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Expression of Mitogen-Activated Protein Kinase Pathways During Postnatal Development of Rat Heart

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Abstract The loss of ability to proliferate (terminal differentiation) and reduction in capability to resist ischemia are key phenomena observed during postnatal development of the heart. Mitogen-activated protein kinases (MAPKs) mediate signaling pathways for cell proliferation/differentiation and stress responses such as ischemia. In this study, the expression of these kinases and their associated kinases were investigated in rat heart ventricle. Extracts of 1-, 10-, 20-, 50-, and 365-day-old rat heart ventricles were probed with specific antibodies and their immunoreactivities were quantified by densitometry. Most of the mitogenic protein kinases including Raf1, RafB, Mek1, Erk2, and Rsk1 were significantly down-regulated, whereas the stress signaling kinases, such as Mlk3, Mekk1, Sek1, Mkk3, and Mapkapk2 were up-regulated in expression during postnatal development. Most MAP kinases including Erk1, JNKs, p38 Hog, as well as Rsk2, however, did not exhibit postnatal changes in expression. The proto-oncogene-encoded kinases Mos and Cot/Tpl 2 were up-regulated up to two- and four-fold, respectively, during development. Pak1, which may be involved in the regulation of cytoskeleton as well as in stress signaling, was downregulated with age, but the Pak2 isoform increased only after 50 days. All of these proteins, except RafB, were also detected in the isolated adult ventricular myocytes at comparable levels to those found in adult ventricle. Tissue distribution studies revealed that most of the protein kinases that were up-regulated during heart development tended to be preferentially expressed in heart, whereas the downregulated protein kinases were generally expressed in heart at relatively lesser amounts than in most of other tissues. J. Cell. Biochem. 71:286–301, 1998.

Key words: heart; development; MAPK; MEK; MEKK

The heart undergoes marked changes in postnatal development from birth to its mature or adult form. The sequence and rate of development varies from species to species and may differ depending on the index under consideration. In rat, most of the adult characteristics of the heart are manifested about 30 to 50 days after birth, and during this postnatal period the heart undergoes dramatic changes in morphological, mechanical, electrophysiological, and biochemical parameters [Rakusan, 1984; Riva and Hearse, 1991; Zak, 1981; Rumyanstsev, 1996]. These changes may be associated with various extracellular signals, such as hormonal, neuronal, or physical stimuli, intracellular expression of cardiac specific genes and functional proteins, and their post-translational modifications [Chien et al., 1993; Komuro and Yazaki, 1993; van-Bilsen and Chien, 1993; Schott and Morrow, 1993; Cummins, 1993].

The growth of cardiac mass in early neonatal development is due to an increase in the number of cardiac myocytes (cell proliferation) and in their individual volume. In rat heart, cardiac myocytes then become terminally differentiated and grow only in size about two weeks after birth, arrested at G_o or G_1 phase of the cell cycle [Capasso et al., 1992; Zak, 1974]. The cardiac mass increases as much as 39-fold by

Abbreviations: AP, alkaline phosphatase; CK2, casein kinase 2; Erk, extracellularly regulated kinase; JNK, c-Jun-N terminal kinase; Pak, p21-activated kinase; Mlk 3, mixed lineage kinase 3; Tak, TGFβ activated kinase; Mapkapk2, MAPK-activated protein kinase 2; Mek, MAP kinase/Erk kinase; Rsk, p90 ribosomal S6 kinase; MAP kinase, mitogen activated protein kinase.

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adulthood, but the number of left ventricular myocytes increases from about 7 X 10^6 cells at birth to 14 X 10^6 and 30 X 10^6 at the age of 11 and 60 days, respectively [Claycomb, 1975; Rukusan, 1984; Anversa et al., 1986]. This number remains constant in the later stage of development. Similarly, the rate of DNA synthesis and the DNA polymerase activity also decline rapidly during the developmental period. While it is not yet clear how many times a cardiac myocyte undergoes mitosis during early postnatal development, it has been suggested that the actual mitosis may take place more than once in view of the number of cells that are also subject to apoptosis during the postnatal development [Misao et al., 1996; Kajstura et al., 1996]. Isolated 5- to 10-day -old neonatal cardiac myocytes undergo one or two cycles of cell division before becoming differentiated in vitro. Adult cardiac myocytes grow only in size (hypertrophy) and occasionally become bi- and/or polynucleated. Under some pathological conditions, cardiac myocytes further grow in size and the number of bi- or poly-nucleated cells increases. It is still unclear how cardiac myocytes become terminally differentiated during postnatal development. Most researchers, however, agree that it is likely due to the biochemical inhibition of mitosis and DNA synthesis rather than the permanent physiological loss in their ability to proliferate. Observations of re-initiation of DNA synthesis and mitotic division of adult cardiac myocytes under certain conditions, such as ischemia [Anversa et al., 1996], pressure overload [Capasso et al., 1993], anemia [Olivetti et al., 1992], phorbol ester treatment [Claycomb and Moses, 1988], and senescent heart in certain strains of rats [Anversa et al., 1991] substantiate this postulation.

Another significant change that occurs during postnatal development is the decrease in resistance to oxygen deprivation with age. The neonatal heart possesses a greater resistance to ischemia than the adult, showing a greater post-ischemic recovery of contractile functions and less cellular damage [Riva and Hearse, 1991]. Several explanations have been advanced for this phenomenon, including the increase in the activity of xanthine oxidoreductase in adult heart which can generate toxic reactive oxygen intermediates during ischemic re-perfusion, differences in calcium handling between neonate and adult, and reduced depletion of ATP in the neonate during ischemia.

MAP kinases are a family of proline-directed Ser/Thr protein kinases, involved in various signaling pathways that mediate cell growth, differentiation, transformation, cellular stress responses, and apoptosis [Pelech and Charest, 1995; Sugden and Bogoyevitch, 1995; Robinson and Cobb, 1997]. Extracellularly-regulated kinase (Erk) 1 and 2 are MAP kinases that are involved in mitogenic signaling in cells. Erk1 and Erk2 are activated by MAP kinase/Erk kinase 1 (Mek1) and Mek2, which are in turn stimulated by Raf isoforms. Other MAP kinase signaling pathways target activation of the p38 kinase (also known as Hog or RK) and stress-activated protein kinases (SAPKs, also referred to c-Jun N-terminal kinases or JNK), which mediate various stress related responses. MAP kinase pathways have been shown to be involved in cardiac hypertrophy and in stress responses such as ischemia and ischemic reperfusion [Bogoyevitch et al., 1994; Bogoyevitch et al., 1996; Knight and Buston, 1996]. However, the expression and regulation of the kinase in these pathways during the postnatal development of the heart have not been studied in detail. Understanding of the regulation of these MAP kinase pathways may be crucial in unraveling the mechanism of age specific responses of mitogenesis and ischemia of the heart. Toward this objective, we initially sought to identify those MAP kinase pathway enzymes that might potentially play significant roles in the developing rat heart. Extracts from ventricular tissues of 1-, 10-, 20-, 50- and 365-day-old rat hearts were immunoblotted with a battery of over 24 different affinity purified antibodies specific for protein kinases and quantified by densitometry. At the same time, the relative expression levels in adult heart were compared to those of 10 different tissues from the same rats.

MATERIALS AND METHODS

Affinity-purified rabbit polyclonal antibodies were either raised against synthetic peptides or purchased, as indicated in Table 1. Goat anti-rabbit IgG and goat anti-mouse IgG conjugated to alkaline phosphatase (AP) or to horseradish peroxidase were purchased from Calbiochem (San Diego, CA). Enhanced chemiluminescence (ECL) detection reagents for immunoblotting were purchased from Amersham Canada (Oakville, ON). Pak3 was expressed in *Escherichia coli* and purified over a glutathione-agarose

column. Other reagents were purchased from Sigma-Aldrich (St. Louis, MI), unless otherwise stated.

Preparation of Rat Tissue Extracts

Hearts from 1-, 10-, 20-, 50- and 365-day-old male Sprague-Dawley rats were rapidly excised, after induction of anesthesia by intraperitoneal injection of pentobarbital (60 mg/kg). The ventricles of the hearts were cut, rinsed with phosphate buffer saline at 4 °C, frozen in liquid nitrogen, and stored at -70 °C until use. The ventricular tissues were pulverized with five strokes of a liquid nitrogen-cooled hand French press and re-suspended in 10 volumes of ice-cold homogenization buffer, containing: 20 mM MOPS, 15 mM EGTA, 2 mM Na_2EDTA, 1 mM Na_3VO_4, 1 mM dithiothreitol, 75 mM β-glycerophosphate, 0.1 mM PMSF, 1 µg/ml aprotinin, 0.7 µg/ml pepstatin, 1 µg/ml leupeptin, 1% Triton X-100 (Membrane Grade), and sonicated with a Branson Probe Sonicator at 4°C with 3 x 30s bursts. The homogenates were ultracentrifuged at 100,000 rpm (240,000 x g) for 11 min in a Beckman TLA-100.2 ultracentrifuge at 4 °C. The supernatants were used for immunoblotting and enzyme assays. Other rat tissues including adipose, brain, intestine, kidney, liver, lung, spleen, testis, skeletal muscle (hind leg tibial muscle), and thymus were also collected from 50-day-old rats and homogenated as described above.

