Identification of a Serine Protease Which Activates the Mouse Heart Adenosine 5′,5‴,P_1,P_4-Tetraphosphate Receptor†

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ABSTRACT: We have previously demonstrated that a serine protease dependent processing step is required for activation of the 30-kDa adenosine 5′,5‴,P_1,P_4-tetraphosphate (Ap_4A) receptor. However, monoclonal antibodies (Mabs) against a 212-kDa polypeptide inhibit Ap_4A binding to its receptor [Walker et al. (1993) *Biochemistry 32*, 1264–1269]. SDS–PAGE followed by autoradiography of [^{3}H]diisopropylfluorophosphate (DIPF) covalently attached to membrane fractions reveals that the serine protease is the 212-kDa polypeptide or a proenzyme. Mabs against the 30-kDa Ap_4A receptor are identified that inhibit Ap_4A binding to its membrane receptor. These Mabs do not recognize the 212-kDa membrane protein but recognize four membrane proteins with molecular masses of 67, 55, 42, and 30 kDa. These data suggest that the precursor for the Ap_4A receptor is a 67-kDa polypeptide which undergoes multiple cleavage events, at least one by the 212-kDa protein.

Diadenylated nucleotides are a novel class of compounds which are emerging as extracellular signal molecules in addition to their known intracellular roles. Ap_4A is the major representative of this type of nucleotide and is found in eukaryotic cells at micromolar concentrations (Zamecnik, 1983; Coste et al., 1987). The localization of Ap_4A inside the cell is mainly nuclear (Weinmann-Dorsch & Grummt, 1986). Ap_4A has been implicated in the initiation of DNA replication through binding to DNA polymerase α (Rapaport et al., 1981) and in the processing of ADP-ribosylated histones (Surowy & Berger, 1983).

Diadenylated nucleotides are found in high concentrations (millimolar) in the dense granules of blood platelets (Flodgaaard & Klenow, 1982; Luthje & Ogilvie, 1983) and adrenal chromaffin cells (Rodriguez Del Castillo et al., 1988), costored with ATP and 5-hydroxytryptamine or catecholamines, respectively. These compounds are released into the extracelular milieu during platelet aggregation (Luthje & Ogilvie, 1983) and chromaffin cell catecholamine secretion stimulation (Pintor et al., 1991).

Extracellularly, Ap_4A modulates diverse physiological functions through binding to distinct receptors. It has been shown that Ap_4A inhibits ADP-induced platelet aggregation (Zamecnik et al., 1992; Busse et al., 1988; Harrison et al., 1975) and influences smooth muscle tone of isolated rabbit arteries (Pohl et al., 1987; Luthje & Ogilvie, 1987). In addition, Ap_4A has been shown to increase basal secretion of catecholamines from isolated chromaffin cells (Casto et al., 1990), elicit excitation of the rat nodose ganglion (Marchenko et al., 1988), and induce intracellular calcium flux (Casto et al., 1992; Morii & Makinose, 1992). We have shown that binding of Ap_4A to mouse brain synaptosomes is saturable and highly specific. Ap_4A binding to the synaposomes activites Ca^{2+} channels with a resultant extracellular Ca^{2+} influx profile that is distinct from that triggered by ATP purinoceptor activation (Pivorun & Hilderman, 1993). This is consistent with the hypothesis that Ap_4A is released from cells and acts as an extracellular modulator by altering tissues in the presence of stress conditions.

We have demonstrated the presence of specific, saturable membrane receptors for Ap_4A in brain, cardiac, liver, kidney, spleen, and adipose tissue (Hilderman et al., 1991). Recently, we have identified the Ap_4A receptor as a 30-kDa polypeptide using a covalent radiolabeled derivative of Ap_4A. Prior to binding Ap_4A, the receptor must be activated, and this activation is dependent upon both divalent cations and a serine protease. We have also prepared Mabs that inhibit Ap_4A binding to its receptor, and these antibodies recognize a 212-kDa membrane protein but not the 30-kDa receptor polypeptide (Walker et al., 1993). This suggests that the 212-kDa polypeptide is either (1) a serine protease that activates the receptor, (2) a precursor molecule for the receptor, or (3) a precursor molecule for both the receptor and serine protease. In this communication, we demonstrate that the 212-kDa polypeptide is indeed the serine protease and that it is unrelated to the 30-kDa receptor which appears to be derived from a 67-kDa membrane protein.

