein A columns (Bio-Rad Laboratories) to purify the immunoglobulin G (IgG). Sera were diluted with an equal volume of 1.5 M glycine–3 M NaCl (pH 8.9) before loading on the columns. The columns were washed with 5 volumes of this buffer before elution with 0.1 M glycine–20 mM NaCl (pH 3.2). IgG-containing fractions were fractionated with 50% (final concentration) saturated ammonium sulfate and centrifuged at 10,000 × *g* to pellet the precipitate. The IgG pellet was resuspended in and dialyzed against phosphate-buffered saline (PBS; 20 mM potassium phosphate, 150 mM NaCl [pH 7.2]).

Western immunoblotting. After electrophoresis on SDS-polyacrylamide gels (23), proteins were electrotransferred to nitrocellulose in a buffer containing 25 mM Tris, 20 mM glycine, and 20% methanol at 150 mA for 12 h or 400 mA for 2 h. After transfer, all subsequent incubations and washes were in Blotto (20), which contains 20 mM KPO_4 (pH 7.2), 150 mM NaCl, 1% (wt/vol) nonfat dry milk (Carnation), and 0.5% Tween-20 (Sigma Chemical Co.). Filters were blocked in Blotto for 1 h at room temperature or overnight at 4°C. Primary antibody was added at a 1:1,000 dilution for 1 to 2 h at room temperature. The filters were washed five times for

30 min each time. Alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit IgG; Sigma) was added at a 1:1,000 dilution for 1 h at room temperature and washed as before. After a brief rinse in 50 mM Tris (pH 9) to remove milk and detergent and to raise the pH, the blots were developed by using the NBT/BCIP system (Kirkegaard and Perry Laboratories).

Metabolic labeling and immunoprecipitation. Before labeling, *Drosophila* S2 cells were preinduced for 20 h at 5×10^6 cells per ml in M3-MTX 500 μM $CuSO_4$. Cells were washed four times in serum-free medium minus methionine and cysteine but containing ascorbate (100 μg/ml) and reduced glutathione (20 μg/ml) and resuspended in this media at 10^7 cells per ml. After 45 min in this medium, [^{35}S]methionine-cysteine (ICN Trans^{35}S-label) was added to 200 μCi/ml, and the cells were labeled for 20 h at 25°C. Phenylmethylsulfonyl fluoride (PMSF) and aprotinin were added to 2 mM and 40 μg/ml, respectively, and the cells were pelleted at 1,500 × *g* for 15 min at 4°C.

Cells were lysed in 50 mM Tris (pH 8.0)–156 mM NaCl–4 mM EDTA–0.1% SDS–1% Triton X-100–0.5% sodium deoxycholate–2 mM PMSF–40 μg of aprotinin per ml for 30 min at 4°C (14). Lysates were clarified by centrifugation at 10,000 × *g* for 15 min at 4°C.

To recover proteins still bound to the petri dish after removal of cells and media, the petri plates were rinsed twice with PBS for 10 min and then with 50 mM Tris (pH 7.4)–500 mM NaCl–10 mM EDTA–1% Tween-20–2 mM PMSF–40 μg of aprotinin per ml for 60 min at 25°C. NaCl–Tween-20 plate washes were centrifuged at 1,500 × *g* for 15 min at 4°C to remove any cellular debris.

For immunoprecipitations, 200-μl samples of media, lysates, or NaCl–Tween-20 plate washes were precleared with 5 μl of normal rabbit serum and two incubations with 40 μl of protein A-Sepharose beads in PBS (50% [vol/vol]). A 1:10 dilution of the lysate treatment and a 1:2.5 dilution of the medium or NaCl–Tween-20 plate wash treatments were prepared in the cell lysate buffer or the NaCl–Tween-20 buffer. *dpp* proteins were immunoprecipitated for 90 min on ice with the addition of 50 μg of an IgG fraction of the polyclonal antibody per 275 μl of dilute sample. Protein A-Sepharose beads (200 μl) were added to the mixtures for 60 min at 4°C, after which the beads were pelleted and washed once in PBS–0.1% Tween-20 and twice in PBS. Immune complexes were eluted at 100°C for 10 min in 2× sample buffer with or without β-mercaptoethanol. The proteins were separated on 10% SDS-polyacrylamide gels, and the gels were treated with En^{3}Hance (Dupont, NEN Research Products) before drying and exposure to Kodak XAR film at −70°C.