TABLE I. Amino Acid Sequences of Synthetic Peptides Used to Raise Antibodies for Various Protein Kinases[a]

Protein kinase	Immunogen sequence	Species of the immunogen	Source
Tpl-2 (Cot)	NT-MEYMSTGSDNKEEIDC	Rat	UBI
	CT-RGHQVIHEGSSTNDPNNSC	Rat	
Erk 1/Erk2	CT-CGGPFTFDMELDDLPKERLKELIFQETARFQPGAPEAP	Rat	UBI
MapkapK2	PCT-SRVLKEDKERWEDVKGC	Mouse	UBI
Mek1	XI-EFQDFVNKCLVKNPAERADLKC	Mouse	
	NT-MPKKKPTPIQLNC	Mouse	
Mekk1	NT-CGSTHFTRMRRRLMAIAD	Mouse	
MKK 3	CT-KTKKTDIAAFVKILGEDSC	Human	UBI
Mlk 3	C20-residue 828–847	Human	SC
Mos	III-ASQRSFWAELNIARLRHDNIVRVVAASTR	Frog	UBI
	M20-residue 371–390	Mouse	SC
p38 Hog	C20-residue 341–360	Mouse	SC
	NT-MSQERPTFYRQEKC	Mouse	UBI
Pak 1	NT-MSNNGLDVQDKPC	Mouse	UBI
	N20-residue 2–21	Rat	SC
Pak2	NT-MEETQQKSNLELLSAC	Human	
Pak 3	NT-MSDSLDNEEKPPAC	Rat	
Raf 1	C20-residue 629–648	Human	SC
RafA	CT-residue 584–597	Human	UBI
RafB	CT-residue 619–632	Human	UBI
Rsk 1	C21-residue 716–735	Human	SC
Rsk 2	PCT-CNRNQSPVLEPVGRS	Mouse	UBI
JNK2	Whole protein	Rat	
JNK1	C17-residue 368–384	Human	SC
Sek1	XI-CLTKDESKRPKYDELLK	Xenopus	UBI
	CT-CKILDQMPATPSSPMYVD	Mouse	
Tak1	CT-CKKQLEVIRSQQQKRQGTS	Mouse	
	M17-residue 563–579	Mouse	SC

[a]The peptides in PBS and Freund's incomplete adjuvant were injected into rabbits and the serum was obtained, after several boosting injections. The antibodies were purified using peptide affinity columns and titred by ELISA. The peptide sequences and their source of species are indicated in the origin column. For commercially purchased antibodies, the peptides are indicated for the corresponding kinase residues with their species origins and the companies from which the antibodies were sourced: SC, Santa Cruz Biotechnology (Santa Cruz, CA); TL, Transduction Laboratory (Mississauga, ON); UBI, Upstate Biotechnology Inc. (Lake Placid, NY).

Preparation of Isolated Rat Ventricular Myocytes

The isolation procedure was modified from the protocol of Rodrigous et al. [1997]. Fifty-day-old rat hearts were excised, perfused retrogradely with Buffer A (Joklik minimal essential medium, containing 2 mM $NaHCO_3$, 1.2 mM $MgSO_4$, and 1 mM DL-carnitine) for 5 min, followed by the same buffer containing 25 µM Ca^{2+} and 75 mU/ml of collagenase (Type II, Worthington Biochemical Corp., Freehold, NJ) for 30 min at 37 °C. The softened ventricular tissue was then cut and teased to small pieces with scalpel, and incubated for 10 min in the same collagenase and Ca^{2+} containing Buffer A with occasional agitation. Dissociated ventricular myocytes were carefully aspirated and passed through a 200 µm mesh silk screen to remove fine tissue debris. The isolated ventricular myocycte were then sequentially re-suspended in Buffer A containing 50 µM, 100 µM, 500 µM, and 1 mM Ca^{2+}. The cells were then pelleted by centrifugation for 60 sec at 45*g*. The cell pellet was carefully loaded onto Buffer A containing 1 mM $CaCl_2$ and 4% BSA to separate viable cells from dead cells. Rod-shape viable cells are larger and asymmetric, and therefore, settled more quickly than non-viable cells. After the settling, the supernatant was discarded and the cells were re-suspended into Buffer A that contained 1 mM $CaCl_2$. The isolated ventricular myocytes were then sonicated with three bursts of 30 sec each on ice-cold homogenization buffer and the homogenates were then fractionated with cytosolic- and detergent-soluble fractions as described in the previous section.

Gel Electrophoresis and Immunoblotting

Extracts were added to SDS-PAGE sample buffer (2.5% SDS, 10 % glycerol, 50 mM HCl, pH 6.8, 0.5 M-mercaptoethanol, and 0.01% bromophenol blue) and boiled at 100 °C for 3 min. Protein concentrations were assayed with Bradford reagent (BioRad, Richmond, CA) and extracts were diluted with 1% SDS to yield identical protein concentrations before adding to the sample buffer. Electrophoresis was performed in 11% SDS-PAGE gels, using a discontinuous buffer system as described. Proteins were then electrophoretically transferred onto nitrocellulose membranes. Membranes were then blocked with 3% skim milk powder in Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.2 % Tween-20; TBST) and, after quickly rinsing the membrane with TBST, exposed to a primary antibody in TBST for 2 h with constant shaking at room temperature. Membranes were washed three times for 10 min with TBST and incubated with horse-radish peroxidase- or alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit or anti-mouse IgG) in TBST for 30 min. After washing membranes three times for 10 min with TBST, Western blots were developed using ECL Western blotting detection system (Amersham) or AP color development as described elsewhere [Sanghera et al., 1996].

Densitometry and Statistical Analysis

For the quantitative analysis of protein expression, the films obtained from the ECL detection system or the color developed membranes were scanned and the intensities of the bands were quantified by the NIH image program. Data are presented as means ± SEM, and analysis of variance (two-way ANOVA) and Tukey's test were performed with an $\alpha < 0.05$ level of significance.

RESULTS

Expression of MAP Kinase Kinase Kinases (MPKKKs) Involved in the Regulation of Erk1 and Erk2: Raf, Mos, and Tpl-2

The Raf family of protein-Ser/Thr kinases was first identified as the normal cellular counterparts of v-raf in murine sarcoma virus 3611. So far, three isoforms (Raf1, RafA, and RafB) have been identified in mammalian systems with distinct cellular distributions and characteristics [Su and Karin, 1996; Boldyreff and Issinger, 1997; Hagemann et al., 1997; Daum et al, 1994]. Raf1 is a ubiquitous cytosolic protein with an apparent molecular mass of 72 to 76 kDa. Activated Raf1 phosphorylates and activates Mek 1 and 2. RafA and RafB are expressed in a more tissue-specific manner and their regulation is less characterized. The expression of Raf1 was investigated by using commercial antibodies raised against the C-terminal region of human Raf1. A protein immunoreacted with apparent molecular mass of 73-kDa (Fig. 1A). The 73-kDa protein was also specifically immunoprecipitated (data not shown) and its level of expression gradually declined by 365-days to about 20% of levels detected shortly after birth (Fig. 1B). Among

adult rat tissues, Raf1 was abundantly expressed in lung, spleen, testis, and thymus but was almost undetectable in the heart (Fig. 1C). Antibody raised against the C-terminus of RafA revealed a 67-kDa protein that did not significantly change in intensity of immunoreactivity during development (Fig. 1D, E). The antibody also specifically immunoprecipitated the 67-kDa protein from ventricular extracts (data not shown). Unlike Raf1, RafA in the heart was abundantly expressed, surpassed only by the liver when compared with 10 different adult tissues (Fig. 1F). RafB C-terminus antibody (CT) specifically but weakly immunoreacted with a doublet of 92-kDa protein in the ventricle and the protein was down regulated to about 40% of the 1-day-old hearts during development (Fig. 1G, H). Even though the ventricle comprise about 80% with cardiomyocytes in mass and volume, other cells such as blood vessels, nerves and adipose tissues may influence the results. To investigate the cell specific expression of these proteins, freshly isolated adult (50-day -old rat) ventricular myocytes were fractionated into cytosolic and particulate fractions. In the isolated ventricular myocytes, the same level of Raf1 and RafA was also detected as in the ventricular tissues (Fig. 2). RafB, however, could not be detected in the isolated ventricular myocyte extracts (data not shown).

In addition to Raf, at least two other protein kinases have been shown to indirectly activate Erk1 and Erk2. The Cot (cancer Osaka thyroid)/Tpl-2 (tumor progression locus 2) oncogene was cloned from Hamster cell transformed with DNA extracts from human thyroid carcinoma cell line [Miyoshi et al., 1991]. It encodes two protein serine kinase isoforms with apparent molecular mass of 52 kDa and 58 kDa, which arise from an alternative initiation mechanism and have different transforming activities [Ohara et al., 1995]. Tpl-2 is a rat homologue which was cloned from cell lines derived from Moloney murine leukemia virus induced thymoma and it shares about 90% amino acid identity to Cot [Salmeron et al., 1996]. Little is known regarding the role and regulation of these proteins, but the over-expression of Tpl-2 in COS-1 and NIH3T3 cells activated both the Erk and JNK pathways with little effects on p38 kinase, probably by direct phosphorylation of Mek1 and Sek1 [Salmeron et al., 1996]. Tpl-2 is found in various tissues, but highly expressed in terminally differentiated granular cells, and mainly localized in cytosolic fractions [Ohara et al., 1995]. Our immunoblotting analysis with both C-terminal and proximal C-terminal (PCT) antibodies for Tpl-2 detected an identical 58-kDa protein with gradually increasing densities up to 4.7-fold in 50-day-old rat ventricles (Fig. 1J, K, L). The protein was also detected in the cytosolic fraction of the isolated ventricular myocytes (Fig. 2). Comparative studies of various adult rat tissues showed that Tpl-2 was expressed in most of the tissues with the highest amount in heart, followed by kidney, liver, and skeletal muscle (Fig. 1M).

Mos is another proto-oncogene-encoded 43-kDa cytoplasmic Ser/Thr protein kinase, which activates the Erk1/2 pathway by direct phosphorylation and activation of Mek1 [Pham et al., 1995]. Immunoblotting with affinity purified antibody, raised against the catalytic subdomain III of Mos, identified a 43-kDa protein with gradual increase in expression as much as two-fold at 50-days of age (Fig. 1N, O). The highest amount of this protein was detected in the skeletal muscle, followed by the heart and then brain (Fig. 1P). This protein also exclusively resided in cytosolic fractions of isolated ventricular myocytes (Fig. 2) at levels comparable to what was observed in ventricular extracts.