MATERIALS AND METHODS

Materials. Swiss mice were obtained from Charles River Laboratories. The animals were housed in an animal facility maintained with a photoperiod of 15L:9D and room temperatures of 22–25 °C. [^{3}H]Ap_4A was purchased from Amersham International and [^{3}H]diisopropylfluorophosphate (DIPF) was purchased from NEN Nuclear. Goat anti-rat IgG conjugated with either alkaline phosphatase or horse radish peroxidase was purchased from Bio-Rad. Immobilon PVDF transfer membranes were purchased from Millipore. Precast polyacrylamide gels (4–20%) were purchased from Gel Tech. Hyperfilm-MP and Amplify were purchased from Amersham. All nucleoties and DIPF were purchased from Sigma Chemical Co. All other reagents were of analytical reagent grade or better.

Isolation of Membrane Extracts from Mouse Hearts. All membrane fractions were prepared as previously described (Hilderman et al., 1991). These membranes were isolated by centrifugation at 40000*g* for 10 min and contain a mixture of plasma membranes, nuclear membranes, mitochrondrial membranes, and other membranes.

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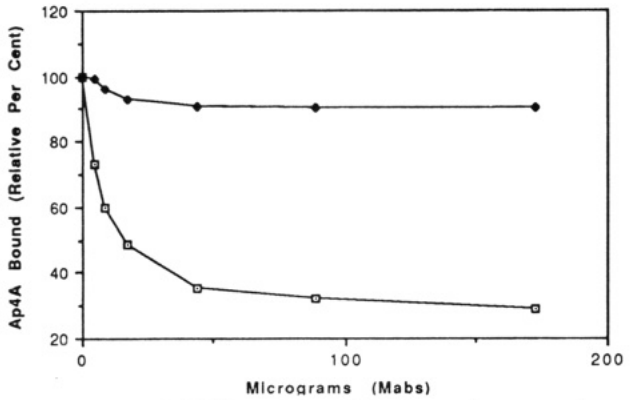

FIGURE 1: TL4 inhibition of Ap_4A binding to heart membrane homogenates. Membrane samples (24 μg) were activated by resuspension in 0.3 mL of binding buffer and incubating the samples for 60 min at 29 °C. The samples were then centrifuged for 10 min at 16000*g*. The pellets were resuspended in 0.3 mL of binding buffer plus varying amounts of TL4 (⊡) or CBC (■) and incubated for 60 min at 4 °C and then centrifuged for 10 min at 16000*g*. The pellets were resuspended in 0.3 mL of binding buffer plus 0.033 μM [^{3}H]-Ap_4A (15 000–20 000 cpm/pmol), and the samples were assayed after incubation for an additional 20 min at 20 °C. All membranes samples were collected on glass fiber discs and counted as described (Hilderman et al., 1991).

Ap_4A Receptor Activation and Binding Assay. Receptor activation is required for Ap_4A binding to its receptor. In this communication, receptor activation is defined as at least a 4–5-fold enhancement of binding of Ap_4A to its receptor. This activation is accomplished by incubating membrane fractions in binding buffer for 60 min at 29 °C prior to assaying for Ap_4A binding (Walker et al., 1993).