Protein sequencing. To sequence the 20-kilodalton (kDa) cleavage product of *dpp*, 5×10^8 2K and 2I cells were induced for 48 h with 500 μM $CuSO_4$ in 120 ml of M3 medium with 1.25% fetal serum. The cells were pelleted at 500 rpm for 5 min, and the medium was made 1 mM in EDTA and 2 mM in PMSF (Boehringer Mannheim Biochemicals). Saturated ammonium sulfate was added to a final concentration of 10%, and the medium was stirred overnight at 4°C. The resulting precipitate was pelleted at 10,000 × *g* for 30 min. The pellet was suspended in 1 M acetic acid–1 mM EDTA–2 mM PMSF and dialyzed against this buffer. The 10% ammonium sulfate supernatant was fractionated further at 20, 30, 40, and 50% ammonium sulfate. The precipitate at each step was recovered and treated as described above. After dialysis, the samples were run on an SDS-polyacrylamide gel, and the gel was stained with silver (33) to visualize the proteins.

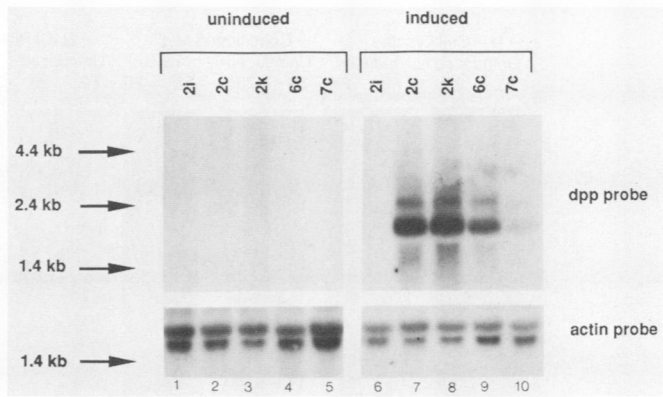

FIG. 2. Northern analysis of *dpp* RNA in S2 cell lines. RNAs from clonal cell lines derived by transfections of *Drosophila* S2 cells with pMTDPP and pHGCO or with pHGCO alone were probed with ^{32}P-labeled *dpp* DNA. ^{32}P-labeled actin DNA was used separately as a probe to confirm even loading of the gel. Lanes: 1 to 5, RNA from uninduced cells; 6 to 10, RNA from cells induced with 500 μM $CuSO_4$ for 24 h; 1 and 6, control cell line 2I; 2 and 7, pMTDPP cell line 2C; 3 and 8, pMTDPP cell line 2K; 4 and 9, pMTDPP cell line 6C; 5 and 10, pMTDPP cell line 7C.

It was determined that the majority of the *dpp* protein precipitated in the 30% cut. This fraction was used for further analysis.

For sequencing, 8% of the 30% ammonium sulfate cut was divided into three lanes of an SDS-polyacrylamide gel, using ultrapure acrylamide (Bethesda Research Laboratories, Inc.), recrystallized SDS, and MilliQ water (Millipore Corp.) in all buffers. Proteins were electrotransferred from this gel to Immobilon P (Millipore) in a 10 mM CAPS [3-(cyclohexylamino)-1-propane-sulfonic acid] buffer at 50 mA for 16 h. After transfer, the membrane was stained with 0.1% Coomassie blue in 50% high-performance liquid chromatography-grade methanol (J. T. Baker Chemical Co.) and destained with 50% methanol (29). A 7-pmol sample of the 20-kDa protein on Immobilon P was sequenced by the University of Wisconsin Biotechnology Center on an Applied Biosystems Pulsed Liquid Phase sequencer (model 447A) with an on-line PTH amino acid analyzer; 20 cycles were achieved.

Two-dimensional gel electrophoresis. Two-dimensional electrophoresis was performed as described previously (34) by Kendrick Laboratories, Inc.

RESULTS

Establishing cell lines that express *dpp*. A *Drosophila* cDNA containing the entire *dpp* protein-coding sequence was inserted into a vector that allows expression of the *dpp* sequences from a *Drosophila* metallothionein promoter, yielding plasmid pMTDPP (Fig. 1a). Clonal lines of MTX-resistant cells were established through two cycles of cloning in soft agar. The expression of *dpp* RNA was measured by Northern (RNA) analysis in control cell lines, which had been transfected with plasmid pHGCO alone, and in cell lines derived from cells cotransfected with pHGCO and pMTDPP (Fig. 2). *dpp* mRNA was not detected in untransfected S2 cells or in the control cell lines in either the absence or presence of copper. A small amount of *dpp* mRNA was found in uninduced cells transfected with pMT DPP, presumably as a result of constitutive expression from the metallothionein promoter. Upon induction with copper,