Expression of MAP Kinase Kinase Kinases (MPKKK) Involved in the Regulation of JNK and p38 Kinase: Paks, Mlk3, and Tak1

The p21-activated kinases (Paks) are a family of Ser/Thr kinases that are activated by trimeric G protein-linked receptors as well as by receptor tyrosine kinases. The details for mechanism of activation of Paks remain to be established. However, the direct binding of Pak to either small G proteins, Cdc42 and Rac 1, via about 60 amino-acid long p21-binding domain (PBD) or a Src-homology 3 (SH3) domain containing the adapter protein, Nck, via the N-terminal proline-rich region of Pak, have been shown to induce Pak phosphotransferase activity which, then, stimulates stress-related protein kinases such as p38 and JNKs [Bagrodia et al., 1995; Zhang et al., 1995; Jakobi et al., 1996; Teramoto et al., 1996; Sells and Chernoff, 1997]. In mammalian systems, at least three isoforms of Paks have been identified: a 67 kDa Pak1

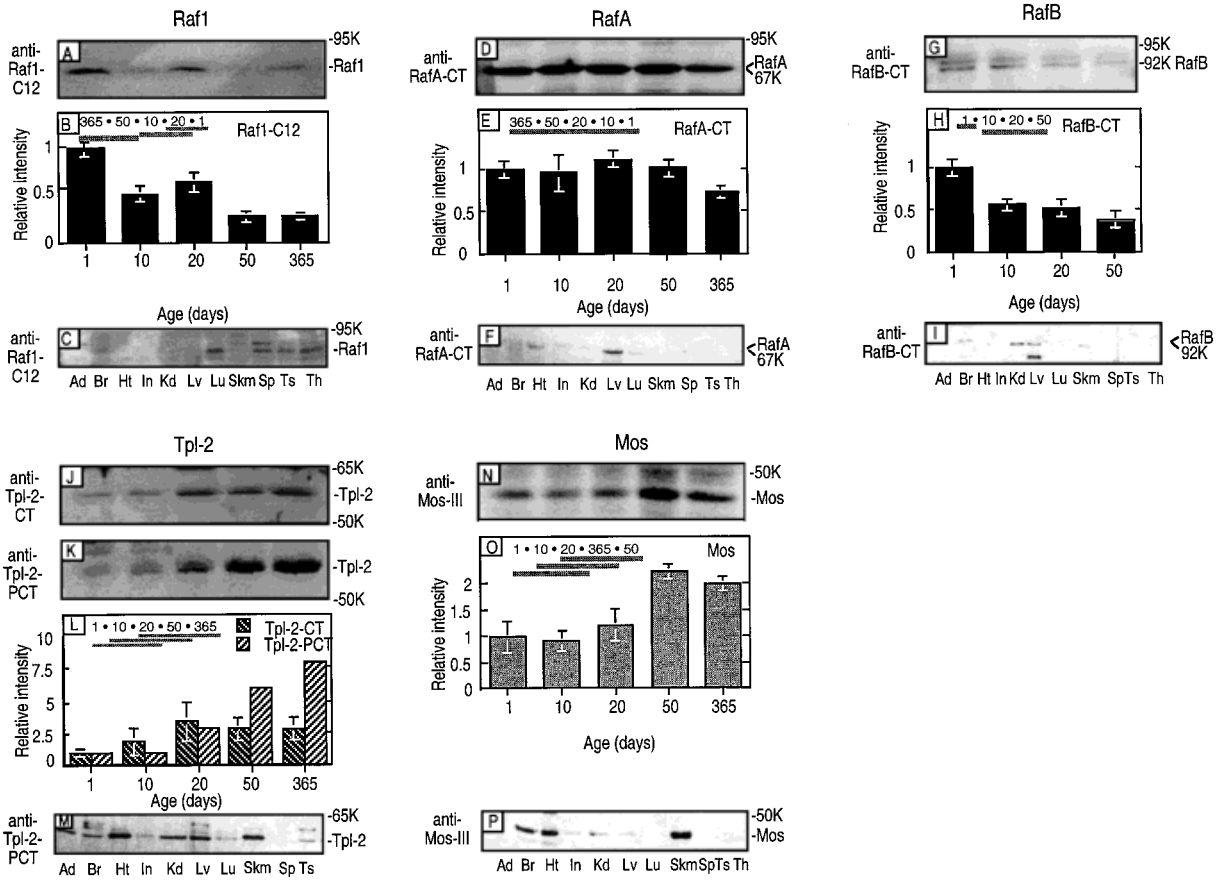

Fig. 1. Expression of MAP kinase kinase kinase (MKKKs) involved in the regulation of Erk1 and Erk2 during postnatal development of rat ventricle. Protein extracts (100 μg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE and immunoblot analysis was performed with various antibodies for Raf1 (**A**), RafA (**D**), RafB (**G**), Tpl-2 (**J, K**), and Mos (**N**). The immunoreactivities were plotted against the relative density as 1one arbitrary unit for 1 -day ventricle and values represent mean ± SEM from three to four separate experiments (**B, E, H, L, O**). Inserted numbers and underscores in the graphs show results of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. For tissue comparison, extracts (50 μg of total protein) of 11 adult rat tissues (adipocytes (Ad), brain (Br), heart (Ht), intestine (In), kidney (Kd), liver (Lv), lung (Lu), skeletal muscle (Skm), spleen (Sp), testis (Ts), and Thymus (Th)) were subjected to 11% SDS-PAGE and immunoblot analysis was performed with Raf1 (C), RafA (F), RafB (I), Tpl-2 (M), and Mos (P) antibodies. (**C**), RafA (**F**), RafB (**I**), Tpl-2 (**M**), and Mos (**P**) antibodies.

(Pakα), a 62 kDa Pak2 (Pakγ, Pak-I), and a 65 kDa Pak3 (Pakβ). Our immunoblotting revealed the presence of p67 Pak1 and p62 Pak2 (Fig. 3) but the virtual absence of Pak3 (Fig. 3H) in rat ventricles. These results support the previous assignments of Pak2 as a ubiquitous protein and Pak1 as a brain and muscle-specific protein [Sells and Chernoff, 1997]. In the heart, the expression of Pak isoforms was differentially regulated during the postnatal development of rat ventricle. Pak1α-C-19 immunoreacted with three different mass of proteins (75 kDa, 67 kDa, and 64 kDa; Fig. 3A), all of which were also specifically immunoprecipitated (data not shown). Another antibody raised against the N-terminus of Pak1, however, only cross-reacted with the 67-kDa protein among those three (Fig. 3B). At present, the identity of those other two bands is unknown. They could be phosphorylated forms of Pak1 or other isoforms which immunoreacted specifically with the C-terminal antibody. We have also immunoblotted with Pak2 and Pak3 antibodies following immunoprecipitation with Pak1α antibody from ventricular extracts; these studies indicated that the 75 kDa and 64 kDa proteins were neither Pak2 nor Pak3 (data not shown). Therefore, we analyzed the expression of 67-kDa protein during development of heart, which displayed a decrease to as much as 30% of newborn

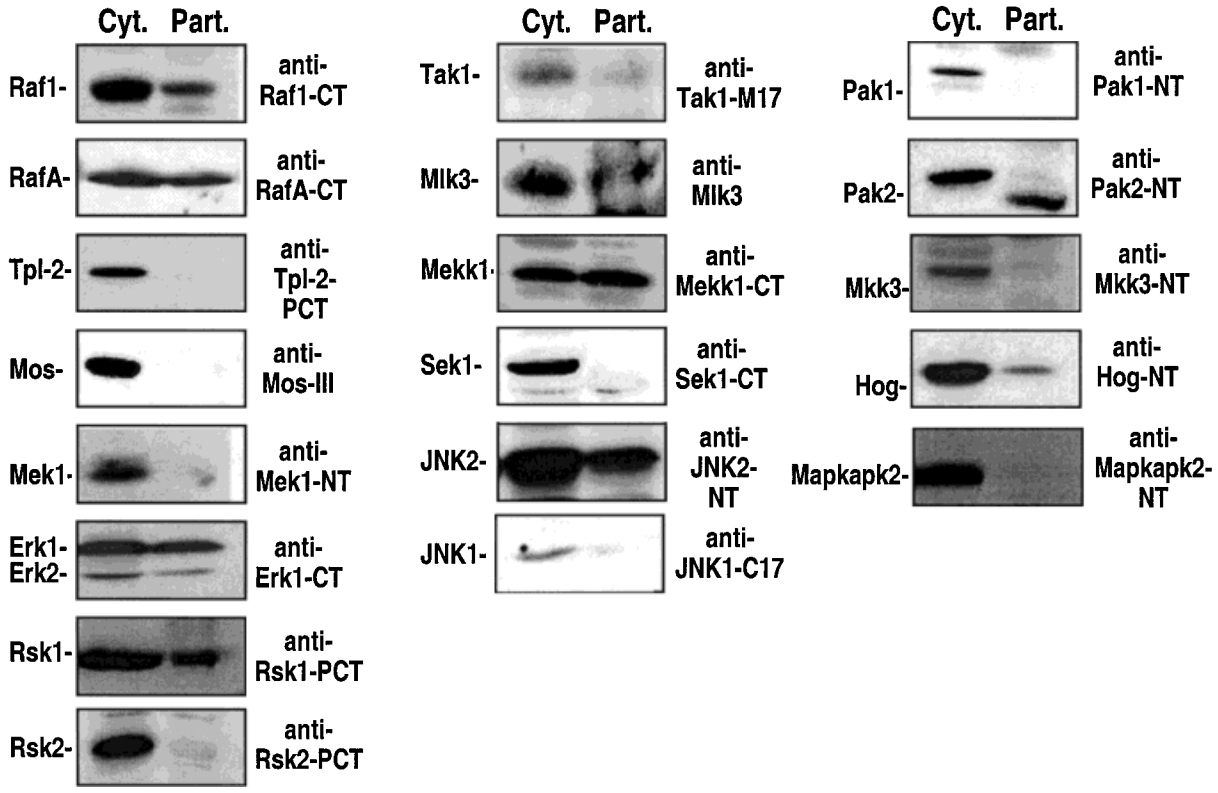

Fig. 2. Subcellular expression of protein kinases involved in MAP kinase pathways in isolated ventricular myocytes. Cytosolic (Cyt.) and particulate extracts (Part.) (100 μg protein) from isolated adult (50-day-old) venticular myocytes were subjected to 11% SDS-PAGE and Western blotted for immunoreactivity against antibodies as indicated in the figure.

levels after 50-days of age (Fig. 3C). Pak1 was expressed abundantly in brain, followed by liver (Fig. 3D). Pak1 and Pak2 were also detected in the isolated ventricular myocytes and mainly resided in the cytosolic fraction (Fig. 2). In the heart, the expression compared to other tissues was very low. In contrast, the expression of Pak2 increased up to 2.3-fold after 50-days of age (Fig. 3E, F). Similar to previous reports, p62 Pak2 was widely expressed in all the tissues investigated (Fig. 3G); the expression in the heart was detectable, but was less than in kidney and liver.