Preparation of Monoclonal Antibodies against the 30-kDa Receptor Protein. The 30-kDa polypeptide was isolated by performing preparative SDS–PAGE electrophoresis (10% polyacrylamide gels) on heart membrane homogenates (4.4 mg). The 30-kDa polypeptide was detected by copper staining. Then, the polypeptide band was excised, lyophilized, and emulsified with 1.0 mL of phosphate-buffered saline, pH 7.4 (PBS), which contains (per liter) 5.46 g of Na_2HPO_4, 1.53 g of NaH_2PO_4, 8.47 g of NaCl, and 1.0 mL of complete Freund's adjuvant. This emulsion was injected into female Wistar rats (Charles River). After two weeks a booster injection was administered intraperitoneally. This booster antigen was emulsified in 1.0 mL of PBS and 1.0 mL of incomplete Freund's adjuvant. A third booster was injected intraperitoneally two weeks later. Thirty days after the third injection, the final booster was administered and the animal was bled by cardiac puncture for isolation of polyclonal antibodies. The spleen cells were isolated and fused with the myeloma cell line P3-X63-AgU1. The cells were cultured and the hybrid clones containing antibodies against the 30-kDa proteins were subcloned and screened as described (Walker et al., 1993). Several Mabs have been isolated, and one clone (TL4) was ultimately chosen for detailed analysis. TL4 was concentrated from culture fluids by ammonium sulfate precipitation and then dialyzed against Tris-buffered saline, pH 7.4 (TBS), which contains 6.06 g of Trizma base and 8.47 g of NaCl. An unrelated Mab prepared against chicken brain N-cadherin was used as a control (kindly supplied by Dr. Jack Lilien) and referred to as CBC.

Western Transfer and Immunoblotting of Mouse Heart Membranes. Western transfer of mouse heart membranes and immunoblotting were performed as described (Walker et al., 1993).

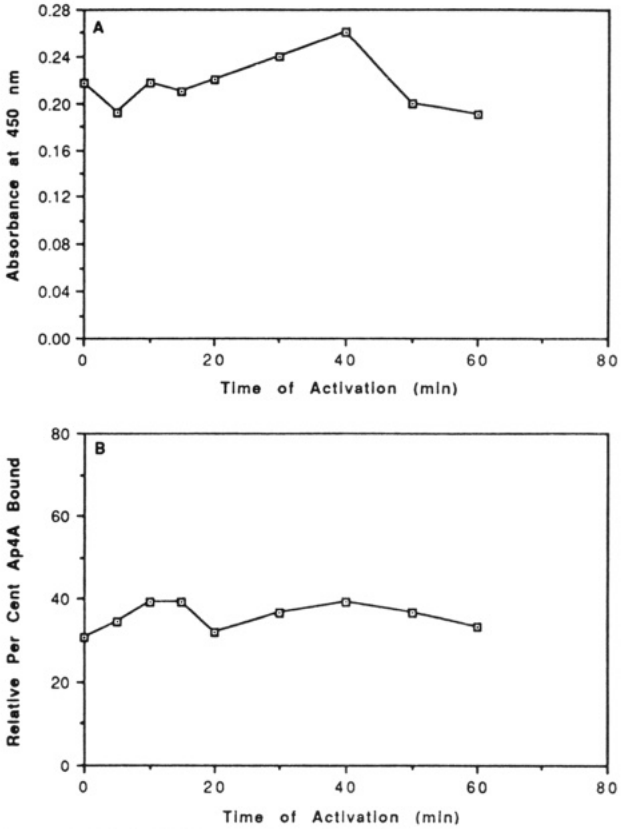

FIGURE 2: TL4 addition at various times during mouse heart receptor activation. All membrane fractions were prepared as described in Materials and Methods. (A) Membrane fractions (100 μg) in binding buffer were incubated for various times at 29 °C prior to binding to poly-L-Lys in microtiter plates as described in Materials and Methods. (B) Membrane fractions (24 μg) were resuspended in 0.3 mL of binding buffer and incubated for various times at 29 °C prior to incubation with TL4 (45 μg) for 60 min at 4 °C. The samples were then reincubated at 29 °C for the corresponding amount of time so that all samples were incubated at 29 °C for a total of 60 min, centrifuged at 16000*g* for 10 min, resuspended in binding buffer with 0.033 μM [^{3}H]Ap_4A (15 000–20 000 cpm/pmol), and the samples were assayed after incubation for an additional 20 min at 20 °C. All membranes samples were collected on glass fiber discs and counted as described (Hilderman et al., 1991).