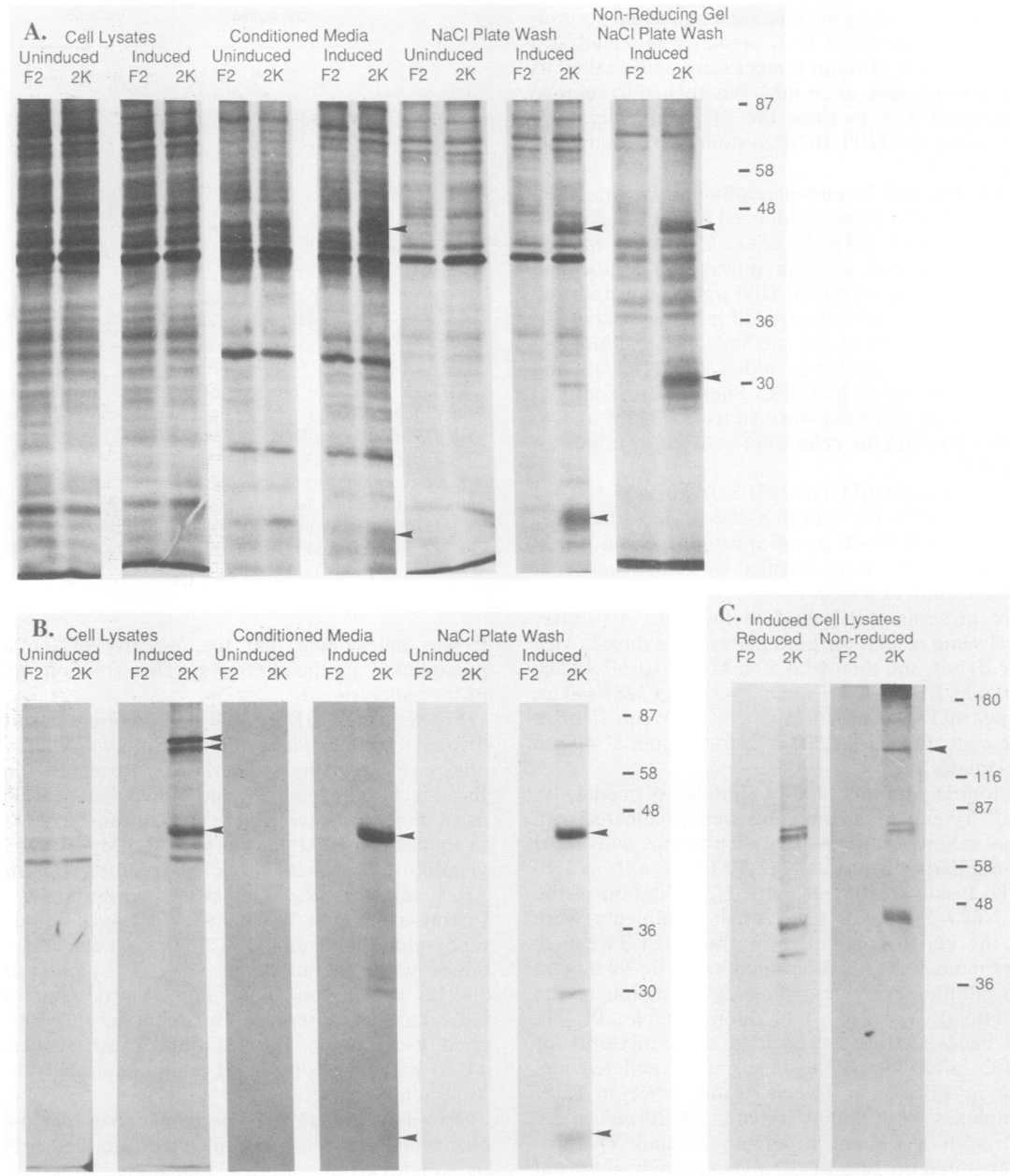

FIG. 3. Evidence for secretion and processing of *dpp* protein in *Drosophila* S2 cells. Uninduced and copper-induced cells from control cell line F2 and pMTDPP-transfected cell line 2K were labeled and proteins were prepared as described in Materials and Methods. All samples were reduced with β-mercaptoethanol before PAGE unless otherwise indicated. The molecular masses (in kilodaltons) of the size standards are indicated to the right of each panel. (A) Total ^{35}S-labeled proteins from F2 and 2K cell lines. Arrowheads point to 47- and 20-kDa proteins specific to *dpp*-expressing cells. The 20-kDa protein was absent and a 30-kDa protein was present on the nonreducing gel of the 2K NaCl–Tween-20 wash. (B and C) Immunoprecipitation of ^{35}S-labeled proteins by antibodies to *dpp*. Proteins were detected in immunoprecipitates from the cell lysates, the media, and the NaCl–Tween-20 plate washes of induced 2K cells but not from control cells or uninduced 2K cells. In panel B, arrowheads point to protein bands at 47, 68, and 70 kDa immunoprecipitated from the cell lysates. A 20-kDa band cannot be seen on the figure but is barely visible on original films of the cell lysates from 2K cells. Proteins of 47 and 20 kDa were immunoprecipitated from the media and the NaCl–Tween-20 wash. The band at 30 kDa varied in intensity between experiments and was most likely due to incomplete reduction of *dpp* homodimer. In panel C, immunoprecipitates from cell lysates were examined under reducing and nonreducing conditions. The arrowhead indicates a band at approximately 140 kDa specific to proteins from *dpp*-expressing cells fractionated under nonreducing conditions. ^{35}S label can be incorporated at three methionines per molecule of the 47-kDa portion of *dpp* and at four methionines and seven cysteines per molecule of the 20-kDa portion of *dpp*.