The mixed lineage kinase 3 (Mlk3), also known as SPRK, is a Ser/Thr protein kinase, with apparent molecular mass of 95 kDa [Ranna et al., 1996]. In ventricular extracts, a 95-kDa protein strongly immunoreacted with an Mlk3 specific antibody raised against its C-terminal 20 residues (Fig. 4A), with increasing intensity up to seven-fold higher than newborn levels by 50-days of age (Fig. 4B). Mlk3 was expressed in various tissues including brain, heart, lung, skeletal muscle, and testis, but it was most extensively expressed in the spleen (Fig. 4C). Mlk3 was also detected in both cytosolic and particulate fractions of isolated ventricular myocytes (Fig. 2).

The TGFβ activated kinase 1 (Tak1) is a 579 amino acid protein with molecular mass of 64 kDa. The kinase domain of Tak1 shares about 30% amino acid homology to Raf1 and Mekk, indicating potential involvement in the JNK and p38 signaling cascades [Fanger et al., 1997; Yamashita et al., 1995]. Western blots with C-terminal antibody specific for Tak1 detected a 72-kDa protein in the ventricular extracts and its expression was significantly decreased up to 80% over the first 50 days after birth (Fig. 4D, E, F). Tak1 was also detected in the cytosolic fraction of the isolated ventricular myocytes (Fig. 2). A similar protein and expression pattern was also detected with another C-terminal antibody (Fig. 4E, F). This protein, however, was not immunoprecipitated by either antibody. Tissue distribution of this pro-

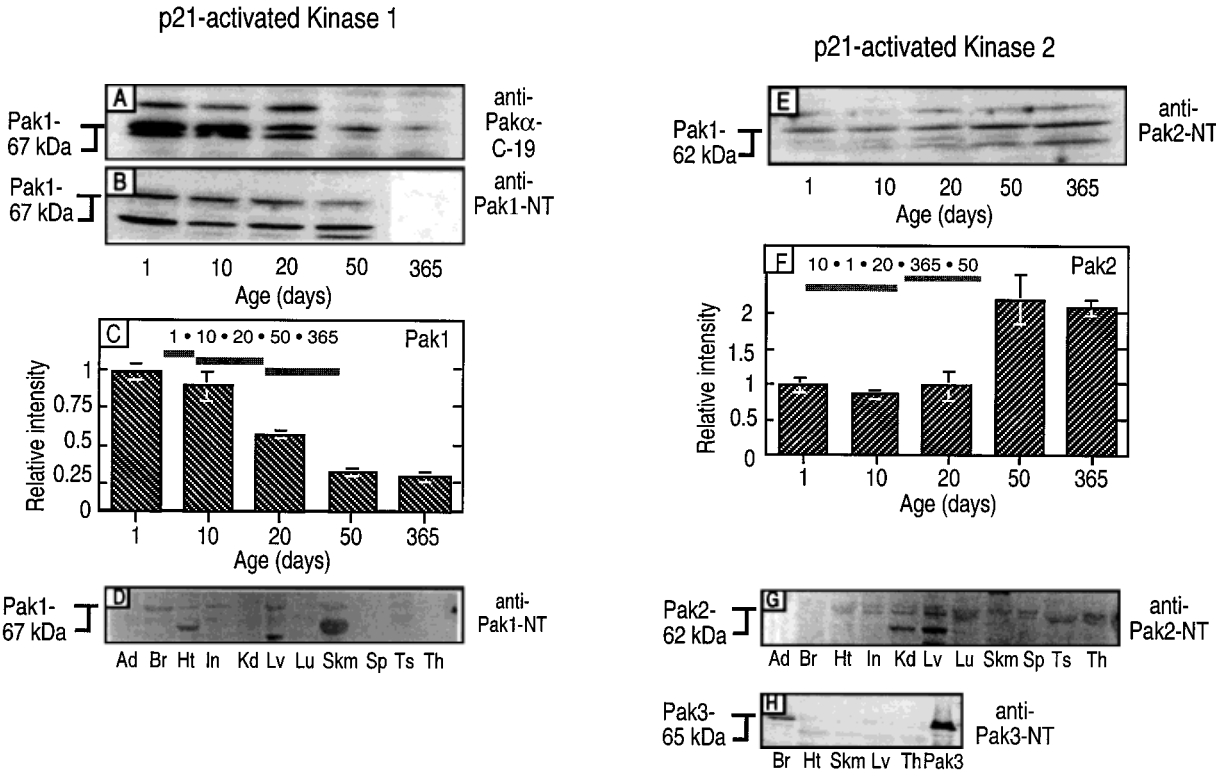

Fig. 3. Expression of p21-activated kinases during postnatal development of rat ventricle. Protein extracts (150 µg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE and Western blot analysis was performed with affinity-purified polyclonal antibodies specific for Pak1 (**A, B**), Pak2 (**E**) and Pak3 (**H**). The immunoreactivities were plotted against the relative density as 1 arbitrary unit for 1 day ventricle and values represent mean ± SEM from three to four separate experiments (**C, F**). Inserted numbers and underscores in the graphs show results of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. In panel **D** and **G,** extracts (50 µg of total protein) of 11 adult rat tissues (see legend to Fig. 1) were subjected to 11% SDS-PAGE and immunoblot analysis was performed with Pak1-NT (D) and Pak2-NT (G) antibodies. In panel H, extracts of five adult rat tissues (see legend to Fig. 1) and thrombin-cleaved recombinant Pak3 were subjected to 11% SDS-PAGE and immunoblot analysis was performed with Pak3-NT antibodies.

tein among other tissues was identical between the two antibodies, which showed the highest amount in brain, followed by lung, liver, thymus, and heart (Fig. 4G, H).

Mekk1 is a mammalian homologue of the yeast protein kinase STE 11 and Byr2 with an apparent molecular mass of 78- to 80-kDa in SDS-PAGE gels [Lange-Carter et al., 1993]. Western blotting with an amino-terminus specific antibody detected a 75-kDa protein, which was also specifically immunoprecipitated by the Mekk1-PNT antibody from ventricular extracts (Fig. 4I). Following birth, the expression in rat heart ventricles increased as much as threefold by 20-days of age (Fig. 4J). The tissue expression pattern of Mekk1 showed that the highest amount was present in the heart (Fig. 4K), which is similar to a previous analysis by Northern blotting [Lange-Carter et al., 1993].

Expression of MAP Kinase Kinases (MAPKKs), MAP Kinases (MAPKs), and Their Substrate Kinases

Expression of Erk1 and 2 and their up- and down-stream kinases. Erk1 and Erk2 are the most studied MAP kinases in the heart, and their activation by various mitogenic stimuli, correlates with hypertrophy of cardiomyocytes. The expression of Mek1, a specific upstream kinase for Erk1 and Erk2 activation, was analyzed by using two Mek1 specific antibodies raised against either N-terminus (Fig. 5A) or catalytic subdomain XI region (Fig. 5B) of Mek1. Both antibodies clearly immunoreacted with a 46-kDa protein that displayed reduced intensity following birth by up to 40-60% in 50- and 365-days (Fig. 5C). This result was consistent with a previous study [Lazou et

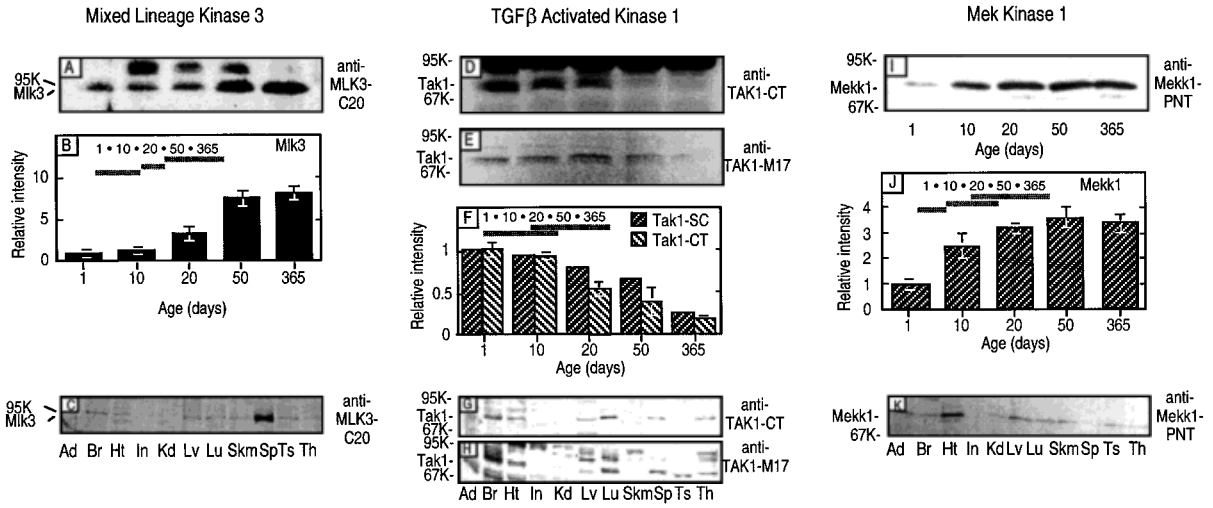

Fig. 4. Expression of MAP kinase kinase kinase (MKKKs) involved in the regulation of JNK and p38 during postnatal development of rat ventricle. Protein extracts (150 μg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE and Western blot analysis was performed with affinity purified polyclonal antibodies specific for Mlk3 (**A**), Tak-1 (**D, E**), and Mekk1 (**I**). The immunoreactivities were plotted against the relative density as one arbitrary unit for 1 day-day-old ventricle and values represent mean ± SEM from three to four separate experiments (**B,F,J**). Inserted numbers and underscores in the graphs show results of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. Extracts (50 μg of total protein) of 11 adult rat tissues (see legend to Fig. 1) were subjected to 11% SDS-SDS-PAGE and immunoblot analysis was performed with Mlk3- Mlk3-C20 (**C**), Tak1-CT (**G**), Tak-M17 (**H**), and Mekk1-PNT (**K**) antibodies.

al., 1994]. Mek1 was also present and predominantly resided in the cytosolic fraction of the isolated ventricular myocytes (Fig. 2). Relative to other rat tissues, the amount of Mek1 in the heart was low, whereas it was abundant in brain, liver, and lung (Fig. 5D).