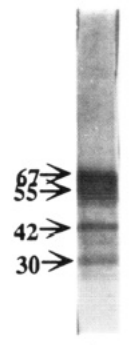

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FIGURE 3: Antibody capture using TL4 of Western transferred mouse heart membrane proteins. Membrane samples (20 μg) were subjected to SDS–PAGE, transferred, blotted, and developed as described in Materials and Methods. TL4 was used at a concentration of 20 μg/mL. Molecular weight values are denoted by the arrows.

Detection of TL4 Binding to Its Membrane Receptor by ELISA. A total of 100 μL of poly-L-Lys (50 μg/mL) in binding buffer was added to microtiter wells and incubated overnight at 4 °C. The plates were washed two times with 100 μL of binding buffer (67 mM Tris-HCL, pH 7.7, and 100 μM $MgCl_2$) prior to the addition of 100 μL of membrane (100 μg) to each well. The samples were incubated for 60 min at

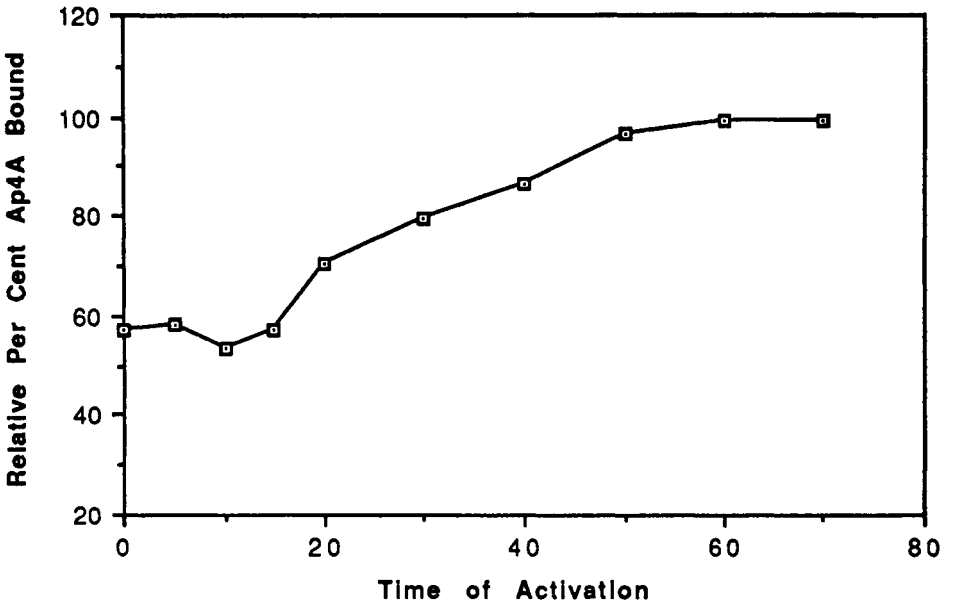

FIGURE 4: PMSF addition at various times during mouse heart receptor activation. All membrane fractions were prepared as described in Materials and Methods. Membrane fractions (24 μg) were incubated at 29 °C in 0.3 mL of binding buffer plus 0.033 μM [^{3}H]Ap$_4$A (specific activity, 15 000–20 000 cpm/pmol) and at various times 2 mM PMSF was added. After 60 min of incubation the samples were collected on glass fiber discs and counted as described (Hilderman et al., 1991).

4 °C, centrifuged at 500*g* for 2 min in a GLCB centrifuge with a plate adaptor. The samples were washed two times with 10% goat serum in binding buffer prior to the addition of 100 μL of TL4 (59 μg) and then incubated for 60 min at 4 °C. The samples were washed three times with 100 μL of 10% goat serum in binding buffer followed by the addition of 100 μL of goat anti-rat IgG conjugated with horse radish peroxidase (1:4000 dilution in 10% goat serum in binding buffer). The samples were incubated for 60 min at 4 °C and washed four times with 100 μL of 10% goat serum in binding buffer. The samples were developed by the addition of 100 μL of a solution containing 4 μL of 30% H_2O_2 and 4 μL of *o*-phenylenediamine in 10 mL of binding buffer followed by a 60-min incubation at room temperature. A MicroTech microtiter plate reader measured absorbency at 450 nm.