the level of *dpp* mRNA increased from 20- to 100-fold in these cells, depending on the specific cell line. One pMT DPP-containing cell line, 2K, was chosen for further analysis because of its high level of induced *dpp* mRNA.

Secretion and processing of *dpp*. ^{35}S-labeled proteins from the 2K cell line and the control F2 cell line were examined by SDS-PAGE and autoradiography (Fig. 3A) and by immunoprecipitation using an IgG fraction of rabbit polyclonal antibody to *dpp* (Fig. 3B). Proteins of 68 and 70 kDa were immunoprecipitated from the cell lysates of induced 2K cells

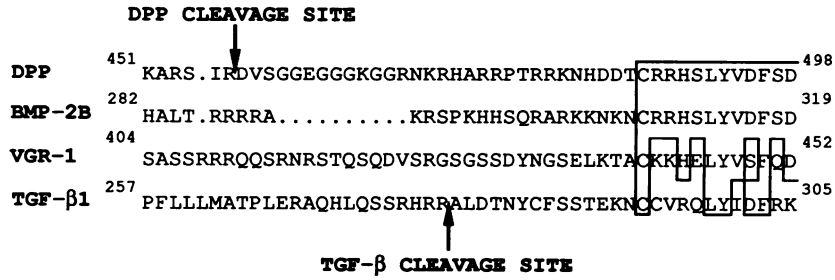

FIG. 4. Alignment of *dpp* (35), BMP2b (52), Vgr-1 (25), and TGF-β1 (10) amino acid sequences in the region of proteolytic cleavage. The BMP2b and Vgr-1 sequences were aligned to the *dpp* sequence by the University of Wisconsin Genetics Computer Group Bestfit program at a gap weight of 10 (11). The corresponding region of the TGF-β1 sequence is also shown. The cleavage of *dpp* after arginine 456 results in a 133-amino-acid carboxy-terminal protein. Cleavage TGF-β results in a 112-amino-acid carboxy-terminal protein. Clusters of arginines in BMP-2b and VgR-1 align with the *dpp* cleavage site and could be sites for the proteolytic processing. A boxed region beginning with the conserved cysteine, the first of the seven cysteines conserved in all four sequences, begins the region of greatest sequence similarity among the four proteins.

but not from F2 cell lysates. The predicted mass of the 588-amino-acid *dpp* polypeptide is 65 kDa. The 2-kDa difference between the two proteins detected may be due to removal of the signal sequence predicted from the *dpp* sequence (35). The absence of immunoprecipitable proteins from the F2 cells or uninduced 2K cells indicates that the antibodies are quite specific for *dpp* epitopes under the immunoprecipitation conditions.

A 47-kDa protein and a 20-kDa protein were also immunoprecipitated from cell lysates of 2K cells and from the conditioned medium of induced 2K cells. These proteins were present in sufficient quantities to be detectable in the total labeled proteins secreted from the induced 2K cells, although there was much less 20-kDa than 47-kDa protein in the medium (Fig. 3A, lane marked conditioned media, induced, 2K). Other members of the TGF-β family are proteolytically processed (10, 24, 28), and it seemed likely that the 47- and 20-kDa proteins secreted from the 2K cells were cleavage products of the full-length 68- and 70-kDa *dpp* proteins. Sufficient quantities of the 47- and 20-kDa proteins were obtained from the media of induced 2K cells to perform amino-terminal sequence analysis (see Materials and Methods). Although the amino terminus of the 47-kDa protein was blocked, the amino-terminal amino acid sequence of the 20-kDa protein (DVSGGEGGGKGGXNKXQPPR . . .) confirmed its identity as the carboxy-terminal portion of *dpp*. The sequence indicates that *dpp* is cleaved after amino acid 456, an arginine (Fig. 4).

The signal intensity from the *dpp* band at 20 kDa was always much lower than the intensity of the *dpp* band at 47 kDa in proteins recovered from the conditioned medium even though the 20-kDa portion of *dpp* could incorporate more [^{35}S]methionine and -cysteine. This finding raised the possibility either that the 20-kDa *dpp* cleavage product was degraded more rapidly than the 47 kDa peptide or that the 20-kDa peptide was adsorbed to the petri dish in which the cells were induced. Examination of proteins eluted from the petri dishes with SDS sample buffer revealed significant quantities of 47- and 20-kDa polypeptides as well as other labeled proteins (data not shown). We tried several milder conditions to remove the 20-kDa polypeptide from the plastic dish and found that washing the dish in a solution containing 500 mM NaCl and 1% Tween-20 solubilized most of the 20-kDa protein (Fig. 3A and 5).