Immunoblotting studies using Erk1 C-terminus (Erk1-CT) antibodies detected both Erk1 and Erk2 in the rat heart ventricle (Fig. 5E). Similar to a previous study [Lazou et al., 1994], the Erk2 expression was reduced by about half over the first 50 days after birth (Fig. 5F), whereas the level of Erk1 remained unchanged. Even though Erk1 and 2 were expressed in all tissues, the level and pattern of expression between Erk1 and Erk2 varied among various adult tissues (Fig. 5G). For example, in brain, both Erk1 and Erk2 were equally expressed but in other tissues, Erk1 was more abundant than Erk2. In the adult heart, the relative levels of Erk1 and Erk2 were low compared to other tissues. In isolated ventricular myocytes, Erk1 and 2 was detected in both cytosolic and particulate fractions (data not shown).

Among the many proposed substrates of Erk1 and Erk2, the p90 ribosomal S6 kinase (Rsk) has been studied extensively in many tissues and cells. Two Rsk isoforms, Rsk1 and Rsk2 were found to be regulated differently in their expression: Rsk1 was down regulated by 50% after 50 days of age (Fig. 5H, I), whereas there was no change in Rsk2 expression (Fig. 5K, L, M). Since both Rsk1 and Rsk2 are similarly sized and might have cross-reacted with the alternate Rsk antibodies, the Rsk isoforms were specifically immunoprecipitated and then immunoblotted. These experiments confirmed the decrease of Rsk1 levels during development with no change in Rsk2 levels, and immunoprecipitated Rsk1 did not react with Rsk2 antibody or vice versa (data not shown). In the isolated ventricular myocytes, Rsk1 was detected in both cytosolic and particulate fractions, whereas Rsk2 was only detected in the cytosolic fraction (Fig. 2). In various tissues, Rsk1 was widely expressed, especially abundant in brain, lung and liver, but much less in heart (Fig. 5J). Rsk2, however, was detected abundantly in heart (Fig. 5N). We did not investigated the specific expression of Rsk3, because commercially available Rsk3 antibodies appeared to cross-react with Rsk1.

Expression of JNKs and a direct upstream kinases. The signaling pathway to stress-activated protein kinases (SAPK, also known as c-Jun N-terminal kinase or JNK) is distinctly regulated by the JNK specific protein kinase, Sek1. Immunoblotting with two antibod-

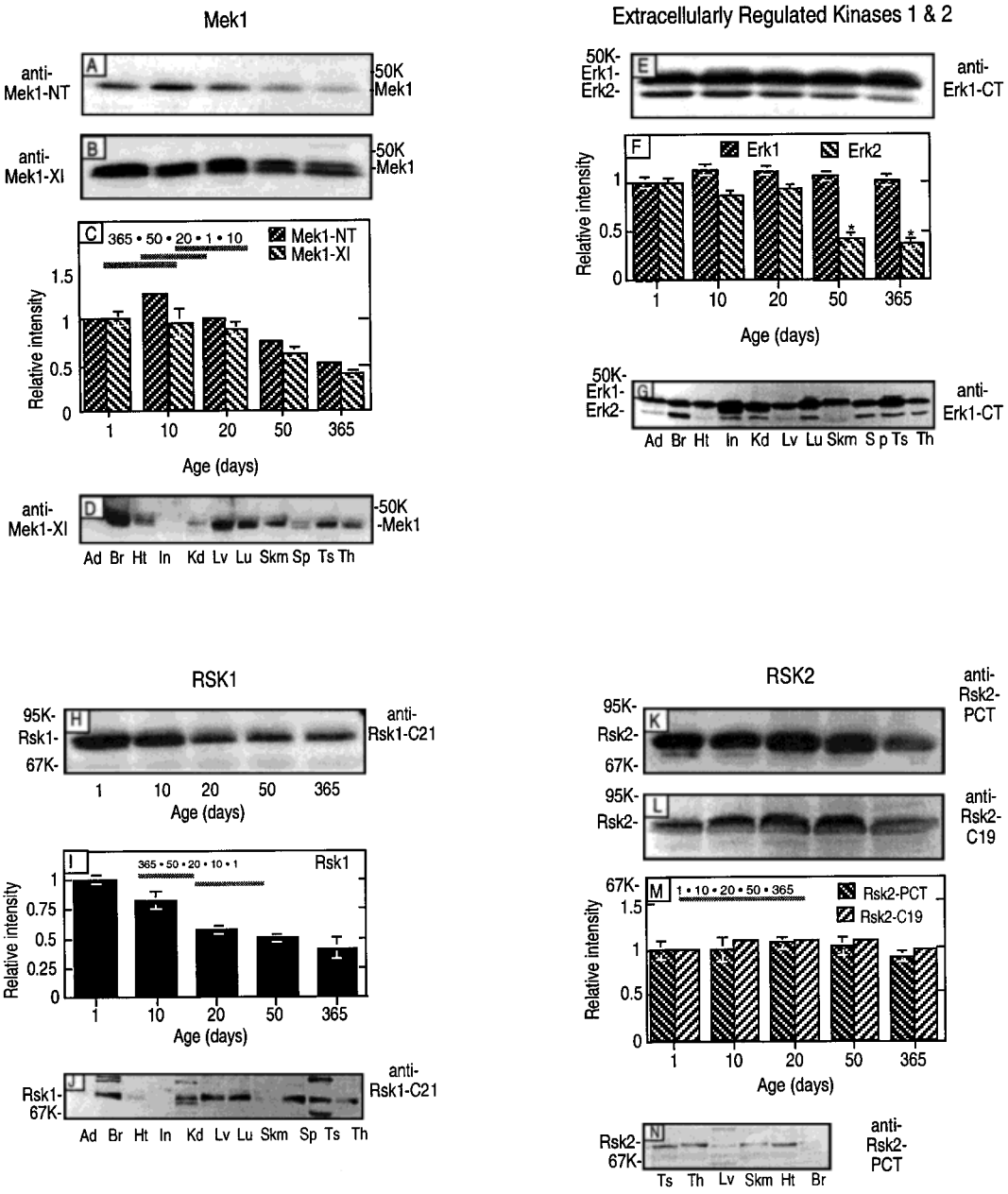

Fig. 5. Expression of Erk1 and Erk2 and their up- and downstream kinases. Protein extracts (15 µg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE and Western blot analysis was performed with affinity purified polyclonal antibodies specific for Mek1 (**A, B**), Erk1/2 (**E**), Rsk1 (**H**) and Rsk2 (**K,L**). The immunoreactivities were plotted against the relative density as 1 arbitrary unit for 1 day-day-old ventricle and values represent mean ± SEM from 3 to 4 separate experiments (**C, F, I, M**). Inserted numbers and underscores in the graphs show results of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. Extracts (50 µg of total protein) of 11 adult rat tissues (see legend to Fig. 1) were subjected to 11% SDS-PAGE and immunoblot analysis was performed with Mek1-XI (**D**), Erk1-CT (**G**), Rsk1-C21, and Rsk2-PCT (**N**) antibodies.

ies, raised against Sek1 catalytic domain XI (Fig. 6A) and C-terminal peptides (Fig. 6B), permitted detection of a 42-kDa protein that increased in expression up to four-fold during development (Fig. 6C). Among 11 different tissues, the heart expressed Sek1 most abundantly (Fig. 6D). In isolated ventricular myocytes, strong immunoreaction was detected exclusively in cytosolic fraction (Fig. 2).

To date, at least two isoforms of JNKs have been identified: JNK1 and JNK2. The JNK2 (SAPKα and SAPKβ) isoforms commonly ex-

hibit apparent molecular masses of 55 kDa, where as JNK1 (SAPKγ) has a molecular mass of 46 kDa [Pelech and Charest, 1995]. In heart, JNK isoforms are activated by several stimuli based on the in situ c-Jun phosphorylation assays [Bogoyevitch et al., 1995, 1996; Komuro et al., 1996; Knight and Buxton, 1996]. Western blotting against JNK antibodies raised against recombinantly expressed whole protein or C-terminal residues detected a 55-kDa JNK2 (Fig. 6E) and 46-kDa JNK1 (Fig. 6F). Neither of these proteins changed in expression during development (Fig. 6G). JNK1 and 2 were also detected in the isolated ventricular myocytes (Fig. 2), and a relatively high amount of JNK1 was detected in heart compared to other adult tissues (Fig. 6H).

Expression of p38 and its direct up- and downstream kinases. p38 Hog (also referred as reactivating kinase (RK), Hog, Mxi2, MAP kinase 2 and cytokine-supressive anti-inflammatory drug binding protein (CSBP) is another family of MAP kinases that are distinct from Erk1/2 and JNK. Mkk3 and Mkk6 target the phosphorylation and activation of p38 Hog [Derijard et al., 1995]. The expression of Mkk3 in heart was investigated with antibody raised against the C-terminus of this kinase. Mkk3, with an expected molecular mass of 35 kDa, was up regulated up to 2-fold during development (Fig. 7A, B). Similarly, the expression of Mkk3 in the heart was higher than in any other tissues investigated (Fig. 7C), and the level in heart ventricles was comparable to that in isolated myocytes, where the kinase predominantly resided in the cytosolic fraction (Fig. 2).