Covalent Attachment of [^{3}H]DIPF and Analysis by SDS–PAGE for Identification of the Serine Protease. Membrane fractions (120 μg) were resuspended into 0.3 mL of binding buffer (67 mM Tris-HCl, pH 7.7, and 100 μM $MgCl_2$) and incubated for 5 min at 29 °C. Then 5 μL of [^{3}H]DIPF (specific activity of 6600 cpm/pmol) was added and the samples were incubated for an additional 5 min at 29 °C. The samples were then centrifuged for 10 min at 40000*g* at 4 °C. The pellets were resuspended into 15 μL of 83.3 mM Tris-HCl, pH 8.0, 6% (w/v) SDS, 1% (v/v) Triton X-100, 4.4 M urea, and 28.1 μg of DTT and boiled for 10 min. Then, 21.6 μg of iodoacetamide was added and the samples were boiled for an additional 5 min prior to the addition of 0.01% (w/v) bromophenol blue. These samples were loaded onto 4–20% continuous polyacrylamide gels and run as described (Laemmli, 1970). After electrophoresis, the gels were fixed for 30 min in a solution of 2-propanol/water/acetic acid (25:65:10 v/v). The gel was stained for 1 h in 2-propanol/acetic acid/water (2.7:16.3 v/v) containing 0.02 M $CuSO_4$ and 0.2 mg/mL of Coomassie brilliant blue R-250. Destaining was for 1 h in 95% ethanol/acetic acid/water (2.4:1.4:16.2 v/v) containing 0.09 M $CuSO_4$. The gels were then placed for 10 min in a 1% glycerol solution prior to soaking the gels for 30 min in the dark with approximately 100 mL of Amplify (Amersham). The gels were then dried at 80 °C, under vaccum for 2 h using a Bio-Rad slab gel dryer. Autoradiography was carried out at −80 °C for 10 days using Hyperfilm-MP.

Protein Determination. Protein concentrations were determined by the Bradford method (Bradford, 1976).

RESULTS

Monoclonal Antibodies against the 30-kDa Ap$_4$A Receptor. To understand the relationship between the 30-kDa receptor and the 212-kDa polypeptide, we raised Mabs to the 30-kDa receptor polypeptide isolated by preparative SDS–PAGE. These antibodies were screened for their ability to immunoblot the 30-kDa receptor following SDS–PAGE and Western transfer and for their ability to block Ap$_4$A binding to its membrane receptor.

One of the Mabs, TL4, inhibits binding of Ap$_4$A to activated heart membrane homogenates while an unrelated Mab (CBC) does not inhibit Ap$_4$A binding (Figure 1). To determine if the TL4 epitope was accessible throughout the activation step, TL4 was added to membrane fractions at various times during activation and Mab binding was assayed using goat anti-rat IgG conjugated with horse radish peroxidase as the secondary antibody. As shown in Figure 2A, TL4 binds equally throughout the entire activation process. Also, TL4 inhibits Ap$_4$A binding to its receptor when the antibody is added at various times during activation (Figure 2B). This is consistent with the notion that the TL4 epitope is accessible throughout activation and that antibody binding to the receptor is not dependent on the activation step.

Because activation requires a proteolytic processing step, we used TL4 to identify potential receptor precursors. TL4 recognizes four polypeptides with molecular masses of 67, 55, 42, and 30 kDa (Figure 3). However, TL4 does not recognize the 212-kDa polypeptide even when SDS–PAGE gels were overloaded with membrane protein (up to 40 μg) and the Western transfers were blotted with TL4 concentrations up to 80 μg (data not shown). This is consistent with the hypothesis that the 212-kDa polypeptide is unrelated to the receptor polypeptide.