Proteins in such an NaCl–Tween-20 wash were resolved by SDS-PAGE and examined by autoradiography (Fig. 3A and 5), by silver staining (Fig. 6), and by Western blot analysis (Fig. 6). When 1.6×10^8 cells in serum-free medium were induced to express *dpp* for 72 h in a 150-mm-diameter petri dish, approximately 740 μg of protein was recovered in the NaCl–Tween-20 wash; the 47- and 20-kDa proteins were highly enriched in this fraction, as indicated by the intensity of the bands on silver-stained gels. Results of silver staining and metabolic labeling experiments indicated that the 47-

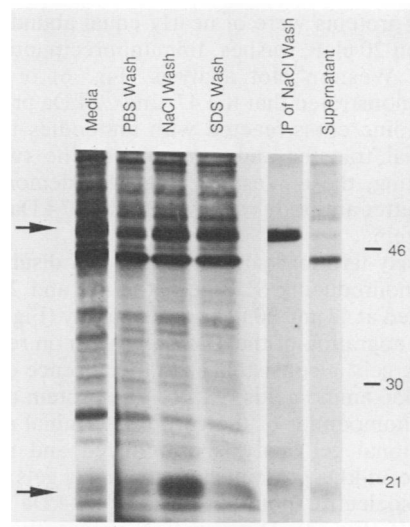

FIG. 5. Recovery of ^{35}S-labeled 20-kDa protein in NaCl–Tween-20 washes of plastic dishes in which the pMTDPP-transfected cell line 2K was induced to express *dpp*. Total labeled proteins in the conditioned medium and in sequential washes of the petri dish with PBS, 500 mM NaCl–1% Tween-20, and SDS sample buffer are shown. Equivalent volumes of the medium and washes were loaded on the gel. Arrows point to protein bands at 47 and 20 kDa detected in the media and the washes of the petri dishes. Most of the 20-kDa protein was recovered from the petri dish in the NaCl–Tween-20 wash. In the next to the last lane are shown proteins that were immunoprecipitated from the NaCl–Tween-20 wash with antibodies to *dpp*. In the rightmost lane are shown proteins that remained in the supernatant after the immunoprecipitation. All of the 47-kDa protein was detected in the immunoprecipitate. The major band at 45 kDa in the last lane represents a major contaminant of the NaCl wash that was left behind in the supernatant after immunoprecipitation of *dpp*. The gel was loaded with 100% of the immunoprecipitate and 15% of the immunoprecipitation supernatant.

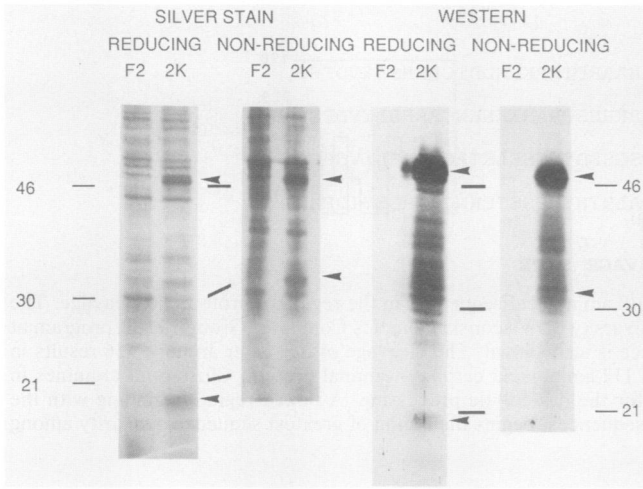

FIG. 6. Analysis of total proteins in NaCl–Tween-20 washes of petri plates in which cells of the pMTDPP-transfected cell line 2K were induced to express *dpp*. Total proteins in an NaCl–Tween-20 plate wash were fractionated by SDS-PAGE and visualized by silver staining or Western blot analysis. Arrowheads point to protein bands at 47 and 20 kDa on the reducing gels and to protein bands at 47 and 30 kDa on the nonreducing gels. We believe that the anti-*dpp* reactive proteins migrating between 47 and 30 kDa were most likely degradation products of the 47-kDa *dpp* fragment that were detected because the color reaction was overdeveloped to detect the 20-kDa portion of *dpp*.

and 20-kDa proteins were of nearly equal abundance in the NaCl–Tween-20 plate washes. Immunoprecipitation (Fig. 3B and 5) and Western blot analysis (Fig. 6) of the eluted proteins demonstrated that the 47- and 20-kDa proteins from the 2K cell line cross-reacted with antibodies to *dpp*. Assuming equal transfer and retention of the two proteins during blotting, these Western blots also demonstrate that there was better antibody reactivity to the 47-kDa than to the 20-kDa protein.