The expression of p38 Hog was investigated by using two antibodies, raised against the N-terminus (NT) and C-terminus (CT) of the kinase. There were no changes in the level of p38 Hog during development (Fig. 7D, E, F). The amount of p38 Hog in the heart was the highest among 11 different adult rat tissues (Fig. 7G). A high level of p38 Hog was also detected in isolated ventricular myocytes, where it was mainly localized in the cytosolic fraction (Fig. 2).

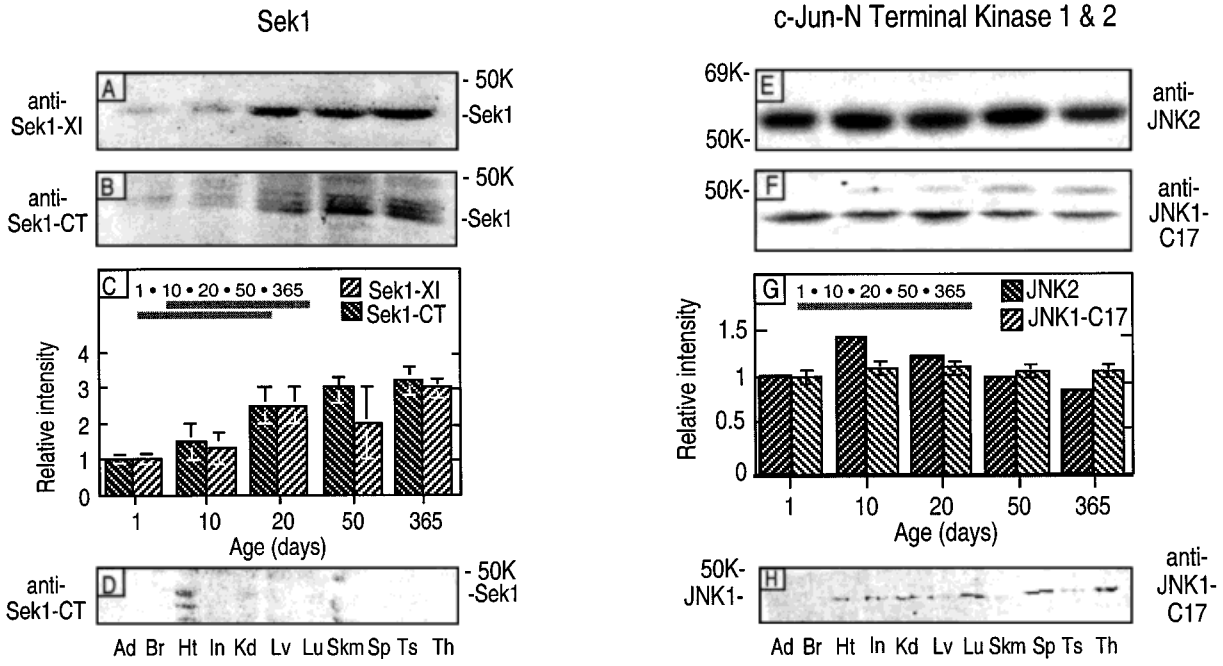

Fig. 6. Expression of JNK and the direct upstream kinase Sek1. Protein extracts (150 μg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE and Western blot analysis was performed with affinity purified polyclonal antibodies specific for Sek1 (**A,B**), JNK (**E,F**). The immunoreactivities were plotted against the relative density as 1 arbitrary unit for 1 day ventricle and values represent mean ± SEM from 3 to 4 separate experiments (**C,G**). Inserted numbers and underscores in the graphs show results of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. Extracts (50 μg of total protein) of 11 adult rat tissues (see legend to Fig. 1) were subjected to 11% SDS-PAGE and immunoblot analysis was performed with Sek1-CT (**D**) and JNK1 (**H**) antibodies.

Mapkapk2 is phosphorylated and activated by p38 Hog. Two affinity purified polyclonal antibodies raised against with the N-terminus (NT) and proximal C-terminus (PCT) of Mapkapk2 immunoreacted with an appropriately sized 60-kDa protein (Fig. 7H, I) that underwent a gradually increase of up to 1.7-fold in expression during the first 50-days after birth (Fig. 7J). The equivalent level of expression was also detected in the isolated adult ventricular myocytes, which mainly resided in the cytosolic fraction (Fig. 2). In comparison with other tissues, abundant amounts of Mapkapk2 were detected in heart but less evident than in liver and skeletal muscle (Fig. 7K).

DISCUSSION

The present study has demonstrated differential regulation of the Raf isoforms during postnatal development of the rat heart. Raf1 and RafB were significantly down regulated in the aging heart, whereas no changes were detected in RafA expression. The expressions of Raf1 and RafA were also detected in isolated ventricular myocytes, but RafB was expressed only in non-myocytes (Fig. 1 and 2). In neonatal ventricular myocytes, Raf1 and RafA were detected by Northern and Western blot analysis [Storm et al., 1990; Bogoyevitch et al., 1995]. Both isoforms were also shown to be activated by endothelin-1 and phorbol ester, but only Raf1 was activated by acidic fibroblast growth factor [Bogoyevitch et al., 1995]. Recent studies with co-expression of Rafs and casein kinase 2 (CK2) in sf-9 insect cells showed that CK2 directly interacts with RafA but not with other isoforms [Boldyreff and Issinger, 1997; Hagemann et al., 1997]. These results indicate that Raf1, RafA, and RafB are regulated by different mechanisms, which may be involved in distinct roles in the activation of Erk1 and Erk2 during postnatal development of the heart.

Tpl-2 and Mos are other protein kinases that may activate Erk1 and Erk2, and possibly JNK but not p38 Hog [Salmeron et al., 1996]. The role and identity of these kinases have not been explored in the heart. Western blotting analysis demonstrated the presence of both these kinases in isolated ventricular myocytes with increased amounts in adult rat ventricles (Fig. 1). In view of the high expression of Tpl-2 in terminally differentiated cells [Ohara et al., 1995], Tpl-2 may be involved in maintaining ventricular myocytes in their terminally differentiated state. Likewise, studies of Mos in *Xenopus* oocytes have shown that the expression of Mos is required for cells to enter meiosis II from meiosis and cell cycle arrest at metaphase II of unfertilized eggs [Roy et al., 1996]. In addition to the expression in germ cells, Mos is expressed in various somatic cells and cell lines [Leibovitch et al., 1991]. Other researchers pre-

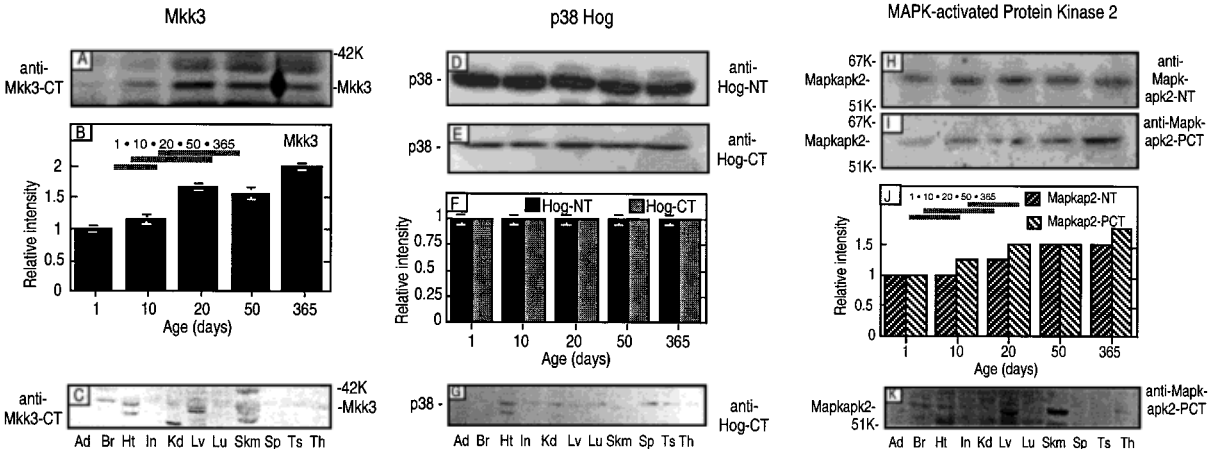

Fig. 7. Expression of p38 Hog and its direct up- and downstream kinases. Protein extracts (150 µg) of 1-, 10-, 20-, 50-, and 365-day-old rat ventricles were subjected to 11% SDS-PAGE and Western blot analysis was performed with affinity purified polyclonal antibodies specific for Mkk3 (**A**), p38 Hog (**D, E**), and Mapkapk2 (**H, I**). The immunoreactivities were plotted against the relative density as one arbitrary unit for 1 day-day-old ventricle and values represent mean ± SEM from 3 to 4 separate experiments (**B,F,J**). Inserted numbers and underscores in the graphs show results of the Tukey multiple comparisons test. At the 0.05 significance level, the means of any two groups underscored by the same line are not significantly different. Extracts (50 µg of total protein) of 11 adult rat tissues (see legend to Fig 1) were subjected to 11% SDS-PAGE and immunoblot analysis was performed with Mkk3-Mkk3-CT (**C**), Hog-CT (**G**), and Mapkapk2-PCT (**K**) antibodies.

viously reported 20- to 40-fold higher levels of Mos in skeletal muscle whose expression was up-regulated during the postnatal development [Leibovitch et al., 1991]. The role of Mos in the heart/cardiomyocytes has not been previously explored but, in Swiss 3T3 cells, the high expression of Mos was involved in the apoptosis of cells in S phase and caused irreversible cell cycle arrest in G_1 phase [Fukasawa et al., 1995]. When these cells were in G_2 phase at the time of Mos expression, the cells completed M phase but failed to undergo cytokinesis, resulting in binucleated cells. Considering the temporal regulation of Mos expression, it may be involved in cell cycle arrest and bi- or polynucleation of adult cardiac myocytes. In fact, the over-expression of Mos causes differentiation of C2C12 myoblast [Leibovitch et al., 1995]. Mos directly phosphorylates a muscle specific transcription factor, MyoD, which induces the heterodimerization of MyoD and E12 proteins [Guillier et al., 1997].