The 212-kDa Membrane Polypeptide Is a Serine Protease. An anti-212-kDa Mab (JW17), which inhibits Ap$_4$A binding,

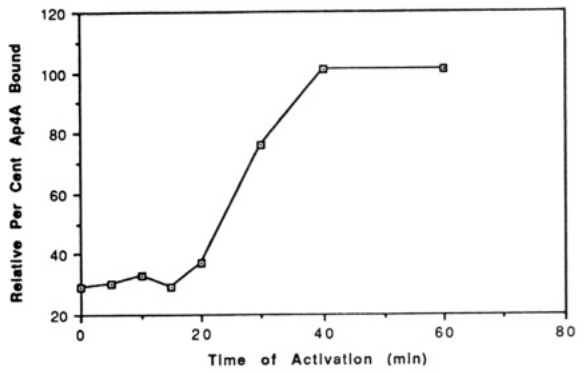

FIGURE 5: JW17 addition at various times during mouse heart receptor activation. All membrane fractions were prepared as described in Materials and Methods. Membrane fractions (24 μg) were preincubated in 0.3 mL of binding buffer at 29 °C for various lengths of time, centrifuged, resuspended in 0.3 mL of binding buffer with 200 μg of JW17, and incubated for 60 min at 4 °C. The samples were then reincubated at 29 °C for the corresponding amount of time so that all samples were incubated at 29 °C for a total of 60 min, centrifuged at 16000*g* for 10 min, resuspended in binding buffer with 0.033 μM [^{3}H]Ap$_4$A (15 000–20 000 cpm/pmol), and the samples were assayed after incubation for an additional 20 min at 20 °C. All membranes samples were collected on glass fiber discs and counted as described (Hilderman et al., 1991).

recognizes only the 212-kDa polypeptide, yet the receptor is a 30-kDa polypeptide [Figure 6B (lane 2) and Walker et al. (1993)]. To further clarify the role of proteolysis in receptor activation, [^{3}H]Ap$_4$A was added at the begining of activation and then phenylmethanesulfonyl fluoride (PMSF) was added at various time intervals. PMSF inhibits Ap$_4$A binding only during the first 20 min of activation (Figure 4). These data suggest that the serine protease activity is required during the first 20 min of the activation. Thus, if the 212-kDa polypeptide is the serine protease, then an anti-212-kDa Mab should only inhibit Ap$_4$A binding during the first 20 min of the activation. Indeed, JW17 inhibits Ap$_4$A binding only when added during the first 20 min of the activation (Figure 5). In contrast, addition of anti-30-kDa antibody inhibits Ap$_4$A binding when added at anytime during the activation step (Figure 2B). This is also consistent with the Ap$_4$A receptor not being the 212-kDa polypeptide.

To further clarify the relation of the 212-kDa polypeptide to the serine protease involved in activation of the Ap$_4$A receptor, [^{3}H]DIPF was incubated with membrane homogenates prior to SDS–PAGE and autoradiography. [^{3}H]DIPF was used in place of PMSF because radiolabeled DIPF is available commerically while radiolabeled PMSF is not available. The covalent attachment of [^{3}H]DIPF is dependent upon membrane concentration (Figure 6A). Autoradiography after SDS–PAGE reveals that [^{3}H]DIPF is covalently attached to only three polypeptides with molecular masses of 212, 63, and 38 kDa (Figure 6B, lane 1). This is consistent with the 212-kDa polypeptide being the serine protease.

DISCUSSION

Mabs raised against the 30-kDa receptor that inhibit Ap$_4$A binding to its receptor recognize four polypeptides of 67, 55, 42, and 30 kDa. This suggests that the 67-kDa protein is the precursor to the 30-kDa receptor and that there may be multiple processing steps involved in receptor activation. We have previously shown that receptor activation is dependent upon both divalent cations and a serine protease. In addition, we have also shown that [^{32}P]8-N$_3$-Ap$_4$A is UV-cross-linked only to the 30-kDa polypeptide (Walker et al., 1993). This