The carboxy-terminal portion of *dpp* is a disulfide-bonded dimer. On nonreducing SDS-gels, the 47- and 20-kDa proteins migrated at 47 and 30 kDa, respectively (Fig. 3A and 6). The similar migration of the 47-kDa protein on reducing and nonreducing gels is consistent with the absence of cysteines in the first 456 amino acids of *dpp*. The protein at 30 kDa is probably a homodimer of the carboxy-terminal portion. On two-dimensional gel analysis of reduced and nonreduced proteins, the 30-kDa protein on nonreducing gels migrated at the same isoelectric point as did the 20-kDa protein on reducing gels (Fig. 7). Dimerization apparently took place before the proteolytic processing of the 68-kDa pro form of *dpp*, since some of the pro form migrated at 140 kDa on nonreducing gels (Fig. 3C).

The 47- and 20-kDa *dpp* proteins are not associated in the NaCl–Tween-20 washes. TGF-β produced in COS cell expression systems is secreted as a latent complex; disruption of the complex to release the biologically active C-terminal dimer requires extremes of pH or heat (4, 13). Before beginning to assay the *dpp* protein for biological activity, we wished to determine whether the *dpp* proteins in the NaCl–Tween-20 wash were in a protein complex. We had observed that amounts of antibody which immunoprecipitated the 47-kDa protein left much of the 20-kDa protein in the supernatant (Fig. 5), indicating that some of the 20-kDa *dpp* protein is not associated with the 47-kDa portion of *dpp* under the nonphysiological conditions of the NaCl–Tween-20 plate wash. To demonstrate the absence of a complex between the 47- and 20-kDa portions of *dpp*, gel filtration chromatography was used to fractionate an NaCl–Tween-20 plate wash. A complex of *dpp* proteins could have a molecular mass of approximately 130 to 140 kDa (two 47-kDa subunits associated with the two 20-kDa subunits in the homodimer). The ^{35}S-labeled *dpp* proteins recovered in a salt wash were fractionated on a Sephacryl G100 column, and the fractions were examined by SDS-PAGE. No labeled protein was recovered in the void volume, and the 47-kDa *dpp* protein came off the column in fractions soon after the bovine serum albumin standard of 68 kDa. The 30-kDa carboxy-terminal dimer came off the column after the 47-kDa protein and before an 18-kDa myoglobin standard. We conclude that the 47-kDa *dpp* protein and the 30-kDa *dpp* protein in the NaCl–Tween-20 plate wash are not associated.

DISCUSSION

Proliferation and differentiation of mammalian cells are regulated by a number of extracellular proteins that are generally referred to as growth factors. During *Drosophila* development, intercellular regulation of cell proliferation and differentiation are also important; for example, the elaboration of positional information specifying segment polarity involves intercellular interactions (16, 48). Thus far, however, relatively few examples of *Drosophila* genes homologous to the genes encoding mammalian growth factors have been reported. The Notch, Delta, and Slit gene products contain sequences that have sequence similarity to epidermal growth factor (21, 22, 40, 51); the *Drosophila wingless* gene encodes a protein, homologous to the murine *int-1* proto-oncogene, that is a secreted factor (37, 48); and the *Drosophila dpp* gene is similar to members of the TGF-β family, especially the human bone morphogenetic proteins and the mouse protein Vgr-1 (25, 52).

The *Drosophila* S2 cell system has been previously used to produce P-element transposase (38, 39), the *Drosophila sevenless* protein (44), and the H-*ras* oncoprotein (19). We have used expression in S2 cells to characterize the biochemical properties of *dpp* and find some similarities to other members of the TGF-β family. The primary sequence of the *dpp* protein begins with a hydrophobic amino acid sequence similar to signal sequences of other secreted proteins (35); our results indicate that the *dpp* protein is secreted from *Drosophila* cells. We could not determine the site at which the signal sequence is removed from the secreted *dpp* protein, since we found that the amino terminus of the 47-kDa *dpp* protein was blocked. *dpp* precursors of 68 and 70 kDa exist intracellularly. We do not yet know the basis for their difference in mass but speculate that it is due to either removal of the signal peptide, i.e., proteolytic processing from the prepropeptide to the propeptide, or other posttranslational modifications such as glycosylation and phosphorylation, which are known to be required for the maturation of other factors in this family (3, 10, 13, 30, 36).