In cardiac myocytes, both Erk1 and Erk2 have been identified and activated by various mitogenic stimuli including angiotensin II, endothelin-1, phenylephrine, adenosine, ATP, fibroblast growth factors, and mechanical stress in both neonatal cell cultures and adult heart tissues or isolated cardiac myocytes [Sadoshima et al., 1995; Yamazaki et al., 1993; Bogoyevitch et al., 1994; Lazou et al., 1994]. Activation of Erk1 and Erk2 is carried out by the 45- to 46-kDa dual specific protein kinases Mek 1 and Mek2 [Crews et al., 1992]. In cardiac myocytes, Mek1 was activated by stimuli that activate Erk1 and Erk2 [Lazou et al., 1993; Bogoyevitch et al., 1994]. Our Western blotting analysis confirmed previous observations of decreased expression of Mek1 and Erk2 during development (Fig. 5). In addition, we also analyzed the expression of Erk1, which showed no change in expression. Similar to the differential expression of Erk isoforms, Rsk isoforms were also different in their expression patterns during development (Fig. 5). Rsk1 was down-regulated up to 50%, the same extent seen for Erk2, where as there were no detectable changes in Rsk2. The specific activities of Erk1 and Erk2 were down regulated with age, while no change was seen in Rsk1 specific activity (data not shown). Even though the regulation of Rsk isoforms by Erks may differ in vitro [Zhao et al., 1996], it is still unclear whether there are functional differences in Erk1 and 2, and Rsk1 and 2. Rsk1 and Rsk2 have been implicated in the meiotic maturation of oocytes and the regulation glycogen synthesis via protein phosphatase1 [Sturgill and Wu, 1991; Blenis, 1991]. In the heart, Rsk1 was reported to be activated by various mitogenic stimuli, such as endothelin-1, angiotensin-II, or mechanical stress [Sadoshima et al., 1995]. However, the role and regulation of Rsk2 in the heart has not been investigated in detail.

Distinct from the Erk1 and Erk2 signaling pathways, several protein kinases have shown to activate JNK and p38 Hog. These protein kinases include Pak, Mlk3, Tak1, and Mekk. Their functional roles and regulation in the heart or cardiomyocytes have not been previously investigated. Pak1 and Pak2 were detected in ventricles and isolated ventricular myocytes, with down regulation of Pak1 and up regulation of Pak2 during development (Fig. 3). The expression of Pak1 and Pak2 without Pak3 concur with previous reports that showed the ubiquitous expression of Pak2, brain- and muscle-specific expression of Pak1, and brain-specific expression of Pak3 [Sells and Chernoff, 1997]. The physiological role and significance of the differential regulation of Pak isoforms during development is currently under investigation in our lab.

Western blotting with Mlk3 antibody revealed the presence of the protein in ventricle and ventricular myocytes of rat heart and its level increased up to seven-fold during development (Fig. 4). Mlk3 has shown to be expressed in many tissues and cell lines, and to interact with Cdc42 and Rac1 [Fanger et al., 1997; Ranna et al., 1996; Teramoto et al., 1996]. Little is known about the regulation and function of this kinase, but over-expression in COS-7 cells could activate JNK signal cascades, probably by direct phosphorylation and activation of Sek1, with little effects on Erk1 and Erk2 and p38 Hog signaling pathways [Teramoto et al., 1996]. Mekk1 is another protein kinase that is preferentially involved in the JNK signaling pathway by phosphorylating and activating Sek1 [Pelech and Charest, 1995]. The mode of Mekk1 activation has not been clearly defined, but it has been proposed to be activated by binding to Ras and Cdc42/Rac [Fanger et al., 1997]. Our Western blotting analysis with a Mekk-specific antibody detected a 75 kDa protein that increased in amount during development (Fig. 4). The functional roles and signifi-

cance of the up regulation of Mlk3 and Mekk1 remain to be established. Since Sek1 expression was also up-regulated concurrently (Fig. 6), it may imply a more prominent role for JNK signaling pathways in the adult as compared to neonatal heart.

The JNKs represent another family member of MAP kinases which are activated by various stress related stimuli, such as heat shock, hyperosmostic conditions, UV radiation, cyclohexamide, anisomycin, phorbol myristic acetate (PMA), and tumor necrosis factor-α (TNF-α). JNK isoforms were activated by mechanical stretch, sorbitol, and endothelin-1 treatment of cultured neonatal ventricular myocytes [Komuro et al., 1996; Bogoyevitch et al., 1995], and in ischemia reperfused whole hearts [Knight and Buxton, 1996; Bogoyevitch et al., 1996]. The activity of JNK is directly regulated by SAPK/Erk kinase 1 (Sek1, also known as MKK4 and JNKK), which shares 45 % amino acid homology with Mek1 and Mek2 [Derijard et al., 1995]. Certain stimuli, such as osmotic shock, taxol, heat shock, sodium arsenite, anisomycin, lipolysaccharide, interleukin 1, and TNF-α, most of which also activate JNK, also lead to activate p38 Hog [Bogoyevitch et al., 1996; Pelech and Charest, 1995]. Activation of p38 Hog is carried out through phosphorylation on both threonine and tyrosine in a TGY motif by dual specific protein kinases, Mkk3 and Mek6. Mkk3 is an approximately 35-kDa kinase that shares about 41 % amino acid sequence with Mek1. In the heart, the p38 Hog signaling pathway is activated by cellular stresses such as hyperosmolarity, ischemia, and ischemia reperfusion [Sadoshima et al., 1996; Bogoyevitch et al., 1996; Knight and Buxton, 1996]. A specific downstream target for p38 Hog is MAPK-activated protein kinase 2 (Mapkapk2), which phosphorylates heat shock protein (hsp) 27 and the transcription factor CREB [Tan et al., 1996; Freshney et al., 1994]. The phosphorylation of hsp27 and CREB induced by cellular stresses has been suggested to stimulate polymerization of actin and production of c-fos mRNA, respectively [Landry and Huot, 1995]. Western blotting analysis of these protein kinases revealed interesting changes in their expression patterns during development of the heart. Both Sek1 and Mkk3 increased in expression in the aging heart, whereas no changes in JNKs and p38 Hog were evident (Figs 6 & 7). Since both of these signaling pathways are activated by various cellular stresses including ischemia, their up regulation during development may play a role in age-specific responses of the stimuli, such as less tolerance to ischemia or hypoxia in adult as compared to neonatal heart. In several non-hemopoietic cells or tissues, the activation of JNK or p38 Hog is implicated in cell death. In addition, the activation of p38 Hog has been suggested to be involved in the mechanism for preconditioning of the heart, protecting damage from prolonged ischemia by previous transient ischemia. Presently, little information is available regarding the differences in preconditioning in between neonatal and adult heart, but the up-regulated expression of these signaling pathways may indicate a substantial role in adult heart.

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REFERENCES

Anversa P, Ricci R, Olivetti G (1986): Quantitative structural analysis of the myocardium during physiologic growth and induced cardiac hypertrophy. J Am Cell Cardiol 7:1140–1149.

Anversa P, Fitzpatrick D, Argani S, Capasso JM (1991): Myocyte mitotic division in the aging mammalian rat heart. Circ Res 69:1159–1164.

Anversa P, Kajstura J, Cheng W, Reiss K, Cigola E, Olivetti G (1996): Insulin-like growth factor-1 and myocyte growth: the danger of a dogma. Part II. Induced myocardial growth: pathologic hypertropy. Cir Res 32:484–495.

Bagrodia S, Derijard B, Davis RJ, Cerione RA (1995): Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. J Biol Chem 270:27995–27998.

Blenis J (1991): Growth-regulated signal transduction by the MAP kinase and Rsks. Canc Cells 3:445–449.

Bogoyevitch MA, Glennon PE, Andersson MB, Clerk A, Lazou A, Marshall C, Parker PJ, Sugden PH (1994): Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes. J Biol Chem 269:1110–1119.

Bogoyevitch MA, Ketterman AJ, Sugden PH (1995): Cellular stresses differentially activate c-Jun N-terminal protein kinases and extracellular signal-regulated protein kinases in cultured ventricular myocytes. J Biol Chem 270:29710–29717.

Bogoyevitch MA, Marshall CJ, Sugden PH (1995): Hypertrophic agonists differentially activate A-Raf and c-Raf in cultured ventricular myocytes. J Biol Chem 270:26303–26310.

Bogoyevitch MA, Gillespie-Brown J, Ketterman AJ, Fuller SJ, Ben-Levy R, Ashworth A, Marshall CJ, Sugden PH (1996): Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. Cir Res 79:162–173.

Boldyreff B, Issinger O (1997): A-Raf kinase is a new interacting partner of protein kinase CK2 subunit. FEBS Letters 403:197–199.

Capasso JM, Bruno S and, Cheng W (1992): Ventricular loading is coupled with DNA synthesis in adult cardiac myocytes after acute and chronic myocardial infarction. Cir Res 71:1376–1389.

Capasso JM, Bruno S, Li P, Zhang X, Darzynkiewicz ZD, Anversa P (1993): Myocyte DNA synthesis with aging: correlation with ventricular loading in rats. J Cellul Physiol 155:635–648.

Chien KR, Zhu H, Knowlton KU, Miller-Hance W, van-Bilsen M, O'Brien TX, Evans SM (1993): Transcriptional regulation during cardiac growth and development. Annu Rev Physiol 55:77–95.

Claycomb WC, Moses RL (1988): Growth factors and TPA stimulate DNA synthesis and alter the morphology of cultured terminally differentiated adult rat cardiac muscle cells. Developmental Biology 127:257–265.

Claycomb WC (1975): Biochemical aspects of cardiac muscle differentiation : d: Deoxyribonucleic acid synthesis and uclear and cytoplasmic deoxyribonuclein acid polymerase activity. J Biol Chem 250:3229–3235.