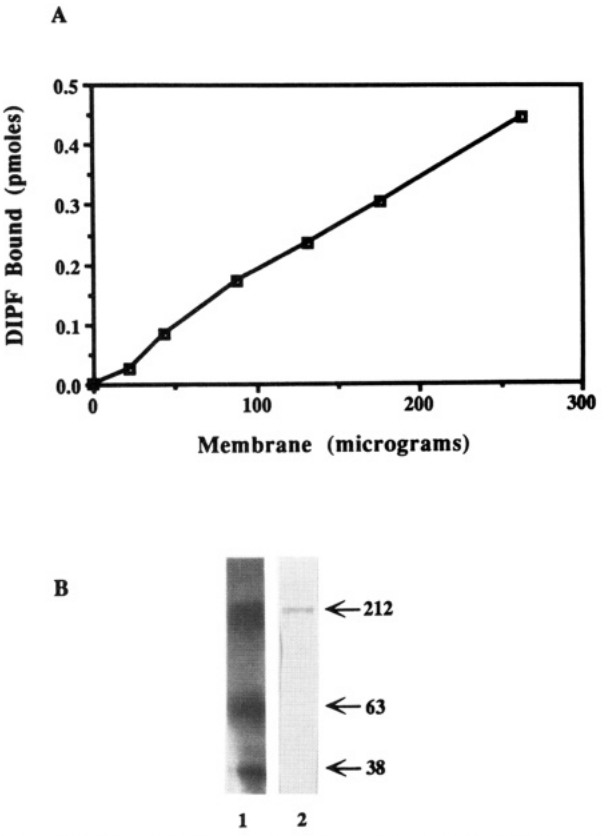

FIGURE 6: SDS–PAGE and autoradiography of [^{3}H]DIPF-Labeled mouse heart membrane homogenates. All membrane fractions were prepared as described in Materials and Methods. (A) Varying amounts of membrane fractions were incubated with [^{3}H]DIPF in 0.3 mL of binding buffer for 5 min at 29 °C as described in Materials and Methods. Then, the samples were centrifuged at 16000*g* and resuspended in 0.3 mL of binding buffer containing 100 μg of BSA. The proteins were precipitated with 3 mL of cold 20% trichloroacetic acid (TCA), collected on glass fiber discs, and counted as described (Hilderman et al., 1991). (B) Lane 1, SDS–PAGE and autoradiography of 176 μg of membrane fraction incubated with [^{3}H]DIPF as described in Materials and Methods; Lane 2, SDS–PAGE and Western transfer of 176 μg of membrane fraction incubated with [^{3}H]DIPF and immunoblotted with 30 μg/mL JW17 as described in Materials and Methods. Molecular weight values are denoted by the arrows.

implies that proteolysis must go to completion before the receptor binds Ap$_4$A.

The serine protease has been identified as a 212-kDa membrane protein (Figure 6); Mabs directed against the 212-kDa polypeptide inhibit Ap$_4$A binding but to not cross-react with the 30-kDa receptor (Walker et al., 1993). In addition, a 212-kDa polypeptide is labeled with [^{3}H]DIPF.

Since the serine protease is a large molecular weight polypeptide, it is conceivable that 212-kDa protein is a proenzyme that is activated by autoproteolysis and that either the 63- or 38-kDa polypeptides are the active form of the serine protease. Other investigators have shown that interleukin-1β-converting enzyme is a cysteine protease that exists as a proenzyme and after autoproteolysis cleaves an IL-1β precursor to produce the active IL-1β (Thronberry et al., 1992; Cerretti et al., 1992). It has also been shown that the endothelin-converting enzyme from human umbilical vein is a membrane-bound metalloprotease (Ahn et al., 1992). Thus, it is possible that the divalent cation is a cofactor for a metalloenzyme required for Ap$_4$A receptor processing or as a cofactor for autoproteolysis of a serine protease proenzyme.

The need for multiple processing steps in Ap$_4$A receptor activation suggests that receptor activation is extremely

complex. In order to characterize the Ap_4A receptor and its processing system, we are in the process of purifying the membrane component(s) that contains the receptor precursors and the receptor processing enzymes. This will allow us to determine the number of proteins involved.

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