dpp is cleaved to release the carboxy-terminal domain, the region most similar in sequence to other proteins in the TGF-β family and the portion of the protein that is the active ligand in the cases examined, i.e., TGF-β and inhibin. The intracellular proteolytic processing of *dpp* occurs after a single arginine. The cleavage site in *dpp* is identical to the consensus cleavage sequence, arginine-X-X-arginine, used in a number of prohormones (1). Proteolytic cleavages of TGF-β and the inhibins occur after dibasic arginine-arginine sequences. Although *dpp* has dibasic residues located in

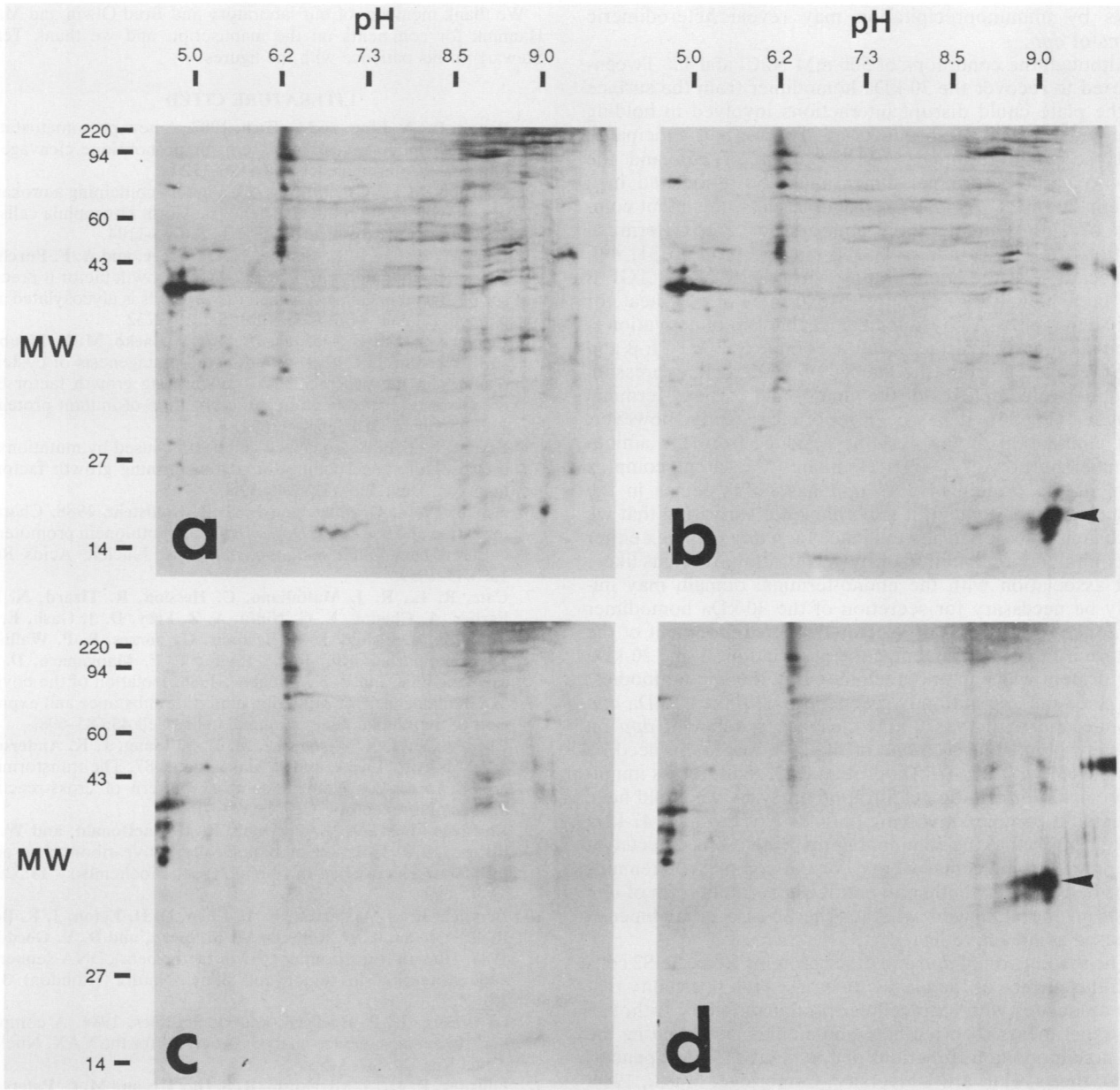

FIG. 7. Two-dimensional gel electrophoresis of ^{35}S-labeled proteins in NaCl–Tween-20 washes. Proteins were recovered from the plates in which cells from control cell line F2 (a and c) and pMTDPP-transfected cell line 2K (b and d) had been induced. The proteins were subjected to two-dimensional gel electrophoresis under either reducing (a and b) or nonreducing (c and d) conditions. The pHs along the isoelectric focusing gradients are indicated at the top of the figure, and the molecular masses (MW) of the protein standards (in kilodaltons) are indicated along the left side. The mobility of the proteins along the pH gradient indicates that the 20-kDa (reduced) and 30-kDa (nonreduced) proteins specific to the 2K cell line have similar isoelectric points (pH 9). The 47-kDa protein, specific to the 2K cells, has a predicted pI of 9.8 and runs to the edge of the focusing gel.