Chien KR, Knowlton KU, Zhu H, Chien S (1991): Regulation of cardiac gene expression during myocardial growth and hypertrophy: molecular studies of an adaptive physiologic response. FASEB J 5:3037–3046.

Crews CM, Alessandrini A, Erikson RL (1992): The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science 258:478–480.

Cummins P (1993): Fibroblast and transforming growth factor expression in the cardiac myocyte. Circ Res 27: 1150–1154.

Daum G, Eisenmann-Tappe I, Fries H, Troppmair J, Rapp UR (1994): The ins and outs of Raf kinases. Trends Biochem Sci 19:474–479.

Derijard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ, Davis RJ (1995): Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science 267:682–685.

Fanger GR, Gerwins P, Widmann C, Jarpe MB, Johnson GL (1997): Mekks, GCKs, MLKs, PAKs, TAKs, and Tpls: upstream regulators of the c-jun amino-terminal kinases? Curr Opin Gen Devel 7:67–74.

Freshney NW, Rawlinson, Guesdon LF, Jones E, Cowley S, Hsuan, J and Saklatvala J (1994): Interleukin-1 activates a novel protein kinase cascade that result in the phosphorylation of Hsp27. Cell 78:1039–1049.

Fukasawa K, Rulong S, Resau J, Pinto da Silva P, Vande Woude GF (1995): Overexpression of *mos* oncogene product in Swiss 3T3 cells induces apoptosis preferentially during S-phase. Oncogene 10:1–8.

Guillier M, M Vandromme, MP Leibovitch and SA Leibovitch (1997): Mos activates myogenic differentiation by promoting heterodimerization of MyoD and E12 proteins. Mol Cell Biol 17:584–593.

Hagemann C, Kalmes A, Wixler V, Wixler L, Schuster T, Rapp UR (1997): The regulatory subunit of protein kinase CK2 is a specific A-Raf activator. FEBS Letters 403:200–202.

Jakobi R, Chen CJ, Tuazon PT, Traught JA (1996): Molecular cloning and sequencing of the cytostatic G protein-activated protein kinase PAK I. J Biol Chem 271:6206–6211.

Kajstura J, Mansukhani M, Cheng W, Reiss K, Krajewski S, Reed JC, Quaini F, Sonnenblick EH, Anversa P (1996): Programmed cell death and expression of the protooncogene bcl-2 in myocytes during postnatal maturation of the heart. Exp Cell Res 219:110–121.

Knight RJ, Buxton DB (1996): Stimulation of c-Jun kinase and mitogen-activated protein kinase by ischemia and reperfusion in the perfused rat heart. Biochem Biophys Res Com 218:83–88.

Komuro I, Yazaki Y (1993): Control of cardiac gene expression by mechnaical stress. Annu Rev Physiol 55:55–75.

Komuro I, Kudo S, Yamazaki T, Zou Y, Shiojima I, Yazaki Y (1996): Mechanical stretch activates the stress-activated protein kinases in cardiac myocytes. FASEB J 10:631–636.

Landry J, Huot J (1995): Modulation of actin dynamic during stress and physiological stimulation by a signaling pathway involving p38 MAP kinase and heat-shock protein 27. Biochem Cell Biol 73: 703–703–707.

Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, Johnson GL (1993): A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science 260:315–319.

Lazou A, Bogoyevitch MA, Clerk A, Fuller SJ, Marshall C, Sugden PH (1994): Regulation of mitogen-activated protein kinase cascade in adult rat heart preparations in vitro. Cir Res 75:932–941.

Leibovitch SA, Guillier M, Lenormand J, Leibovitch M (1991): Accumulation of the c-mos protein is correlated with post-natal development of skeletal muscle. Oncogene 6:1617–1622.

Leibovitch MP, B Solhonne, M Guillier, P Verrelle, SA Leibovitch and P Verrelle (1995): Direct relationship between the expression of tumor suppressor H19 mRNA and c-mos proto-oncogene during myogenesis. Oncogene 10:251–260.

Misao J, Hayakawa Y, Ohno M, Kato S, Fujiwara T, Fujiwara H (1996): Expression of bcl-2 protein, an inhibitor of apoptosis, and bax, an accelerator of apoptosis, in ventricular myocytes of human hearts with myocardial infarction. Circulation 94:1506–1512.

Miyoshi J, Higashi T, Mukai H, Ohuchi T, Kakunaga T (1991): Structure and transforming potential of the human cot oncogene encoding a putative protein kinase. Mol Cell Biol 11:4088–4096.

Ohara R, Hirota S, Onoue H, Nomura S, Kitamura Y, Toyoshima K (1995): Identification of the cells expressing *cot* proto-oncogene mRNA. J Cell Sci 108:97–103.

Olivetti G, Quaini F, Lagrasta C, Ricci R, Tiberti G, Capasso JM, Anversa P (1992): Myocyte cellular hypertrophy and hyperplasia contribute to ventricular wall remodeling in anemia-induced cardiac hypertrophy in rats. Am J Pathol 141:227–239.

Pham CD, Arlinghaus RB, Zheng C, Guan KL, Singh B (1995): Characterization of MEK1 phosphorylation by the v-Mos protein. Oncogene 10:1683–1688.

Pelech SL, Charest DL (1995): MAP kinase-dependent pathways in cell cycle control. In Meijer L, Guidet S, Tung HYL (eds): "Progress in Cell Cycle Research." New York: Plenum Press, 1995, pp 33–52.

Rakusan K (1984): Cardiac growth, maturation, and aging. In Zak R (ed): "Growth of the heart in health and disease." New York: Raven Press, pp 131–164.

Ranna A, Gallo K, Godowski P, Hirai S, Ohno S, Zon L, Kyriakis JM, Avruch J (1996): The mixed lineage kinase SPRK phosphorylates and activates the stress-activated protein kinase activator, SEK-1. J Biol Chem 271:19025–19028.

Riva E, Hearse DJ (1991): "The Developing Myocardium." New York: Futura Publishing Co, pp 1–127.

Robinson MJ, Cobb MH (1997): Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 9:180–186.

Rodrigous B, Severson DL (1997): Preparation of cardiomyocytes. In McNeill JH (ed): "Biochemical techniques in the heart III." Boca Raton: CRC Press, pp 99–113.

Roy LM, Haccard O, Izumi T, Lattes BG, Lewellyn AL, Maller JL (1996): Mos proto-oncogene function during oocyte maturation in Xenopus. Oncogene 12:2203–2211.

Rumyantsev PP (1996): Interrelation of the proliferation and differentiation processes during cardiac myogenesis and regeneration. Intern. Rev Cytol 51:187–273.

Sadoshima J, Qiu Z, Morgan JP, Izumo S (1996): Tyrosine kinase activation is an immediate and essential step in hypotonic cell swelling-induced ERK activation and c-fos gene expression in cardiac myocytes. EMBO J 15:5535–5546.

Sadoshima J, Qiu Z, Morgan JP, Izumo S (1995): Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 90-kDa kinase in cardiac myocytes. The critical role of Ca^{2+}-dependent signaling. Cir Res 76:1–15.

Salmeron A, Ahmad TB, Carlele GW, Pappin D, Narsimhan RP, Ley SC (1996): Activation of MEK-1 and SEK-1 by Tpl-2 protooncoprotein, a novel MAP kinase kinase kinase. EMBO J 15:817–826.

Sanghera JS, Weinstein SL, Aluwalia M, Girn J, Pelech SL (1996): Activation of multiple proline-directed kinases by bacterial lipopolysaccharide in murine macrophages. J Immunol 156:4457–4465.

Schott R, Morrow LA (1993): Growth factors and angiogenesis. Cir Res 27:1155–1161.

Sells MA, Chernoff J (1997): Emerging from the Pak: the p21-activated protein kinase family. Trends Cell Biol 7:162–167.

Storm SM, Cleveland JL, Rapp UR (1990): Expression of raf family proto-oncogenes in normal mouse tissues. Oncogene 5:345–351.

Sturgill TW, Wu J (1991): Recent progress in characterization of protein kinase cascades for phosphorylation of ribosomal protein S6. Biochim Biophys Acta 1092:350–357.

Su B, Karin M (1996): Mitogene-activated protein kinase cascades and regulation of gene expression. Curr Opin Immunol 8:402- 8:402–411.

Sugden PH, Bogoyevitch MA (1995): Intracellular signalling through protein kinases in the heart. Cardiov Res 30:478–492.

Tan Y, Rouse J, Zhang A, Cariati S, Cohen P, Comb MJ (1996): FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. EMBO J 15:4629–4642.

Teramoto H, Coso OA, Miyata H, Igishi T, Miki T, Gutkind JS (1996): Signal from the small GTP-binding protein Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinases pathway. J Biol Chem 271: 27225–27228.

van-Bilsen M, Chien KR (1993): Growth and hypertrophy of the heart: towards an understanding of cardiac specific and inducible gene expression. Cardiov Res 27:1140–1149.

Yamashita K, Shirakabe K, Shibuya H, Irie K, Oshi I, Ueno N, Taniguchi T, Nishida E, Matsumoto K (1995): Identification of a member of the MAPKKK family as a potential mediator of TGF-β signal transduction. Science 270:2008–2011.

Yamazaki T, Tobe K, Hoh E, Maemura K, Kaida T, Komuro I, Tamemoto H, Kadowaki T, Nagai R, Yazaki Y (1993): Mechanical loading activates mitogen-activated protein kinase and S6 peptide kinase in cultured rat cardiac myocytes. J Biol Chem 268:12069–12076.

Zak R (1974): Development and proliferation capacity of cardiac muscle cells. Circ Res 34 and 35:II-17-II-26.

Zak R (1984): Overview of the growth process. In Zak R (ed): "Growth of the heart in health and disease." New York: Raven Press, pp 1–24.

Zhang S, Han J, Sells MA, Chernoff J, Knaus UG, Ulevitch RJ, Bokoch GM (1995): Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J Biol Chem 270:23934–23936.

Zhao Y, Bjorbek C, Moller DE (1996): Regulation and interaction of $pp90^{rsk}$ isoforms with mitogen-activated protein kinases. J Biol Chem 271:29773–29779.