analogous positions about 100 amino acids from the carboxy terminus of the precursor, none of these are used in the S2 cell system. Instead, cleavage occurs after the arginine at position 456, 132 amino acids from the carboxy terminus. BMP2b and Vgr-1 have tetrabasic and tribasic residues, respectively, that align with the *dpp* cleavage site (Fig. 4). MIS is cleaved after a single arginine; however, the position of the MIS cleavage site aligns with the cleavage sites of the TGF-βs and inhibins (36).

The carboxy-terminal portion of *dpp* is isolated as a disulfide-bonded dimer from the surface of the plastic dishes in which the 2K cells are induced to express *dpp*. On the basis of our inability to detect any other proteins in sufficient quantities to comprise a second subunit of a *dpp* heterodimer and the fact that the dimer has the same isoelectric point as the 20-kDa monomer, we believe that the 30-kDa *dpp* protein is a homodimer of the carboxy-terminal portion of *dpp*. It is possible that in embryonic or imaginal disk cells expressing *dpp*, another protein is produced in sufficient quantities to form a heterodimeric complex with the 20-kDa portion of *dpp*. Heterodimeric forms of TGF-βs and inhibins exist (8, 24). The homodimeric and heterodimeric forms of inhibin have opposite effects on follicle-stimulating hormone release (17). Recovery of *dpp* protein from embryos or imaginal

disks by immunoprecipitation may reveal heterodimeric forms of *dpp*.

Although the conditions of 500 mM NaCl and 1% Tween-20 used to recover the 30-kDa homodimer from the surface of the plate could disrupt interactions involved in holding together the cleavage products of *dpp*, we had anticipated that the amino-terminal 47-kDa portion of *dpp* and the 30-kDa carboxy-terminal dimer might be associated in a protein complex. As isolated from platelets, the latent complex of TGF-β includes the amino- and carboxy-terminal portions of TGF-β and a TGF-β-binding protein (31, 49). Dissociation of the latent complex to release active TGF-β can be achieved by exposure to extremes of pH, heat, or ionic detergents. A physiological mechanism of activation is removal of carbohydrate from the complex (30). TGF-β and MIS recovered from the media of COS cell expression systems are complexes of the amino- and carboxy-terminal domains (13, 36). It has been reported recently, however, that elimination of the cysteine residues from the amino-terminal portion of TGF-β eliminates the latent complex form of the protein (4). As *dpp* has no cysteines in the amino-terminal domain, it is perhaps not surprising that we were unable to obtain any evidence for a *dpp* complex either by coimmunoprecipitation or by gel filtration. It seems likely that association with the amino-terminal domain may initially be necessary for secretion of the 30-kDa homodimer but, once the proteins are secreted, the environment of the culture dish could destabilize the association. Some 20-kDa *dpp* protein was immunoprecipitated by the *dpp* antibodies, but since the *dpp* antibody reacts both with the 47-kDa *dpp* peptide and with the 20-kDa cleavage product of *dpp* on Western blots (Fig. 5), none of the 20-kDa protein need be complexed with the 47-kDa protein to account for its immunoprecipitation. In the gel filtration analysis, we could have detected a complex involving only 10% of the total 47-kDa protein applied to the column, but no protein was detected at the predicted molecular weight for the complex. It remains to be determined whether an extracellular latent form of *dpp* exists in the animal or whether the 30-kDa homodimer is released as an active ligand.

The association of *dpp* and other proteins from the S2 cells with the surface of the plastic dish raises the possibility that *dpp* associates with extracellular proteins in vivo. Although the experiments reported here and studies documenting the cell nonautonomous functions of *dpp* in vivo (F. A. Spencer, Ph.D. thesis, Harvard University, Cambridge, Mass., 1984; L. M. Posakony, Ph.D. thesis, Harvard University, Cambridge, Mass., 1986) both indicate the role of *dpp* as a secreted factor, association with extracellular proteins may limit the region of *dpp* actions to areas immediately adjacent to its site of synthesis. Examination of *dpp* protein distribution in *Drosophila* embryos and imaginal disks is feasible with use of the available antibodies (G. E. F. Panganiban and F. M. Hoffmann, unpublished data). Of great interest is how the presence of *dpp* protein affects the behavior of cells or their patterns of gene expression; the availability of *dpp* protein isolated from the S2 cell expression system permits us to examine these cellular responses.

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